Chapter 3

Absence of HIV-1 Superinfection at Year 1 after Infection between 1985 and 1997 Coincides with Reduction in Sexual Risk Behavior in the Seroincident Amsterdam Cohort of Homosexual Men

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Abstract

**Background** HIV-1 superinfection incidence rates differ among cohorts and as yet, only 2 homosexual men cohorts have been screened. Here, we investigated the incidence of HIV-1 superinfection in the first year after infection in the homosexual participants of the Amsterdam Cohort Studies on HIV infection and AIDS who seroconverted between 1985 and 1997.

**Methods** We analyzed diversity in env C2-C4 in sera of therapy-naïve participants using a heteroduplex mobility assay (HMA); HMA heteroduplexes were considered indicators for potential dual infections, in which case the env C2-C4 PCR products were cloned and sequenced. Sequences were subjected to phylogenetic analysis. Data on sexual behavior of participants were collected from 1 year before seroconversion until the end of the investigated time period.

**Results** From 89 seroconverters with detectable viral load (>1,000 copies/ml), env PCR products were generated from SC and 1 year later. Heteroduplexes were observed in 68 of 89 patients, and from all these 68 patients, a median of 9 molecular clones per time point was sequenced. Phylogenetic analysis did not reveal evidence for superinfection; one patient was HIV-1 co-infected. Shortly after HIV diagnosis, the number of sexual partners decreased, the frequency of anal intercourse declined, while condom use increased.

**Conclusions** The incidence of HIV-1 superinfection early after seroconversion in this cohort is low. Risk reduction shortly after HIV-1 diagnosis in the early HIV-1 epidemic in the Netherlands might have contributed to the absence of HIV-1 superinfection in this study.

Introduction

Natural immune responses after primary HIV-1 infection have been thought to protect against HIV-1 superinfection *in vivo* [1, 2]. However, as early as 1995 dual infections have been reported in patients for whom superinfection could not be excluded [3-5]. Dual infection comprises co- and superinfection, with co-infection defined as (nearly) simultaneous infection with multiple viral strains at or around seroconversion and superinfection with acquisition of another viral strain after immune response to an initial infection [6]. Since 2002, more than 20 publications have reported close to 50 well-documented cases of HIV-1 superinfection [7-26] including even 2 cases of triple infection with HIV-1 [27, 28].

As the HIV-specific immune response develops in the course of infection, acutely HIV-1 infected individuals were considered to be more susceptible to superinfection than chronically infected individuals. Overall, approximately half of the published cases of superinfection were acquired within approximately one year after seroconversion (SC) [9, 10, 12, 15-24]. However, we recently reported superinfection in a long-term elite controller more than 13 years after primary
infection [29], indicating that mechanisms halting disease progression may not be able to protect against superinfection.

The rate of HIV-1 superinfection remains controversial, with reported rates close to the rate of initial infection [15, 17, 22, 24] and absence of superinfection in other studies [30, 31]. Mostly, commercial sex worker (CSW) cohorts have been screened for superinfection, which has resulted in the detection of almost half of all published cases of superinfection [13, 16-18, 22, 24]. In addition, four injecting drug user cohorts where studied [9, 11, 20, 30], revealing 7 cases. In 2 studies cohorts of men who have sex with men (MSM) have been screened for superinfection [14, 15], revealing a total of 4 superinfection cases.

In our exploratory study, we screened HIV-1 positive therapy-naïve homosexual male participants of the ACS with documented SC dates between 1985 and 1997 for HIV-1 superinfection at the first year after SC and we analyzed sexual behavioral data for the time periods before and after SC.

Patients and Methods

Patient population and sample selection

The Amsterdam Cohort Studies on HIV Infection and AIDS (ACS) among homosexual men are conducted in accordance with the ethical principles set out in the Declaration of Helsinki. Enrolment started in October 1984 and up to December 31, 2008, 2,383 participants have had at least one visit, with 1,588 testing HIV seronegative, 585 testing HIV seropositive and 210 HIV seroconverters. Clinical and epidemiological data is collected, CD4+ T cell numbers and plasma viral load are determined, serum and peripheral blood mononuclear cells are stored at 3-monthly intervals. Written informed consent is obtained from every participant.

