Title:

Chronic and early antiretroviral therapy impact HIV serological assay sensitivity leading to more false negative test results in HIV diagnosis

Running title:

HIV diagnosis challenged by treatment

Authors:

Karolien Stoffels[1,10,11], Fien Vanroye[2,10], Virginie Mortier[3], Laurent Debaisieux[4], Marie-Luce Delforge[4], Melissa Depypere[8], Géraldine Dessilly[5], Dolores Vaira[6], Ellen Vancutsem[7], Sigi Van den Wijngaert[1], Kristel Van Laethem[8,9], Koen O.A. Vercauteren[2], Chris Verhofstede[3], Katrien Fransen[2]

[1] AIDS Reference Laboratory, Centre Hospitalier Universitaire St. Pierre, Brussels, Belgium
[2] HIV/STD Reference Laboratory, Clinical Virology Unit, Department of Clinical Sciences, Institute of Tropical Medicine, Antwerp, Belgium
[3] AIDS Reference Laboratory, Department of Diagnostic Sciences, Ghent University, Ghent, Belgium
[4] AIDS Reference Laboratory, Université Libre de Bruxelles, Brussels, Belgium
[5] AIDS Reference Laboratory, Medical Microbiology Unit, Université Catholique de Louvain, Brussels, Belgium
[6] AIDS Reference Laboratory, Centre Hospitalier Universitaire de Liège, Liège, Belgium
[7] AIDS Reference Laboratory, Vrije Universiteit Brussel VUB, Brussels, Belgium
[8] AIDS Reference Laboratory, Katholieke Universiteit Leuven, University Hospitals Leuven, Leuven, Belgium
[9] Laboratory of Clinical and Epidemiological Virology, Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium
[10] These two authors contributed equally to this work
[11] Corresponding author: arl@stpierre-bru.be

Summary

Antiretroviral treatment may cause false negative results in HIV diagnostic assays: false negativity was demonstrated for seroconverters with early treatment initiation in 12.0 to 31.3%, and for patients on suppressive treatment for more than 9 years in 2.1% to 4.9%.

1. Abstract

This retrospective study evaluated the reactivity of three HIV confirmatory assays (INNO-LIA®, Geenius™ and MP) and seven HIV rapid tests, on samples from two different study populations in Belgium.

For the early-treated cohort (83 HIV-1 adult patients treated within 3 months after infection), HIV-1 diagnosis was not obtained in at least one confirmatory assay in **12.0%** (10/83) and in an HIV rapid test in **31.3%** (26/83). Confirmation assay sensitivities ranged 87.5-95.2%, whereas rapid test assay sensitivities ranged 75.9-100%. The time to treatment initiation or the length of time on treatment did not have a statistical influence on the probability to obtain a false negative test result. The fastest reversion was demonstrated after 4 months of treatment.

Among the long-term treated cohort (390 HIV-1 patients with ≥9 years of undetectable viral load), false-negative test results were found in at least one HIV confirmatory assay for **2.1%** (8/390) of the patients and in a HIV rapid tests for **4.9%** (19/390). Confirmation assay sensitivities ranged 98.1-99.5%, whereas rapid test sensitivities ranged 96.2-100%. Longer treatment increased non-reactivity of the HIV rapid tests (p=0.033).

Undetectable viral load decreases the sensitivities of HIV diagnostic tests and further monitoring of the performance of serological assays is advised.

Keywords: HIV, HIV-diagnosis, HIV confirmatory assays, HIV rapid tests

2. Introduction

Depending on the test used, the HIV window period can range from a few days to 3 weeks [1]. In Belgium, a low HIV prevalence country, HIV screening is performed in clinical laboratories by Enzyme Immuno Assays (EIA), and in some local healthcare centers and non-governmental organizations by rapid tests as Point of Care (POC) testing. Samples from all patients with a reactive screening test result are further analyzed by specialized AIDS Reference Laboratories. In resource-constrained settings or remote places, HIV diagnosis is usually established by an algorithm of solely rapid tests [2]. In both situations, confirmation of a true infection in patients on antiretroviral therapy (ART) is challenging. ART may suppress virus replication for years and reduced antigen presence may result in waning of the host's antibody production [3, 4]. This might lead to a partial or complete loss of antibody detection which is well known for HIV infected newborns or young children taking ART [5-8]. However, only a few clinical cases of seroreversion or incomplete seroconversion have been reported in HIV-1 patients starting ART as adult [9-12]. Shortening the period to which the patient is exposed to replicating and disseminating viruses tends to diminish the immune response [4, 13]. Indeed, most cases of seroreversion at adult age are documented for patients initiating ART during the acute phase of HIV infection [13-18]. The implementation of continuous HIV Pre-Exposure Prophylaxis (PrEP) presents diagnostic settings with even more of a challenge to prove an established HIV infection, as immunoglobulin-based assays would remain non-reactive or become delayed reactive due to the interrupted antibody response. Viral load testing could only confirm the HIV infection in cases of viral escape due to treatment cessation or to PrEP resistance.

Even though WHO does not recommend retesting for diagnosis once a patient is on ART [19], it can occur in case of non-disclosure when visiting a new health care facility. In 2017, 7.2% (386/5331) of the requested confirmation tests in Belgium were not performed because the

patient was already registered as HIV infected in the addressed AIDS Reference Laboratory (unpublished data). In the past 10 years, at least 4 cases were recorded as having an indeterminate confirmation result due to years of ART intake in routine HIV clinical diagnostic settings in Belgium. This triggered an in depth investigation on a national level to assess the current situation. In this retrospective study, the sensitivity of the HIV confirmation assays currently used in Belgium was investigated, as well as some rapid tests used worldwide, using samples from adult patients on fully suppressive therapy for at least 9 years. These assays were also evaluated using samples from adults who started HIV treatment during the acute or early phase of infection.

