Chapter 2

Broadly reactive neutralizing activity in HIV-1 infected injecting drug users

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Abstract

Objective
Vaccine elicited potent and broadly reactive neutralizing humoral immunity may provide protection against HIV-1 acquisition. Several studies have focused on a better understanding of broadly reactive neutralizing activity (bNAc) in natural infection, but only in homosexual and heterosexual transmission cases. The prevalence and characteristics of bNAc in injecting drug users (IDUs) remains to be established.

Design
A retrospective cohort study on the prevalence of bNAc in HIV-1 infected individuals, who reported injecting drug use as the only risk factor.

Method
Serum samples from 50 male and 35 female participants of the Amsterdam Cohort Studies (ACS) were screened across a heterologous 6-viral panel. For comparison, similar data from the ACS on men who have sex with men (MSM) were available from previous studies.

Results
HIV-1 infected IDUs showed a significantly lower prevalence and potency of bNAc as compared to MSM, which was no longer evident when women were excluded from the IDU group. Interestingly, 6% of male IDUs were elite neutralizers as compared to only 0.3% amongst MSM. Multivariate analysis with gender, transmission route, viral load and CD4⁺ count at setpoint as co-variates, revealed all except viral load independently associated with bNAc in serum. There was no association between HIV-1 env sequence diversity and bNAc.

Conclusions
Our data indicate that IDU have a lower prevalence for bNAc and that the emergence of bNAc may be dependent on multiple factors. The lower prevalence of bNAc in women may potentially translate into gender dependent ability of future HIV vaccines to elicit these humoral immune responses.
Introduction

During HIV-1 infection, neutralizing antibodies are mounted within the first three months of infection [1, 2]. However, the autologous virus can rapidly escape neutralization, as most of these antibodies are strain specific. Broadly reactive neutralizing activity (bNAc), defined as the ability to neutralize various heterologous viruses from different subtypes, develops within 1-3 years post-seroconversion (post-SC), but only in about 10 – 30% of HIV-1 infected individuals [3-10]. In 1% of the HIV-1 infected individuals, so called “elite neutralizers”, bNAc is elicited that neutralizes the majority of HIV-1 subtypes with very high breadth and potency [7, 8, 10]. Although natural bNAc does not protect from disease progression [5], in several nonhuman primate studies, passive transfer of known broadly reactive neutralizing antibodies (bNAbs) could completely block infection by a chimeric simian-human immunodeficiency virus (SHIV) [11-15] as well as reduce viral load to undetectable levels in chronic infected humans and macaques [16-18]. The presence of bNAc in humans indicates that there are no fundamental barriers to their induction, and a vaccine should at least be capable of eliciting this type of neutralizing activity in order to provide full protection against HIV-1 acquisition [19-21].

The most predictable clinical markers for the development of bNAc are duration of infection, high viral load and a low CD4⁺ T-cell count, especially during primary infection [4-6, 22-25]. Also, specialized Env-specific follicular helper CD4⁺ T-cells (Tfh) in the lymph nodes are associated with bNAc in non-human primates [26] as well as circulatory Tfh in humans [27]. Virological markers such as viral diversity, diversification and envelope glycoprotein (Env) characteristics are also suggested to be potential contributors to breadth [8, 28, 29]. History of past antiretroviral use, age, gender, ethnicity and human leukocyte antigen (HLA) class II alleles did not correlate with the development of bNAc in a previous study [4, 30].

Most of the studies to identify bNAc were performed in individuals who had become infected via homo- or heterosexual HIV-1 transmission. The prevalence of bNAc in injecting drug users (IDUs) remains to be established. This is relevant as another route of HIV-1 exposure, as well as the immuno-modulatory effect of drug use [31-33], may influence the development of bNAc, and therefore potentially also after vaccination. Furthermore, women have been shown to have higher CD4⁺ T-cell counts at seroconversion [34], which might influence the formation of bNAc as well. Here we studied the prevalence and potency of bNAc in a mixed gender cohort of HIV-1 infected individuals who reported injecting drug use as their only HIV-1 risk factor. Data were compared with similar data previously obtained in our cohort of HIV-1 infected men who had sex with men (MSM) [5, 22, 30].
Methods

Ethics Statement
The ACS are being conducted in accordance with the ethical principles set out in the declaration of Helsinki and all participants provided written informed consent. The study was approved by the institutional Medical Ethics Committee of the Academic Medical Center, University of Amsterdam.