RNA isolation, RT-PCR and PCR amplification of HIV-1 envelope C2-C4

Viral RNA was isolated from 140μl serum (Qiamp Viral Mini Kit, Qiagen) and eluted in 50μl. Ten microliter of viral RNA, containing a median of 1,386 RNA copies (range, 56 – 728,000), was reverse transcribed (SuperScript™ First-strand synthesis system, Invitrogen) into cDNA using the sequence-specific primer Seq2 (5’-TCCTCCATATCTCCTCCTCCAGGTC-3’). 5μl cDNA was subjected to first-round PCR in a volume of 25μl (primers Seq2, Seq3 (5’-TATGGGATCAAAGCCTAAAGCATG-3’)). Two microliter of first-round PCR product was subjected to second-round PCR (primers Seq5 (5’-GTCAACTCAACTGCTTAAATGTC-3’), Seq6 (5’-ATCTAATTGTCCACTGATGGGAGG-3’)) in a volume of 25μl generating a 549 nucleotide fragment of env C2-V3-C3-V4-C4 (HXB2R positions 7012-7560). Amplification for both PCR was: 1 cycle 94°C, 5 min, 35 cycles 94, 50 and 70°C of respectively 45, 30 and 90 sec and a final step of 10 min, 70°C.
**Heteroduplex mobility assay (HMA) of HIV-1 envelope C2-C4**

Heteroduplexes were generated by denaturing 5µl second-round PCR product of each time point (TP) separately and a mix of PCR products of the 2 time points from each patient at 95°C, 2 min in 10x heteroduplex annealing buffer [32]. Specimens were immediately transferred to wet ice allowing formation of homo- and heteroduplexes within the quasispecies of patient’s env sequences and resolved on a 5% non-denaturing polyacrylamide gel.

**Molecular cloning and sequencing of HIV-1 envelope C2-C4**

Second-round PCR products were cloned into the pGEM T Easy Vector System (Promega), transformed into competent DH5α E.coli (Invitrogen) and plated on LB agar using blue/white screening. White colonies were picked at random (4-32 colonies per reaction). Cloned PCR products were amplified (vector primers T7 (5’-TAATACGACTCACTATAGGG-3’) and SP6 (5’-GATTTAGGTGACACTATAG-3’)) with above-described PCR program. After purification of PCR products (ExoSAP-IT, USB), T7 and SP6 primers were used for sequencing (Big Dye terminator v1.1 cycle sequencing kit, Applied Biosystems). Sequences were determined with an automated DNA sequencer (Applied Biosystems). When sequence diversity did not reflect the heteroduplex pattern obtained in the HMA, an additional 4 nested PCR reactions were performed, cloned and plated. 2 clones per plate were picked, PCR with SP6/T7 primers was performed, and PCR products were sequenced.

**Sequence analysis**

Clonal sequences were aligned per patient using the ClustalW algorithm [33] and alignments were manually edited in BioEdit (BioEdit, v7.0.5.3; Ibis Biosciences). Alignments were visually inspected for the presence of mismatches, insertions and deletions.

**Phylogenetic analysis**

For phylogenetic analysis, all patients’ alignments were merged. Published sequences from samples isolated in The Netherlands were downloaded (http://www.hiv.lanl.gov) to serve as a local control panel. Additionally, a similarity search was performed using BLAST [34], retrieving the most similar sequences to those generated during this study. This panel of highly related but epidemiologically unlinked sequences was merged with the local control panel and the multiple patients’ alignments. The resulting alignment was subjected to ClustalW and manually edited. The corresponding nucleotide substitution model was chosen with Modeltest v3.7 [35] using the hierarchical likelihood tests (hLRTs). Subsequently, phylogenetic analyses were performed using PAUP* [36]. A Neighbour-Joining (NJ) tree was inferred, followed by a heuristic search for a Maximum Likelihood (ML) tree making use of the best-fit substitution model starting with the NJ tree. Statistical
support for nodes was generated with bootstrapping on the NJ tree (1,000 repeats). The ML tree was rooted with a non-B subtype sequence.

