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Hepatitis C virus reinfection and spontaneous clearance of reinfection - the InC^3 study

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FOOTNOTE PAGE

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ABSTRACT

Background: We aimed to characterize the natural history of hepatitis C virus (HCV) reinfection and spontaneous clearance following reinfection (reclearance), including predictors of HCV reclearance.

Methods: Data were synthesised from nine prospective cohorts evaluating HCV infection outcomes among people who inject drugs (InC^3 study). Participants with primary HCV infection were classified as achieving viral suppression if they had at least one subsequent negative HCV RNA test. Those with a positive HCV RNA test following viral suppression were investigated for reinfection. Viral sequence analysis was used to identify reinfection (heterologous virus with no subsequent detection of the original viral strain).

Results: Among 591 participants with acute primary HCV infection, 118 were investigated for reinfection. Twenty-eight participants were reinfected (12.3/100 person-years, 95%CI: 8.5-17.8). Peak HCV RNA was lower in reinfection than primary infection (p=0.011). The reclearance proportion at six months after reinfection was 52% (95%CI: 33-73%). Adjusting for study site, females with *IFNL4* (formerly *IFNL3* and *IL28B*) rs12979860 -CC genotype were more likely to reclear (HR:4.16, 95%CI: 1.24-13.94, p=0.021).

Conclusions: Sex and *IFNL4* genotype are associated with spontaneous clearance after reinfection.

INTRODUCTION

Spontaneous clearance of primary hepatitis C virus (HCV) occurs in 25% of individuals [1]. However, reinfection following spontaneous clearance suggests that natural immunity is short-lived or has limited breadth or magnitude, with implications for vaccine development [2].

HCV reinfection studies in people who inject drugs have produced contradictory results [2], with variation in reinfection rates and proportions of reinfections clearing spontaneously [3-12]. These discrepancies may be attributed to methodological limitations, including variations in frequency of follow-up testing and data capture [2, 13], and classification of viral recurrence as reinfection without confirmation that viraemic episodes are genetically distinct [12].

The International Collaboration of Incident HIV and HCV in Injecting Cohorts (InC^3) Study, pooling data from nine prospective cohorts from Australia, Canada, the Netherlands, and the United States mainly following people who inject drugs [14], enables assessment of HCV reinfection in well-characterized HCV-infected participants. The study aims were: 1) to characterize the natural history of spontaneous clearance following primary infection (primary clearance), HCV reinfection and spontaneous clearance following reinfection (reclearance) in the InC^3 study; and 2) to assess (i) differences in peak HCV RNA in reinfection compared to primary infection; (ii) predictors of HCV reclearance; and (iii) differences in the time to primary clearance and reclearance.

PARTICIPANTS AND METHODS

Study population and design

The InC^3 Study has been described previously [14]. All cohorts follow participants at regular intervals using standardized methods (Table 1). Participants were recruited and followed between 1985 and 2010. For the current study, only individuals with documented acute primary HCV and >2 subsequent HCV RNA tests were included.

Primary HCV infection means an individual's first HCV infection. Documented acute primary HCV is defined as either: 1) HCV seroconversion with an HCV antibody negative test followed by an HCV antibody or RNA positive test within two years; or 2) evidence of symptomatic infection (defined by jaundice or ALT elevation >400 U/L, and positive HCV RNA or antibody test and recent high-risk exposure). All participants provided written informed consent and cohort protocols were approved by local institutional human research review committees.

Laboratory testing

Choice of HCV RNA testing and HCV sequencing methods and regions varied between, but not within, cohorts. Qualitative and quantitative HCV RNA, HCV genotype and serotype, and interferon lamda 4 (*IFNL4*) rs12979860 genotype (formerly known as interferon lambda 3 [*IFNL3*] and interleukin-28B [*IL28B*]) assays have been described previously [15]. Regions of the virus sequenced to confirm reinfection are listed in Supplementary Table 1. PCR amplification, primers and sequencing methods have been described previously [16-20]. Cohort sites used distance [6, 7, 11] or phylogenetic methods [5] to distinguish heterologous from homologous virus within viral subtypes.

Estimated date of primary HCV infection

The estimated date of primary HCV infection was determined using the flowchart in Figure 1A

HCV antiviral treatment

The natural history of HCV reinfection and reclearance may differ following antiviral treatment compared to spontaneous clearance of primary HCV infection. Therefore this analysis was limited to studying HCV reinfection and reclearance in the absence of a history of antiviral treatment. Individuals who were treated >26 weeks after the estimated date of primary infection were censored from the treatment date. Individuals treated for HCV were excluded if the estimated duration of primary infection <26 weeks to reduce misclassification bias due to uncertainty around subsequent spontaneous clearance without treatment (n=37).

Primary HCV infection outcomes

After acute primary HCV, those with one subsequent undetectable HCV RNA test and those with two consecutive undetectable HCV RNA tests (≥28 days apart) were classified as having viral suppression and primary clearance, respectively. Those with viral suppression at final follow-up were excluded because the final outcome could not be determined (n=34). Those with detectable HCV RNA following viral suppression or primary clearance were classified as having reoccurring viraemia. Among those with reoccurring viraemia, viral genotype/subtype and viral sequence analysis were used to distinguish reinfection (heterologous virus with no subsequent detection of the original viral strain), intercalation (homologous virus), and indeterminate cases (viral sequencing unavailable, or heterologous virus with subsequent detection of the original viral strain). If primary clearance occurred

prior to detection of seroconversion so no HCV RNA could be isolated for the primary infection, serotyping was performed to classify primary infection.

Estimated dates and times of primary clearance, viral suppression and reinfection

Methods for determining the estimated dates of primary clearance, viral suppression and reinfection are illustrated in Figure 1 (panels B-D). In participants with evidence of primary clearance prior to reinfection, the time to reinfection was calculated as the time from the date of primary clearance to the date of reinfection. In participants with viral suppression prior to reinfection, the time to reinfection was calculated as the time from the date of viral suppression to the date of reinfection (Figure 1C). The time to reappearance of viraemia in intercalation and indeterminate intermittent viraemia was calculated similarly to the time to reinfection.

HCV reinfection outcomes

Reclearance was defined as two consecutive negative HCV RNA tests (≥28 days apart) following reinfection. The date of reclearance was calculated similarly to the date of primary clearance and the time to reclearance was calculated as the time from the date of reinfection to the date of reclearance. For those without clearance, follow-up time was calculated from the date of reinfection until the date of the last therapy-naïve detectable HCV RNA test. Persistent reinfection was defined as continuous viraemia with the confirmed reinfecting virus for greater than six months.

Classification of peak HCV RNA in primary infection and reinfection

Peak HCV RNA was defined as the maximum quantitative RNA measured within three months of the date of infection for both primary infection and reinfection (Supplementary Information 1).

