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Filename	Chapter 3.3: Frequent HCV reinfection and superinfection among injecting drug users in Amsterdam argues against HCV protective immunity

SOURCE (OR PART OF THE FOLLOWING SOURCE):

Type	Dissertation
Title	Molecular epidemiology of hepatitis C virus
Author	T.J.W. van de Laar
Faculty	Faculty of Medicine
Year	2008
Pages	176
ISBN	9789071382611

FULL BIBLIOGRAPHIC DETAILS:
http://dare.uva.nl/record/288571

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CHAPTER 3.3

Frequent HCV reinfection and superinfection among injecting drug users in Amsterdam argues against HCV protective immunity

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Submitted for publication.

Abstract

Background/aims: To expand limited data on HCV protective immunity in humans, this study investigates the occurrence of HCV reinfection and superinfection among HCV seroconverters participating in the Amsterdam Cohort Studies among drug users from 1985 through 2005.

Methods: HCV seroconverters (n=59) were tested for HCV RNA at 5 different time points: the last visit before seroconversion (t=-1), the first visit after seroconversion (t=1), six months after (t=2), one year after (t=3), and the last visit predating November 2005 (t=4). If HCV RNA was present, part of the NS5B region was amplified and sequenced. Additional phylogenetic analysis and cloning was performed to establish HCV reinfection and superinfection.

Results: Multiple HCV infections were detected in 24/59 (41%) seroconverters, of whom 7 had HCV reinfections, 14 were superinfected, and 3 had combinations of reinfection, superinfection and/or coinfection. In total, we identified 94 HCV infections, varying from 1 to 4 infections per seroconverter. Multiple HCV infections were observed in 10/25 seroconverters with spontaneous HCV clearance (11 reinfections, 3 superinfections) and in 14/34 seroconverters without viral clearance (20 superinfections, 1 coinfection).

Conclusions: HCV reinfection and superinfection are common among actively injecting drug users, this finding argues against HCV protective immunity and will complicate future vaccine development.

Introduction

Injecting drug users (DU) are at high risk for hepatitis C virus (HCV) infection through the shared use of needles and injection equipment. The reported HCV seroprevalence among injecting DU ranges from 30 to 90% in Europe, North-America, and Australia [1-3]. Over decades, persistent HCV viremia can cause chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [4]. Spontaneous resolution of the virus occurs in 15% to 40% of those infected [5]. However, both HCV reinfection and HCV superinfection have been documented among individuals with ongoing risk behaviour [6-8], suggesting that neither viral clearance nor ongoing HCV infection consistently protect against new HCV infection. Evidence for partial protective immunity against HCV infection is derived largely from chimpanzee studies. Chimpanzees that previously cleared HCV and were rechallenged with homologous HCV strains, generally showed lower levels of HCV viremia and self-limited infection [9-11]. Whether humans previously infected with HCV gain protection against a second HCV infection is unclear [6,12-14]. Partial HCV protective immunity in humans has been suggested [12,13], but limitations in study design cause us to interpret these findings with caution.

In Amsterdam, low levels of injection and a combined harm reduction approach of adequate methadone therapy and full participation in needle exchange programs have substantially reduced HCV incidence among injecting DU [15,16]. Despite this decline in HCV incidences and improved success rates of HCV treatment, injecting DU still have limited access to HCV antiviral treatment [17]. Effectiveness of HCV treatment in this population is often questioned, as poor treatment adherence and potential HCV superinfection might negatively affect HCV treatment outcome. Moreover, successfully treated DU remain at risk for HCV reinfection when they continue risk behaviour [18].

Insight into HCV protective immunity after previous exposure contributes to vaccine development and also provides health guidance for preventive strategies in populations at risk. As HCV reinfection and superinfection implies that no protective immunity was obtained as a result of previous HCV infection, we studied the occurrence of HCV reinfection and superinfection in a well-defined cohort of injecting DU from the moment of HCV seroconversion until the end of follow-up. This study uniquely combines longitudinal data from prospectively identified HCV seroconverters with an epidemiologic and phylogenetic approach.