3. Material and Methods

A retrospective study on two HIV-1 infected study groups was performed in Belgium. **The first study group consisted of HIV-1 infected patients treated long-term.** The inclusion criteria were patients with all plasma HIV-1 RNA viral load results below the quantification limit of the routine viral load test used at the time of sample collection for at least 9 years, and with a maximum interval of 18 months between 2 consecutive determinations. The patient's age at start of the undetectable viral load period was set at a minimum of 17 years. **The second group were HIV-1 infected adults initiating fully suppressive ART at early infection.** The inclusion criteria were HIV-1 diagnosis in 2010 or later, laboratory documented acute HIV-1 infection (i.e. HIV confirmation test negative or indeterminate, but with a positive p24 antigen test or detectable RNA viral load), start of ART within 3 months of diagnosis and no viral load blips once undetectable viral load was achieved. For both groups, the **most recent sample collected from each patient** was evaluated by the routine confirmation tests used in Belgium and by a selection of frequently used rapid tests. Collection dates were between 2011 and 2018 with 80% collected in 2016 or 2017. When an indeterminate, HIV negative or non-reactive result was observed in any of the evaluated assays, look-back samples (one sample per year) were tested until an HIV-1 positive result was obtained. Where possible, the evolution was further investigated with look-forward samples (collected in 2019). Any possible immune dysfunction of the patient was excluded using other laboratory test results and clinical data for each patient with an HIV test reversion. All tests were performed according to manufacturer's instructions and in compliance with the clinical laboratory's quality regulations ISO15189. The **confirmation tests** analyzed in this study were INNO-LIA® HIV I/II Score (FujiRebio), Geenius™ HIV 1/2 Confirmatory Assay (BioRad) using the Reader's interpretation (Geenius) and HIV Blot 2.2 (MP Biomedicals), further referred to as INNO-LIA, Geenius and MP Blot 2.2. **Rapid tests** were selected based on their use

in Belgian help centers and on global use (data provided by the World Health Organization): ABON™ HIV1/2/O Tri-Line Rapid Test Device (ABON Biopharm Hangzhou Co.Ltd.) (Abon), Alere Determine™ HIV 1/2 (Abbott) (Determine), First Response® HIV 1.2.0 Card Test (Premier Medical Co.Ltd.) (First response), INSTI™ HIV-1/HIV-2 AntibodyTest (BioLytical Laboratories) (INSTI), SD Bioline HIV 1/2 3.0 (Abbott) (SD Bioline), HIV 1/2 STAT-PAK® Assay (Chembio Diagnostic Systems Inc.) (StatPak) and HIV 1+2 Rapid Test (WANTAI Bio-Pharm) (Wantai). Except for Abon and Wantai, all were CE IVD labeled. False-negative test results were defined as negative or indeterminate for the HIV confirmation assays and non-reactive for the rapid tests.

HIV-1 viral load was determined with the automated systems from Roche cobas®, Abbott m2000 RealTime System or Siemens VERSANT®, depending on the site where the patient was followed. Subtype was determined by consensus with COMET HIV-1 v2.3 [20] and REGA HIV subtyping tool v3.41 [21] based on PR and RT sequences from de Pol region by in-house techniques, ViroSeq HIV-1 Genotyping System (Abbott) or TRUGENE HIV-1 Genotyping Assay (Siemens).

Statistical analysis was performed on the comparison of following groups: HIV-1 result in both INNO-LIA and Geenius assays versus indeterminate or negative result in at least one these two confirmatory assays. For the rapid tests, sample comparison groups were identified as HIV reactive in all seven rapid tests versus a non-reactive result in at least one rapid test. Statistical significance was set at <0.05 and depending on sample size and statistical distribution, the Fisher's exact test, Mann-Whitney U test or Chi Square test was retained using SPSS (IBM SPSS Statistics for Windows, Version 23.0. Released 2015; IBM Corp, Armonk, NY). Data concerning time periods were grouped into quartiles for analysis. Univariate logistic regression was used to investigate the influence of treatment duration in the long term treated group and multivariate

logistic regression to investigate whether treatment duration or time to ART initiation influenced the serological test outcomes in the early treated group.

This **study** is representative for the whole of Belgium as all seven AIDS Reference Laboratories eligible for HIV confirmation and follow-up in Belgium participated. The study was performed on encoded remnant samples, excluding patients who opted-out for sample use in scientific research. Ethics committee approval was obtained at the leading investigator's center, University Medical Center St-Pieter in Brussels n°O.M.007 and registered as approval CE/17-11-11.

4. Results

Long term ART: HIV confirmatory assays

A total of 390 patients were included in the **first group of patients with a long period of undetectable HIV-1 viral loads**, ranging between 8.6 and 20.9 years with an average of 13.0 years. HIV-1 infection could not be confirmed on the most recent sample available in 1.5% (6/390) and 0.5% (2/390) for INNO-LIA and Geenius, respectively. All samples with an indeterminate result in INNO-LIA were reported as HIV-1 positive by Geenius, and vice versa. A completely negative profile (i.e. none of the HIV-1 specific bands positive) was not observed. MP HIV Blot 2.2 was evaluated on 54 of these 390 samples, of which 1.9% (1/54) scored as indeterminate (**table 1**). This sample had an indeterminate test result in INNO-LIA and an HIV-1 positive result in Geenius. In total, eight different patients (8/390 = 2.1%) could not be confirmed as HIV-1 infected by at least one of these three confirmatory assays.

