Genetic diversity of HIV in seminal plasma remains higher than in blood after short-term antiretroviral therapy

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ABSTRACT

Objective To provide insight on viral kinetics and genetic diversity of HIV in seminal plasma at baseline and 1 month after initiating antiretroviral therapy (ART).

Patients and methods Blood and seminal samples from patients with newly diagnosed HIV were obtained before ART initiation (T0) and 1 month after ART initiation (T1). HIV env genetic diversity was studied using deep sequencing Nextera and V3 chemistry in a MiSeq Illumina platform. The number of viral quasispecies (5% cut-off) and Shannon Index were used to analyse diversity.

Results Forty-seven ART-naive patients were recruited between September 2016 and November 2018. At enrolment, the number of quasispecies in blood (median 4 (IQR 2–5)) was lower than in the seminal compartment (median 6, (IQR 4–8)) (p<0.001); the Shannon Index was also higher (p<0.001) in the seminal compartment than in blood (1.77 vs 0.64). At T1, for the 13 patients with detectable HIV in both blood/seminal plasma, viral diversity remained higher (p=0.139) in seminal plasma (median 2 (IQR 1–4.5)) than in blood (median 1 (IQR 1–1.5)). Integrate inhibitors (INI)-based regimens achieved higher levels of undetectability and led more frequently to lower variability (p<0.001) than protease inhibitors (PI) or non-nucleoside reverse transcriptase inhibitors (NNRTI).

Conclusion We provide here further evidence of a larger genetic diversity in seminal plasma, both at diagnosis and short term after ART initiation. Our results strengthen previous findings on HIV diversity in seminal plasma. In addition, INIs decrease variability more rapidly than PI and NNRTI in both blood and seminal plasma.

INTRODUCTION

Infection with the HIV is one of the epidemics affecting the global population. Sexual route, followed by parenteral route, are the main transmission routes of HIV, and semen is the vector responsible for majority of sexual transmissions globally. Currently, quantification of viral load in blood is used in clinical practice to assess therapy effectiveness and disease control. However, it is known that the kinetics of the HIV viral replication in the blood compartment differs from its kinetics in the seminal compartment. Several authors have reported the presence of viral DNA in seminal fluid with undetectable viral load in blood.1,2 The HIV DNA can be found in seminal leucocytes as non-integrated with linear or circular form or as integrated provirus.3 Nevertheless, cell-free HIV in seminal plasma seems to be actively infective4 and infectivity is based on viral load. The presence of virus in this compartment can be intermittent, regardless of the time lapse from a negative result in the blood sample.5 Similarly, seminal positive result for HIV RNA was found despite a negative result for HIV RNA was found in blood.6 Gene sequencing of proviral protease and DNA envelope genes has shown different variants in seminal plasma and blood.7

The presence of HIV in seminal plasma seems to depend on various factors that may influence its presence in this compartment, even when it is undetectable in blood. Thus, the presence of a symptomatic sexually transmitted disease, such as acute urethritis,8 cytomegalovirus infection9 or herpes simplex virus type 2,8 increases the risk of HIV transmission without showing association with previous or asymptomatic urethritis. Similarly, seropositivity to herpes simplex virus type 2, the increase in cytokines in seminal plasma, the increase in activation of mucosal T cells and the number of compartmentalised viral quasispecies3 seem to influence the presence of the virus in seminal plasma. Time to virological suppression varies based on the ART regimen and compartment; being integrase inhibitors (INIs) and rilpivirine-based ART regimens the best ones in seminal plasma1,9 followed by protease inhibitors (PI).

Despite the above described, the seminal compartment is not well understood to date. Therefore, in the present study, we investigated viral kinetics and quasispecies in patients with HIV infection initiating antiretroviral therapy (ART).
patients. All approached patients agreed to participate in the study. Before entering the cohort, ethics approval was obtained from the Ethics Committee of San Cecilio University Hospital in Granada (Spain) (1184N-16). Patients signed a written informed consent to participate in the study. No patient had other sexually transmitted diseases at the time of this study.

Methods

Blood and seminal samples were obtained before ART initiation (T0) and 1 month (T1) after ART initiation. At diagnosis, age, sex, concomitantly sexually transmitted infections, HIV viral load (RNA copies/mL), baseline resistance and CD4 cell count (cells/µL) were recorded (retrieved from their medical records). Treatment regimen was recorded at the study entry (T0). HIV viral load, CD4 count and HIV env diversity were recorded at T0 and at T1.