Study population and phenotype
To analyze the association between the route of infection and the presence of bNAc in HIV-1 infected individuals, we first screened serum samples from participants of the Amsterdam Cohort Studies on HIV-1 infection and AIDS (ACS) for the presence of bNAc. The study population consisted of a total of 299 HIV-1 infected MSM [30, 35] and 85 HIV-1 infected IDUs (50 men and 35 women) [36, 37]. Participants were eligible to participate in this study when they were therapy naïve and when a serum sample was available ~3 years post imputed or documented date of seroconversion, when bNAc generally can be detected [3-7, 24]. For MSM this was on average 34 months (range, 21–37 months) and for IDUs on average 36 months (range, 23–55 months). Sera were screened for neutralizing activity on a panel of 6 viruses and the geometric mean ID$_{50}$ titer (GMT) across this panel was calculated [median GMT= 64 (range 20-978)] [7].

U87/Pseudovirus assay for testing of HIV-1 broadly reactive neutralizing activity in serum
Data on HIV-1 neutralizing activity in serum for MSM were available from our previous studies (n=299) [5, 22, 30] and newly generated for the IDUs. In short; sera were tested for bNAc in a pseudovirus assay involving six Tier-2 viruses in a single round of viral infection as developed by Monogram Biosciences. This six viral panel covered 93% of the variation in neutralization of a larger pseudovirus panel (n=15) [7]. Previously we have shown that classification of bNAc in patient serum samples as determined on an independent 23 viral panel or on the smaller six viral panel used in this study, was highly correlated (Spearman r=0.91, P<0.0001) [5]. The GMT across the 6 viral panel was calculated per individual.

Heatmap analysis based on broadly reactive neutralizing activity
A heatmap was made with a web-tool on the HIV database website (http://www.hiv.lanl.gov/content/sequence/HEATMAP/heatmap.html) which uses the heatmap tool "heatmap.2" of the gplots package of the statistical environment R (A Language and Environment for Statistical Computing). Kmeans clustering was performed on the rows and the columns and the rows/columns that fall in the same cluster are
represented by the same colors on the row/column side bar. Bootstrapping was performed with 1000 iterations. Complete cluster analysis was performed on base10 data and output was given as a heatmap with a 9 colors “brewer” palette. Neutralizing ID$_{50}$ titers were used to generate the heatmap.

**Statistical analysis**

Group differences were calculated with a Mann-Whitney test, and Spearman correlations with bNAC and various variables were performed in GraphPad prism 4 (GraphPad Software, La Jolla, California, USA). A multivariate regression analysis was performed using SPSS on both the IDU and MSM cohort with the logarithmic transformed GMT as dependent factor and mode of transmission and gender; viral load and CD4$^+$ T-cell count at setpoint as potential predictors. Mode of transmission and gender were grouped as MSM male, IDU male and IDU female, as the MSM cohort did not include females, therefore gender and mode of transport could not be separated as separate variables. The correlation of diversity and bNAC were calculated with Spearman correlations. The effect bNAC on disease progression was analysed in a Kaplan-Meier and Cox proportional hazard analysis using clinical AIDS (1993 CDC definition) as endpoint. bNAC groups were divided in 3 groups: those who neutralized ≤ 1; 2 or 3; or ≥ 4 viruses at an ID$_{50}$ titer ≥ 100. Left truncation of follow-up time was performed for time between imputed seroconversion date and first seropositive visit using S-Plus 8 (Insightful Corporation, Seattle, Washington, USA). P-values < 0.05 were considered significant.

**Diversity analysis**

The HIV envelope gp160 gene was PCR amplified from DNA isolated from PBMCs that were infected in vitro with a single clonal HIV-variant and subsequently sequences as described previously [38]. Nucleotide sequences were aligned using ClustalW in the software package of BioEdit [39], and edited manually. Sequence nucleotide diversity within each individual was calculated for 23 MSM and 15 IDU infected individuals with median GMTs of 64 (range 20-782) and 47 (range 23-978), respectively, with the Kimura-2 parameter substitution model in the software package MEGA 6. For our analyses we only used Env sequences from within the first year post-SC.