A full HIV-1 profile (i.e. all HIV-1 specific bands positive) was observed in 56.4% (220/390), 17.2% (67/390) and 3.7% (2/54) of the samples in INNO-LIA, Geenius and MP Blot 2.2, respectively. The band capturing the gp41 antibodies was the only one detected in all assay test results (**table 2**). Overall weakening of the band strength was observed over time. For 37.9% (148/390) of the samples, the INNO-LIA result of the most recent sample could be compared with the original INNO-LIA result around time of diagnosis (mean of 12 years of fully suppressive ART). The band score weakened with a score of 1.4 on average over all 5 bands taken together (**table 3**). Looking at the duration of undetectable viral load for all 390 samples, the INNO-LIA indeterminate samples were spread over the four quartiles, while for Geenius, both indeterminate samples were found in Q4 (14.3 to 20.9 years). Taking both assays together, comparison between the HIV-1 group and the indeterminate group (in either INNO-LIA or Geenius) did not reveal a statistical significant influence of treatment duration (p=0.707), or any other parameter (age,

year start treatment). The HIV-1 subtype could not be compared because only 30.3% (118/390) of the population was subtyped, of which only 2 samples were from the indeterminate group.

Long term ART: HIV rapid tests

From the seven evaluated rapid tests, only three tests were able to detect all samples as HIV reactive: Abon, Determine and Wantai (**table 1**). The sensitivity of the StatPak was the lowest with 3.8% (15/390) false negative test results, of which 33.3% (5/15) were sampled in Q2 (after 11.3 to 12.3 years of undetectable viral) and 46.7% (7/15) in Q4 (after 14.3 to 20.9 years). Taking all rapid tests together, treatment duration showed a statistical significant influence on the probability to obtain a false negative test result in at least one of the seven rapid tests (p=0.033).

Long term ART: Analysis over time

Yearly look-back samples could be analyzed for 18 patients from the 19 with at least one false negative test result. The first reversion was identified after a mean of 9.5 [5.8;14.5], 11.75 [7.7;15.8] and 12.6 [6.7;19.2] years of undetectable plasma viral load for INNO-LIA (5/18), Geenius (2/18) and rapid tests (15/18), respectively (**figure 1**).

Early ART initiation after infection: HIV confirmatory assays

The **second study group consisted of 83 adults with acute HIV-1 infection treated within 3 months of diagnosis**. ART was started at a mean age of 37.3 years and between 0 and 88 days after diagnosis (mean 23.5 days, median 14.0 days). The studied samples were collected after 2.9 years on ART on average and detection of HIV-1 infection failed for 4.8% (4/83) and 8.4% (7/83) of the patients using INNO-LIA and Geenius, respectively (**table 4**). Analysis of look-back and supplementary look-forward samples showed the first reversion in four cases for the INNO-LIA assay after an average of 1.4 years treatment. For the Geenius assay, the first reversion was observed in six cases (including two INNO-LIA reversions) after a mean of 2.4 years of ART, with

further evolution towards a negative result in one case while it remained indeterminate in the INNO-LIA assay (Ac-07). Three additional cases never evolved to an HIV-1 positive result and remained indeterminate. The fastest reversion was demonstrated after 13 and 11 months of ART with the INNO-LIA and Geenius assays, respectively (**table 5**). One sample was HIV negative in INNO-LIA while indeterminate in Geenius (Ac-02). Taking both assays together with 9/83 patients with at least one false negative test result, time on ART (p=0.460) or time to ART initiation (p=0.727) did not influence the confirmatory test outcomes in this early treated study group. Mean viral load at diagnosis for the samples with an indeterminate or negative confirmatory test result (5.92 log copies/ml) was significantly lower compared to the mean viral load of HIV-1 positive confirmations (6.63 log copies/ml) (p=0.031 Mann Whitney U test). Other parameters were not found to be statistically significant (age, CD4 count at diagnosis, subtype B vs non-B, integrase strand transfer inhibitor dolutegravir or elvitegravir in first line treatment). The gp41 capturing band was most frequently present. Recent samples from 16 patients from this cohorte were additionally tested by MP Blot 2.2, of which 12.5% (2/16) did not result in a HIV-1 diagnosis. In total, 10 different patients (10/83 = 12.0%) could not be confirmed as HIV-1 infected by at least one of the three confirmatory assays after a mean of 2.2 years of treatment.

Early ART initiation after infection: HIV rapid tests

Again, rapid tests Abon, Determine and Wantai showed a 100% HIV detectability while the StatPak assay was the least performant, with a sensitivity of 75.9% (**table 1**). Overall, the same percentage of HIV-1 non-reactive specimens was found in all quartiles, ranging from 0 to 88 days until treatment initiation after acute diagnosis. The length of time on ART (p=0.974) or the time to ART initiation (p=0.967) did not influence the rapid test outcomes. All other investigated parameters did not reveal statistical significant differences between the group with at least one non-reactive test and the group with a consistent reactive test result (CD4 count at diagnosis,

viral load at diagnosis, age, subtype B vs non-B, use of integrase strand transfer inhibitor dolutegravir or elvitegravir in first line treatment). Look-back samples demonstrated the HIV rapid test reversion for 14 patients after mean of 2.4 years of treatment (13/14 StatPak, 4/14 INSTI, 1/14 SD Bioline and 1/14 First Response). Additionally, samples from 6 patients never evolved to a reactive test result for StatPak (6 patients), INSTI (1/6) and SD Bioline (1/6).

