Corso Precongressuale D
NEXT GENERATION SEQUENCING:
SUE APPLICAZIONI IN VIROLOGIA

HIV resistenze, applicazioni e vantaggi

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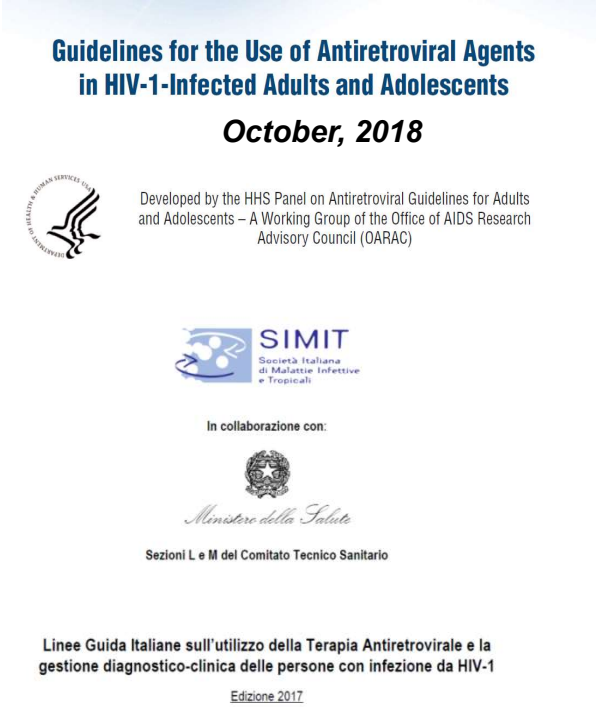
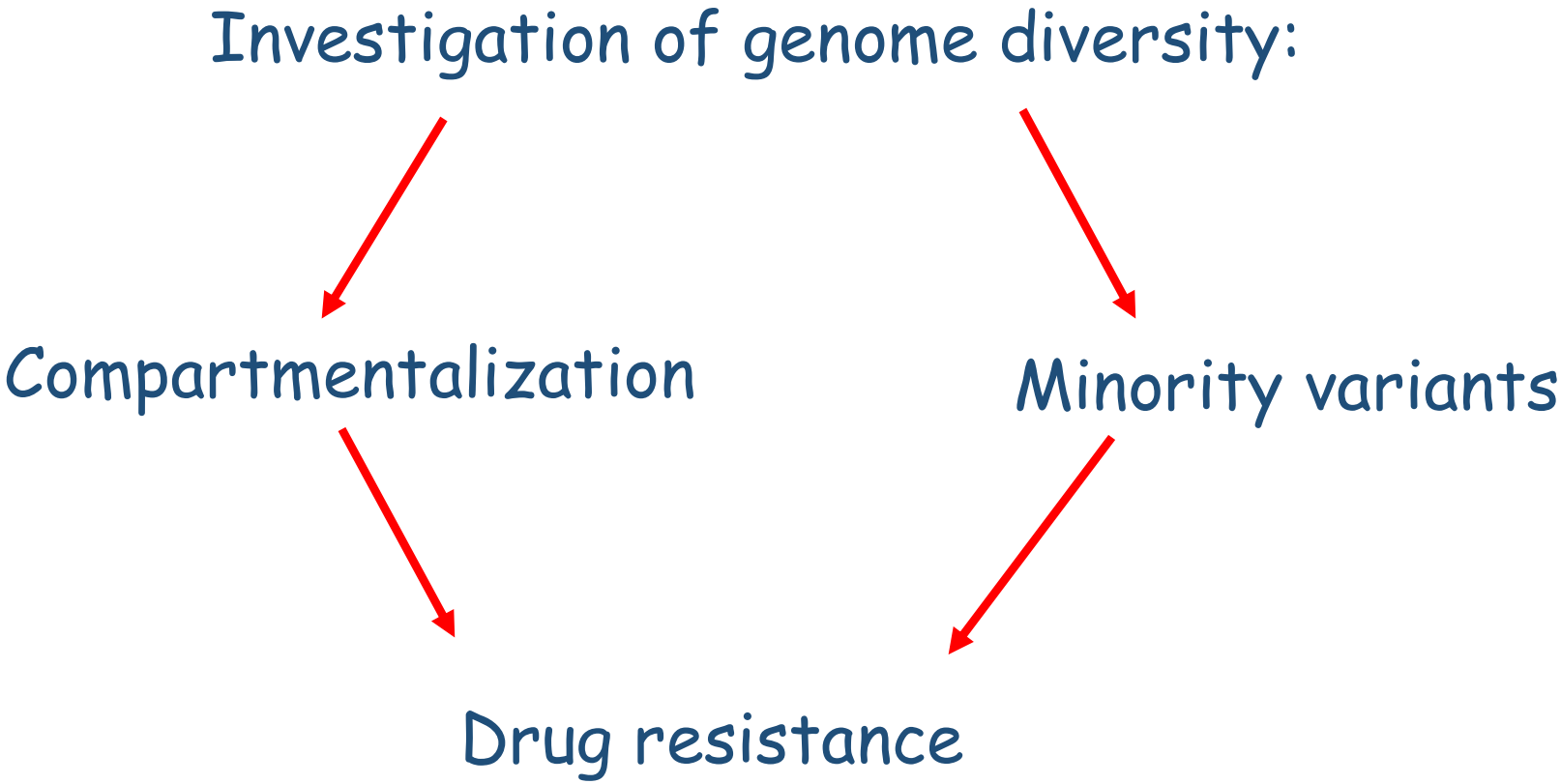
Genotypic resistance testing in HIV-1 infected patients is now recommended to guide the choice of antiretroviral therapy in clinical practice both in drug-naïve and drug-treated patients



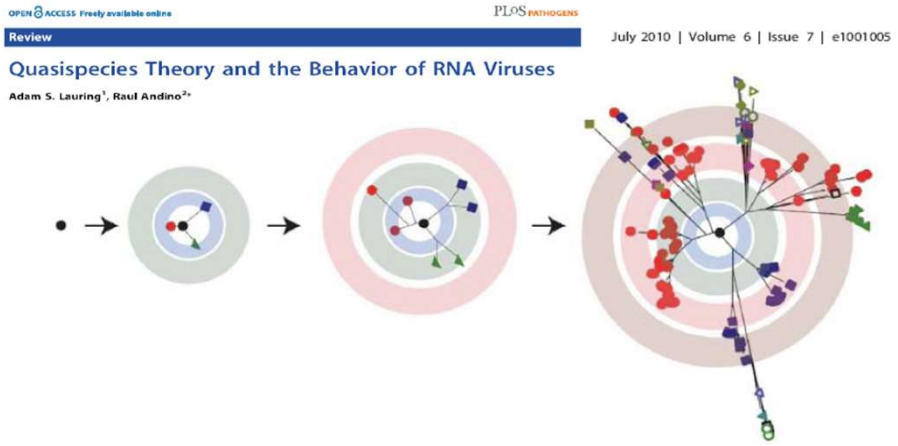
The arrival of a variety of NGS platforms has revolutionized the field of virology

The assessment of the complexity of the viral population has been performed by analyzing regions of the genome exhibiting different degrees of genetic variability and to a lesser extent the full-length viral genome

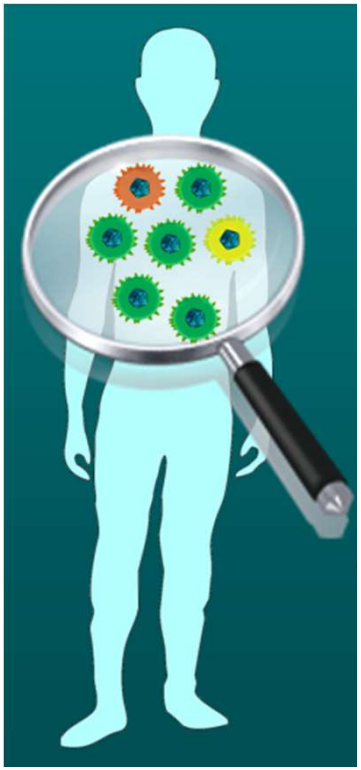
This is of particular interest in virology since the high genetic variation observed in many viruses results in the establishment of a swarm of genetically related but distinct viral variants within the host, named quasispecies.


Investigation of genome diversity:
Compartmentalization
Minority variants
Drug resistance

Viral quasispecies:
a concept derived from the observation that, in an individual infected for a long time, there is a continued evolution of the virus, as a result of spontaneous mutations and selective pressures exerted by host's immunity and drug therapy.