Materials and methods

Study population and sample selection

This study includes all participants that have seroconverted for HCV during follow-up in the Amsterdam Cohort Studies (ACS) among DU in the past 20 years. The ACS is an open, prospective cohort study of both injecting and non-injecting DU, initiated in December 1985 [19]. Recruitment is still ongoing and takes place via local methadone posts, sexually transmitted diseases clinics, and by word of mouth. At ACS visits every 4-6 months, participants complete a standardised questionnaire about personal health, risk behaviour and socio-economic situation; blood is drawn for HIV-testing and storage at -80°C. For our study, we determined the HCV status of all 1276 ACS participants who had at least two visits between December 1985 and November 2005, using a third generation commercial microparticle EIA system (AxSym HCV version 3.0; Abbott. We screened the first available serum sample for HCV antibodies, and if negative the last available sample. On finding HCV seroconversion, samples taken at intervening visits were tested to determine the interval of seroconversion [16]. The moment of seroconversion was calculated as the midpoint between the last HCV-seronegative visit and the first HCV-seropositive visit.
For each HCV seroconverter, samples taken at five time points were selected, if available: the last visit before HCV antibody seroconversion (t=-1), the first visit with a positive HCV antibody test (t=1), visit approximately six months later (t=2) and one year later (t=3), and the last ACS visit predating November 2005 (t=4). A subset of 19 HCV seroconverters from the ACS identified in an earlier study had more than 5 samples available [20].

Definitions: reinfection, coinfection and superinfection

Spontaneous viral clearance was assumed if two or more consecutive visits, at least 4 months apart, showed HCV antibodies without detectable HCV RNA. During our study period, none of the participating DU had received HCV antiviral treatment.
HCV reinfection refers to a situation in which a primary HCV infection is spontaneously cleared (i.e. two consecutive PCR negative visits, as above) prior to subsequent infection with an HCV strain of an homologous or heterologous lineage. In contrast, *HCV coinfection* and *HCV superinfection* both refer to HCV dual infections.
Coinfection is defined as infection with two or more heterologous HCV strains simultaneously or within a window period too narrow for the first HCV infection to have resulted in detectable HCV antibodies.
Superinfection is defined as a subsequent infection with a heterologous HCV strain in the presence of a previous HCV strain and the antibodies that it has generated. All changes in HCV strains over time without a demonstrated spontaneous clearance in between, were considered as superinfections [18].

HCV RNA-quantification

HCV serum RNA was quantified by an in-house realtime PCR assay based on the 5'-UTR region of the HCV genome [20]. In brief, RNA extraction was performed using the Boom method [21], in which 200 µl of serum and 15 µl of internal control were added to 900 µl of lysis buffer and 20 µl of size-fractioned coarse silica particles. RNA was eluted in a volume of 100 µl and transcribed to cDNA as detailed elsewhere [22]. Realtime PCR mixes (25 µl total volume) contained 12.5 µl of 2x LC480 probes master, 0.6 µM of forward and reverse primers (HCV47F: 5'-GTGAGGAACTACTGTCTTCACG-3', HCV312R: 5'-ACTCGCAAGCA CCCTATCAGG-3'), and 0.2 µM of labelled HCV and IC taqman probes (HCV-P129: 5'-FAM-CTCCCGGGAGAGCCATAGTGGTCTGCG-MGB-NFQ-3', HCV-IC: 5'-VIC-ATGGCCACAGC GCCGCGGTGTTAGTGC-MGB-NFQ-3'). Realtime PCR was performed on a Roche LC480 using the following cycling conditions: 2 min. at 50°C and 10 min. at 95°C followed by 50 cycles of: 20 sec. at 95°C, 20 sec. at 55°C, and 1 min. at 72°C. Quantification of viral RNA was performed by using standard curves which were produced by linear regression analysis of dilution series of plasmid DNA.

Dominant HCV variant detection: The NS5B PCR

HCV RNA-positive samples for each DU were selected to document changes in the dominant HCV viral variant in individuals over time. From each sample, 3 µl cDNA was used as input for a nested multiplex PCR which amplifies 449 nucleotides of the HCV NS5B region (nt 8546-8994) [23]. Except for the modifications made to use cDNA instead of RNA as PCR input, conditions and primers of the NS5B PCR were those we described earlier [24].

Sequencing and phylogenetic analysis

Sequencing reactions were performed as described earlier [24]. First, NS5B PCR products were ethanol-precipitated. Sense and antisense strands were separately cycle sequenced using the BigDye Terminator system (version 1.1; Perkin Elmer). They were subsequently purified using DyExe spin kits (Qiagen) and analyzed on an ABI-310 genetic analyzer (Applied Biosystems). Sequence alignment of the NS5B fragments was performed using the BioEdit software package [25]. Viral genotype was confirmed after phylogenetic analysis of the NS5B sequences obtained (GenBank accession number FJ024088 to FJ024273) along with established GenBank reference sequences [26]. Mega software (version 3.1; available at http://www.megasoftware.net) was used to construct a phylogenetic tree by the neighbour-joining method, using the Tamura-Nei substitution model with γ-distribution (α=0.40). Bootstrap values (n=1000) were calculated to analyze the robustness of tree topology.