5. Discussion

Monitoring of the diagnostic HIV assays is essential as antibody response fades on continuous, efficient ART [3, 4]. The combination of highly efficient new ART molecules, the 'test and treat'-strategy, PrEP and increased effective treatment duration may influence the performance of HIV antibody-based diagnostic assays [22, 23]. Long term suppression of plasma viral load is an easy marker for sample selection in the surveillance of possible negativation of HIV diagnostic assays. It must, however, be kept in mind that other processes might continue stimulating immune responses, for example, virus release in lymph nodes due to lack of penetration of the administered ART to the lymphatic tissues, while maintaining undetectable plasma viral load [24]. This may be an explanation for the fact that we only could observe a statistical link between length of treatment and test result reversion for the rapid tests in the chronic treated group. As at least one confirmatory assay (INNO-LIA or Geenius) had a reactive gp41 band for each tested sample in this study, the more sensitive and gp41 based EIA screening tests are assumed to have a reactive result on the study samples (not assessed). The quantification of HIV-1 DNA could not be tested in the samples with confirmatory reversion because whole blood or buffy coat was not available. In any case, HIV-DNA testing is not a valid alternative as it is not cost-effective enough to be performed in routine analysis for all negative or indeterminate confirmation test results. As long as routine EIA screens remain reactive after many years of suppressive treatment, a negative confirmatory assay result might be disputed by the clinician who could further question the patient (e.g., initial non-disclosure by the patient) but nevertheless the danger of missed diagnosis remains. Moreover, seroreversion on 3rd and 4th generation EIA has already been proven on samples from patients initiating ART in the early acute infection stage [17, 25]. The risk of having low HIV-1 antibody levels due to ART uptake during early HIV-1 infection entails a high risk for misdiagnosis of these patients [26, 27].

In this evaluation, especially the early-treated HIV seroconverters were prone to false negative results in HIV confirmatory assays and HIV rapid tests. A baseline factor associated with non-positivity of the confirmatory assays was the lower viral load at time of diagnosis, but not the CD4 count, differing from de Souza et al., where both factors were statistically significant in the evaluation of EIA assays and western blot [17]. In contrast, initial viral load in non-reactive rapid tests was slightly higher than in the reactive group (p=0.283 Mann-Whitney U test). Although 2nd generation HIV integrase strand transfer inhibitor administration during acute HIV infection might accelerate seroreversion, this could not be demonstrated for HIV confirmatory assays (p=0.312) or HIV rapid tests (p=0.132). Future monitoring is however recommended, based on the recent observation that two seroconverting patients with plasma viral loads of >7.0 log copies/ml obtained undetectable viral load at their follow-up consultation only 12 and 35 days after bictegravir/emtricitabine/tenofovir alafenamide initiation, with a positive but incomplete Geenius profile and thus a suspected aborted antibody response (unpublished data, author's experience). Bictegravir/emtricitabine/tenofovir alafenamide was not administered to the patients of the investigated study cohorts.

HIV POC testing is commonly used in resource constrained and non-clinical settings. While they are easy to use and results are obtained faster than with other serology screening tests, some of the 3rd generation rapid tests fail to detect HIV-1 antibodies in early HIV infection [23, 28]. The impact of efficient antiretroviral treatment was most clearly seen on the StatPak performance in both study groups. Possible inhibition caused by a particular antiretroviral drug was not found, but was only superficially investigated. There was no common factor and the same ART was taken at the time of a reactive and a non-reactive StatPak result. The second worst performing POC test was INSTI. In the seroconverters cohort, a non-reactive INSTI result was obtained in 8.4% (7/83) of the samples after an average of 3.3 years of treatment, which is perfectly in line

with the 9.1% (4/44) INSTI non-reactivity after 3 years of suppressive ART reported by a French study group [18]. Time from infection to treatment initiation was statistically not linked with non-reactivity of the rapid tests, something that was also observed by the same French research. The limitations of this retrospective study are the limited number of specimens with an indeterminate, negative or non-reactive test result and the use of stored frozen specimens even though freeze-thaw cycles were kept to a minimum. Additionally, all 7 different HIV rapid tests were performed with one single production lot, impeding assessment of lot-to-lot variation. Missed (treated) HIV infections by the current diagnostic tests might lead to life detrimental situations, for example, drug-drug interactions, blood or tissue donation to immune compromised patients. It might also lead to AIDS status if the patient decides to stop all ART after a non-reactive self-test or rapid test, something that has already been observed at least once in Belgium (unpublished data) and such cases could contribute to HIV epidemiological expansion. Other markers to identify an established HIV infection in an easy, fast and cost-effective way will be required in the upcoming years.