Sexual (risk) behavior
Behavioral data were collected from structured questionnaires conducted at 6-monthly intervals, representing behavior in the preceding 6 months. To investigate changes in sexual behavior after SC, we summarized self-reported sexual (risk) behavior among study participants during the 12 months before SC and from SC onwards until the 2nd time point analyzed. Sexual behavior for the present study consisted of the median number of male sexual partners, the median number of insertive and receptive anal sexual partners, and inconsistent condom use during insertive and receptive anal intercourse. Differences in sexual behavior before and after SC were tested by Wilcoxon signed ranks test for median number of (anal) partners per period and generalized estimating equation logistic regression (univariate) for inconsistent condom use (SPSS statistical software package v15).

Results

Study cohort and selected samples
141 homosexual men participating in the ACS with a documented SC date between 1985 and 1997 (median date 1987, Figure 1) were screened for availability of serum samples with a detectable viral load (>1,000 copies/mL plasma) close to SC and 1 year later.

Figure 1: Year of seroconversion of Amsterdam Cohort Studies on HIV and AIDS (ACS) homosexual participants of this study (n=89) and yearly HIV incidence of all homosexual ACS participants between 1985 and 1997 (n=141). Bars show the number of study participants seroconverting in the respective year. Line shows HIV incidence (expressed as cases per 100 person years (PY)) in the homosexual ACS participants for the respective year. (Source: The Amsterdam Cohort Studies on HIV Infection and AIDS. A summary of the results 2001-2009. [Link to source].)
For 46 patients, VL was undetectable or serum samples were unavailable either at the first or second time point leaving 95 patients for analysis (Figure 2). The first time point was at a median of 2 months after SC (range, 4 months before - 8 months after SC) and the second time point at a median of 14 months after SC (range, 8 to 19 months after SC). The median difference between the 2 time points was 12 months (range, 6 - 21 months). PCR fragments of env C2-C4 from both time points could be generated for 89 out of 95 patients and were studied for heterogeneity using HMA (Figure 2).

Figure 2: Approach for patient selection and identification of HIV-1 superinfection in homosexual seroconverters of the Amsterdam Cohort Studies on HIV Infection and AIDS (ACS). Patients were screened for serum samples with detectable VL (>1,000 copies/mL) available at seroconversion (SC) and 12 months post-SC. Samples were subsequently analyzed by heteroduplex mobility assay (HMA) on PCR products of the C2-C4 region of the viral envelope. Viral copy number was determined by limiting-dilution PCR for patients whose samples showed no heteroduplexes in the HMA. From the other patients, samples were further analyzed by clonal sequencing and phylogenetic analysis. TP: time point. VL: viral load. CI: confidence interval. PY: person-year.
Heterogeneity in env C2-C4 as detected by HMA and sequence analysis

Heteroduplexes of env C2-C4 PCR fragments generated from each patient’s serum sample obtained close to SC or 1 year later and/or (additional) heteroduplexes in the mixture of the PCR fragments were considered an indication for a possible dual infection in a patient. In PCR products from sera of 21 patients, heteroduplexes were not observed (Figure 2). As low copy numbers may account for the absence of heteroduplexes, serial end-point dilution PCR was performed on samples of all 21 homoduplex-only patients. For these 21 patients, the number of analyzed env fragments was indeed too low (median 2.92 copies per 5μl input in the first-round PCR, range, 0.35 - 9.88) to firmly exclude the presence of dual infection. Heteroduplexes were observed in PCR products from 68 out of 89 patients (Figure 2). To determine whether these heteroduplexes were the result of dual infection, second-round PCR products of both time points from these 68 patients were cloned and 4-32 colonies per time point were sequenced. If sequence variability (mismatches, insertions and deletions in sequence alignments) did not match the HMA heteroduplex pattern, another 4 nested PCR products were generated, cloned and 2 colonies per cloning reaction were picked and sequenced. Sequences were added to initial alignments and sequence variability was again compared to the HMA. After this, both methods (HMA and molecular cloning followed by sequencing) gave concordant results. In total, 1261 sequences were kept for phylogenetic analysis (median of 9 sequences per time point, range, 2 to 22). Visual inspection of alignments indicated the presence of 2 different virus strains in one patient at the first time point, indicating HIV-1 co-infection.