Minority HCV variant detection: The cloning PCR

The cloning PCR is a single round PCR targeting a second part of the NS5B region (337 bp, nt 8279-8615) [23]. Only one set of primers is used for HCV RNA amplification of all genotypes. In brief, 25 µl of cDNA was added to 25 µl reaction mixture containing PCR II Buffer (Applied Biosystems), 200 µmol/L of each dATP, dCTP and dGTP, plus 400 µmol/L dUTP (Applied Biosystems), 0.1 µg/µL bovine serum albumin (Roche Diagnostics), 0.9 µM sense primer 5'-TATGAYACCCGCTGYTTTGACTC-3', 0.9 µM antisense primer 5'-TAYCTVGTCATAGCCTCCGTGAA-3', 0.5 U Uracil N Glycolase (Applied Biosystems) and 2.5 U Amplitaq Gold (Applied Biosystems). The final $MgCl_2$ concentration was 2.5 mmol/L. PCR cycling conditions were: 2 min. at 50°C and 10 min. at 95°C followed by 45 cycles of: 20 sec. at 95°C, 20 sec. at 55°C and 60 s at 72°C, with a final incubation of 5 min at 72°C. The cloning PCR product was cut from the agarose gel, resolved in 900 µl lysis buffer and purified using a shortened Boom-isolation protocol [21]. According to the manufacturer's protocol, purified amplicons were ligated into PCR II vector using the TOPO TA Cloning kit (Invitrogen). The plasmid was used to transform competent *Escherichia coli* cells, which were plated on LB agar plates containing ampicillin (100 µg/ml) and Xgal (5-bromo-4-chloro-3-indolyl-B-galactoside). Based on Xgal selection, 50-100 white clones, containing the PCR insert, were picked for each time-point and grown overnight at 37°C on LB agar plates. For each clone, the PCR insert was amplified using M13 primers, according to the TOPO TA Cloning kit protocol, and then sequenced.

Results

Study population

Of the 1276 DU with two or more visits in the ACS, 1259 (99%) had serum available for antibody screening. Of these, 803/1259 (63.8%) were HCV-antibody positive at entry, and an additional 59 DU seroconverted for HCV during ACS follow-up [16]. Of these 59 DU, 58% was male; their median age at HCV seroconversion was 29 years (IQR, 25-34 years), and the median ACS follow-up time since HCV seroconversion was 7.85 years (interquartile range [IQR], 3.30 – 12.1 years]. The median year of HCV seroconversion was 1991 [IQR: 1989-1994], at a median of 2.23 years [IQR: 0.93-6.49 years] after initiation of drug injection. Only one HCV seroconverter denied ever injecting. HCV/HIV coinfection occurred in 14/59 (23%) HCV seroconverters; HCV infection preceded HIV infection in 7/14, 5 contracted both viruses during the same brief period, and two DU were HIV-positive before they acquired HCV.

Dominant HCV variant detection

The 59 HCV seroconverters were tested for HCV RNA on a total of 323 visits, varying from 2 to 10 visits per DU. At 208/323 (64%) of these visits, HCV RNA was detected; log HCV viral loads varied from 2 to 6.14 IU/ml. Amplification and sequencing of the dominant HCV variant succeeded in 169/176 (96%) samples with a viral load exceeding 1000 IU/ml and in 15/32 (47%) samples with a viral load below 1000 IU/ml. Sequencing analysis of HCV RNA-positive samples revealed that at least 24/59 (41%) of HCV seroconverters had evidence for multiple HCV infections over time. According to our definitions, 7 HCV seroconverters had HCV reinfections, 14 were superinfected, 2 had evidence of both reinfection and superinfection, and 1 was coinfected and subsequently superinfected. In total, 94 different HCV infections were identified: 59 initial infections, 11 reinfections, 23 superinfections and 1 coinfection. Of the total, 75/94 (80%) were genotyped and sequenced, finding a HCV genotype distribution of 1a (45%), 3a (33%), 4d (10%), 1b (5%), 2a/b (5%) and 4a (1%).