In conclusion, assays used to confirm an HIV infection and make the differentiation between a real and a false reactive EIA HIV screening test, were not 100 % reliable for patients on ART. A false negative test result was observed in at least one of the three tested assays (INNO-LIA, Geenius and MP Blot 2.2) in 2.1% (8/390) of the adult patients with undetectable plasma viral load for at least 9 years and in 12.0% (10/83) of the seroconverters treated within 3 months. At the same time, at least one of the seven rapid tests generated a non-reactive result in 4.9% (19/390) of the patients on long term efficient ART and in 31.3% (26/83) of the seroconverters. Most non-reactive results were obtained with StatPak. Patient non-disclosure might have a significant clinical impact when entering care in regions where diagnosis is based on an algorithm of rapid tests. Future monitoring is necessary as most samples analyzed in this study were

287 collected in 2016-2017 and new, highly effective ART molecules have become available since
288 then.

289

6. Acknowledgements

All seven participating Belgian AIDS Reference Laboratories are supported by the Belgian Ministry of Social Affairs through a fund within the Health Insurance System. No conflict of interest for each author.

Footnote:

- No conflict of interest
- Funded by Ministry of Social Affairs within the Health Insurance System
- The results from this manuscript comparing all test results performed on the same sample has not been presented before

7. References

1. Delaney KP, Hanson DL, Masciotra S, Ethridge SF, Wesolowski L, Owen SM. Time Until Emergence of HIV Test Reactivity Following Infection With HIV-1: Implications for Interpreting Test Results and Retesting After Exposure. Clinical Infectious Diseases **2016**; 64:53-9.

2. World Health Organization. Consolidated Guidelines on HIV Testing Services: 5Cs: Consent, Confidentiality, Counselling, Correct Results and Connection 2015. Available at: https://www.ncbi.nlm.nih.gov/books/NBK316021/. Accessed 16 March 2020.

3. Falkensammer B, Freissmuth D, Hubner L, Speth C, Dierich MP, Stoiber H. Changes in HIV-specific antibody responses and neutralization titers in patients under ART. Front Biosci **2007**; 12:2148-58.

4. Keating SM, Pilcher CD, Jain V, et al. HIV Antibody Level as a Marker of HIV Persistence and Low-Level Viral Replication. The Journal of infectious diseases **2017**; 216:72-81.

5. Hainaut M, Alexandra Peltier C, Goetghebuer T, et al. Seroreversion in Children Infected with HIV Type 1 who are Treated in the First Months of Life Is Not a Rare Event. Clinical Infectious Diseases **2005**; 41:1820-1.

6. Zanchetta M, Anselmi A, Vendrame D, et al. Early therapy in HIV-1-infected children: effect on HIV-1 dynamics and HIV-1-specific immune response. **2008**; 13:47.

7. Sherman G, Lilian R, Coovadia A. The Performance of 5 Rapid HIV Tests Using Whole Blood in Infants and Children Selecting a Test to Achieve the Clinical Objective. The Pediatric infectious disease journal **2012**; 31:267-72.

8. Kuhn L, Schramm DB, Shiau S, et al. Young age at start of antiretroviral therapy and negative HIV antibody results in HIV-infected children when suppressed. **2015**; 29:1053-60.

9. Gutiérrez M, Soriano V, Bravo R, Vallejo A, Gonzalez-Lahoz J. Seroreversion in Patients with End-Stage HIV Infection. **1994**; 67:238-9.

10. Metadilogkul O, Jirathitikal V, Bourinbaiar AS. Serodeconversion of HIV antibody-positive AIDS patients following treatment with V-1 Immunitor. J Biomed Biotechnol **2009**; 2009:934579-.

11. Ellenberger DL, Sullivan PS, Dorn J, et al. Viral and immunologic examination of human immunodeficiency virus type 1-infected, persistently seronegative persons. The Journal of infectious diseases **1999**; 180:1033-42.

12. Cornelissen M, Jurriaans S, Prins JM, Bakker M, van der Kuyl AC. Absence of seroreversion in 80 HAART-treated HIV-1 seropositive patients with at least five-years undetectable plasma HIV-1 viral load. AIDS Res Ther. Vol. 3, **2006**:3.

13. Killian MS, Norris P, Rawal B, et al. The Effects of Early Antiretroviral Therapy and Its Discontinuation on the HIV-Specific Antibody Response. AIDS research and human retroviruses **2006**; 22:640-7.

14. Jurriaans S, Sankratsing S, Prins M, et al. HIV-1 seroreversion in an HIV-1-seropositive patient treated during acute infection with highly active antiretroviral therapy and mycophenolate mofetil. Inflammatory Bowel Diseases - INFLAMM BOWEL DIS **2004**; 18:1607-8.

15. Kassutto S, Johnston MN, Rosenberg ES. Incomplete HIV Type 1 Antibody Evolution and Seroreversion in Acutely Infected Individuals Treated with Early Antiretroviral Therapy. Clinical Infectious Diseases **2005**; 40:868-73.

16. Hare CB, Pappalardo BL, Busch MP, et al. Seroreversion in Subjects Receiving Antiretroviral Therapy during Acute/Early HIV Infection. Clinical Infectious Diseases **2006**; 42:700-8.

17. de Souza MS, Pinyakorn S, Akapirat S, et al. Initiation of Antiretroviral Therapy During Acute HIV-1 Infection Leads to a High Rate of Nonreactive HIV Serology. Clinical Infectious Diseases **2016**; 63:555-61.

18. Stefic K, Novelli S, Mahjoub N, et al. Nonreactive Human Immunodeficiency Virus Type 1 Rapid Tests After Sustained Viral Suppression Following Antiretroviral Therapy Initiation During Primary Infection. The Journal of infectious diseases **2018**; 217:1793-7.