Statistical analyses

Wilcoxon signed-rank tests were used to evaluate the median within-participant difference between peak log HCV RNA in reinfection compared to primary infection. It was hypothesised that the peak log RNA would be lower in reinfection compared to primary infection [6]. Participants with at least one quantitative HCV RNA test in the first three months of primary HCV infection and reinfection were included.

Predictors of HCV reclearance were assessed using Cox proportional hazards regression. Models included shared frailty terms for study site to capture unobserved heterogeneity between sites which may have contributed to the time to reclearance. Potential interactions between study site – categorised as BBAASH (study site contributing largest number of reinfection events) versus other sites – and hypothesised predictors were evaluated. Hypothesized predictors were determined *a priori* based on established predictors of primary clearance; including age [24], sex [1, 15, 25, 26], *IFNL4* genotype (SNP rs12979860; CC vs. CT/TT) [27-29], the combined effect of sex and *IFNL4* genotype (female rs12979860-CC vs. others) [15, 26], HCV genotype of reinfection (genotype 1 vs. non-1) [15, 30], and reinfection with the same vs. different HCV genotype to the primary infection. It was hypothesised that participants reinfected with the same HCV genotype would have greater propensity toward reclearance [31, 32]. The combined effect of sex and *IFNL4* genotype was investigated by comparing females with rs12979860-CC genotype and all other participants

because despite this study combining the largest number of HCV reinfections studied to date, there were not sufficient numbers of reinfections to investigate the interaction between sex and *IFNL4* genotype. The effect of HIV infection [33] was not assessed due to small numbers of HIV-infected participants. The effects of jaundice and elevated ALT were not assessed because most of the participating studies did not collect this information at the time of HCV reinfection.

Differences in time to primary clearance and reclearance in participants with reclearance were assessed using gap-time unrestricted proportional hazards regression (appropriate for analysis of predictors of time-to-event outcomes with multiple events [34]). The hypothesis was that time to primary clearance would be longer than the time to reclearance [6].

For all investigations, sensitivity analyses were performed to assess the effect of excluding participants with HIV at reinfection (n=3), those reinfections defined on the basis of serotyping (n=2), those with viral suppression rather than primary clearance prior to reinfection (n=7), and stratifying by study site (BBAASH versus others). For the analysis of differences between peak HCV RNA in primary HCV infection and reinfection, sensitivity analysis of the effect of defining peak RNA as the peak within one month of infection rather than three months were conducted (Supplementary Information 1). For the analysis of predictors of reinfection, sensitivity analyses excluding participants with fewer than two HCV RNA tests after reinfection was also assessed.

Characteristics at the time of primary HCV infection and reappearance of viraemia were analysed by primary infection outcome using Kruskal-Wallis, Chi-squared, and Fisher's exact tests, as appropriate. Proportions of reinfections resulting in spontaneous clearance and

persistent infection six-months after reinfection were estimated using Kaplan-Meier survivor functions. Finally, classifications of reoccurring viraemia after primary clearance versus recurring viraemia after viral suppression were compared using Chi-squared tests. Statistically significant differences were assessed at $p<0.05$; p-values are two-sided. All analyses were performed using Stata v11.0 (College Station, TX, United States). Participant timeline figures were prepared in R [35, 36].

RESULTS

Participant characteristics

Of 662 participants with acute primary HCV infection, 591 had a defined infection outcome (Figure 1D and Table 2). At the time of primary infection, the median age was 26 years and 36% were female. Most participants had injected drugs (96%). A small minority (7%) were infected with HIV.

Primary HCV infection characteristics and outcomes

Among those with known HCV genotypes during primary infection (n=518, 88%), the most common genotypes were 1 (n=288; 56%) and 3 (n=176, 34%; Table 2). Following primary infection, 252 participants had viral suppression (at least one undetectable HCV RNA test), of whom 146 (58%) had primary clearance (at least two consecutive undetectable HCV RNA tests ≥28 days apart). Overall,118 (47%) had reoccurring viraemia, 72 after viral suppression and 46 after primary clearance (Figure 1D). Among those with reoccurring viraemia, viral sequence analysis was used to distinguish reinfection (n=28), intercalation (n=31), and indeterminate cases (viral sequencing not available, n=55; heterologous virus with subsequent detection of the original viral strain, n=4). Reinfection was more common after primary clearance (46% of cases) than viral suppression (10% of cases, $p<0.001$; Figure 1D).

Study retention and frequency of HCV testing

In the 28 reinfected participants, the median (interquartile range [IQR]) length of follow-up after the date of primary HCV infection was 4.6 (3.2-7.3) years. The median (IQR) number of HCV RNA tests in this time was 17 (9-35) and the median test interval 48 (33-122) days. The median test interval prior to primary clearance (49, IQR: 32-140) was similar to the test interval during the risk period for reinfection and until reclearance or the end of follow-up (median: 53, IQR: 33-138). In participants without reinfection, the median (IQR) length of follow-up was 1.5 (0.7-2.8) years; the median (IQR) number of HCV RNA tests was 5 (3-9); and the median (IQR) test interval was 84 (38-140) days. HCV RNA assay lower limits are provided in Supplementary Table 3.

HCV reinfection

Twenty-eight participants had at least one reinfection (Figure 1D), and the incidence rate was 12.3 per 100 PY (95%CI: 8.5-17.8; calculated by including participants with persistent primary clearance and reinfection; Table 1 shows incidence rates stratified by study site). Fifteen reinfections were with a different viral genotype to primary infection, three were with a different viral subtype, and 10 were with the same genotype and subtype (Table 3).

Peak HCV RNA in reinfection compared to primary infection

Fifteen reinfections had quantitative HCV RNA measurements available within the first three months of both primary infection and reinfection. The peak HCV RNA was lower in reinfection (median: 3.4 log IU/mL, IQR: 2.6-6.5) than primary infection (median: 6.7 log IU/mL, IQR: 5.3-7.0, median difference: 1.46 log IU/mL, IQR: 0.34-4.18, p=0.011).

Quantitative HCV RNA timelines for three of the reinfection participants illustrating a range of trajectories are included in Supplementary Figure 1.

Reinfection outcomes

For 23 of the 28 reinfection cases, follow-up was sufficient to classify the outcome (at least two subsequent study visits). Of the nine reinfection participants without reclearance but with sufficient follow-up, the median estimated duration of reinfection at the end of follow-up was 66.9 months (range: 10.1-226.6). All participants had HCV genotype data on more than one time point following reinfection. Excluding those with changes in genotype or subtype (n=3), the Kaplan-Meier estimate for reclearance proportion at six months after reinfection was 52% (95%CI: 33-73%).