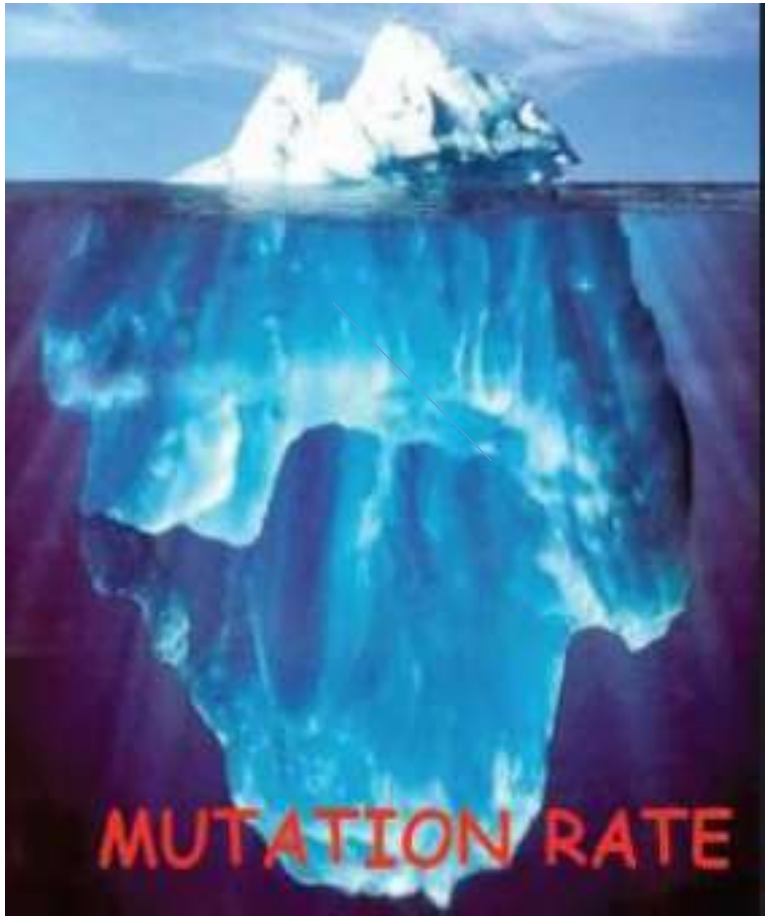


RNA viruses: error prone nature of the RNA-dependent RNA polymerase, 7×10^{-4} to 5.4×10^{-3} mutations per site per infectious cycle



NGS or Sanger?

The assessment of viral genome variability within the host and detection of low-abundance antiviral drug-resistance mutations using NGS approaches have been the foundation of the new era of the molecular epidemiology of viral diseases



The Sanger methodology detects only mutations with a frequency greater than 20%

With NGS the strains present at frequencies lower than 0.1% are detected

Traditional population-based (Sanger) sequencing of HIV is able to detect quasi-species representing on average at least 20% of a viral population.

This has led to concerns that resistant viral variants present as minor species (below 20%) are not being detected, with consequent **under-representation of both primary** (or transmitted) **and secondary** (or drug-induced) **resistance**.

Table 4 Characteristics NGS technologies used in HIV sequencing

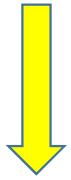
Platform	Systems	Reads Lenth, Output, Time	Error type %	Advantage	Disadvantage	Library Amplification	Principle
Illumina	MiSeq (v2. or v.3)	300bp PE, 15Gb, 1-2 Days	Substitution 0,1%	High sequence yield, Streamlined library preparation, Low input DNA concentration required,	Short sequence size complicates Haplotype reconstruction Substitution error	PCR amplification of adapter ligated fragments	Sequencing by synthesis. Bridge-PCR on flow cell surface
	NextSeq 500 High	75bp SE,150bp PE, 129Gb, 1 Day	Substitution <1%				
	NextSeq 500 Mid	75bp SE,150bp PE, 16-40Gb, 1 Day	Substitution <1%				
	HiSeq 2500 v2Rapid	36 bp SE 250bpPE, 9-150 Gb,3 Days	Substitution 0,1%				
	HiSeq 2500 v3	36bp PE,100bp PE, 47-300Gb,2-11 Days	Substitution 0,1%				
	HiSeq 2500 v4	36bp PE,125bp PE, 64-500Gb,1-6 Days	Substitution 0,1%				
	HiSeq 3000/4000	2x150bp, 105-750Gb, 1-3,5Days	Substitution 0,1%				
Thermo Fisher SOLID	Solid 5500 Wildfire	50-75bp SE, 80-160Gb, 6 Days	AT bias, ≤0,1%	Two base encoding corrects for intrinsic error	Long run time	Emultion PCR on microbeads (PCR on FlowChip for 5500Wmodels)	Sequencing by ligation
	Solid 5500XL	50-75bp SE, 160-320Gb, 10 Days	AT bias, ≤0,1%				
Thermo Fisher Ion Torrent	Ion PGM 314	200-400bp SE, 30-100Mb, 1 Day	Indel, 1%	Low expensive equipment	Homopolymer error (eg K65R, K103N in RT)	Emultion PCR on microbeads	Semiconductor based sequencing
	Ion PGM 316	200-400bp SE, 300Mb-1Gb, 3-5h	Indel, 1%				
	Ion PGM 318	200-400bp SE, 600Mb-2Gb, 4-7h	Indel, 1%				
	Ion Proton	Up to 200bp SE, 10Gb, 2-4h	Indel, 1%				

PE: paired-end; SE single end; bp: base-pairs; Mb: megabyte; Gb: gigabyte; h: hours.

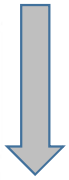
Marino-Merlo F et al. Appl Microbiol Biotechnol. 2018

Why use next generation sequencing in management of HIV-1 infection? What are the advantages?

- Next generation sequencing allows to detect hidden minority resistance variants and theoretically to prevent their selection during treatment
- Cost: to date it is not so much expensive
  - If something, it is cheaper than Sanger



- Labour intensity: to date it doesn't need hard working



- Requires bioinformatic support: to date there are available tools

In the last years, investigators from different parts of the globe have started to show how ultra-deep sequencing can reveal details about drug resistance and tropism not detectable by the methods currently used in labs and clinics…

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J Antimicrob Chemother
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Journal of Antimicrobial Chemotherapy

'Sentinel' mutations in standard population sequencing can predict the presence of HIV-1 reverse transcriptase major mutations detectable only by ultra-deep pyrosequencing

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Objective

This proof-of-concept study aims at identifying in drug-naïve patients whether few selected mutations, **T69S**, **L210M** and **K103R**, found at **3** **reverse transcriptase (RT) resistance positions** and **easily detected by standard population sequencing** (GRT), can act as "***sentinel mutations"*** by predicting **hidden minor species with primary drug resistance mutations** (DRMs).

OPEN ACCESS Freely available online

Minority HIV-1 Drug Resistance Mutation Present in Antiretroviral Treatment–Naïve Populations and Associate with Reduced Treatment Efficacy

Jeffrey A. Johnson[1*], Jin-Fen Li[1], Xierong Wei[1], Jonathan Lipscomb[1], David Irlbeck[2], Charles Craig[3], Ar

Background

Transmitted HIV-1 drug resistance can compromise initial antiretrovira
therefore, its detection is important for patient management. The absence o
selection pressure in treatment-naïve persons can cause drug-resistant viru
levels undetectable by conventional bulk sequencing (minority drug-resist
used sensitive and simple tests to investigate evidence of transmitted d

antiretroviral drug-naïve persons and assess the clinical implications of minori
variants.

Methods and Findings

We performed a cross-sectional analysis of transmitted HIV-1 drug resista
control study of the impact of minority drug resistance on treatment respons
sectional analysis, we examined viral RNA from newly diagnosed ART-naïve p
and Canada who had no detectable (wild type, $n = 205$) or one or more r
mutations ($n = 303$) by conventional sequencing. Eight validated real-time P
were used to test for minority drug resistance mutations (protease L9
transcriptase M41L, K70R, K103N, Y181C, M184V, and T215F/Y) above nat
frequencies. The sensitive real-time PCR testing identified one to three minority
mutation(s) in 34/205 (17%) newly diagnosed persons who had wild-type virus
genotyping; four (2%) individuals had mutations associated with resistance to t
Among 30/303 (10%) samples with bulk genotype resistance mutations we fo
minority variant with a different drug resistance mutation. For the case-co

assessed the impact of three treatment-relevan
separate group of 316 previously ART-naïve persons with no evidence of dr
bulk genotype testing who were placed on efavirenz-based regimens. We foun
persons who experienced virologic failure had minority drug resistance mutat
however, minority resistance was found in only 2/221 (0.9%) treatment succes

Johnson et al Plos Med 2008

Fraction of Treatment Success or Failure Versus the Presence of Detectable Minority Drug Resistance Mutations for the 316 Treatment Study Participants Evaluated

Mutation Status	Treatment Success ($n = 221$)	Treat ($n =$
No detectable drug resistance mutation	219 (99.1%)	88 (92
Minority drug	2 (0.9%)	7 (7.4

Fisher exact test, p= 0.0038

•In the baseline samples of this previously ART-naive group we were again able to identify minority treatment relevant mutations.

• The minority mutations were significantly associated with virologic failure using the Fisher exact test (p =0.0038) and in a logistic model.