HCV seroconverters with spontaneous HCV clearance

Spontaneous HCV viral clearance occurred in 25/59 (42%) HCV seroconverters, including 2 DU that were HIV-positive at the moment of HCV seroconversion. In 10/25 (40%) of HCV seroconverters with spontaneous viral clearance, we found evidence for multiple HCV infections over time (figure 1a). Of these, 7 HCV seroconverters had HCV reinfections, 2 had HCV reinfection followed by HCV superinfection (DU 12905; DU 18917), and 1 had HCV superinfection but spontaneously resolved both viral strains (DU 16786). In 4/10 (40%) of the seroconverters with spontaneous viral clearance and evidence for multiple HCV infections, no HCV viremia was detected at their last follow-up visit. Although our numbers are small, this finding suggests that the proportions of DU who develop persistent HCV infection after HCV reinfection and after initial HCV infection are similar. However, whereas 3/6 HCV seroconverters with evidence of persistent HCV reinfection were HIV-positive, none of the four HCV seroconverters that cleared a second or even third HCV infection were coinfected with HIV.

HCV seroconverters without spontaneous HCV clearance

Chronic HCV infection without evidence of previous viral clearance occurred in 34/59 (58%) HCV seroconverters. According to our definitions, 14/34 (41%) seroconverters had HCV superinfections over time (figure 1b). From 6/14, we isolated three or even four distinct HCV viral strains. In DU 18934, the dominant viral strain switched from HCV genotype 4a to genotype 1a, subsequently to genotype 3a and eventually to a heterologous strain of genotype 1a (denoted as 1a*). DU 12962 had clear evidence of HCV coinfection. In the last visit predating HCV antibody presence, two distinct HCV strains were detected using the NS5B PCR, one was HCV subtype 1b and one was HCV subtype 4d. HCV subtype 4d

became the dominant viral strain, however it submerged after superinfection with HCV genotype 1a. No particular pattern in the switch of HCV strains was observed; for example the dominant HCV strains switched from genotype 1a to 3a as often as from 3a to 1a.

Sequencing and phylogenetic analysis

A phylogenetic tree was constructed of all 184 HCV sequences obtained from the 59 HCV seroconverters (Figure 2). Minor genetic variations between HCV isolates obtained from one host, were classified as intrahost HCV evolution. However, 4 seroconverters had clear evidence of phylogenetically distinct HCV infections with strains of the same genotype (Figure 2). HCV strains obtained from 2 other seroconverters were separated by bootstrap value > 70, and genetic variation exceeded intrahost evolution observed in other participants. Although reinfection with a very similar HCV strain for these 2 seroconverters is plausible, intrahost genetic evolution cannot be excluded (Figure 2). Based on RNA patterns and phylogenetic analysis we identified 24 seroconverters with multiple HCV infections. Sequencing confirmed the presence of multiple HCV infections in all 14 seroconverters without viral clearance. However, in 7/10 seroconverters with viral clearance sequencing of either the initial or the reinfecting strain failed. In 5 of 7, no primary viremia was detected as both the primary infection and its natural viral clearance occurred within the interval of HCV seroconversion. In two of the 7, amplification and sequencing of the reinfecting strain failed as a result of low viremia (<1000 IU/ml).

Minority HCV variant detection

Two patients, DU 18898 and DU 18917, were selected for minority HCV variant detection. DU 18898 was selected because of the presence of two genotype switches (1b → 4d → 1a) within a period of 15 months. Based on the NS5B sequence results during dominant HCV variant detection, the seroconversion sample (t=1; genotype 1b) was suspected of HCV dual infection. DU 18917 was selected because, after resolution of the primary HCV 1a infection, we observed a reinfection with a different strain of HCV genotype 1a (1a*) followed by a superinfection with HCV 3a, and eventually a back-switch of the dominant viral strain to the previous HCV 1a* strain, suggesting 3a/1a* superinfection. For DU 18898, we selected, amplified, and sequenced 40-56 clones representing four HCV RNA-positive visits during the 15-month interval of multiple HCV genotype change; except for quasispecies variations, no dual infections were detected (data not shown). For DU 18917, 35 clones from the first and last HCV RNA samples were selected, amplified, and sequenced. Except for variations in quasispecies, all clones belonged to the dominant subtype 1a and heterologous 1a*. For the three intermediate time points, we sequenced 80 to 100 clones and found evidence of superinfection only in the third intermediate sample. Of these 100 clones, 98 were of subtype 1a* and 2 clones were subtype 3a (data not shown).