Time to reclearance

The median time to reclearance after reinfection was 3.0 months (IQR: 2.0-4.4). In the same participants, the median time to primary clearance was 5.5 months (IQR: 2.6-11.2).There was a tendency towards shorter time to reclearance than primary clearance this did not reach statistical significance (HR: 1.86, 95%CI: 0.70-4.91, p=0.211).

Predictors of HCV reclearance

In shared frailty (for cohort site) but otherwise unadjusted Cox proportional hazards analysis of participants with reinfection, female participants with *IFNL4* rs12979860 -CC genotype were fourfold more likely to reclear at any given time compared to other participants (HR: 4.16, 95%CI: 1.24-13.94, p=0.021, Table 4). There were no other statistically significant factors associated with reclearance.

Multiple HCV reinfection

Of the 14 participants with reclearance, five had further reinfections. Three participants had two reinfections, and two had three reinfections. Overall, 35 reinfections were observed (Figure 2).

HCV intercalation

Compared to reinfection, intercalation was more likely to occur after a shorter HCV RNA undetectable period ($p<0.001$, Table 5). Similarly, intercalation tended to be observed earlier following primary infection (timing of reappearance of viraemia after the estimated date of primary HCV infection in intercalation, $p=0.002$, Table 5). However, considerable variation was observed.

Indeterminate intermittent viraemia

Of the 59 cases of indeterminate intermittent viraemia, four were classified on the basis of sequencing of heterologous virus with subsequent detection of the original viral strain, and the remaining 55 were classified on the basis of insufficient viral sequencing. The duration of the HCV RNA undetectable period preceding reappearance of viraemia and the timing of reappearance of viraemia in the latter 55 cases were similar to that in intercalation (Table 5).

Sensitivity analyses

Results were not sensitive to any of the factors tested (Supplementary Tables 3-5).

DISCUSSION

This study characterizes the natural history of viral suppression, primary clearance, HCV reinfection and reclearance in the largest sample of participants (mostly people who inject drugs) with well-defined primary HCV infection and reinfection reported to date. Peak HCV RNA at the time of HCV reinfection was lower than in primary infection, providing further evidence of protective immunity in humans. Six months after reinfection, the clearance proportion was 52% (95%CI: 33-73%). The combined effect of female sex and rs12979860 -CC *IFNL4* genotype was predictive of reclearance following reinfection.

Reclearance was predicted by a combined effect of sex and *IFNL4* genotype. To the best of our knowledge, this is the first study to investigate predictors of reclearance. The propensity toward reclearance was four times greater among females with the rs12979860 -CC *IFNL4* genotype. This is particularly notable given that by definition all reinfection participants have already cleared one HCV infection and therefore would be expected to have greater tendencies toward spontaneous clearance *a priori*. In the context of primary clearance, similar findings have been reported with respect to female sex [1, 25] and *IFNL4* genotype [27, 28] predicting clearance independently and in combination [15, 26], including within the InC^3 study population [15]. The *IFNL4* gene region encodes the interferon-λ3 protein and is involved in viral control, although the precise mechanism remains unknown. It is possible that female sex influences HCV clearance through a mechanism related to general sex-based differences in immunity [37, 38]; however, the pathways by which these differences affect HCV control require elucidation. Further research is required to assess whether the combined effect of *IFNL4* genotype and sex on primary clearance and reclearance is simply the product of the two independent effects or there is a synergistic effect [15, 26]. The importance of

gender and *IFNL4* genotype in both primary HCV infection and reinfection suggests that these factors have a crucial role in long-term protection from persistent HCV infection. Although *IFNL4* genotype and sex are fixed genetic traits, the fact that spontaneously clearing infections are controlled better with subsequent exposures suggests the existence of an adaptive component. A better understanding of the mechanisms behind the immune response in females with the *IFNL4* rs12979860 -CC genotype has the potential to provide insights for vaccine development.

The findings presented here suggest partial protective immunity following primary clearance of HCV. Peak HCV RNA level was lower in reinfection than primary infection. Reclearance following reinfection was observed in half of the participants, with the time to reclearance tending to be shorter following reinfection than primary clearance. These findings are consistent with previous findings by Osburn and colleagues, and are not sensitive to stratification or adjustment by study site (BBAASH vs. others) [6]. Mathematical modeling studies have shown that the test interval influences the proportions of reinfections resulting in reclearance and persistence [13]. In this study, heterogeneity in test intervals between study sites limited the interpretation of these proportions. Nonetheless, the identification of persistent reinfections indicates that while primary HCV infection appears to confer protection against persistent HCV reinfection in some cases, there are limits to this protection. Chimpanzee studies indicate inadequate cross-strain protection in some cases [31]; however, this study did not find a higher probability of reclearing in participants reinfected with the same genotype as their primary infection. Further studies to characterize the viral genomes of the primary and reinfection strains, and to resolve the detailed characteristics of the immune responses against these viruses, including both neutralizing antibodies and HCV-specific T cells, are warranted.

The identification of diverse outcomes of HCV reinfection illustrates the complexity of HCV natural history. Among the 23 participants with follow-up after HCV reinfection, approximately one third experienced persistent reinfection and one third experienced multiple reinfections after resolution of their first reinfection. The remaining third was composed of participants with reclearance but without further reinfections (possibly partly due to shorter follow-up), and participants with changes in viral genotype or subtype following reinfection. This report adds to the few cases of multiple consecutive reinfection that have been reported previously [5, 6, 11], highlighting the ongoing risk of reinfection among those who continue to be exposed to HCV and emphasizing the need for education about reinfection risk in these groups and delivery of HCV antiviral treatment to those who become reinfected.

In contrast to reinfection, intercalation was usually observed within the first two years of primary HCV infection, consistent with previous reports of fluctuations in HCV RNA in early HCV infection [39, 40]. However, intercalation cases were also observed later in HCV infection, and after lengthier HCV RNA undetectable periods, as has previously been reported [6, 12, 21, 41]. This highlights the importance of viral sequencing for classification of HCV reinfection. Intercalation may occur as a result of transient control of viral replication by the host immune response, but further research is required to develop more detailed understanding of such events.

Limitations

Despite bringing together the largest number of well-defined HCV reinfection events following spontaneous clearance or viral suppression reported to date, the number of events is low for detailed statistical analysis. Therefore, our analysis of predictors of HCV reclearance

could not be adjusted for the effect of potential confounders. Participants with identified reinfections were typically followed longer and more frequently than other participants, and a large proportion of reoccurring viraemia events could neither be classified as reinfections nor intercalations. These factors suggest that the true reinfection rate in the InC[3] population is likely to be higher than the observed rate. While standard methods were used to classify outcomes of infection, there were differences between cohort sites in terms of methods of recruitment, test intervals, HCV RNA monitoring methods, and the region of HCV sequenced to assess reinfection. In some of the participating cohorts, data on HCV-related risk behaviors were not collected; therefore, risk behaviors could not be assessed as predictors of HCV reinfection or reclearance. The analysis of time to HCV reclearance vs. primary clearance only included participants with reclearance during the study period. While the participants with persistent reinfection were all followed for at least ten months, indicating that future reclearance would be unlikely, late clearance can occur so there is a small risk of bias from excluding right-censored data (Supplementary Information 1) [41].