**Multiple virus transmission analysis**

To assess the stringency of the IDU and MSM transmission barrier we analysed the viral env sequences from the first available timepoint within 3 months of seroconversion (n=12), to characterize the complexity of initial infection. Sequences were analysed by a previously described model of neutral virus evolution and using Poisson-Fitter a statistical test on the frequency distribution of the Hamming distance was performed and tested for star-phylogeny [40]. Under a model of random evolution, monophyletic low-diversity lineages are expected to display a star-like phylogeny with a distribution of mutations.
conforming to a Poisson distribution [41, 42]. The failure of the model to fit a Poisson distribution can then be interpreted as either transmission of multiple viral variants or early selective pressure.

**Results**

**Broadly reactive neutralizing activity in injecting drug users and men who have sex with men**

The prevalence and potency of bNAc was first determined in our IDU cohort, depicted in a heatmap together with previously generated data from the MSM cohort (Fig. 1) [5]. Potency of bNAc was determined with the GMT values across the 6-virus panel, and was strongly correlated with both the number of viruses neutralized (Spearman r = 0.85, P < 0.001) as well as the number of viruses that were neutralized with neutralization titers higher than 100 (Spearman r = 0.92, P < 0.001) (data not shown).

Of the 384 HIV-1 infected individuals 18% developed bNAc in their sera, defined by their ability to neutralize ≥ 4 viruses of the 6-virus panel, at ID_{50} titers > 100 (Table 1). The prevalence of bNAc in the IDUs was lower compared to prevalence in the ACS of MSM (18% and 27%, respectively) as established in a previous study [30]. Within the IDU cohort, there was no significant difference in prevalence of bNAc between women and men (17% and 20%, respectively). Also with regards to neutralization potency, IDUs had significantly lower GMT values compared to MSM (P = 0.0009) (Fig. 2A). The IDU cohort showed an abnormal distribution of GMT, with few outliers at the top of the range [median GMT= 41 (range 20-978)], whereas the MSM cohort had a more normal distribution of the GMT [median GMT= 68 (range 20-782)]. Interestingly, the IDU cohort had significantly more elite neutralizers (GMT > 500; range 510-978; all male) [7], three out of the total of 85 individuals (3.5%), as compared to only one out of 299 individuals (0.3%) with a GMT of 754 among the MSM (P = 0.035, Fisher’s exact test) (Table 1).

As the IDU cohort is a mixed-gender population, we repeated our analyses after exclusion of women (n=35), which allowed a comparison of only the men of both the IDU (n=50) and MSM cohorts (n=299) (Fig. 2B). After the exclusion, the difference in GMT and prevalence of bNAc between the IDUs and MSM was lost. This suggested that women determined the observed differences between the IDU and MSM cohorts. Therefore, we wanted to compare GMT between all the men (N=349) and women (n=35) irrespective of the route of HIV-1 transmission (Fig. 2C). HIV-1 infected men had a higher GMT in serum than HIV-1 infected women (P = 0.0008). However, when comparing the GMT between men and women within the IDU cohort, we observed no significant difference (Fig. 2D) although a similar trend, a lower GMT in women, was observed.
Figure 1. Heatmap and clustering analysis of broadly reactive neutralizing activity in serum in IDUs and MSM. ID$_{50}$ values of sera from 85 HIV-1 infected injecting drug users at ~35 months post (imputed) seroconversion (rows) against 6 viral isolates (columns) are shown. Darker colors represent more potent neutralization. Kmeans clustering was performed on the rows and the columns and the rows/columns that fall in the same cluster are represented by the same colors on the row/column side bar. Darker colors indicate more potent neutralization. Patients with broadly reactive neutralizing activity (bNAc) cluster together in the top of the heatmap and patients with no bNAc cluster in the bottom of the heatmap. Men who have sex with men (MSM) (light gray) and injecting drug users (IDU) male (medium gray) and female (dark gray) are color coded on the y-axis.
Table 1. Prevalence of broadly reactive neutralizing activity in the HIV-1 infected MSM and IDU cohorts