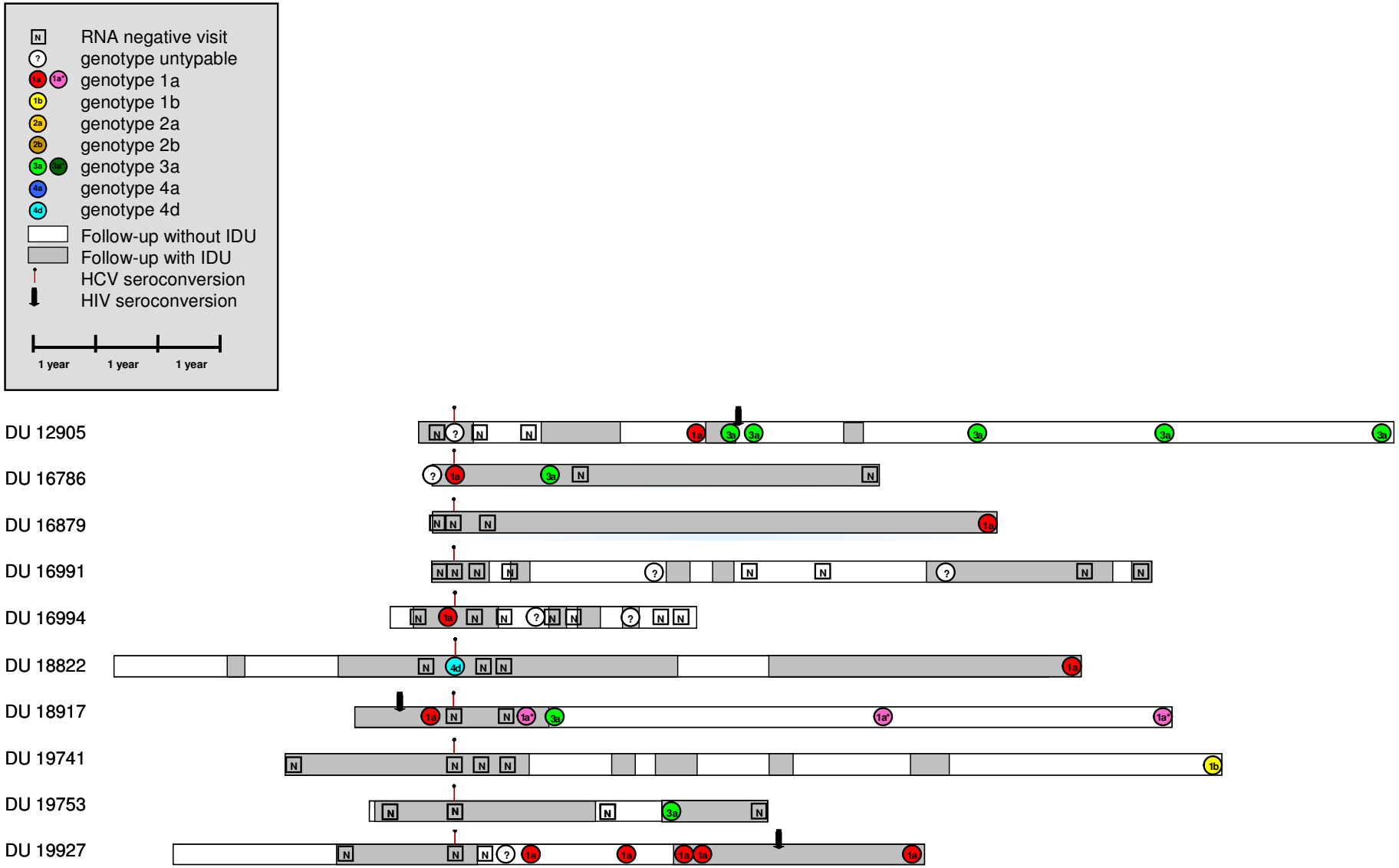

Figure 1a: 10 HCV seroconverters with viral clearance and multiple HCV infections