Conclusions

This is the first study to investigate predictors of HCV reclearance. Similar to primary clearance, there appears to be a combined effect of gender and *IFNL4* genotype on reclearance of HCV, suggesting that these factors together have considerable impact on long-term protection from persistent HCV infection.. This study also highlights the complexity of acute HCV infection and reinfection and the factors that contribute to viral clearance. These findings suggest that HCV reinfection is associated with lower levels of viraemia and a possible shorter time-course to spontaneous reclearance, supporting a role for immunologic memory in conferring partial protection against persistent infection. Nonetheless, there is

considerable heterogeneity in reinfection outcomes, and participants with ongoing exposure to HCV risk developing persistent reinfection.

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TABLES

Table 1: Incidence rate of reinfection by cohort site

	n[a]	Reinfections	Test interval (months)	Test interval acute primary HCV infection (months)[b]	PY	Rate per 100 PY (95% CI)
Total	128	28			227.8	12.3 (8.5-17.8)
Cohort site						
ACS (The Netherlands)	13	4	6	6	67.0	6.0 (2.2-15.9)
ATAHC (Australia)	25	2	-[c]	3	31.0	6.5 (1.6-25.8)
BAHSTION (United States)	12	1	-[d]	1[e]	11.3	8.9 (1.2-62.9)
BBAASH (United States)	27	15	1	1	41.2	36.4 (21.9-60.4)
HEPCO (Canada)	7	0	6	1[f]	19.7	0.0 (-)
HITS-c (Australia)	3	0	6	0.5-1[g]	2.7	0.0 (-)
HITS-p (Australia)	18	2	6	0.5-1[g]	23.5	8.5 (2.1-34.1)
N2 (Australia)	4	2	3	3	7.4	27.1 (6.8-108.5)
UFO (United States)	19	2	3	1	24.0	8.3 (2.1-33.3)

[a]Includes participants with persistent spontaneous clearance and reinfection. Participants with intercalation are excluded because they are assumed not to have spontaneously cleared infection. Participants with indeterminate reoccuring viraemia are excluded because the reinfection outcome is unknown. [b]Many cohort sites have a more frequent test interval after identification of acute primary HCV infection. [c]Eligibility is on the basis of recent HCV seroconversion or confirmed acute HCV infection. [d]Eligibility of seronegative or acute HCV participants is on the basis of recent exposure to HCV or suspected or acute HCV infection. [e]For six months, after which test interval is reduced. [f]For 24 weeks, after which normal test schedule resumes. [g]For three months among participants with early acute infection (i.e., HCV RNA is still present), after which normal test schedule resumes. UFO, UFO STUDY; ATAHC, Australian Trial in Acute Hepatitis C; BAHSTION, Boston Acute HCV Study: Transmission, Immunity and Outcomes Network; BBAASH, Baltimore Before and After Acute Study of Hepatitis; HEPCO, St. Luc Cohort, HEPCO; HITS-c, Hepatitis C Incidence and Transmission Study-Community; HITS-p, Hepatitis C Incidence and Transmission Study-Prison; N2, Networks 2; ACS, Amsterdam Cohort Studies.

Table 2: Characteristics, Exposures, and Risk Behaviors of 591 Participants with Acute Primary Hepatitis C Virus Infection[a]

Characteristic at Time of Incident Primary HCV Infection	Persistent HCV No. (%)[b]	Cleared or Intermittent HCV Infection, No. (%)				
		Total[b]	Reinfection[c]	Intercalation[c]	Indeterminate Intermittent Viraemia[c]	Persistent Cleared[c]
Overall	373	218	28 (13%)	31 (14%)	59 (27%)	100 (46%)
Site						
ACS (The Netherlands)	18 (5%)	24 (11%)	4 (17%)	1 (4%)	10 (42%)	9 (38%)
ATAHC (Australia)	84 (23%)	29 (13%)	2 (7%)	1 (3%)	3 (10%)	23 (79%)
BAHSTION (United States)	21 (6%)	21 (10%)	1 (5%)	3 (14%)	6 (29%)	11 (52%)
BBAASH (United States)	52 (14%)	59 (27%)	15 (25%)	20 (34%)	12 (20%)	12 (20%)
HEPCO (Canada)	48 (13%)	18 (8%)	0 (0%)	0 (0%)	11 (61%)	7 (39%)
HITS-c (Australia)	6 (2%)	3 (1%)	0 (0%)	0 (0%)	0 (0%)	3 (100%)
HITS-p (Australia)	63 (17%)	20 (9%)	2 (10%)	1 (5%)	1 (5%)	16 (80%)
N2 (Australia)	10 (3%)	7 (3%)	2 (29%)	3 (43%)	0 (0%)	2 (29%)
UFO (United States)	71 (19%)	37 (17%)	2 (5%)	2 (5%)	16 (43%)	17 (46%)
Median age, yrs (IQR)[d]	27 (23-34)	26 (22-30)	24 (20-30)	25 (24-28)	26 (21-30)	26 (23-32)
Sex						
Male	259 (69%)[e]	118 (54%)	14 (12%)[e]	16 (14%)[e]	38 (32%)	50 (42%)[e]
Female	113 (30%)	100 (46%)	14 (14%)	15 (15%)	21 (21%)	50 (50%)
Missing	1 (0%)	0 (0%)	0 (-)	0 (-)	0 (-)	0 (-)
Ethnicity/Race						
European origin	305 (82%)	177 (81%)	26 (15%)	25 (14%)	48 (27%)	78 (44%)
Other	41 (11%)	23 (11%)	2 (9%)	4 (17%)	6 (26%)	11 (48%)
Missing	27 (7%)	18 (8%)	0 (0%)	2 (11%)	5 (28%)	11 (61%)
History of injecting drug use	358 (96%)	210 (96%)	28 (13%)	30 (14%)	59 (28%)	93 (44%)
HIV infection[d]						
No	328 (88%)	199 (91%)	25 (13%)	28 (14%)	55 (28%)	91 (46%)
Yes	30 (8%)	12 (6%)	3 (25%)	1 (8%)	2 (17%)	6 (50%)
Missing	15 (4%)	7 (3%)	0 (0%)	2 (29%)	2 (29%)	3 (43%)
Median (IQR) peak log HCV RNA (log(IU/mL))[f]	5.5 (4.5-6.4)	5.8 (4.0-7.0)	6.7 (5.3-7.0)	5.7 (5.1-6.8)	5.3 (2.9-6.5)	5.9 (3.2-7.0)
HCV genotype[d]						
Genotype 1	174 (47%)[g]	114 (52%)	18 (16%)	24 (21%)[g]	27 (24%)	45 (39%)
Genotype 2	22 (6%)	10 (5%)	1 (10%)	2 (20%)	2 (20%)	5 (50%)
Genotype 3	130 (35%)	46 (21%)	8 (17%)	5 (11%)	9 (20%)	24 (52%)
Genotype 4	2 (1%)	4 (2%)	1 (25%)	0 (0%)	3 (75%)	0 (0%)
Genotype 6	4 (1%)	0 (0%)	0 (-)	0 (-)	0 (-)	0 (-)
Mixed genotype	10 (3%)	2 (1%)	0 (0%)	0 (0%)	0 (0%)	2 (100%)
Unknown genotype	31 (8%)	42 (19%)	0 (0%)	0 (0%)	18 (43%)	24 (57%)
Recent injecting[h]						
No	57 (15%)[i]	19 (9%)	2 (11%)	2 (11%)	1 (5%)[i]	14 (74%)[i]
Yes	238 (64%)	113 (52%)	9 (8%)	5 (4%)	40 (35%)	59 (52%)
Missing	5 (1%)	6 (3%)	1 (17%)	1 (17%)	0 (0%)	4 (67%)
Not collected at cohort site	73 (20%)	80 (37%)	16 (20%)	23 (29%)	18 (23%)	23 (29%)
Recent injecting						