Phylogenetic analysis demonstrates absence of HIV-1 superinfection

The merged patients’ alignments were combined with a reference set comprising 264 local control sequences and 242 globally sampled, highly similar sequences. The NJ tree was constructed with PAUP* v4.0 under the GTR model. The ML tree was inferred with the selected model (GTR+G, K=9) and with estimated substitution parameters based on hLRTs, the heuristic search applied the nearest neighbor interchange (NNI) algorithm. The absence of superinfection in this study was inferred from the ML tree topology: each patient’s sequences clustered separately, with sequences from both time points together in one cluster per patient (ML tree, see supplementary material). As an exception, one patient appeared to be HIV-1 co-infected: sequences isolated from the 1st time point were retrieved in 2 distinct clusters, of which one cluster also contained the 2nd time point sequences (see patient 61 in ML tree, supplementary material). Hence, the patient was infected with 2 distinct virus strains at or around SC of which one strain was detectable at SC but undetected at the 2nd time point. All HIV-1 sequences from study participants were subtype B.

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**Incidence of initial HIV-1 infection and HIV-1 superinfection in the ACS**

The yearly incidence rate of initial HIV-1 infection in homosexual men of the ACS between 1985 and 1997 ranged from 0.23 to 7.59 per 100 person-years (PY) (Figure 1), with an overall incidence rate in this period of 2.25 per 100 PY (95% CI: 1.9-2.7). The majority of our study participants seroconverted between 1985 and 1987 with yearly incidence rates of 7.59, 3.83 and 2.58 per 100 PY respectively, and an overall incidence rate of 4.45 per 100 PY (95% CI: 3.5-5.5) (Figure 1). The absence of HIV-1 superinfection in 68 homosexual men of the ACS resulted in an overall HIV-1 superinfection incidence rate of 0 per 100 PY with a 95% CI of 0-5.0 (Figure 1). Although we did not detect HIV-1 superinfection, given the overlapping 95% CI, we cannot assume that the incidence rates of the initial HIV-1 infection and the subsequent HIV-1 superinfection are significantly different in this group.

**Low sexual (risk) behavior before SC declines after HIV diagnosis**

We next wanted to explore reasons for the absence of superinfection in the ACS at 1 year after infection. Self-reported behavioral data were available from 243 questionnaires for 86 of 89 seroconverters in the periods before SC and after SC until the 2nd time point investigated. Data available from before and after SC of 72 seroconverters were included for analysis of median number of (anal) partners. The median number of total sexual partners decreased significantly from 12.3 (Inter Quartile Range (IQR): 4.5-24.4) in the 12 months before SC to 6 (IQR: 1.6-15.4) after HIV diagnosis ($P<0.001$) until the 2nd time point investigated in this study (Table 1).

<table>
<thead>
<tr>
<th>Number of sexual partners (median)$^1$</th>
<th>Before SC$^2$</th>
<th>After SC$^2$</th>
<th>$P$ Value$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sexual partners$^4$</td>
<td>12.3</td>
<td>6.0</td>
<td>3.0E-04</td>
</tr>
<tr>
<td></td>
<td>(4.5-24.4)</td>
<td>(1.6-15.4)</td>
<td></td>
</tr>
<tr>
<td>Insertive anal sexual partners</td>
<td>3.0</td>
<td>1.0</td>
<td>6.7E-03</td>
</tr>
<tr>
<td></td>
<td>(1.0-7.0)</td>
<td>(0.0-4.0)</td>
<td></td>
</tr>
<tr>
<td>Receptive anal sexual partners</td>
<td>1.5</td>
<td>1.0</td>
<td>3.8E-02</td>
</tr>
<tr>
<td></td>
<td>(0.5-4.8)</td>
<td>(0.0-3.0)</td>
<td></td>
</tr>
</tbody>
</table>

$^1$Information was available for both time points from 72 of the 89 study participants. $^2$Median (inter-quartile range). $^3$P value from the Wilcoxon Signed Rank Test. $^4$Total sexual partners also includes non-anal sexual partners.