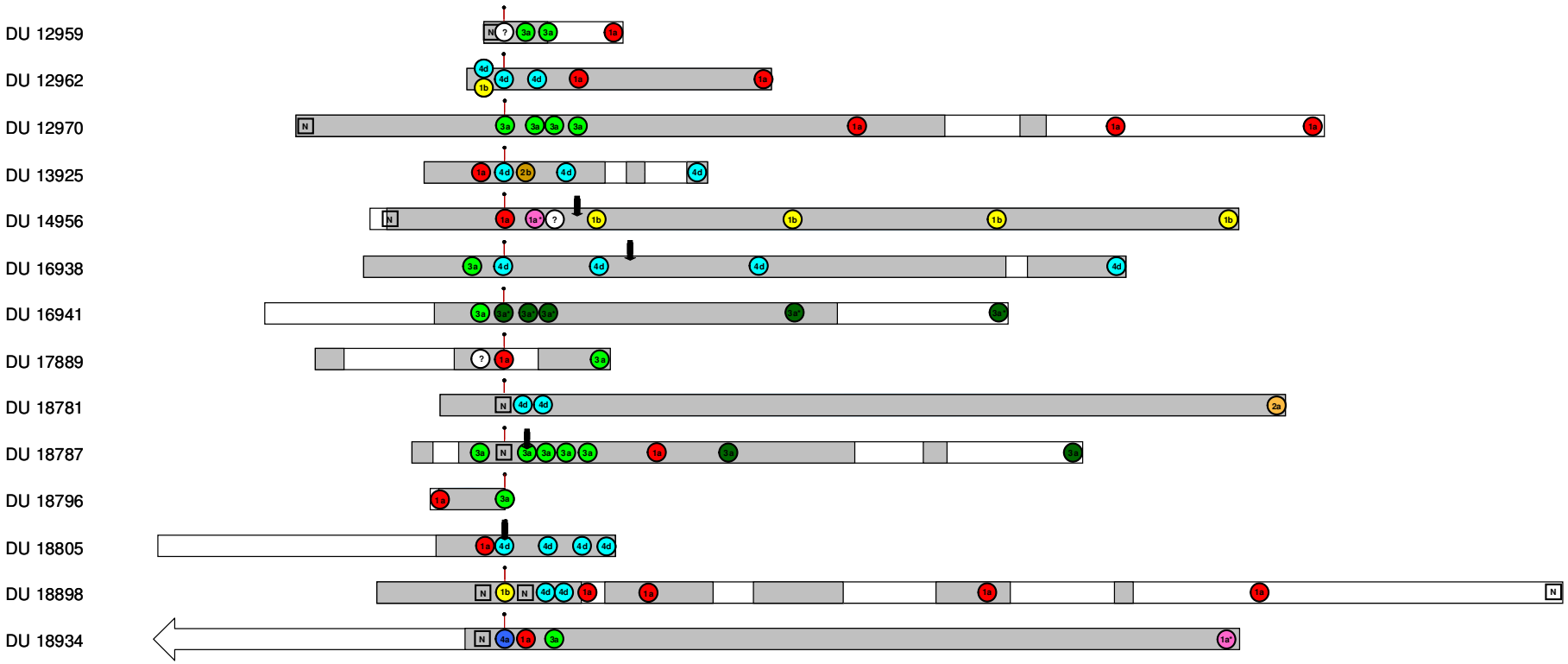

Figure 1b: 14 HCV seroconverters without HCV viral clearance and multiple HCV infections