19. World Health Organization. Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection

Recommendations for a public health approach - Second edition. Available at: https://www.who.int/hiv/pub/arv/arv-2016/en/. Accessed 16 March 2020.

20. Struck D, Lawyer G, Ternes A-M, Schmit J-C, Bercoff DP. COMET: adaptive context-based modeling for ultrafast HIV-1 subtype identification. Nucleic Acids Res **2014**; 42:e144-e.

21. Pineda-Peña A-C, Faria NR, Imbrechts S, et al. Automated subtyping of HIV-1 genetic sequences for clinical and surveillance purposes: Performance evaluation of the new REGA version 3 and seven other tools. Infection, Genetics and Evolution **2013**; 19:337-48.

22. Shahar E, Shapiro A, Baskin L, Oz ZK. Antiretroviral therapy-induced negative HIV antibody test following diagnosis of HIV infection. **2019**; 33:1804-5.

23. Fransen K, de Baetselier I, Rammutla E, et al. Performance of serological and molecular tests within acute HIV infection. Journal of Clinical Virology **2017**; 93:81-4.

24. Fletcher CV, Staskus K, Wietgrefe SW, et al. Persistent HIV-1 replication is associated with lower antiretroviral drug concentrations in lymphatic tissues. Proc Natl Acad Sci U S A **2014**; 111:2307-12.

25. Manak M, Peel S, Malia J, et al. Loss of HIV Serological Markers Following Early Treatment of Acute HIV Infection. CROI (Conference on Retroviruses and Opportunistic Infections). Boston, **2014**.

26. Johnson CC, Dalal S, Baggaley R, Taegtmeyer M. A public health approach to addressing and preventing misdiagnosis in the scale-up of HIV rapid testing programmes. Journal of the International AIDS Society **2017**; 20:22190.

27. Bock P, Phiri C, Piwowar-Manning E, et al. Understanding low sensitivity of community-based HIV rapid testing: experiences from the HPTN 071 (PopART) trial in Zambia and South Africa. Journal of the International AIDS Society **2017**; 20:21780-.

370 28. Patel P, Bennett B, Sullivan T, Parker MM, Heffelfinger JD, Sullivan PS. Rapid HIV screening: Missed
371 opportunities for HIV diagnosis and prevention. Journal of Clinical Virology **2012**; 54:42-7.

372

8. Tables and figures

Table 1. Sensitivity of HIV confirmatory assays and HIV rapid tests in long-term treated HIV-1 patients and early-treated HIV-1 seroconverters

		CE IVD label	Long-term treated HIV-1 patients [a]	Early-treated HIV-1 seroconverters [b]
HIV Confirmatory assays	**INNO-LIA®** HIV I/II Score (FujiRebio)	Yes	98.5% (384/390)	95.2% (79/83)
	Geenius™ HIV 1/2 Confirmatory Assay (BioRad)	Yes	99.5% (388/390)	91.6% (76/83)
	HIV Blot 2.2 (**MP** Biomedicals)	Yes	98.1% (53/54) [c]	87.5% (14/16) [d]
HIV Rapid tests	**ABON**™ HIV1/2/O Tri-Line Rapid Test Device (ABON Biopharm Hangzhou Co.Ltd.)	No	100.0% (390/390)	100.0% (83/83)
	Alere Determine™ HIV 1/2 (Abbott)	Yes	100.0% (390/390)	100.0% (83/83)
	HIV 1+2 Rapid Test (**WANTAI** Bio-Pharm)	No	100.0% (390/390)	100.0% (83/83)
	First Response® HIV 1.2.0 Card Test (Premier Medical Co.Ltd.)	Yes	99.7% (389/390)	96.4% (80/83)
	SD Bioline HIV 1/2 3.0 (Abbott)	Yes	99.5% (388/390)	92.8% (77/83)
	INSTI™ HIV-1/HIV-2 AntibodyTest (BioLytical Laboratories)	Yes	98.7% (385/390)	91.6% (76/83)
	HIV 1/2 **STAT-PAK®** Assay (Chembio Diagnostic Systems Inc.)	Yes	96.2% (375/390)	75.9% (63/83)

[a] HIV-1 test result from patients with undetectable HIV-1 viral load for ≥9 years
[b] HIV-1 test result from patients with acute HIV-1 infection treated within 3 months
[c] only 54 samples from the 390 selected were analyzed
[d] only 16 samples from the 83 selected were analyzed

382

383 **Table 2. Long-term treated HIV-1 patients: clinical and performance characteristics**

	INNO-LIA® HIV I/II Score (FujiRebio)		**Geenius™** HIV 1/2 Confirmatory Assay (BioRad)		**All 7 HIV rapid tests**		
	HIV-1	**Indeterminate**	**HIV-1**	**Indeterminate**	**Reactive**	**Non-reactive in at least 1 rapid test**	**Total**
Number of samples	98.5% (384/390)	1.5% (6/390)	99.5% (388/390)	0.5% (2/390)	95.1% (371/390)	4.9% (19/390)	390
Mean age at start undetectable viral load period (years)	40.9	37.9	40.9	30.3	40.8	41.7	40.9
Time between first undetectable viral load and most recent sample tested (years)							
Mean	13.0	12.0	13.0	15.3	12.9	14.3	13.0
Q1							11.3
Q2 (median)	12.3	12.3	12.3	15.3	12.3	13.8	12.3
Q3							14.3
Q4 (max)							20.9
Band profile confirmatory assay					N/A	N/A	
Presence of **gp160**	N/A	N/A	100.0% (388/388)	0.0% (0/2)			**99.5%** (388/390)
Presence of **gp120**	95.3% (366/384)	0.0% (0/6)	N/A	N/A			**93.8%** (366/390)
Presence of **gp41**	100.0% (384/384)	100.0% (6/6)	100.0% (388/388)	100.0% (2/2)			**100.0%** (780/780)
Presence of **p31**	70.6% (271/384)	16.7% (1/6)	22.7% (88/388)	0.0% (0/2)			**46.2%** (360/780)
Presence of **p24**	91.4% (351/384)	0.0% (0/6)	53.4% (207/388)	0.0% (0/2)			**71.5%** (558/780)
Presence of **p17**	80.7% (310/384)	16.7% (1/6)	N/A	N/A			**79.7%** (311/390)