All blood and seminal plasma were obtained by venipuncture and masturbation, respectively, and stored at −80°C until transfer to the laboratory. Quantitative nucleic acid test for use on the Cobas 6800/8800 Systems (Roche Diagnostics) with a limit of detection 20 copies/mL was used for HIV RNA viral load test in both blood and seminal plasma. The diversity of HIV was studied in all viraemic samples using an in house, deep sequencing protocol, using the Nextera and V3 chemistry in a MiSeq Illumina platform. We amplified a 500 bp of the HIV type 1 (HIV-1) env C2V3C3 region for these studies.11 We selected env because this is the HIV gene with the largest variability.12–15

Briefly, HIV RNA was extracted from blood and seminal plasma samples using the Magna Pure Compact System (Roche) following the manufacturer’s instructions. This system is based on a magnetic-bead technology. RNA was eluted into 50 µL of the kit buffer from sample volumes of 400 or 1000 µL according to availability. Seminal plasma was obtained from semen samples by centrifugation at 2500 rpm for 10 min. After extraction, RNA was purified using Ampure Agencourt RNAclean XP (Beckman Coulter) before cDNA synthesis. Quantification was performed with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Delaware, USA). The nucleic acid yield was determined by measuring absorbance ratios spectrophotometrically. The measurement included A260/280 nm for protein contamination and A260/230 nm for salt and phenol contamination.

The Transcriptor One-Step RT-PCR (Roche) kit was used for cDNA synthesis. All PCRs were performed in 25 µL reaction volumes containing 12.5 µL 2X KAPA HiFi Hotstart ready mix (KAPA Biosystems, Woburn, Massachusetts, USA), 5 µL of each forward and reverse primers (1 µM) and 2.5 µL of extracted RNA (10 ng). The PCR conditions were the following: initial denaturation at 95°C for 3 min, followed by cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s, with a final extension at 72°C for 5 min. PCR clean-up was performed using Ampure XP beads (Beckman Coulter, Indianapolis, Indiana, USA) to purify the env ampiclon away from free primers and primer dimer species. Then, the next step was the index PCR, which attaches dual indices and Illumina sequencing adapters using the Nextera XT Index Kit (Illumina, San Diego, California, USA). The conditions of the second PCR were the following: 95°C for 3 min; 8 cycles of 95°C for 30 s, 55°C for 30 s and elongation at 72°C for 30 s, with a final extension at 72°C for 5 min. The pooled PCR products were purified using AMPure XP beads (Beckman Coulter) before quantification. The resulting amplicons were sequenced at MiSeq Platform (Illumina), using a paired-end (2 × 300nt) sequencing system. All experiments were run on an equimolar concentration of amplicons.

The FASTA files obtained were demultiplexed to obtain a FASTA file for each sample and for the strand. Ugene tool16 was used for quality assessment of raw reads, which were assembled, aligned and screened based on the minimum length of 200 bp and a maximum length of 500 bp. Sequences were filtered and chimera were removed using Usearch,17 accepting only those with Q>30 and a seed cluster of 30. Finally, the number of quasispecies in each sample was investigated using BLAST. Only the quasispecies representing more than 5% of the population were recorded; this limit was used to skew any method error, since this is the standard limit for resistance analysis.18

The diversity of the HIV quasispecies was calculated using the Shannon Index as previously reported19 using the entropy tool from Los Alamos HIV (https://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy_one.html). This numerical score is a representative number and relative frequency of the HIV quasispecies found in each sample.

We were able to investigate HIV viral tropism using the HIV genotypheno tool20 because our env fragment encompassed the V3 loop. The number and location of potential N-linked glycosylation sites for each sequence were estimated using the N-GLYOSITE web tool from the Los Alamos HIV database (http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html).

Normality of the variables has been analysed using the Shapiro-Wilks test for the statistical analysis. Baseline characteristics are expressed as proportions for categorical variables and as medians and IQR for continuous variables. Mann-Whitney U non-parametric test has been used to compare differences in the number of quasispecies between blood and seminal plasma. Wilcoxon test was used for the differences in the number of quasispecies before and after therapy. Wilcoxon test was applied, correcting the p value obtained by the number of comparisons, to analyse whether the decrease in the number of quasispecies was significant based on the type of regimen. Pearson χ2 test was used for qualitative variables. To correlate the number of quasispecies with initial viral blood load (T0) and the nadir CD4 T-cell count, the Spearman’s correlation coefficient was used. A p<0.05 was considered significant. Data have been analysed using the IBM SPSS software Statistics 19.