frequency[h]						
No recent injecting	41 (11%)	16 (7%)	1 (6%)	2 (13%)	1 (6%)	12 (75%)
Daily or more	127 (34%)	48 (22%)	7 (15%)	3 (6%)	13 (27%)	25 (52%)
Less than daily but at least weekly	72 (19%)	48 (22%)	3 (6%)	1 (2%)	19 (40%)	25 (52%)
Less than weekly	55 (15%)	19 (9%)	0 (0%)	0 (0%)	8 (42%)	11 (58%)
Missing	5 (1%)	7 (3%)	1 (14%)	2 (29%)	0 (0%)	4 (57%)
Not collected at cohort site	73 (20%)	80 (37%)	16 (20%)	23 (29%)	18 (23%)	23 (29%)
Primary drug injected recently[h]						
Heroin/other opioids[j]	122 (33%)	72 (33%)	5 (7%)	3 (4%)	26 (36%)	38 (53%)
Psychostimulants[j]	88 (24%)	34 (16%)	3 (9%)	1 (3%)	14 (41%)	16 (47%)
Other	6 (2%)	0 (0%)	0 (-)	0 (-)	0 (-)	0 (-)
Missing	84 (23%)	32 (15%)	4 (13%)	4 (13%)	1 (3%)	23 (72%)
Not collected at cohort site	73 (20%)	80 (37%)	16 (20%)	23 (29%)	18 (23%)	23 (29%)
Recent receptive needle sharing[h]						
No	162 (43%)	77 (35%)	7 (9%)	1 (1%)	26 (34%)	43 (56%)
Yes	75 (20%)	30 (14%)	3 (10%)	3 (10%)	11 (37%)	13 (43%)
Missing	63 (17%)	31 (14%)	2 (6%)	4 (13%)	4 (13%)	21 (68%)
Not collected at cohort site	73 (20%)	80 (37%)	16 (20%)	23 (29%)	18 (23%)	23 (29%)

[a]Participants who did not have intermittent viraemia or spontaneous clearance but were HCV RNA undetectable at their final HCV RNA test (that is, viral suppression at last test, n=34) and participants treated within 26 weeks of infection (n=37) were excluded from this table, [b]Percentages indicate column percentages, [c]Percentages indicate row percentages, [d]At the time of primary HCV infection, [e]Statistically significant differences in the sex distribution between the group with persistent infection and the groups with persistent spontaneous clearance, reinfection, and intercalation, [f]In the first three months of primary HCV infection. [g]Statistically significant difference in the genotype distribution (genotype 1 vs other genotypes) between the group with persistent infection and the group with intercalation. [h]Reported at the interview prior to primary HCV infection diagnosis, recent indicates last 1-6 months prior to interview. [i]Statistically significant differences in the recent injecting distribution between the group with indeterminate intermittent viraemia and the groups with persistent infection and persistent spontaneous clearance. [j]Heroin/other opioids includes heroin, other opioids, and speedball; psychostimulants includes amphetamines (including methamphetamines) and cocaine. UFO, UFO STUDY; ATAHC, Australian Trial in Acute Hepatitis C; BAHSTION, Boston Acute HCV Study: Transmission, Immunity and Outcomes Network; BBAASH, Baltimore Before and After Acute Study of Hepatitis; HEPCO, St. Luc Cohort, HEPCO; HITS-c, Hepatitis C Incidence and Transmission Study-Community; HITS-p, Hepatitis C Incidence and Transmission Study-Prison; N2, Networks 2; ACS, Amsterdam Cohort Studies.

Table 3: Viral genotype at primary HCV infection and reinfection in the 28 participants with reinfection

Characteristic at Time of HCV Infection	Primary HCV infection (n=28)	HCV reinfection (n=28)
HCV genotype		
1	18 (64%)	17 (61%)
2	1 (4%)	4 (14%)
3	8 (29%)	6 (21%)
4	1 (4%)	1 (4%)
HCV genotype change between primary infection and reinfection		
Different genotype	-	15 (54%)
Different subtype		3 (11%)
Same genotype	-	10 (36%)

Table 4: Cox proportional hazards regression analysis of predictors of reclearance of HCV adjusted for cohort site using a shared frailty model

	Reclearances n	Reclearance rate (/100 py)	HR (95% CI)	p-value	Shared frailty for cohort site theta(p-value)
Overall	14	56.6			
Age at reinfection					
≤25	7	45.1	1.00		
>25	5	60.9	1.72(0.45-6.53)	0.426	0.72(0.119)
Gender[a]					
Male	5	36.0	1.00		
Female	9	82.9	2.45(0.72-8.29)	0.150	0.74(0.125)
IFNL4 rs12979860					
CC	10	69.8	2.00(0.56-7.18)	0.285	1.18(0.053)
CT/TT	4	38.3	1.00		
Combined effect of gender and IFNL4[b]					
Female and CC	7	126.9	**4.16(1.24-13.94)**	**0.021**	**1.08(0.063)**
Other	7	36.4	1.00		
Reinfection HCV genotype					
Genotype 1	9	53.2	1.00		
Other	5	63.9	2.79(0.62-12.51)	0.181	1.26(0.041)
Reinfected with a different genotype to primary infection					
Yes	6	53.1	4.69(0.90-24.36)	0.066	2.22(0.017)
No	8	59.5	1.00		

[a]Missing data for two participants with reclearance. No missing data for participants without reclearance.