•These results suggest that minority transmitted resistance mutations can be clinically important

Johnson et al Plos Med 2008

Minority resistant quasispecies are associated with high risk of virologic failure in patients receiving an initial NNRTI-based ART regimen.

Low-Abundance Drug-Resistant Viral Variants in Chronically HIV-Infected, Antiretroviral Treatment–Naive Patients Significantly Impact Treatment Outcomes

Birgitte B. Simen,[1,a] Jan Fredrik Simons,[1,a] Katherine Huppler Hullsiek,[3] Richard M. Novak,[4] Rodger D. MacArthur,[5] John D. Baxter,[6] Chunli Huang,[1] Christine Lubeski,[1] Gregory S. Turenchalk,[1] Michael S. Braverman,[1] Brian Desany,[1] Jonathan M. Rothberg,[1] Michael Egholm,[1] and Michael J. Kozal[2] for the Terry Beirn Community Programs for Clinical Research on AIDS

[1]454 Life Sciences, a Roche Company, Branford, and [2]Yale University School of Medicine and Veterans Affairs Connecticut Healthcare System, New Haven, Connecticut; [3]Division of Biostatistics, University of Minnesota, Minneapolis, Minnesota; [4]University of Illinois College of Medicine at Chicago, Department of Medicine, Chicago, Illinois; [5]Wayne State University School of Medicine, Department of Medicine, Detroit, Michigan; [6]Cooper University Hospital, University of Medicine and Dentistry, New Jersey–Robert Wood Johnson Medical School, Camden, New Jersey

(See the editorial commentary by Shafer, on pages 610–2.)

Background. Minor (i.e., <20% prevalence) drug-resistant human immunodeficiency virus (HIV) variants may go undetected, yet be clinically important.

Objectives. To compare the prevalence of drug-resistant variants detected with standard and ultra-deep sequencing (detection down to 1% prevalence) and to determine the impact of minor resistant variants on virologic failure (VF).

Methods. The Flexible Initial Retrovirus Suppressive Therapies (FIRST) Study ($N = 1397$) compared 3 initial antiretroviral therapy (ART) strategies. A random subset ($n = 491$) had baseline testing for drug-resistance mutations performed by use of standard sequencing methods. Ultra-deep sequencing was performed on samples that had sufficient viral content ($N = 264$). Proportional hazards models were used to compare rates of VF for those who did and did not have mutations identified.

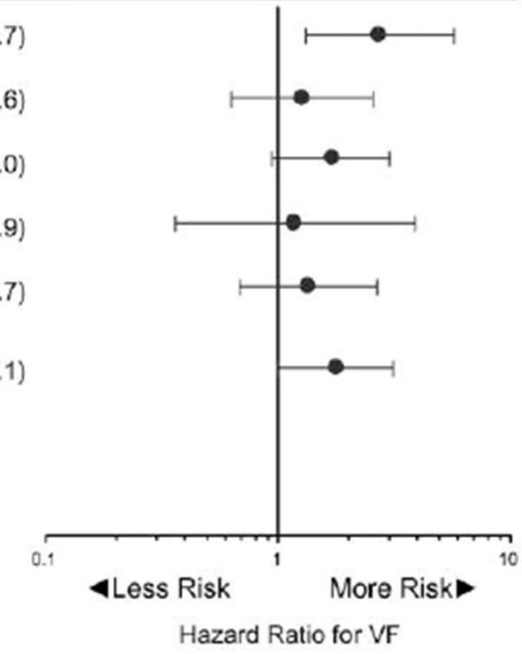
Results. Mutations were detected by standard and ultra-deep sequencing (in 14% and 28% of participants, respectively; $P < .001$). Among individuals who initiated treatment with an ART regimen that combined nucleoside and nonnucleoside reverse-transcriptase inhibitors (hereafter, "NNRTI strategy"), all individuals who had an NNRTI-resistance mutation identified by ultra-deep sequencing experienced VF. When these individuals were compared with individuals who initiated treatment with the NNRTI strategy but who had no NNRTI-resistance mutations, the risk of VF was higher for those who had an NNRTI-resistance mutation detected by both methods (hazard ratio [HR], 12.40 [95% confidence interval {CI}, 3.41–45.10]) and those who had mutation(s) detected only with ultra-deep sequencing (HR, 2.50 [95% CI, 1.17–5.36]).

Conclusions. Ultra-deep sequencing identified a significantly larger proportion of HIV-infected, treatment-naive persons as harboring drug-resistant viral variants. Among participants who initiated treatment with the NNRTI strategy, the risk of VF was significantly greater for participants who had low- and high-prevalence NNRTI-resistant variants.

Simen et al JID 2009

A.

Baseline Mutation Pattern	Participants, no.	VF Events, no.	VF	
			P	HR (95%CI)
Any NNRTI	11	11	.007	2.7 (1.3-5.7)
Any NRTI	14	10	.51	1.3 (0.6-2.6)
Any NNRTI or NRTI	22	18	.07	1.7 (1.0-3.0)
Any PI	4	3	.79	1.2 (0.4-3.9)
Any PI or NRTI	15	11	.38	1.4 (0.7-2.7)
Any Mutation	23	19	.05	1.8 (1.0-3.1)
No Mutations	61	40		
Overall	84	59		



B

Figure 2. Hazard ratios (HR) for virologic failure (VF) by baseline drug-resistance mutation pattern and initial antiretroviral therapy (ART) regimen. *A,* Initial ART regimen, nonnucleoside reverse-transcriptase inhibitor (NNRTI) and nucleoside reverse-transcriptase inhibitor (NRTI). *B,* Initial ART regimen, protease inhibitor (PI) and NRTI. *C,* Initial ART regimen, NNRTI + PI + NRTI. Baseline mutation pattern is described in terms of mutations detected with ultra-deep sequencing using the Stanford HIV Drug-Resistance Database Mutation algorithm. VF was defined as plasma HIV RNA level >1000 copies/mL at or after the 4-month visit. CI, confidence interval.

Simen et al JID 2009

Low-Frequency HIV-1 Drug Resistance Mutations and Risk of NNRTI-Based Antiretroviral Treatment Failure

A Systematic Review and Pooled Analysis

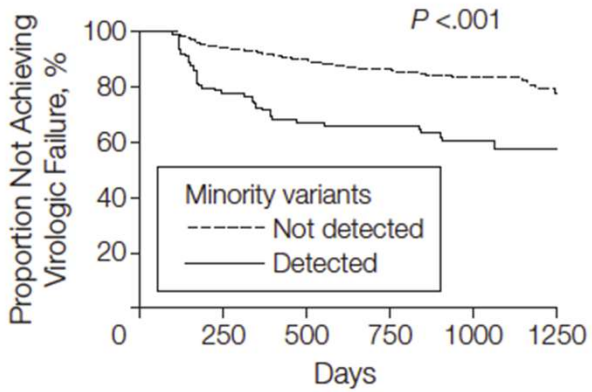
Systematic Review and Baseline Characteristics

Ten studies with 985 patients were identified as meeting the inclusion and exclusion criteria.

The median CD4 cell count was 229 cells/mm3 and mean plasma HIV-1 RNA level was 5.0 log10 copies/mL.

All studies evaluated the presence of K103N. Other commonly evaluated minority variants included Y181C (N=435) and the NRTI mutations M184V (N=228) and K65R (N=163).

Figure 2. Kaplan-Meier Curves for Proportion of Patients Without Virologic Failure by Presence of Drug-Resistant HIV-1 Minority Variants



No. at risk

Minority variants						
Not detected	691	620	455	398	344	46
Detected	117	86	60	53	37	7

- Minority drug-resistant variants were found in 14% (117/808).
- 35% of those with detectable minority variants experienced virologic failure as compared to 15% of those without minority variants.