After HIV awareness, the median number of insertive and receptive anal sexual partners declined significantly, respectively from 3 (IQR: 1-7) to 1 (IQR: 0-4) ($P<0.01$) and from 1.5 (IQR: 0.5-4.8) to 1 (IQR: 0-3) ($P<0.05$, Table 1).

Data from 74 participants were included for analyzing inconsistent condom use during insertive anal intercourse and from 86 participants for analyzing inconsistent condom use during receptive anal intercourse. After HIV diagnosis, the inconsistent
The use of condoms during insertive anal sex decreased from 37.1% to 29.2% (OR: 0.68, 95% CI: 0.37-1.26) but this decline did not reach statistical significance (P=0.213). Inconsistent condom use during receptive anal sex did decrease significantly from 56.9% to 33.9% (OR: 0.35, 95% CI: 0.21-0.58, P<0.001, Table 2). Overall, unsafe sexual behavior declined significantly after awareness of HIV-positive status. As the 68 participants for whom superinfection could be ruled out might constitute a subgroup, we also performed the analysis of the behavioral data for just this group. Data was available for 65 of the 68 participants and yielded the same results as above, but with slightly higher P-values (data not shown).

### Table 2. Inconsistent condom use during anal intercourse for study participants before and after seroconversion (SC).

<table>
<thead>
<tr>
<th>Inconsistent condom use</th>
<th>Before SC</th>
<th>After SC</th>
<th>OR (^2)</th>
<th>(P) Value (^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>During insertive anal intercourse</td>
<td>37.1% (26/70)</td>
<td>29.2% (28/96)</td>
<td>0.68 (0.37-1.26)</td>
<td>2.1E-01</td>
</tr>
<tr>
<td>During receptive anal intercourse</td>
<td>56.9% (66/116)</td>
<td>33.9% (41/121)</td>
<td>0.35 (0.21-0.58)</td>
<td>&lt;1.0E-04</td>
</tr>
</tbody>
</table>

\(^1\) Of the 89 study participants, information was available for 74 men with 166 observations for insertive sex and for 86 men with 237 observations for receptive sex. \(^2\) Odds Ratio (95% confidence interval). \(^3\) \(P\) value from univariate generalized estimating equation logistic regression.

### Discussion

In the ACS, we observed a low incidence rate of HIV-1 superinfection in the first year after SC (yearly incidence rate: 0/100 PY, 95% CI: 0-5.0) in homosexual men in the period 1985-1997. To our knowledge, this is the first report that links the absence of HIV-1 superinfection with a decline in sexual risk behavior. HIV-1 env C2-C4 sequence heterogeneity in serum of cohort participants was pre-screened with HMA and multiple molecular clones for 68 of 89 patients were sequenced, not showing evidence for superinfection; 1 case of HIV-1 co-infection was observed. For 21 of 89 patients, superinfection could not be ruled out due to too low input of DNA genome copies. Similar to other publications, our study may underestimate the actual incidence of superinfection. We analyzed only 2 time points per patient, targeting the first year after SC. Transient superinfection and/or superinfections after the first year of infection were therefore not detected. Secondly, recombined strains differing in genome parts other than the env C2-C4 region were not detected. Finally, superinfecting strains constituting less than 1.4% variation to any other viral strain within the viral quasispecies remained undetected due to the threshold of genetic difference for generation of HMA heteroduplexes (Rachinger et al, manuscript submitted for publication). However, our approach is equivalent to other studies that did report superinfection [10, 12, 14-16, 18, 27], as we applied
HMA and clonal sequencing instead of population sequencing - which is less sensitive to detect minor strains (Rachinger et al, manuscript submitted for publication). While samples were from the early stages of the Dutch HIV epidemic, HIV-1 variants circulating then were sufficiently different to allow discrimination between variants from any 2 investigated individuals in the cohort (minimum genetic distance between patients of 3%, data not shown).

An explanation for the absence of superinfection could be the level of risk behavior. Half of all documented superinfection cases have been identified with screenings of female cohorts with high-risk for HIV-1 infection [13, 16-18, 22, 24]. Risk behavior was reported as 1-2 sexual partners per week [22] and for 2 superinfected women respectively 3 and 30 sex partners per week were described [16]. Our cohort however, reported a median of only 6 sexual partners per participant per 12 months following SC implying a 10 to 30-fold lower number of sexual partners compared to these female sex worker cohorts.