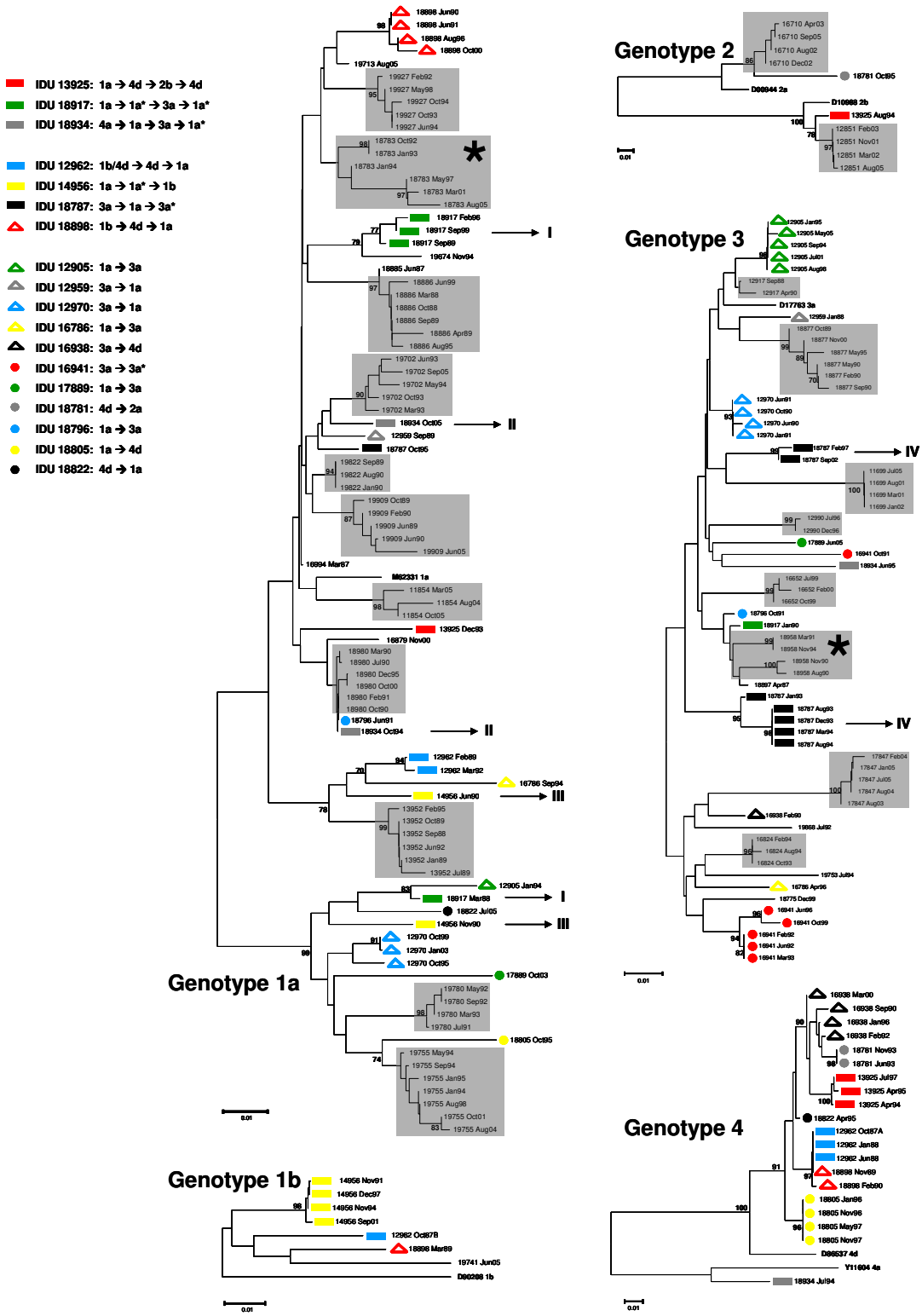

Figure 2: HCV NS5B phylogenetic tree comparing longitudinal samples from 59 drug users with a documented seroconversion. Coloured squares, triangles and circles represent seroconverters with phylogenetically distinct HCV infections over time. Seroconverters with multiple infections of the same genotype are denoted I to IV. Shaded boxes show intrahost evolution among seroconverters without evidence for HCV reinfection and superinfection; (*) might be superinfected with a highly similar strain

Discussion

In a large longitudinal cohort of DU in Amsterdam, we demonstrated that 41% of DU with a documented HCV seroconversion during follow-up, experience multiple HCV infections over time. Both HCV reinfection and HCV superinfection were common in this high-risk population, suggesting that neither previous viral clearance nor ongoing HCV infection provide significant protective immunity against HCV. Traditional HCV incidence calculations based on HCV antibody seroconversion, with the assumption that HCV seropositive individuals no longer are susceptible, underestimate the true HCV incidence and do not properly reflect HCV transmission dynamics within a network of high-risk individuals [27].