[b]Test of proportional hazards p-value: 0.990

Table 5: Time from the estimated date of primary infection until the reappearance of viraemia (days), and duration of preceding HCV RNA undetectable period (number of tests and days)

	n	Median	IQR	Range
Timing of reappearance of viraemia[a]				
- Intercalation	31	250	174-433	113-2219
- Reinfection	28	546	312-984	93-3747
- Indeterminate cases without sufficient viral sequencing	55	231	140-399	46-3167
- Indeterminate cases with viral sequencing[b]	4	173	168-645	166-1114
Number of HCV RNA undetectable tests[c]				
- Intercalation	31	1	1-2	1-6
- Reinfection	28	3	2-7	1-15
- Indeterminate cases without sufficient viral sequencing	55	1	1-2	1-18
- Indeterminate cases with viral sequencing[b]	4	1	1-6	1-10
Duration of HCV undetectable period[d]				
- Intercalation	7	88	32-221	31-1293
- Reinfection	21	210	131-412	28-1031
- Indeterminate cases without sufficient viral sequencing	22	119	63-282	28-2707
- Indeterminate cases with viral sequencing[b]	1	603	-	-

[a]Statistically significant differences between the reinfection group and the intercalation group (p=0.002), and the reinfection group and indeterminate group without sufficient viral sequencing (p<0.001)
[a]Classified as indeterminate on the basis of heterologous virus with subsequent detection of the original viral strain
[b]Preceding reappearance of viraemia; statistically significant differences between the reinfection group and the intercalation group (p=0.001), and the reinfection group and indeterminate group without sufficient viral sequencing (p<0.001)
[c]Among cases with at least two HCV RNA undetectable tests, at least 28 days apart, calculated as the period from the first undetectable test until the last undetectable test

FIGURE LEGENDS

Figure 1:Timing and classification of HCV primary infection, viral suppression, spontaneous clearance and reinfection

Panel A. Flowchart for determining the estimated date of primary HCV infection.

Panel B. Estimated dates of and times to primary clearance and viral suppression

Figure notes for panel B: All timelines represent participants with confirmed primary HCV infection followed by either viral suppression or primary clearance. The timelines begin at the estimated date of primary infection. After primary infection, HCV RNA test results are depicted on the timeline by squares. Black squares represent HCV RNA detectable tests and white squares represent HCV undetectable tests. Primary clearance is distinguished from viral suppression by the number of HCV RNA undetectable tests (white squares). (i) and (ii) depict primary clearance (as indicated by the two consecutive HCV RNA undetectable tests) and (iii) and (iv) depict viral suppression (one HCV RNA undetectable test). In timelines (ii) and (iv) HCV RNA was undetectable at the time of primary infection detection, in which case the first squares (HCV RNA tests) in the timeline are white (undetectable). The estimated date of primary clearance is illustrated using a white triangle, and the estimated date of viral suppression is illustrated using a grey triangle. These dates are both estimated as follows: if HCV RNA is detectable at detection of primary infection (cases i and iii), the estimated date of primary clearance or viral suppression is the midpoint between the HCV RNA detectable test prior to primary clearance or viral suppression and the first HCV RNA undetectable tests. If HCV RNA is undetectable at detection of primary infection (cases ii and iv), the estimated date of primary clearance or viral suppression is the midpoint between the estimated date of primary infection (the beginning of the illustrated timeline) and the first HCV RNA undetectable test. In all four cases, the time to primary clearance or viral suppression is the

time from the estimated date of infection until the estimated date of primary clearance or viral suppression.

Panel C. Estimated dates of and times to reinfection

Figure notes for panel C: Both timelines represent participants with confirmed primary HCV infection followed by either viral suppression or primary clearance and confirmed reinfection. The timelines begin at the estimated date of primary clearance or viral suppression. HCV RNA test results are depicted on the timeline by squares. Black squares represent HCV RNA detectable tests and white squares represent HCV undetectable tests. Primary clearance is distinguished from viral suppression by the number of HCV RNA undetectable tests (white squares). (i) depicts primary clearance (as indicated by the two consecutive HCV RNA undetectable tests) and (ii) depicts viral suppression (one HCV RNA undetectable test). The estimated date of reinfection is the midpoint between the last HCV RNA undetectable test and the first HCV RNA detectable test. The time to reinfection is the time from the estimated date of primary clearance or viral suppression until the estimated date of reinfection.

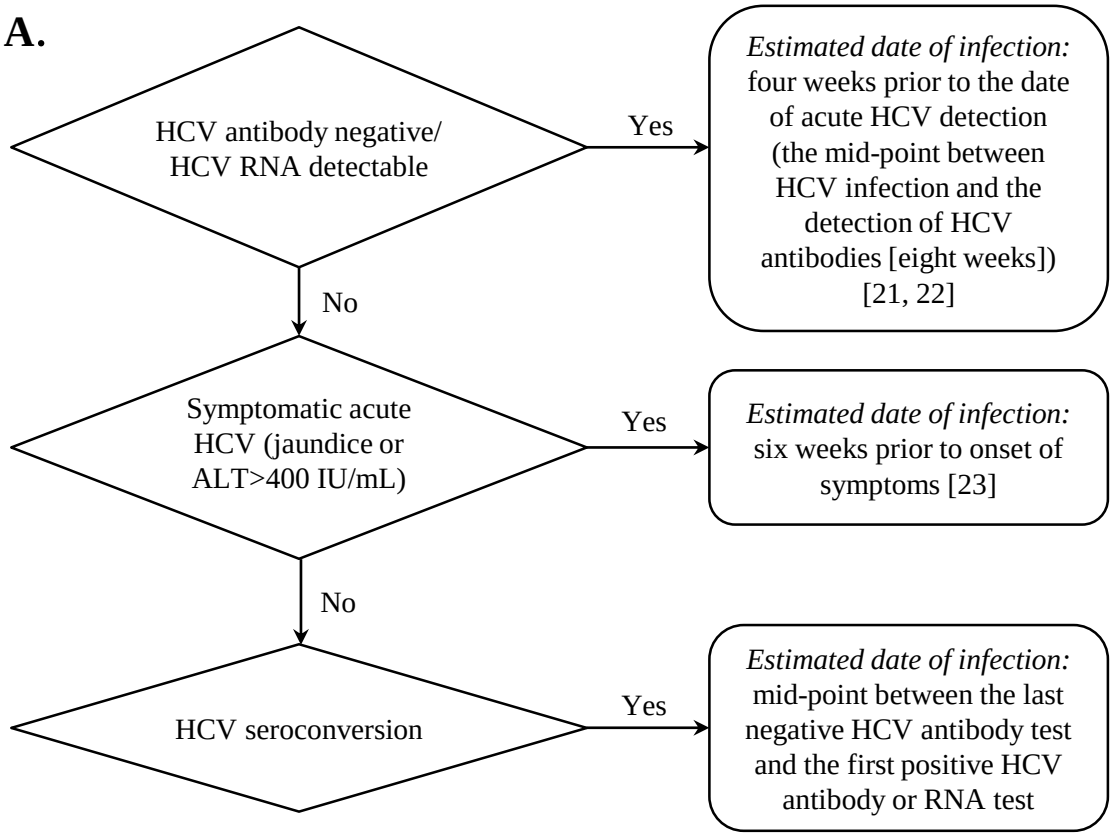
Panel D: Flowchart of reinfection classification