Li et al JAMA 2011

PRE-EXISTING LOW-LEVELS OF THE K103N HIV-1 RT MUTATION AI THRESHOLD IS ASSOCIATED WITH VIROLOGICAL FAILURE IN TRE

DD Goodman[1], NA Margo[2], DJ McColl[2], MD Miller[2], K Borroto-Esoda[1] an

[1]Gilead Sciences, Inc., Durham, NC, USA; [2]Gilead Sciences, Inc., Foster City, CA, USA

Antivir Ther **2009; 14 Suppl 1:A43 (abstract no. 41)**

RESULTS: AS-PCR results were obtained for 476/485 samples. Overall, 16/476 (3 K103N by AS-PCR but not by population sequencing, increasing the percentage of t patients with NNRTI resistance to 7.5% (38/509). Six of 16 patients (37.5%) with lo showed VF (n=5 in the 3TC+ZDV group and n=1 in FTC+TDF group) and statistica strong correlation between pre-existing low-level K103N and VF within the 3TC+Z (P=0.005). The size of the K103N subpopulation at baseline correlated with VF. Wi

5,535 copies/ml of K103N). The presence of K103N above 2,000 copies/ml strongl (5/6 with VF versus 1/10 without VF; P=0.008). For the one FTC+TDF patient witl and VF, only 0.8% K103N (1,254 copies/ml) was detected; however, this patient ha

CONCLUSIONS: Utilization of AS-PCR resulted in an overall increase in detecti treatment-naïve patients as compared with detection by population sequencing only low-levels of K103N was associated with risk of VF within the 3TC+ZDV group. T

Quantity, rather than just presence, of minority resistant quasispecies, plays a game in regulating the efficacy of NNRTI-based first line therapies

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Low-frequency HIV-1 drug resistance mutations can be
significant but must be interpreted with cautio

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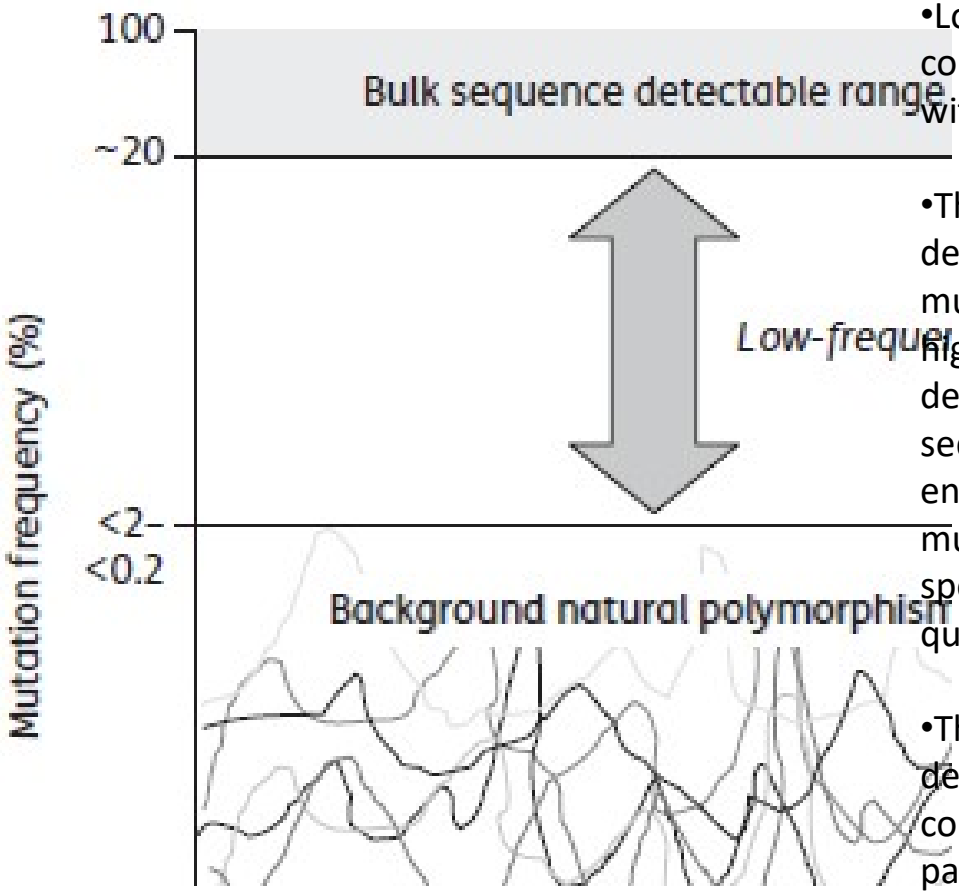
With drug-resistant HIV-1 present in at least 10%–20% of new infections in Western countries and
patients failing antiretroviral therapy (ART), monitoring HIV-1 drug resistance is becoming increas
tant for assessing its impact on therapeutic measures of virus control and for guiding treatment. Th
limitations of conventional bulk genotyping often lead to an underestimation of the total burden o
ance in a patient, as resistant variants escape detection when present at low frequency within the
species. Using sensitive resistance testing methods, a few investigators have linked low-frequency r
poor treatment outcomes, while other studies have shown no correlation. Understanding the tec
ations of sensitive testing methods and the relevance of the amount of a particular resistance
the context of different ART regimens will help to define the clinical benefit of low-frequenc



•Low-frequency HIV-1 variants containing mutations associated with drug resistance.

•The low-frequency range is defined at the upper end by mutations that are present at high frequency and can be detected by bulk population sequencing, and at the lower end by the natural background of mutations that emerge spontaneously within the viral quasispecies.

•The background reactivity was determined using samples collected from HIV-1-infected patients in the years preceding the introduction of ART.

An Italian case of transmitted integrase inhibitor resistance in a drug-naïve patient: a refined analysis by ultra-deep-454 pyrosequencing

Ada Bertoli[1], **Daniele Armenia[1]**, Maria Mercedes Santoro[1], Maria Concetta Bellocchi[1], Luca Carioti[1], Claudia Alteri[1], Maurizio Mariani[2], Francesca-Ceccherini Silberstein[1], Alessandro Grimaldi[2], Carlo Federico Perno[1,3], Giovanna Picchi[2]

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[3]Tor Vergata University Hospital, Rome, Italy

Clinical Case ID 17310: anamnesis

- In February 2016, a 45-years-old man, in previous good-health conditions, referred to an outpatient clinic complaining neck lymph nodes enlargement since 5 years, with worsening during the past 6 months.

- During the past five years blood test had shown normal levels of Anti-streptolysin titer (TAS), Reactive-C-protein, White blood cells; Toxoplasma serology was negative, with previous CMV and HBV infection*; HCV and HIV screening, performed 2 years before, were both negative.

- After visit, screenings for HIV and syphilis were prescribed.

Methods

Diagnosis:

- ✓ IV generation serological screening test (ARCHITECT HIV Ag/Ab Combo assay)
- ✓ Immunoblotting
- ✓ HIV-1 RNA quantification

Genotyping:

- ✓ Protease, reverse transcriptase (RT) and integrase genotypic resistance tests (GRT) were performed after diagnosis, firstly through Sanger sequencing and after screening trough 454 GS-Junior ultra-deep pyrosequencing (UDPS)
- ✓ Plasma, PBMC and cerebrospinal fluid (CSF) compartments were evaluated

Resistance evaluation:

- ✓ Resistance to protease inhibitors, RT inhibitors (RTIs) and INIs was evaluated by Stanford HIV Drug-resistance list 2015
- ✓ Mutational-load (% resistant variant x HIV-RNA) in plasma and CSF was calculated per each resistance mutation

Tropism determination

- ✓ HIV-1 co-receptor usage was determined through gp120-V3-loop Sanger sequencing by using the geno2pheno algorithm
  (false positive rate [FPR %] set at 10%)

Clinical Case ID 17310: diagnosis (March 2016)

- **The patient was positive for HIV-1** (IV generation serological screening test confirmed with immunoblotting)
- **Patient was also positive for syphilis** (VDRL positive, TPHA :1/1280).

- **HIV-RNA: 92,470 copies/mL**
- **CD4 cell count: 567 cell/µL**
- **Co-morbidities: hypertriglyceridemia**

At diagnosis genotypic resistance test (GRT) on protease (PR) and reverse trascriptase (RT) was performed by Sanger sequencing (as indicated by current guidelines)

Tabella 6 - Impiego del test di resistenza in PR e RT nella gestione del paziente naïve alla cART.

IMPIEGO	RACCOMANDAZIONE (FORZA/EVIDENZA)	RAZIONALE	RIFERIMENTI BIBLIOGRAFICI
In tutti i pazienti naïve che iniziano una cART.	[AI]	Negli ultimi anni, la prevalenza in Italia di farmaco-resistenze per le tradizionali classi di antivirali (inibitori della PR e inibitori della RT) in pazienti naïve alla cART varia dal 6 al 10% essa è maggiore nei pazienti infettati con virus di sottotipo B rispetto a quelli infettati con ceppi non-B.	[3,12,16,24-26]
Su un campione il più vicino possibile alla diagnosi di infezione, sia in pazienti con infezione acuta, sia in pazienti con infezione cronica.	[AII]	L'effettuazione del test in prossimità della diagnosi permette di apprezzare in tempo reale il trend di trasmissione delle mutazioni di resistenza.	
Sarebbe utile ripetere il test in pazienti naïve anche al momento dell'inizio della terapia cART.	[BII]	Il test in pazienti naïve al momento dell'inizio della terapia può dare indicazioni riguardo l'evolutività del virus e la cinetica di scomparsa delle mutazioni (in soggetti con resistenza trasmessa). In pazienti in cui si riconosca una perpetuazione di comportamenti a rischio è da considerare un possibile rischio di superinfezione con ceppi di HIV farmaco-resistenti.	