384 N/A = Not Applicable

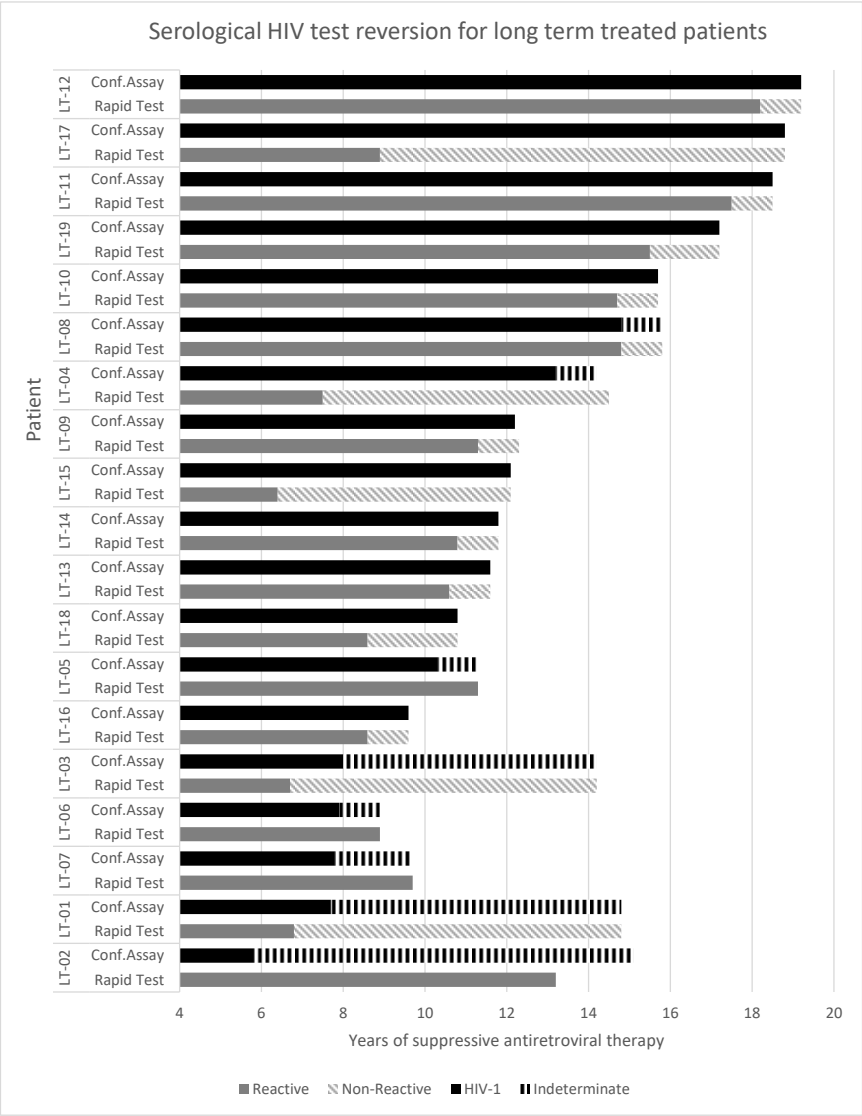
385

386 **Table 3. Long-term treated HIV-1 patients: Weakening of band intensities in INNO-LIA confirmatory**
387 **assays after a mean of 12 years antiretroviral therapy**

Average band scores INNO-LIA	gp120 ENV1	gp41 ENV1	p31 POL	p24 GAG	p17 GAG	Complete HIV-1 band profile (%) [a]	Average presence of the HIV-1 profile (%) [b]
Around time of diagnosis (A)	2.9	3.5	2.4	2.9	2.5	87.8% (130/148)	97.1
After averagely 12y ART (B)	2.0	2.5	1.3	2.0	1.7	57.4% (85/148)	89.5
Difference in band score between A and B	-1.27	-1.22	-1.54	-1.46	-1.52	-30.4%	-7.6

388 [a] Number of samples with a complete HIV-1 band profile in test result (all HIV-1 bands positive)
389 [b] Overall average of the HIV-1 profile test result: 100% = all 5 bands positive, 80% = 4/5 bands positive etc.
390

391 **Figure 1. Long-term treated: Reversion time identified by analysis of look-back samples (1 sample per**
392 **year)**
393



394

Table 4. Early-treated HIV-1 seroconverters: clinical and performance characteristics