RESULTS

Forty-seven ART-naive patients with HIV-infection from the different participating locations (Virgen del Rocio University Hospital, Virgen de las Nieves University Hospital, San Cecilio University Hospital and Jaén University Hospital) were recruited for the study between September 2016 and November 2018. Patients had a median age of 30 years, a mean viral load of 29 2513 copies (log 4.56) and a mean CD4 count of 494 cells/µL. At enrolment (T0), 4 patients (8.51%) had clinical signs of acute HIV infection, 6 (12.8%) patients initiated a non-nucleoside reverse transcriptase inhibitors (NNRTI)-based regimen, 10 (21.3%) patients initiated a PI-based regimen and 31 (65.6%) patients initiated an INI-based regimen. Table 1 shows the baseline characteristics of the patients.

At baseline, we were able to sequence 47 blood and seminal samples. The mean number of sequences studied in blood was 68 458 (range 38 307–79 352). The mean number of sequences studied in seminal plasma was 65 435, (range 54 675–73 669). Using the prespecified 5% cut-off, the number of quasispecies in blood was lower than in the seminal compartment (p<0.01). In fact, a median of 4 (IQR 2–5) different quasispecies was found in the blood compartment, while a median of 6 (IQR 4–8), different
quasispecies were found in the seminal tract. All patients except one showed a higher number of quasispecies in seminal plasma than in the blood compartment. No statistically significant differences in viral diversity were found between patients with and without acute HIV infection. Similarly, the Shannon Index was higher (p<0.001) in the seminal compartment than in blood (1.77 vs 0.64). Patients were infected predominantly with CCR5 viruses (80.9% in blood and 83% in seminal plasma). Interestingly, we found a different tropism from blood to seminal plasma.

To further characterise the evolution of viral diversity in both the seminal and the systemic compartment, we studied env quasispecies in 33 patients for whom paired T0 and T1 samples from blood and seminal plasma were available. Table 3 shows the number of viral quasispecies for each patient and the median of these values. At T1 (1 month after ART treatment), a significant decrease (p<0.001) in both blood (T0: 3 (2–5) vs T1: 0 (0–1)) and seminal plasma (T0: 6 (4–7.5) vs T1: 0 (0–2)) was found. HIV-RNA was undetectable in both blood and seminal plasma in 16 patients (48.5%). Discordant results were found in 4/33 patients (12.1%) characterised by detectable HIV-1 RNA in the seminal plasma but undetectable HIV-1 RNA in the blood plasma.

Patients treated with INI were more likely to reach undetectability in both blood and seminal plasma (p<0.001) than patients treated with a PI or with an NNRTI-based regimen, even from viral blood loads (T0) higher than other regimens (INI 47 423 (4502–3397 50), IP 8220 (4730–42 700), NNRTI 46 300 (32 050–83 650)). Table 4 shows the quasispecies according to the prescribed treatment regimen regarding the 33 patients, we found that INI led to a decrease in the number of quasispecies in both samples (p<0.001); this decrease was not found with PI or with NNRTI. Regarding the number of quasispecies at T1 in the 13 patients with positive viral load in seminal plasma and blood, viral diversity remained higher in seminal plasma (median 2 (IQR 1–4.5)) than in blood (median 1 (IQR 1–1.5)), p=0.139.

### Table 1 Baseline characteristics of patients

| Characteristic                           | Value          |
|-----------------------------------------|----------------|
| N=47                                    |                |
| Age* (years)                            | 30 (26–41)     |
| Acute HIV Infection, n (%)              | 4 (8.51%)      |
| Viral load (Log copies/mL)*             | 4.48 (3.98–5.06) |
| CD4 count (cells/µL)*                   | 494.5 (301.75–641.5) |
| HIV Treatment, n (%)                    |                |
| NNRTI-based                             | 6 (12.8%)      |
| PI-based                                | 10 (21.3%)     |
| INI-based                               | 31 (65.6%)     |
| Previous infections                     |                |
| Chlamydia                               | 4 (8.7%)       |
| Syphilis                                | 9 (19.1%)      |
| Gonorrhea                               | 0 (0%)         |
| HCV                                     | 2 (4.3%)       |
| HPV                                     | 10 (21.3%)     |
| *Median (IQR, 25th–75th percentile).    |                |
| HCV, hepatitis C virus; HPV, human papillomavirus; INI, integrase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; PI, protease inhibitors. |