<table>
<thead>
<tr>
<th></th>
<th>bNAC ≥4 virus (IC₅₀ &gt; 100)</th>
<th>Elite neutralizer GMT &gt; 500</th>
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<tbody>
<tr>
<td>MSM</td>
<td>299</td>
<td>27%</td>
</tr>
<tr>
<td>IDU</td>
<td>85</td>
<td>19%</td>
</tr>
<tr>
<td>IDU Male</td>
<td>50</td>
<td>20%</td>
</tr>
<tr>
<td>IDU Female</td>
<td>35</td>
<td>17%</td>
</tr>
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MSM, men who have sex with men; IDU, injecting drug users

Clinical and viral factors associated with broadly reactive neutralizing activity

A high viral load and low CD4⁺ T-cell count, especially during primary infection, are the strongest predictors for the development of bNAC in studies reported to date [4-6, 22-25]. Here we analysed the association between the CD4⁺ T-cell count and the viral load at setpoint and the development of bNAC within the MSM and the IDU cohort. Mean CD4⁺ T-cell counts and mean viral load were similar between the two cohorts when the IDU males and IDU females were combined (data not shown).

In the MSM cohort we could confirm a negative correlation between the CD4⁺ T-cell count at setpoint (+/- 18 months post-SC) (Spearman r = -0.24, P < 0.001), and a positive correlation between the viral load at setpoint, and the development of bNAC (Spearman r = 0.14, P = 0.014). However in the IDU cohort, only a trend was observed between the CD4⁺ T-cell count at setpoint and bNAC (Spearman r = -0.20, P = 0.133), while no correlation with viral load at setpoint was observed.

Viral diversity has previously been shown to correlate with the development of bNAC [28], therefore we analysed the earliest available Env sequences (within one year post-SC) from 23 MSM and 15 IDU infected individuals with known SC dates (Fig. 3A). We did not observe any correlation between the presence of bNAC and the Env diversity in these individuals, nor when we analysed the MSM and IDUs as separate groups (Spearman r=0.04, P=0.83, and r=0.29, P=0.29, respectively). As viral evolution occurs in most HIV-1 infected individuals, time since infection can have an influence on viral diversity measured in our individuals. Therefore, we analysed only the Env sequences obtained from within the first three months post-SC (MSM=8 and IDU=5), including one MSM elite neutralizer, but again found no correlation (data not shown).

Several studies have shown that IDUs are more likely to be infected with multiple variants during transmission (multiple virus transmission; MVT) as compared to sexual HIV-1 transmission [43, 44]. First, we compared the Env sequence diversity from only those obtained within the first three months post-SC, and found that in the IDU infected individuals this was higher, although not significant, compared to the MSM, which may point to possible MVT (Fig. 3B). As there was an uneven distribution of bNAC in the IDU
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Figure 2: The potency of broadly reactive neutralizing activity in the HIV-1 infected MSM and IDU cohorts
Serum samples from men who have sex with men (MSM) (n=299) and injecting drug users (IDUs) (n=85) from the Amsterdam Cohort Studies on HIV-1 infection and AIDS (ACS) screened for the capability to neutralize viruses from different HIV-1 subtypes in a 6-viral panel. (A) Each dot represents one individual’s geometric mean ID$_{50}$ titer (GMT) across the 6-viral panel for both the IDU (black) and MSM (gray) cohorts. (B) Same as A, but after the exclusion of women. (C) GMT between the male (gray) and female (black) individuals, irrespective of the route of HIV-1 transmission. (D) Same as C, but only within the IDU cohort. Open dots represent the elite neutralizers. Differences between the different cohorts were determined using a Mann-Whitney t-test and P-values are shown. Horizontal bars represent the median value per group. ID$_{50}$, 50% inhibitory dilution.

cohort, we speculated that some of these individuals, especially the ones with high GMTs, could have MVT, which contributed to this observation. Based on phylogenetic analyses, inspection of env sequences coupled with a previously described model of neutral virus evolution [41, 42] we characterized the complexity of infection for each subject. Four of
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Figure 3: HIV-1 gp160 sequence diversity and multiple variant transmission for IDU and MSM infected individuals.