	INNO-LIA® HIV I/II Score (FujiRebio)		**Geenius™** HIV 1/2 Confirmatory Assay (BioRad) using Reader		**All 7 HIV rapid tests**		
	HIV-1	**Indeterminate or negative**	**HIV-1**	**Indeterminate**	**Reactive**	**Non-reactive in at least 1 rapid test**	**Total**
Number of samples	95.2% (79/83)	4.8% ((3IND+1NEG)/83)	91.6% (76/83)	8.4% (7/83)	68.7% (57/83)	31.3% (26/83)	83
Mean age at start ART (years)	37.7	29.5	37.6	34.4	37.3	37.4	37.3
Mean viral load at diagnosis (log cp/ml) [a]	6.61	5.50	6.61	6.02	6.65	6.38	6.56
Mean CD4 count at diagnosis (#/µl)	447	571	438	601	439	483	453
Start ART after diagnosis (days)							
Mean	23.6	21.8	23.6	22.1	23.8	22.8	23.5
Q1							7.0
Q2 (median)	14.0	9.5	14.0	12.0	14.0	13.5	14.0
Q3							38.5
Q4 (max)							88.0
Time between start ART and most recent sample tested (years)							
Mean	2.9	2.6	2.9	2.4	2.9	2.9	2.9
Q1							1.3
Q2 (median)	2.5	2.6	2.7	1.8	2.6	2.2	2.5
Q3							4.1
Q4 (max)							8.7
HIV-1 subtypes B [b]	52.1% (38/73)	66.6% (2/3)	53.6% (37/69)	42.9% (3/7)	53.8% (28/52)	50.0% (12/24)	52.6% (40/76)
Band profile confirmatory assay					N/A	N/A	
Presence of **gp160**			100.0% (76/76)	0.0% (0/7)			**91.6%** (76/83)
Presence of **gp120**	62.0% (49/79)	0.0% (0/4)					**59.0%** (49/83)
Presence of **gp41**	100.0% (79/79)	75.0% (3/4)	100.0% (76/76)	100.0% (7/7)			**99.4%** (165/166)
Presence of **p31**	16.5% (13/79)	0.0% (0/4)	3.9% (3/76)	0.0% (0/7)			**9.6%** (16/166)
Presence of **p24**	94.9% (75/79)	0.0% (0/4)	31.6% (24/76)	0.0% (0/7)			**59.6%** (99/166)
Presence of **p17**	43.0% (34/79)	0.0% (0/4)					**41.0%** (34/83)

[a] cp/ml = copies/ml in plasma

[b] based on PR and RT sequences from Pol region; 96.4% (80/83) of the samples could be subtyped

Table 5. Early-treated HIV-1 seroconverters: reversion of the confirmation test result and their STAT-PAK® test results

Patient ID (REV/NP) [a]	HIV-1 subtype	Start treatment after diagnosis (days)	Time after treatment initiation (days)	HIV-1 viral load (cp/ml) [b]	CD4 (#/µl)	INNO-LIA® HIV I/II Score [c]	Geenius™ HIV 1/2 Confirmatory Assay using Reader [c]	HIV 1/2 STAT-PAK® Assay [d]
AC-01	B	12	598	<20	500	HIV-1	IND	NR
(G REV)			38	79	671	HIV-1	HIV-1	R
			-12	970 000	587	NEG	HIV-1	R
AC-02	B	12	1322	<20	1193	NEG	IND	NR
(G REV, I REV)			658	<20	1353	IND	HIV-1	NR
			332	<20	1724	HIV-1	IND	NR
			29	<20	1251	HIV-1	HIV-1	R
			-12	306 000	867	IND	IND	/
AC-03	A1	8	1407 – 84	<20	900 – 1605	HIV-1	IND	NR
(G NP)			27	50	364	HIV-1	IND	NR
			-8	415 000	527	NEG	NEG	/
AC-04	B	7	1125	<20	791	IND	IND	NR
(G REV, I REV)			786	<20	676	IND	IND	NR
			390	<20	640	IND	HIV-1	NR
			0	333000	484	HIV-1	IND	R
			-7	Unknown (p24 Ag pos)	unknown	NEG	NEG	/
AC-05	02_AG	47	672	<40	1140	HIV-1	IND	NR
(G REV)			575	<40	1362	HIV-1	IND	/
			98	<40	1036	HIV-1	HIV-1	R
			-47	314 000	1004	IND	IND	/
AC-06	G	7	1188 – 440	<40	679 – 829	IND	IND	NR
(I REV; G NP)			29	83	1040	HIV-1	IND	NR
			-5	Unknown (p24 Ag pos)	316	/	/	NR
AC-07	Unknown	61	1373	<40	1853	IND	NEG	/
(G REV, I REV)			1019	<40	1112	IND	IND	/
			788	<40	1174	IND	HIV-1	NR
			611	<40	1212	IND	/	/
			123	100	1365	HIV-1	/	NR
			29	unknown	618	/	/	R
AC-08	B	54	106	<40	834	HIV-1	IND	NR
(G NP)			-48	223000	727	NEG	NEG	NR
AC-09	01_AE	15	1925	<20	639	HIV-1	IND	R
(G REV)			1526	<20	848	/	HIV-1	/
			-15	$>10^7$	182	NEG	/	/

[a] G = Geenius™ HIV 1/2 Confirmatory Assay using Reader(BioRad) ; I = INNO-LIA® HIV I/II Score (FujiRebio) ; REV = confirmatory assay Reversion ; NP = confirmatory assay Never became Positive

[b] cp/ml = copies/ml in plasma

[c] IND = indeterminate ; NEG = HIV negative ; / = not performed

[d] NR = non-reactive ; R = reactive ; / = not performed