Previous reports have suggested the existence of partial protective HCV immunity in chimpanzees and humans [9-13,28]. Although HCV reinfection was observed in 3-12% of anti-HCV-positive DU, those who had previously cleared HCV infection were two to four times less likely to develop new episodes of HCV viremia, compared to DU without previous HCV infection [12,13]. As those studies were conducted in DU with prevalent HCV infection, they suffered from two major methodological flaws. First, the period of high-risk behaviour which led to primary HCV infection and subsequent anti-HCV seroconversion most likely proceeded the study period by several years. As a result, previously HCV-infected DU were older and had less active drug use compared to HCV-uninfected DU. Indeed, their median age was 41-47 years, compared to 29 years in our DU. Moreover, and 35-58% of participants in those studies had quit injecting before the actual start of the study period, being therefore at minimal risk of HCV reinfection. Second, long-lasting anti-HCV seroconversion supports detection of all initial HCV infections, whereas HCV reinfection requires detection of new HCV viremia. Compare to initial infection, new viremia is generally of lower level and of shorter duration [12]; thus the probability of detecting HCV reinfection strongly depends on the frequency and interval of HCV testing. Absence of persistent viremia may reflect partial protective immunity but alternatively could relate to the same host factors that influenced HCV viral clearance following the initial HCV infection.

The data in this study are concordant with the only other study performed in a similar HCV seroconverter cohort of young actively injecting DU [6]. No protective HCV immunity was observed; the incidence of HCV reinfection was similar to the incidence of initial HCV infection, causing 50% of DU who previously cleared the virus to experience a second viremic episode. However, our study has several limitations. Specimens taken from DU with fluctuating low levels of HCV viremia following HCV seroconversion might have been misclassified as HCV RNA-negative, leading to overestimation of the rate of viral clearance with subsequent HCV-reinfection. Confirmation of reinfection by sequencing analysis of both the initial and reinfecting strain failed in the majority of cases. To minimise the risk of such misclassification, we defined viral clearance as two consecutive HCV RNA-negative visits at least 4 months apart. However, underestimation of the rate of HCV reinfection was also

very likely for three reasons. First, characterisation of reinfecting strains was generally hampered by short episodes of low viremia suggesting that additional reinfections were missed as a result of too low frequency of sampling and RNA levels below detection limit. Second, in the context of a steady injection partner, reinfection with a HCV strain almost identical to the initial strain, cannot be excluded. Third, superinfection requires detection of at least two divergent viral strains at the same time point, and our method did not exclude the possibility of rapid viral clearance of the initial strain followed by reinfection with a second strain. Despite these limitations the study clearly shows that in a high-risk population, the proportion of individuals that experience multiple HCV infections over time is much higher than was previously assumed [8,29,30], both among individuals who were able to clear the virus and those who were not.

The high rates of HCV reinfection and superinfection might have serious clinical implications. Although our numbers are small and might be skewed by HIV/HCV coinfection, the persistence of HCV after reinfection is similar to that after initial HCV infection. Hence. patients with spontaneous viral clearance should be aware of the ongoing risk of HCV reinfection. Moreover, HCV vaccine development needs to be based on multiple HCV epitopes, but even then the question remains whether protective immunity will be acquired for all different or even similar viral variants. Multiple infections with divergent viral variants of the same genotype were observed, but not as often as would be expected based on the high prevalence of genotype 1a in our population. Whether partial HCV immunity is obtained for HCV strains of the same subtype, or whether our method failed to detect reinfections with highly similar strains, remains unclear. Moreover, little is known about the possible negative impact of HCV superinfection on, for example, HCV viral load, immunologic escape, natural course of HCV infection or treatment outcome. HCV treatment failure induced by undetected underlying difficult-to-treat genotypes at start of therapy has been reported [31] but seems to be rare [32]. The same holds true for the development of HCV recombinant strains [33]. HCV superinfection has been associated with exacerbation of chronic HCV [34] and immunologic progression of HIV-related diseases in DU coinfected with HIV and HCV [35], but these results need to be confirmed.

In conclusion, both HCV reinfection and superinfection are common among actively injecting DU, suggesting that neither prior virological clearance nor ongoing HCV infection provide immunological protection against HCV. This will complicate future vaccine development, causing prevention to rely heavily on precautionary measures preventing the further spread of HCV.

Acknowledgements

The authors would like to thank all doctors and nurses of the Amsterdam Cohort Studies for data collection and blood sampling, the DU for their participation, Dr D. van Baarle for her contribution to the manuscript, and L Philips for editing the manuscript. The Amsterdam Cohort Studies on HIV infection and AIDS, a collaboration between the Health Service Amsterdam, the Academic Medical Center of the University of Amsterdam, Sanquin Blood Supply Foundation and the University Medical Center Utrecht, are part of the Netherlands HIV monitoring Foundation and financially supported by the Netherlands National Institute for Public Health and the Environment. Website: http://www.amsterdamcohortstudies.org Additional funding was provided by the Netherlands Organisation for Health Research and development (grant 912-03-005).

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