(A) Scatter plot of individual’s geometric mean IC$_{50}$ titer across the 6 virus panel (y-axis) versus the viral diversity of Env (x-axis), is shown for 23 MSM (grey) and 15 IDU (black) HIV-1 infected individuals. Env sequences were obtained within the first year post-SC. (B) Early sequence diversity of gp160 is shown for 5 IDU versus 8 MSM infected individuals, env sequences were obtained within the first 3 months post-SC. Horizontal bars represent the median diversity per group. Each dot represents one individual, and the open dots represents the elite neutralizers. (C) Highlighter plot comparing env sequences for MSM elite neutralizer with ticks indicating nucleotide mismatches (left panel). This individual displays a phylogenetic pattern consisting with multiple viruses transmission with each lineage color-coded (right panel). (D) Highlighter plot comparing env sequences from a MSM infected individual showing a mutational and phylogenetic pattern associated with infection by a single virus. Both individuals are representative data for MVT and single transmission in our analyzed individuals.

The IDU infected individuals (4/5, 80%) displayed high env diversity (data not shown) and demonstrated phylogenetic evidence of MVT and did not conform to a model of random virus diversification while one IDU demonstrated a single low-diversity lineage characteristic of acute infection founded by a single virus. Unfortunately, the earliest sample date from two of the IDU elite neutralizers were only available at 7 and 8 months post-SC and are considered too late into infection for the model to be reliably used as the viral diversity will obscure the true number of virus lineages founding infection. Interestingly, the elite neutralizer within the the MSM group (n=7) was the only subject, that had evidence for MVT (Fig. 3C), while the remaining MSM subjects displayed phylogenetic evidence for a single variant virus (Fig. 3D). However, individuals with MVT
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Table 2. Factors associated with the presence of broadly reactive neutralizing activity in serum.

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<th>Univariate Analysis</th>
<th>Multivariate analysis</th>
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<tbody>
<tr>
<td></td>
<td>P-value</td>
<td>B-coefficient</td>
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<tr>
<td>Differences among MSM, IDU male, IDU female</td>
<td>0.002</td>
<td>-0.157</td>
</tr>
<tr>
<td>CD4 count at setpoint</td>
<td>0.002</td>
<td>-0.163</td>
</tr>
<tr>
<td>VL at setpoint</td>
<td>0.011</td>
<td>1.34</td>
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MSM, men who have sex with men; IDU, injecting drug users

did not have higher GMT than individuals with no MVT (data not shown). Moreover, we observed a significant enrichment of individuals with MVT in the IDU compared to the MSM cohort (IDU 4/5, MSM 1/7, p = 0.022). However, given that the number of sequences obtained from each individual was quite low there is a likelihood of missing viral variants, thus these estimates represent the very minimum estimates with greater sequencing depth needed to give more sensitive and precise estimates on the number of variants initiating infection.

The mode of transmission, gender, viral load and CD4$^+$ T-cell count at setpoint were all significantly associated with the potency of bNAc, as described above. To determine whether these parameters were independently associated with bNAc potency, we performed a multivariate model analysis on the combined IDU and MSM cohorts using all parameters as covariates (Table 2). As the MSM cohort consisted of males only, not allowing mode of transmission to be analysed as an independent variable, we combined the variables of gender and transmission into the following groups: MSM male, IDU male and IDU female. Under the multivariate model, viral load at setpoint was no longer independently associated with bNAc. However, CD4$^+$ T-cell count at setpoint (P = 0.017) and combined gender and mode of transmission (P = 0.001) were still associated with bNAc. In line with this we observed that within the IDU cohort, women had higher mean CD4$^+$ T-cell counts, although not higher than the MSM cohort (Fig. 4), while the viral load at setpoint was not statistically significant between men and women in the IDU cohort.

bNAc has previously been shown to have no effect on disease progression in this MSM cohort [5]. Individuals in the IDU cohort that neutralized a majority of the viruses (≥ 4) at ID$_{50}$ titers higher than 100 (n = 16) had a similar time to AIDS’93 as compared to IDU with intermediate (neutralizing 2 or 3 viruses, n = 24) or those with no bNAc (neutralizing 1 or none of the viruses, n = 45) (data not shown), indicating that also in IDU, bNAc had no protective effect on the clinical course of infection.
Discussion

In this study we have shown that individuals infected with HIV-1 via injecting drug use had lower potency of \( b_{\text{NAc}} \) in serum than the HIV-1 infected MSM cohort. This effect can be explained by the presence of women in the IDU cohort, who were shown to have a lower prevalence and potency of \( b_{\text{NAc}} \) as compared to men in both the MSM and IDU cohorts. The MSM and IDU cohorts are similar with respect to calendar period in which participants became infected (1982 – 1997), and subtype B as the only HIV-1 clade. Moreover, there were no significant differences in age, disease course [45], \( \text{CD}^+ \) T-cell count and viral load at setpoint when looked at the group as a whole.