	Assessment	At HIV diagnosis	Prior to starting ART
Virology	Confirmation of HIV Ab pos	+	
	Plasma HIV-VL	+	+
	Genotypic resistance test and sub-type	+	+/-
	R5 tropism (if available)		+/-

EACS European AIDS Clinical Society

Panel's Recommendations
For Antiretroviral Therapy-Naive Patients:
• HIV drug-resistance testing is recommended for persons with HIV infection at entry into care to guide selection of the initial antiretroviral therapy (ART) regimen (**AII**). If therapy is deferred, repeat testing may be considered at the time of ART initiation (**CIII**).
• Genotypic testing is recommended as the preferred resistance testing to guide therapy in antiretroviral (ARV)-naive patients (**AIII**).
• In special circumstances (e.g., in patients with acute or recent [early] HIV infection and in pregnant HIV-infected women, ART initiation should not be delayed while awaiting resistance testing results; the regimen can be modified once results are reported (**AIII**).
• Standard genotypic drug-resistance testing in ARV-naive persons involves testing for mutations in the reverse transcriptase (RT) and protease (PR) genes. If transmitted integrase strand transfer inhibitor (INSTI) resistance is a concern, providers should ensure that genotypic resistance testing also includes INSTI genotype testing (**BIII**).

Plasma GRT: presence of transmitted 3TC/FTC resistance

March 2016
VL: 92,000 cps/ml
CD4: 460 cells/µl

Subtype: F1

Resistance mutations

PR: L10V, M36I, L63A, V77I, L89M

RT: M184M/V

Due to the presence of TDR, to complete viral genotypic profile, integrase (IN) GRT and tropism determination were performed

Plasma GRT: presence of transmitted 3TC/FTC resistance and also Raltegravir/Elvitegravir resistance

March 2016
VL: 92,000 cps/ml
CD4: 460 cells/µl

Subtype: F1

Resistance mutations

PR: L10V, M36I, L63A, V77I, L89M

RT: M184M/V

IN: T97A, N155N/H

Tropism: X4 tropic (geno2pheno false positive rate : 3.8%)

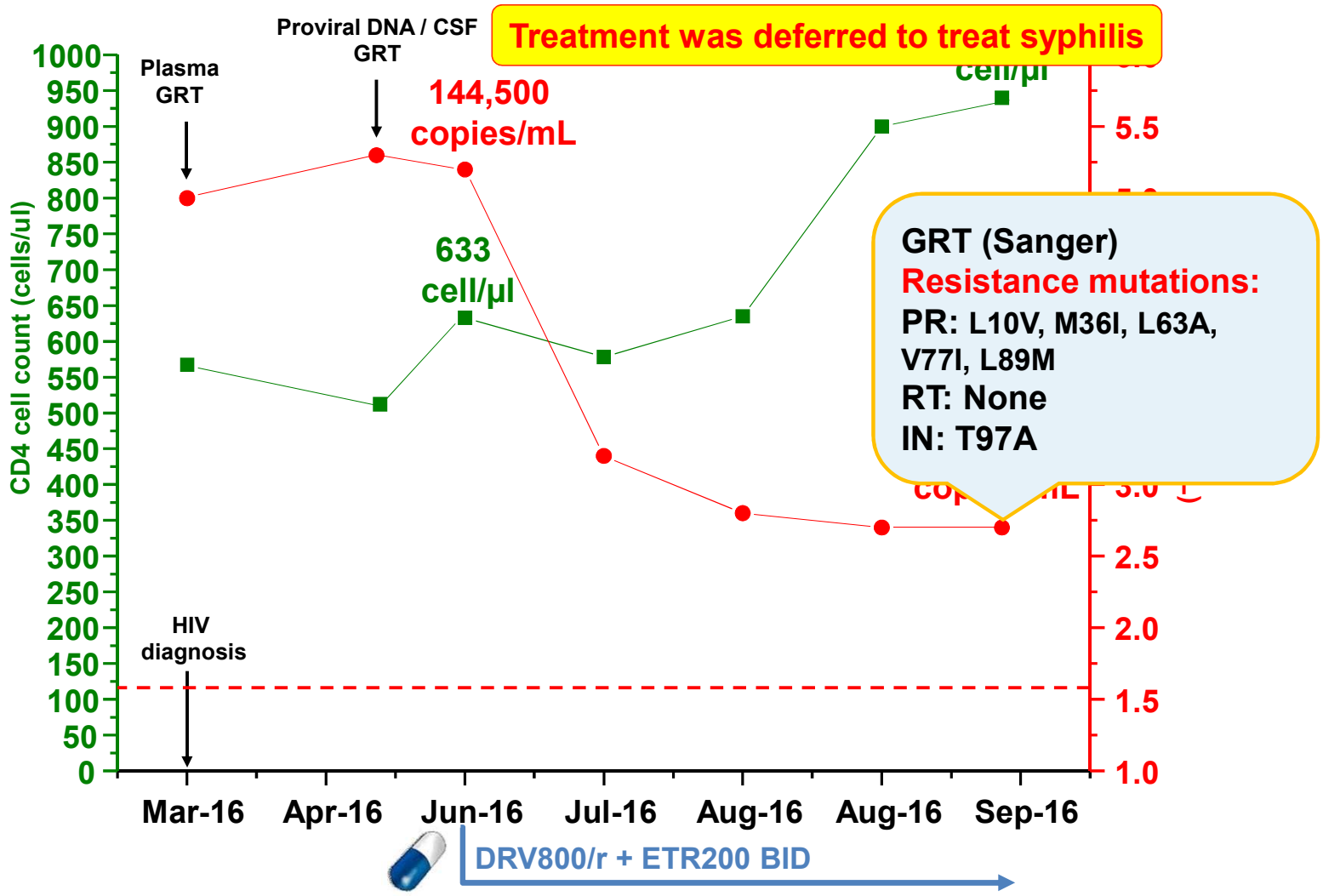
Thus UDPS was also performed…

Overview of UDPS GRT before cART start

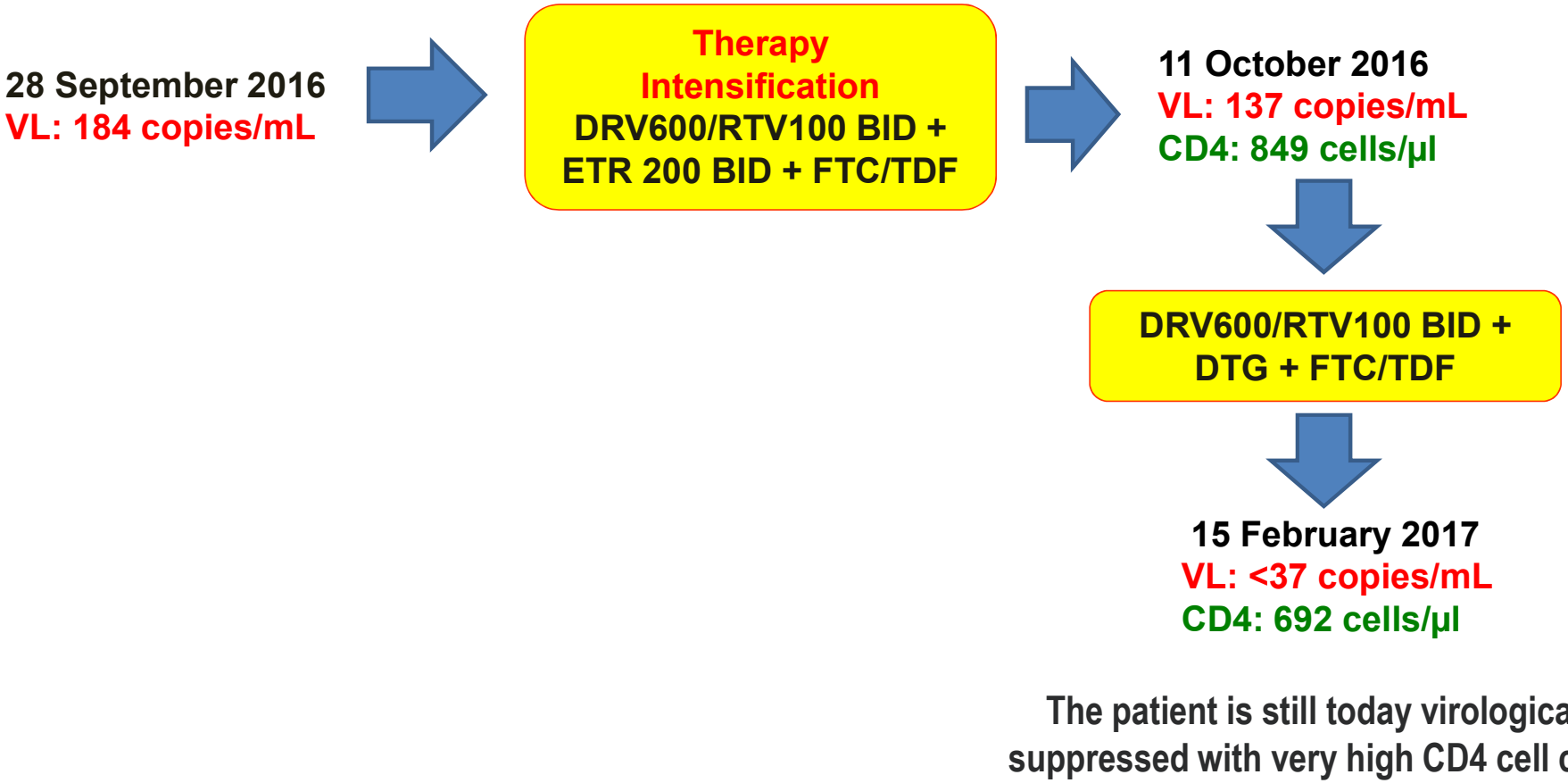
GRT from plasma	GRT from PBMC	GRT from CSF
March 2016 **VL: 92,000 cps/mL** **CD4: 460 cells/µl** **UDPS Coverage** **(mean ± SD seq/position):** **PR= 1,793 ± 651** **RT= 4,549 ± 1,500** **IN= 1,310 ± 688** **Resistance mutations:** **PR:** L10V (100%), M36I (100%), L63A (100%) V77I (100%), L89M (100%) **RT: M184V (39.4%; 36,248 cps/mL)** **IN:** T97A (91.9%, 84,548 cps/mL), **N155H (38.8%; 35,696 cps/mL)**	**May 2016** **Plasma VL: 196,700 cps/mL** **CD4: 512 cells/µl** **UDPS Coverage** **(mean ± SD seq/position):** **PR= ND** **RT=6,640 ± 1,558** **IN=1,180 ± 1,154** **Resistance mutations:** **PR:** ND **RT: M184I (0.8%)**, **M184V (9.4%)**, V179D (5.4%) **IN:** T97A (100%), **N155H (11.3%)**	**May 2016** **CSF VL: 21,427 cps/mL** **CD4: 512 cells/µl** **UDPS Coverage** **(mean ± SD seq/position):** **PR= 6,729 ± 2,612** **RT= 3,343 ± 1,597** **IN= 2,035 ± 1,267** **Resistance mutations:** **PR:** L10V (100%), M36I (100%), L63A (100%) V77I (100%), L89M (100%) **RT:** V179D (10.9%; 2,336 cps/mL) **IN:** T97A (97.0%; 20,784 cps/mL)