### Table 2 Virological characteristics in blood and seminal plasma

| Characteristic                           | Blood, n=47 | Seminal plasma, n=47 | P value |
|-----------------------------------------|-------------|----------------------|---------|
| Quasispecies*                           | 4 (2–5)     | 6 (4–8)              | <0.001  |
| Shannon Index*                          | 0.64 (0.41–1.01) | 1.77 (1.21–2.32) | <0.001  |
| Tropism                                 | 0.789       |                      |         |
| CCR4                                    | 9 (19.1%)   | 8 (17%)              |         |
| CCR5                                    | 38 (80.9%)  | 39 (83%)             |         |
| Sequences studied*                      | 68 963 (57 699–79 352) | 62 577 (54 335–73 669) | 0.155   |
| *Median (IQR, 25th–75th percentile).    |              |                      |         |
| CCR5, chemokine coreceptor type 5; CCR4, chemokine receptor type 4. |
**DISCUSSION**

Understanding HIV viral genetic diversity in seminal plasma of patients with HIV infection is of utmost importance to elucidate HIV sexual transmission. To our knowledge, our study is one of the largest studies analysing paired blood and seminal plasma samples from ART-naive patients with HIV-1 infection. We found a consistent larger number and variability of HIV-1 env viral quasispecies in seminal plasma than in blood by using deep sequencing. After starting first-line treatment, we also found that HIV-1 env viral diversity was higher in seminal plasma than in blood, and HIV remained detectable in seminal plasma in more patients than in blood.

Sexual contact is currently the main route of transmission of HIV-1. The male genital tract has been characterised as a reservoir, a compartment and a drug sanctuary. HIV compartmentalisation and diversity in seminal plasma has been previously studied using various methodologies. Ghosh et al. analysed the genotypic resistance pattern in patients with HIV established on ART with a median of seven previous antiretroviral treatments (range 3–15), showing different genotypic variability.

In summary, we provide here strong evidence of a larger genetic variability in seminal plasma than in blood at diagnosis and short term after ART initiation. Our results strengthen previous findings on HIV diversity in seminal plasma. Furthermore, INIs decrease the number of quasispecies more efficiently than PI and NNRTI in both blood and seminal plasma.

**Table 4 Number of quasispecies according to the prescribed treatment regimen**

|                  | Blood | INI | PI | NNRTI | *Median (IQR, 25th–75th percentile). |
|------------------|-------|-----|----|-------|-------------------------------------|
|                  | N     | T0* | T1*|       |                                     |
| Blood            | 33    | 22  | 7  | 4      |                                     |
| INI              | 22    | 3   | 4  | 4      |                                     |
| PI               | 7     | 2   | 5  | 5      |                                     |
| NNRTI            | 4     | 3   | 6  | 6      |                                     |
| **Seminal plasma** | **33** | **22** | **7** | **4** | **1 (0–0.25)** |
| INI              | 22    | 6   | 5  | 6      |                                     |
| PI               | 7     | 5   | 7  | 6      |                                     |
| NNRTI            | 4     | 6   | 6  | 6      |                                     |

|                  | Blood | INI | PI | NNRTI | *Median (IQR, 25th–75th percentile). |
|------------------|-------|-----|----|-------|-------------------------------------|
|                  | N     | T0* | T1*|       |                                     |
| Blood            | 33    | 3   | 1  | 0      |                                     |
| INI              | 22    | 2   | 1  | 0      |                                     |
| PI               | 7     | 5   | 2  | 0      |                                     |
| NNRTI            | 4     | 6   | 1  | 0      |                                     |

**Key messages**

- There is a greater viral HIV diversity in seminal plasma than in blood before treatment.
- There is a greater viral HIV diversity in seminal plasma than in blood 1 month after treatment.
- Integrate inhibitors decrease the viral load in seminal plasma more rapidly than protease inhibitors or non-nucleosides, regardless of the viral load found before starting treatment.
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