Recreational drugs have been shown to have various immune-modulating properties depending on the frequency and type of drug used [31, 32]. It has been suggested that endogenous opioids can suppress B-cell proliferation [33] and we can therefore not exclude the suppressive or activating effects these drugs can have on priming of B cells.

A previous study on clinical variables that associate with \( b_{\text{NAc}} \) did not show a difference in \( b_{\text{NAc}} \) prevalence that was associated with gender of the infected individual [4, 7, 46-49]. In our present study we had no women who acquired HIV-1 through sexual contact and we therefore cannot exclude heterosexual transmission as a confounder.

The most predictable clinical marker for the development of \( b_{\text{NAc}} \) is a high viral load and a reduced \( \text{CD}^+ \) T-cell count, especially during primary infection [4, 5, 22-24]. The overall \( \text{CD}^+ \) T-cell count and viral load at setpoint of the MSM and IDU cohorts were similar, however within the IDUs we did not observe a correlation between either the \( \text{CD}^+ \) T-cell count or viral load at setpoint and the development of \( b_{\text{NAc}} \). Nevertheless, in the combined cohorts, both viral load and \( \text{CD}^+ \) T-cell count at setpoint were strongly
associated with GMT. Viral diversity within the first year of infection, another proposed marker for bNAC development, did not correlate with bNAC development. Conflicting results have been reported on the influence of viral diversity. A study in a cohort of subtype A infected individuals showed that early viral diversity was associated with the development of bNabs [28]. Our lab has also shown that diversity over the first 5 years of infection correlated with bNAC [8], with diversity and bNAC tracking hand in hand over time, although it is unclear whether diversity drives bNAC, or antibody pressure leads to escape and diversity. The effect of very diverse sequences, such is the case with superinfection, on bNAC is also unclear with some studies pointing to increase bNAC with SI [50], whereas others, including our own lab, observed no increase in bNAC after SI (Cornelissen et al. submitted)[47]. These conflicting results suggest that the role of viral diversity in the induction of bNabs is not black or white, and is probably a co-dependency factor.

Moreover, CD4+ T-cell count at setpoint and combined gender and transmission route, but not viral load at setpoint predicted the presence of bNAC. Within the IDU cohort women had a higher CD4+ T-cell count than men. It has previously been shown that both IDU and women have a higher CD4+ T-cell count at SC as compared to MSM [34], though, here only the women had higher CD4+ T-cell counts. However, all these variable were independently associated with bNAC. Therefore, the development of bNAC remains multifactorial.

Interestingly, the IDU cohort had a higher proportion of elite neutralizers. As a macaque study showed that a higher degree of viral heterogeneity could be observed in intravenous infected animals compared to animals infected via sexual transmission [51], and viral diversity has been associated with bNAC [28], we speculated that MVT possibly occurred in these elite neutralizers. Unfortunately, we had no viral sequences from very early during infection from the IDU elite neutralizers, and were not able to conduct MVT analyses on them. However, when we analysed viral diversity of samples obtained in the first three months post-SC, we found that all but one IDU demonstrated evidence consisting with a pattern associated with MVT. Curiously, the MSM elite neutralizer was the only MSM predicted the have MVT. While this is only one individual, this could have contributed to the high bNAC in this individual. Comparing the number of MVT in the analysed IDU and MSM infected individuals, we observed a significant higher number of MVT in the IDUs, which implies that MVT is more common in IDUs. These findings are in concordance with others, where MVT have been observed in up to 60% of the individuals [43, 52].

We and others have shown previously that bNAC did not have an effect on disease progression in MSM or heterosexually transmitted women [5, 22, 23, 28]. Here we show that bNAC also did not have a protective effect in IDU.

In conclusion, we have found that individuals infected via HIV-1 contaminated needles had general lower bNAC titers than HIV-1 infected MSM. However, this difference was mainly
due to the women in this cohort. These data may imply that the efficacy of future HIV-1 vaccine candidates may be different in men and women. Testing of vaccine candidates, that could potentially also induce different vaccine elicited responses in different genders and risk populations should therefore continuously be encouraged in designing clinical studies.

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