Underlined: mutations detected only by UDPS

Clinical Case: ID 17310 Patient infected with HIV-1 F1 subtype	Age: 45	Sex: M	Risk Factor: Sexual	1st Seropositivity: 2016



Clinical Case: ID 17310 Patient infected with HIV-1 F1 subtype	Age: 45	Sex: M	Risk Factor: Sexual	1st Seropositivity: 2016



The patient is still today virologically suppressed with very high CD4 cell count

What about the usefulness NGS in HIV infected patients under treatment?

Clinical Infectious Diseases Advance Access published May 29, 2

MAJOR ARTICLE

Improved Prediction of Salvage Antiretr Therapy Outcomes Using Ultrasensitive Drug Resistance Testing

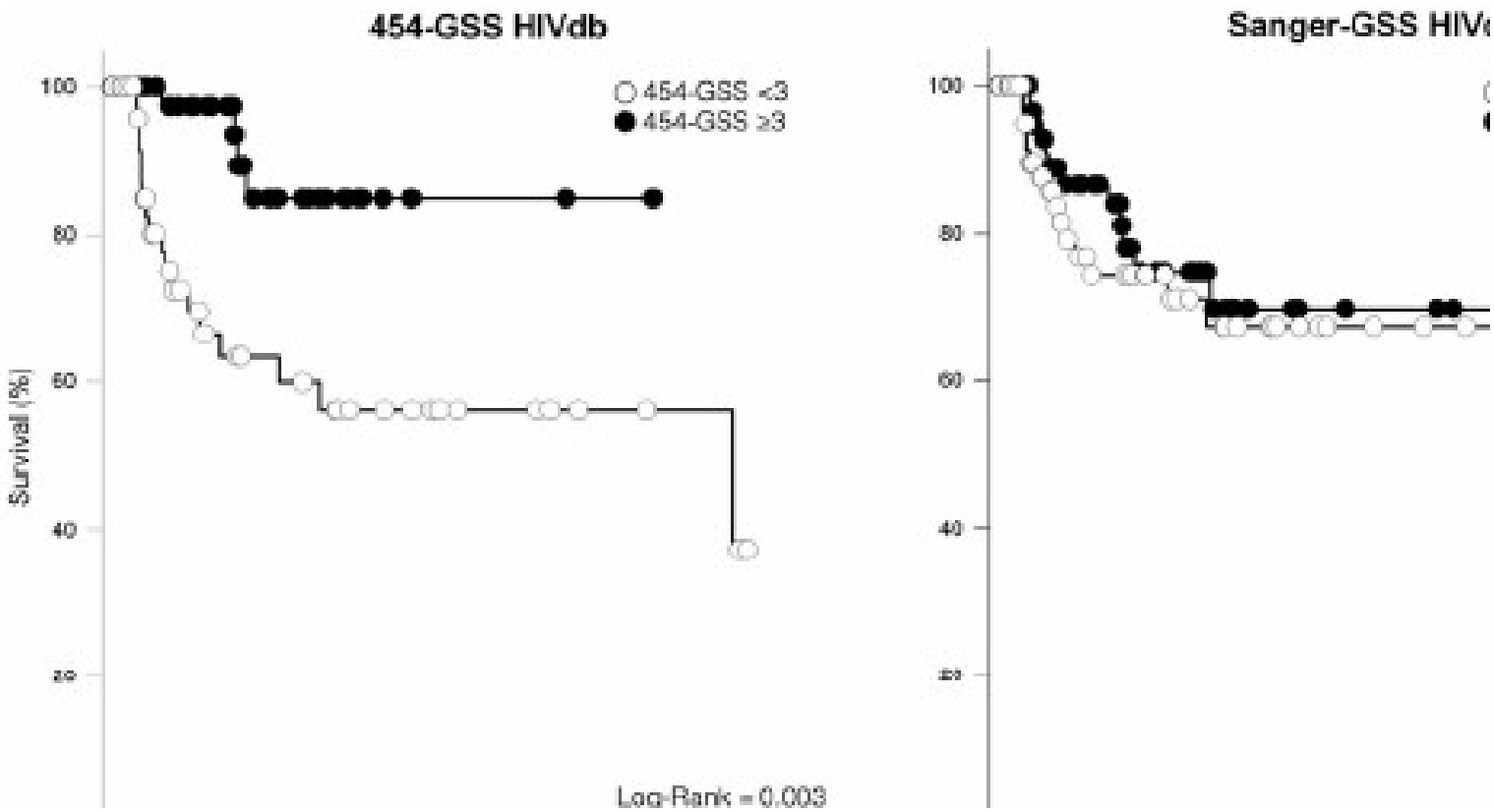
BACKGROUND: The **clinical relevance** of **ultrasensitive** human immunodeficiency virus type 1 **(HIV-1) genotypic** resistance testing **in** antiretroviral treatment **(ART)-experienced individuals remains unknown**.

METHODS: This was a retrospective, multicentre, cohort study in ART-experienced, HIV-1-infected adults who initiated salvage ART including, at least 1 ritonavir-boosted protease inhibitor, raltegravir or etravirine. Presalvage ART Sanger and **454 sequencing** of plasma HIV-1 were used to generate separate genotypic sensitivity scores (GSS) using the HIVdb, ANRS, and REGA algorithms. Virological failure (VF) was defined as 2 consecutive HIV-1 RNA levels ≥200 copies/mL at least 12 weeks after salvage ART initiation, whereas subjects remained on the same ART. The ability of Sanger and 454-GSS to predict VF was assessed by receiver operating characteristic (ROC) curves and survival analyses.

RESULTS: The study included 132 evaluable subjects; 28 (21%) developed VF. Using HIVdb, **454 predicted VF better than Sanger sequencing** in the ROC curve analysis (area under the curve: 0.69 vs 0.60, Delong test P = .029). **Time to VF was shorter for subjects with 454-GSS < 3 vs 454-GSS ≥ 3 (Log-rank P = .003) but not significantly different between Sanger-GSS < 3 and ≥3.** Factors independently associated with increased risk of VF in multivariate Cox regression were a 454-GSS < 3 (HR = 4.6, 95 CI, [1.5, 14.0], P = .007), and the number of previous antiretrovirals received (HR = 1.2 per additional drug, 95 CI, [1.1, 1.3], P = .001). Equivalent findings were obtained with the ANRS and REGA algorithms.

CONCLUSIONS: **Ultrasensitive HIV-1 genotyping improves GSS-based predictions of virological outcomes of salvage ART relative to Sanger sequencing**. This may improve the clinical management of ART-experienced subjects living with HIV-1. Clinical Trials Registration.

Time to virological failure was significantly shorter for pluri-experienced HIV-1 infected patients with a suboptimal genotypic susceptibility (GSS<3) predicted by 454-GSS performed at baseline of salvage regimen.



Pou et al CID 2014

Nowadays, performing genotypic resistance (GRT) is crucial also in patients with low or undetectable plasma viral load.

The evaluation of drug resistance can be also helpful in clinical practice to plan drug switches for intolerance, toxicity or simplification in suppressed HIV-1 infected patients with good virologic control.