Figure 2: Timeline of reinfection events from the estimated date of primary HCV infection.

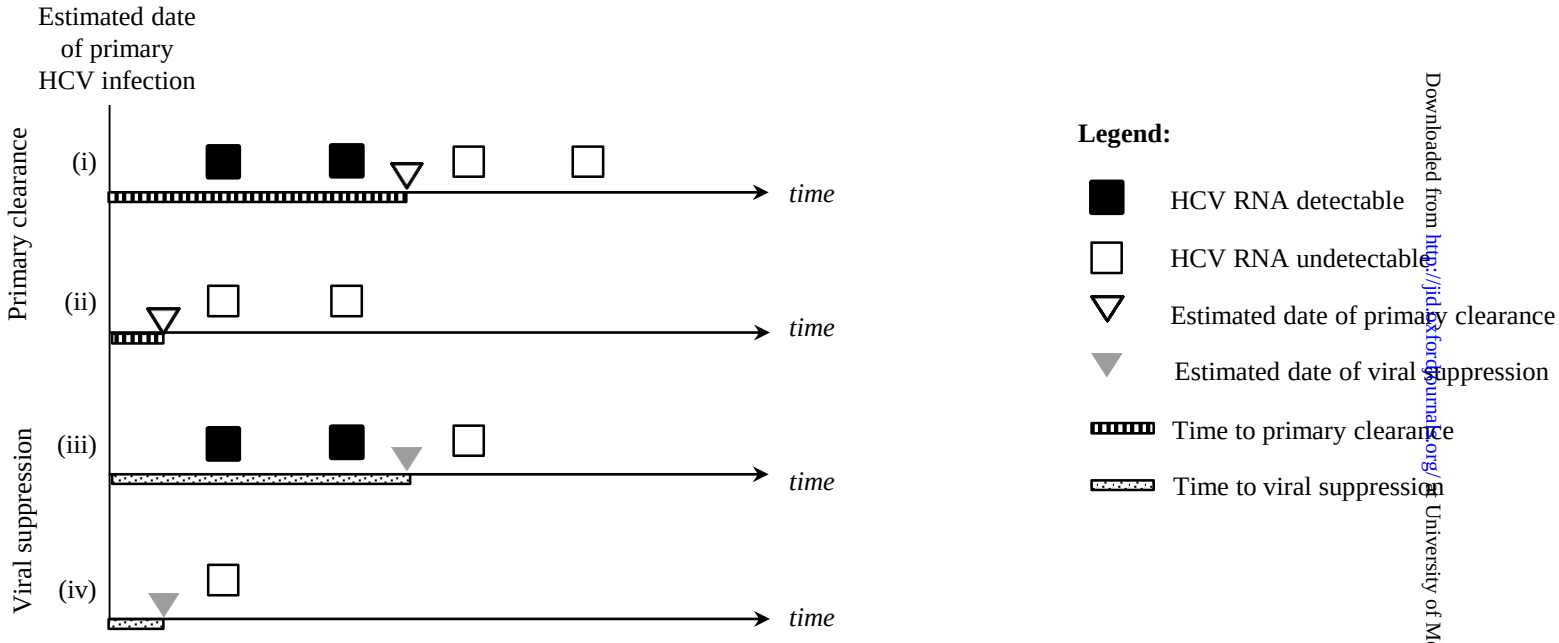
Figure notes: Timelines illustrate HCV RNA and HCV genotyping results in the 28 participants with reinfection. Each box represents one HCV RNA test. Empty boxes are tests where HCV RNA was undetectable whereas filled boxes (black or coloured) indicate that HCV RNA was detectable. HCV genotype results are indicated using colour and box labels. Distinct strains within a single viral genotype and subtype that were confirmed by viral sequencing are illustrated using different shades of the same colour. R=Reinfection event. Reinfection events are defined by the appearance of a new viral genotype, subtype or a

distinct strain confirmed by viral sequencing following a period of undetectable HCV RNA. The primary infections of participants UFOVT0134 and AUS206 were serotyped.

A.

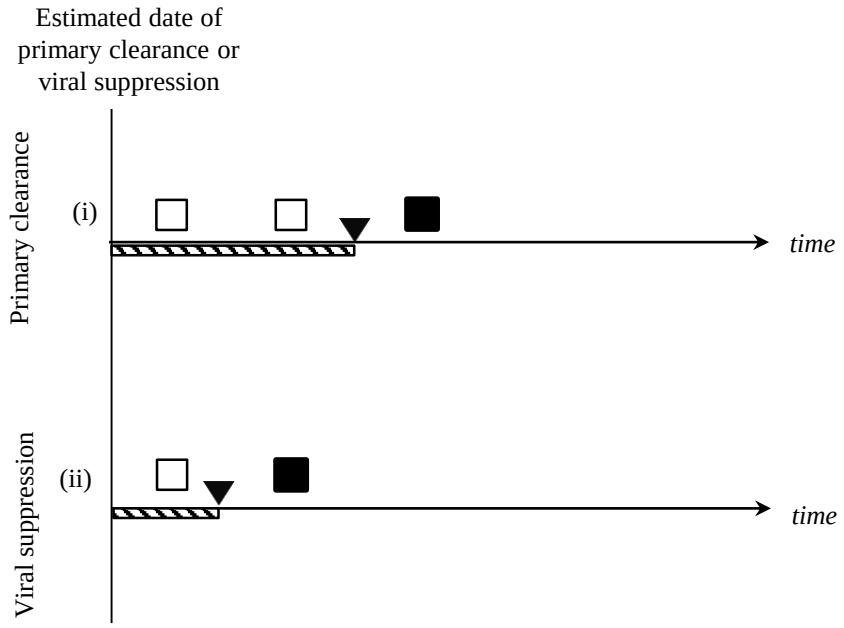


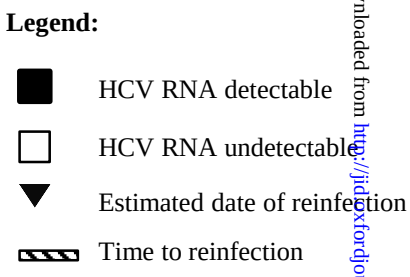
B.