Here we report on the absence of HIV-1 superinfection in a cohort of gay men during the early Dutch HIV epidemic. A low incidence rate was also reported from a US gay cohort superinfection screening, with 1 case of superinfection detected by analyzing at least 4 samples from 32 Multiple AIDS Cohort Studies participants at 6-monthly intervals [14]. Smith et al [15] detected in another US cohort 3 cases of superinfection in 76 gay men in samples spanning the first 6-12 months post-SC (incidence rate of 5.0 per 100 PY, 95% CI 1.7-13.3) between 1997 and 2004, a time period where HAART became widely available in high income countries and where resurgence of unprotected anal intercourse with more partners was reported.

At the beginning of the HIV epidemic, safer sex strategies, such as consistent condom use during anal intercourse and reduction of number of sexual partners, were greatly promoted to halt HIV-1 transmission and these risk elimination messages resulted in a decline in HIV-1 transmission rates in gay communities [37, 38]. Interestingly, the absence of HIV-1 superinfection in our cohort in the first year of HIV-1 infection coincided with a significant reduction in sexual risk behavior after being diagnosed with HIV-1 infection. Being aware of their HIV-positive status, our study participants reported significantly fewer sexual partners and fewer partners in anal sex and significantly more frequent condom use. As a result of early preventive activities in the Netherlands, the number of partners also declined in seronegatives, but the reduction was by far more pronounced in seroconverters following HIV-1 diagnosis [37]. These behavioral changes are all described in the context of risk elimination messages during the early HIV-1 epidemic. In a case-control study, seroconverters of the ACS were more likely to have had more sexual partners, specifically more partners for receptive anal intercourse than controls [39]. The
overall HIV-1 incidence rate over the period 1985-1997 was 2.25/100 PY (95% CI: 1.9-2.7) in the homosexual male cohort of the ACS (the yearly incidence rates ranged from 0.23 to 7.59 per 100 PY, Figure 1). However, the majority of study participants seroconverted in 1985, 1986 and 1987 (Figure 1) with yearly HIV-1 incidence rates of 7.59, 3.83 and 2.58 per 100 PY, respectively (overall 4.45/100 PY, 95% CI: 3.5-5.5).

The incidence of HIV-1 superinfection depends most likely on the number of unprotected sexual acts, sexual techniques, but also on HIV-1 prevalence within sexual networks. Given that HIV-1 prevalence was high in homosexual men in Amsterdam in the early epidemic (estimated HIV-1 prevalence of 31-39% between 1985 and 1987), behavioral factors not related to HIV-1 prevalence are more likely to have played a role in our cohort [38]. Further research is required to elucidate the impact of each factor on acquisition of HIV-1 superinfection.

In conclusion, the absence of persisting superinfection in our study cohort may relate to the low risk behavior after SC as a consequence of risk elimination messages during the early HIV epidemic. Studies of seroconverter cohorts with substantial follow-up, in which continuous and/or increased sexual risk behavior with prolonged time since HIV-1 diagnosis might occur, are needed to precisely estimate the incidence of superinfection among homosexual men who do not reduce sexual risk behavior after HIV-1 diagnosis.

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**References**


Supplementary Material

Figure S1: Maximum likelihood tree of env sequences generated from serum samples derived at around seroconversion and approximately one year later of 68 seroconverters of the Amsterdam Cohort Studies. The tree includes only one sequence per time point for clarity of display (_1: time point 1, at around seroconversion, _2: time point 2, at approximately one year later). The tree topology supports absence of HIV-1 superinfection in all patients. Sequences from each patient cluster together, except for the co-infected patient 61, of which sequences isolated from the 1st time point were retrieved in 2 distinct clusters (represented by sequences 61_1a and 61_1b, boxed), of which one cluster also contained the 2nd time point sequences (represented by sequence 61_2, boxed). The tree is rooted at its midpoint. All patient clusters are supported by bootstrap values above 70%, based on 1000 replicates of NJ calculations. Scale is represented as nucleotide changes per site.