Switch Strategies for Virologically Suppressed Persons

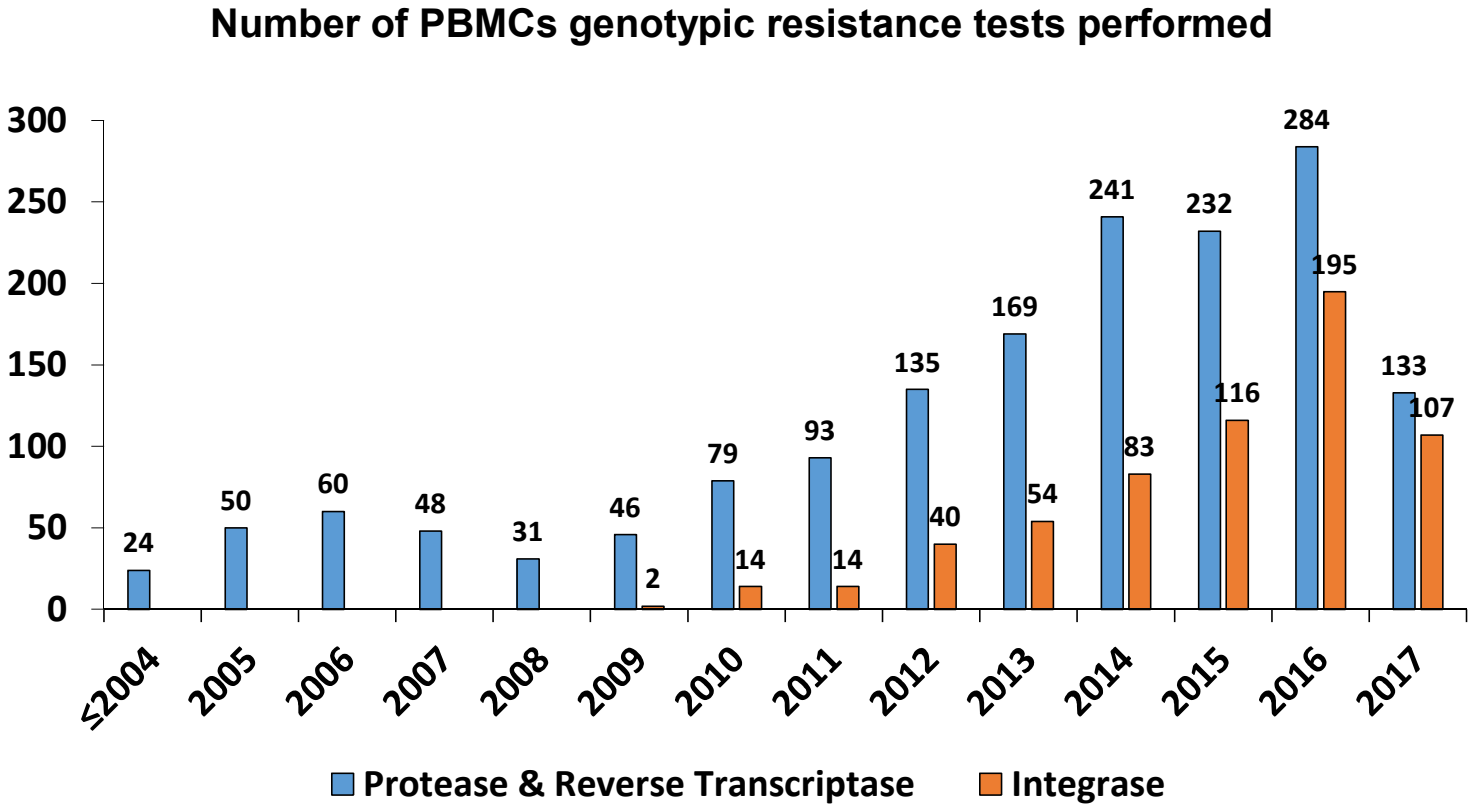
A complete ARV history with HIV-VL, tolerability issues and cumulative genotypic resistance history should be analysed prior to any drug switch.

Switch Strategies for Virologically Suppressed Persons

A complete ARV history with HIV-VL, tolerability issues and cumulative genotypic resistance history should be analysed prior to any drug switch.

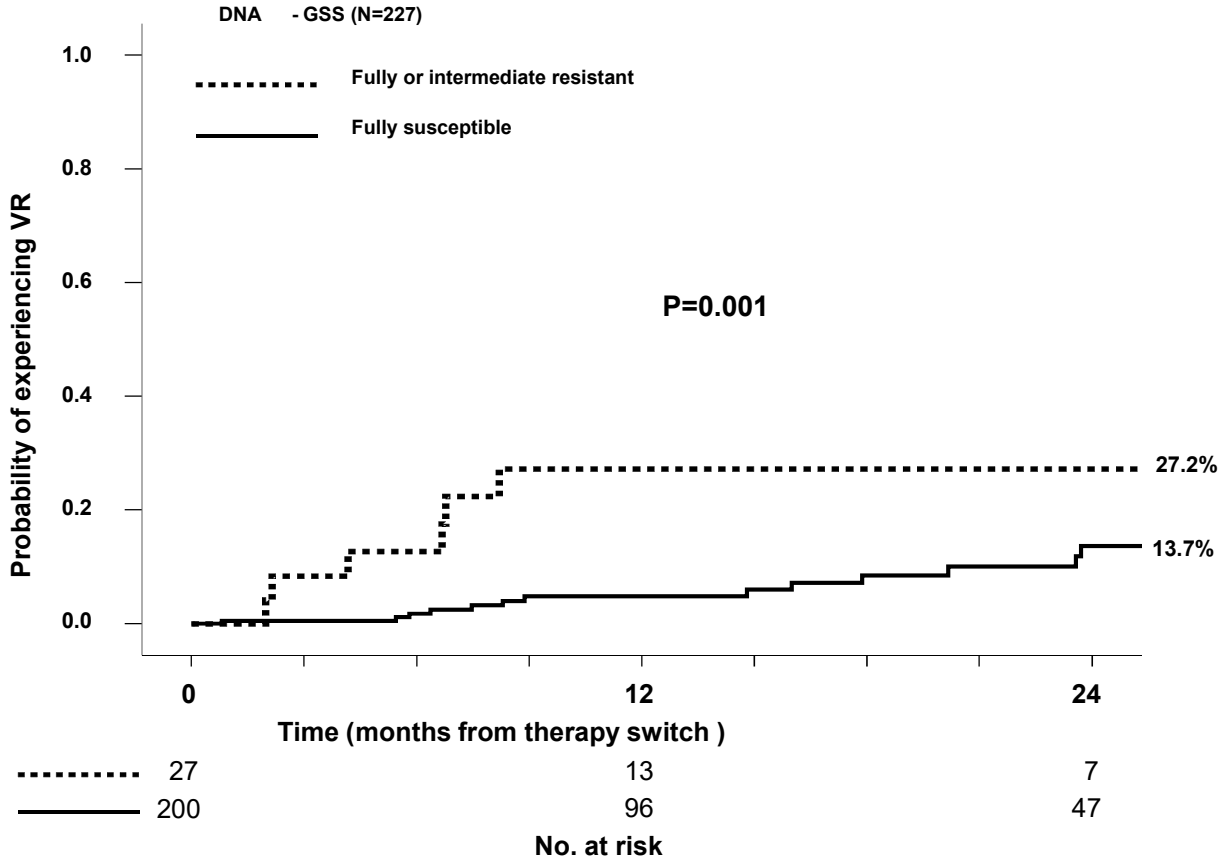
In this contest, the genotypic test on HIV-DNA represents an added value for drug resistance detection

Increased requests of HIV DNA GRT in clinical practice over the recent years



Armenia D unpublished data, data updated in June 2017

Virologically suppressed patients showing an intermediate or fully resistant virus at PMBC GRTs performed before a therapy switch had a higher probability of experiencing virological rebound compared to those carrying a fully susceptible virus



Armenia D et al. Journal of Clinical Virology 2018

- Ultra-deep sequencing improves the detection of drug in cellular DNA from HIV-infected patients on ART suppressed viraemia

JAG 2018

- Analysis of drug resistance mutations in whole blood DNA from HIV-1 infected patients by single genome and ultradeep sequencing analysis

JV METH 2018

- Utility of HIV-1 DNA genotype in determining antiretrovir in patients with low or undetectable HIV RNA viral

JAC 2018

Narjis Boukli[1]†, Anders Boyd[2]*†, Marianne Collot[1], Jean-Luc Meynard[3], Pierre-Marie

International Journal of
Environmental Research and Public Health

MDPI

Brief Report

Sanger and Next Generation Sequencing Approaches to Evaluate HIV-1 Virus in Blood Compartments

Andrea Arias, Pablo López, Raphael Sánchez, Yasuhiro Yamamura and Vanessa Rivera-Amill *