Notes: All timelines represent participants with confirmed primary HCV infection followed by either viral suppression or primary clearance. The timelines begin at the estimated date of primary infection. After primary infection, HCV RNA test results are depicted on the timeline by squares. Black squares represent HCV RNA detectable tests and white squares represent HCV undetectable tests. Primary clearance is distinguished from viral suppression by the number of HCV RNA undetectable tests (white squares). (i) and (ii) depict primary clearance (as indicated by the two consecutive HCV RNA undetectable tests) and (iii) and (iv) depict viral suppression (one HCV RNA undetectable test). In timelines (ii) and (iv) HCV RNA was undetectable at the time of primary infection detection, in which case the first squares (HCV RNA tests) in the timeline are white (undetectable). The estimated date of primary clearance is illustrated using a white triangle, and the estimated date of viral suppression is illustrated using a grey triangle. These dates are both estimated as follows: if HCV RNA is detectable at detection of primary infection (cases i and iii), the estimated date of primary clearance or viral suppression is the midpoint between the HCV RNA detectable test prior to primary clearance or viral suppression and the first HCV RNA undetectable tests. If HCV RNA is undetectable at detection of primary infection (cases ii and iv), the estimated date of primary clearance or viral suppression is the midpoint between the estimated date of primary infection (the beginning of the illustrated timeline) and the first HCV RNA undetectable test. In all four cases, the time to primary clearance or viral suppression is the time from the estimated date of infection until the estimated date of primary clearance or viral suppression.

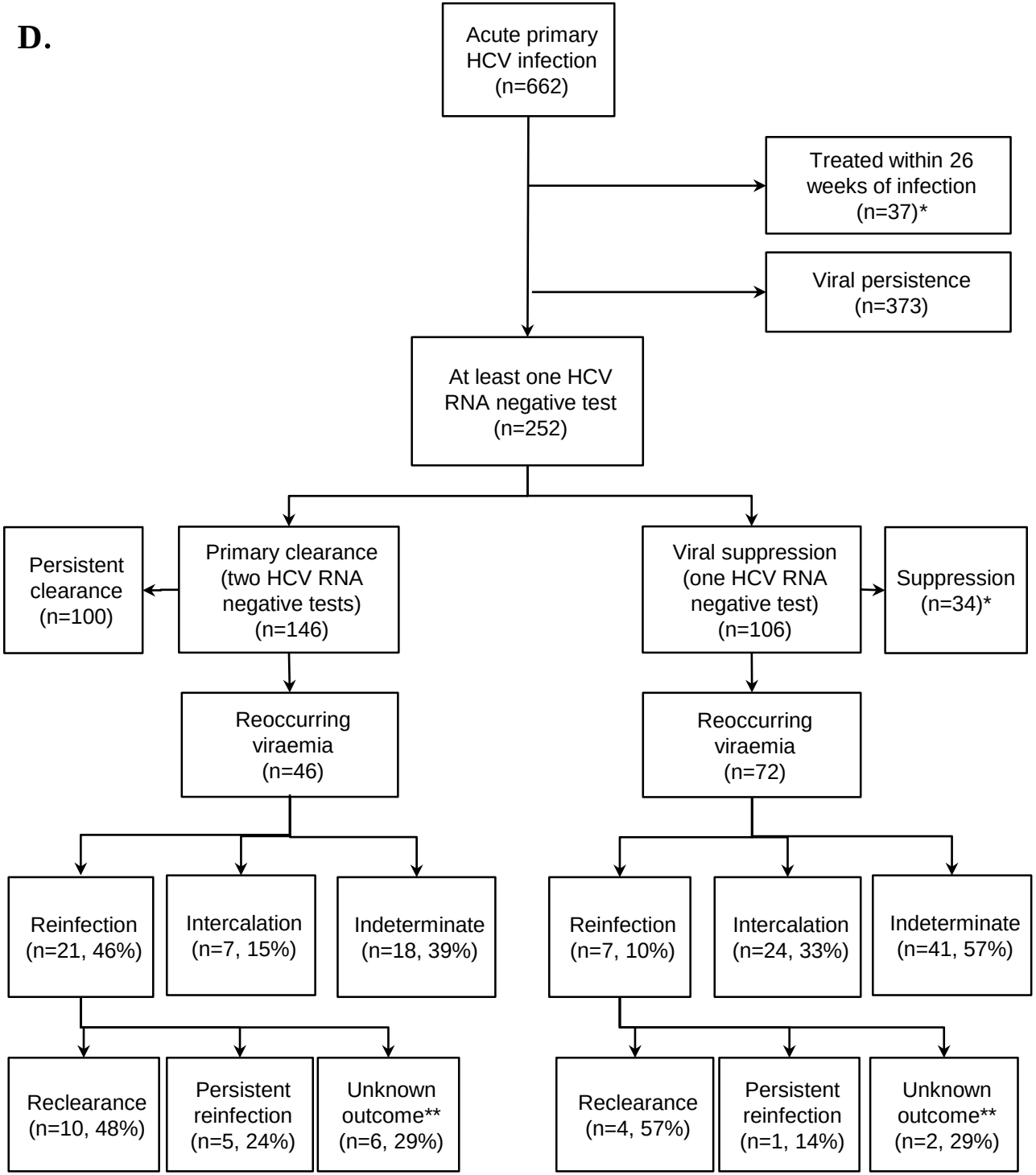
C.





Notes: Both timelines represent participants with confirmed primary HCV infection followed by either viral suppression or primary clearance and confirmed reinfection. The timelines begin at the estimated date of primary clearance or viral suppression. HCV RNA test results are depicted on the timeline by squares. Black squares represent HCV RNA detectable tests and white squares represent HCV undetectable tests. Primary clearance is distinguished from viral suppression by the number of HCV RNA undetectable tests (white squares). (i) depicts primary clearance (as indicated by the two consecutive HCV RNA undetectable tests) and (ii) depicts viral suppression (one HCV RNA undetectable test). The estimated date of reinfection is the midpoint between the last HCV RNA undetectable test and the first HCV RNA detectable test. The time to reinfection is the time from the estimated date of primary clearance or viral suppression until the estimated date of reinfection.

D.



*Primary HCV infection outcome unknown
**Reinfection outcome unknown: includes five cases with insufficient follow-up to determine outcome and three cases with change in genotype after reinfection