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Abstract: The implementation of antiretroviral treatment combined with the monitoring of drug resistance mutations improves the quality of life of HIV-1 positive patients. The drug resistance mutation patterns and viral genotypes are currently analyzed by DNA sequencing of the virus in the plasma of patients. However, the virus compartmentalizes, and different T cell subsets may harbor distinct viral subsets. In this study, we compared the patterns of HIV distribution in cell-free (blood plasma) and cell-associated viruses (peripheral blood mononuclear cells, PBMCs) derived from ART-treated patients by using Sanger sequencing- and Next-Generation sequencing-based HIV assay. $CD4^+CD45RA^-RO^+$ memory T-cells were isolated from PBMCs using a BD FACSAria instrument. HIV *pol* (protease and reverse transcriptase) was RT-PCR or PCR amplified from the plasma and the T-cell subset, respectively. Sequences were obtained using Sanger sequencing and Next-Generation Sequencing (NGS). Sanger sequences were aligned and edited using RECall software (beta v3.03). The Stanford HIV database was used to evaluate drug resistance mutations. Illumina MiSeq platform and HyDRA Web were used to generate and analyze NGS data, respectively. Our results show a high correlation between Sanger sequencing and NGS results. However, some major and minor drug resistance mutations were only observed by NGS, albeit at different frequencies. Analysis of low-frequency drugs resistance mutations and virus distribution in the blood compartments may provide information to allow a more sustainable response to therapy and better disease management.

J Antimicrob Chemother
doi:10.1093/jac/dky315

Journal of Antimicrobial Chemotherapy

Ultra-deep sequencing improves the detection of drug resistance in cellular DNA from HIV-infected patients on ART with suppressed viraemia

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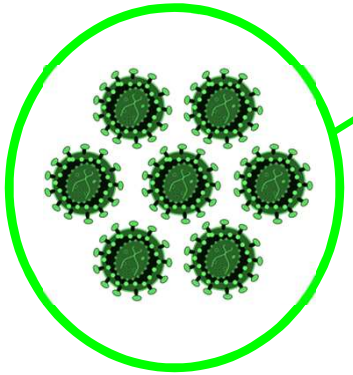
Background: Standard genotypic tests performed on HIV DNA from patients on suppressive ART, with previous resistance-associated mutations (RAMs) detected in their plasma, underestimate resistance. We thus compared ultra-deep sequencing (UDS) with bulk sequencing of DNA to detect RAMs previously identified in plasma.

Methods: We sequenced the DNA of 169 highly treatment experienced patients with suppressed viraemia (ANRS 138-EASIER trial). Protease (PR) and reverse transcriptase (RT) genes from HIV DNA were sequenced by bulk sequencing and UDS, comparing 1% and 20% as thresholds of detection for UDS.

Results: Patients were highly treatment experienced (13.6 years). UDS of DNA was successful for the RT and PR genes in 133 (79%) and 137 (81%) patients, respectively. The detection of RAMs was similar by bulk sequencing and UDS with a 20% cut-off. However, the detection of RAMs by UDS with a 1% cut-off was significantly higher than that of bulk sequencing for RT codons D67N (65.4% versus 52.3%), M184V (66.2% versus 52.3%), L210W (48.9% versus 36.4%) and T215Y (57.9% versus 42.1%) and PR codons M46I (46% versus 26%), I54L (12.4% versus 3.9%), V82A (44.5% versus 29.9%) and L90M (57.7% versus 42.5%).

Conclusions: Genotypic resistance testing of cellular HIV DNA of well-controlled patients should use UDS technology with a sensitivity threshold of 1% to improve the detection of the resistant reservoir.

HIV-1 coreceptor usage



R5 Virus

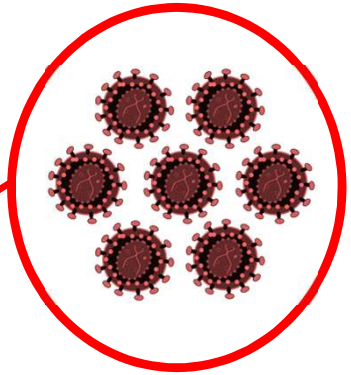
They use only CCR5 co-receptor.

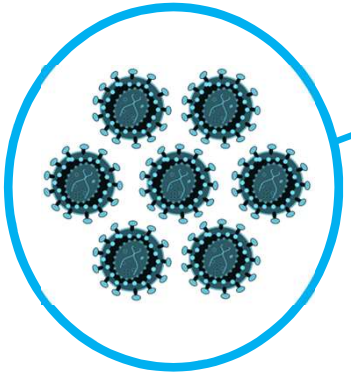
They are predominant at trasmission and in early stages of infection

X4 Virus

They use only CXCR4 co-receptor.

They emerge in late stages of infection. They are associated with a more rapid decline of CD4+ T cell and disease progression

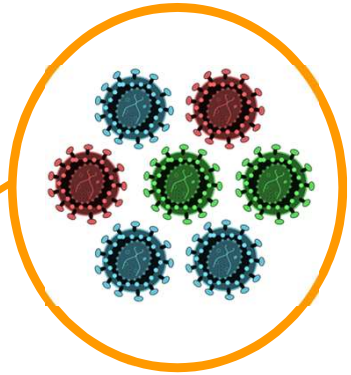




Dual tropic Virus

They use either CCR5 or CXCR4

Viral mixed tropic population



Genotypic Tropism Assays

The main genetic determinant of co-receptor tropism in the HIV envelope in the V3-loop region in gp120.

Genotypic approaches are based on:

- Amplifying the envelope gene
- Sequencing the V3 loop and, sometimes, additional regions, such as V2
- Interpreting the obtained data with different algorithms available online (www.geno2pheno.org or Position-Specific Scoring Matrix [PSSM])

Paredes and Clotet Eur. Infect. Dis. 2008

OPEN ACCESS Freely available online

PLOS | ONE

The Genotypic False Positive Rate Determined by V3 Population Sequencing Can Predict the Burden of HIV-1 CXCR4-using Species Detected by Pyrosequencing

Valentina Svicher[1], Valeria Cento[1], Gabriella Rozera[2], Isabella Abbate[2], Maria Mercedes Santoro[1], Daniele Armenia[1], Lavinia Fabeni[2], Alessandro Bruselles[2], Alessandra Latini[3], Guido Palamara[3], Valeria Micheli[4], Giuliano Rizzardini[4], Caterina Gori[2], Federica Forbici[2], Giuseppe Ippolito[2], Massimo Andreoni[5], Andrea Antinori[2], Francesca Ceccherini-Silberstein[1]*, Maria Rosaria Capobianchi[2], Carlo Federico Perno[1,2]

1 Department of Experimental Medicine and Surgery, University of "Tor Vergata" Rome, Italy, **2** I.N.M.I. "L. Spallanzani", Rome, Italy, **3** "San Gallicano" Hospital, Rome, Italy, **4** "L. Sacco" Hospital, Milan, Italy, **5** University Hospital "Tor Vergata", Rome, Italy

Abstract

Objective: The false-positive rate (FPR) is a percentage-score provided by Geno2Pheno-algorithm indicating the likelihood that a V3-sequence is falsely predicted as CXCR4-using. We evaluated the correlation between FPR obtained by V3 population-sequencing and the burden of CXCR4-using variants detected by V3 ultra-deep sequencing (UDPS) and Enhanced-Sensitivity Trofile assay (ESTA).

Methods: 54 HIV-1 B-subtype infected-patients (all maraviroc-naïve), with viremia >10,000copies/ml, were analyzed. HIV-tropism was assessed by V3 population-sequencing, UDPS (considering variants with >0.5% prevalence), and ESTA.

Results: By UDPS, CCR5-using variants were detected in 53/54 patients, irrespective of FPR values, and their intra-patient prevalence progressively increased by increasing the FPR obtained by V3 population-sequencing (rho = 0.75, p = 5.0e-8). Conversely, the intra-patient prevalence of CXCR4-using variants in the 54 patients analyzed progressively decreased by increasing the FPR (rho = −0.61; p = 9.3e-6). Indeed, no CXCR4-using variants were detected in 13/13 patients with FPR>60. They were present in 7/18 (38.8%) patients with FPR 20–60 (intra-patient prevalence range: 2.1%–18.4%), in 5/7 (71.4%) with FPR 10–20, in 4/6 (66.7%) with FPR 5–10, and in 10/10(100%) with FPR<5 (intra-patient prevalence range: 12.1%–98.1%).

Conclusions: FPR by V3 population-sequencing can predict the burden of CXCR4-using variants. This information can be used to optimize the management of tropism determination in clinical practice. Due to its low cost and short turnaround time, V3 population-sequencing may represent the most feasible test for HIV-1 tropism determination. More sensitive methodologies (as UDPS) might be useful when V3 population-sequencing provides a FPR >20 (particularly in the range 20–60), allowing a more careful identification of patients harboring CXCR4-using variants.

The Genotypic False Positive Rate Determined by V3 Population Sequencing
Can Predict the Burden of HIV-1 CXCR4-using Quasispecies

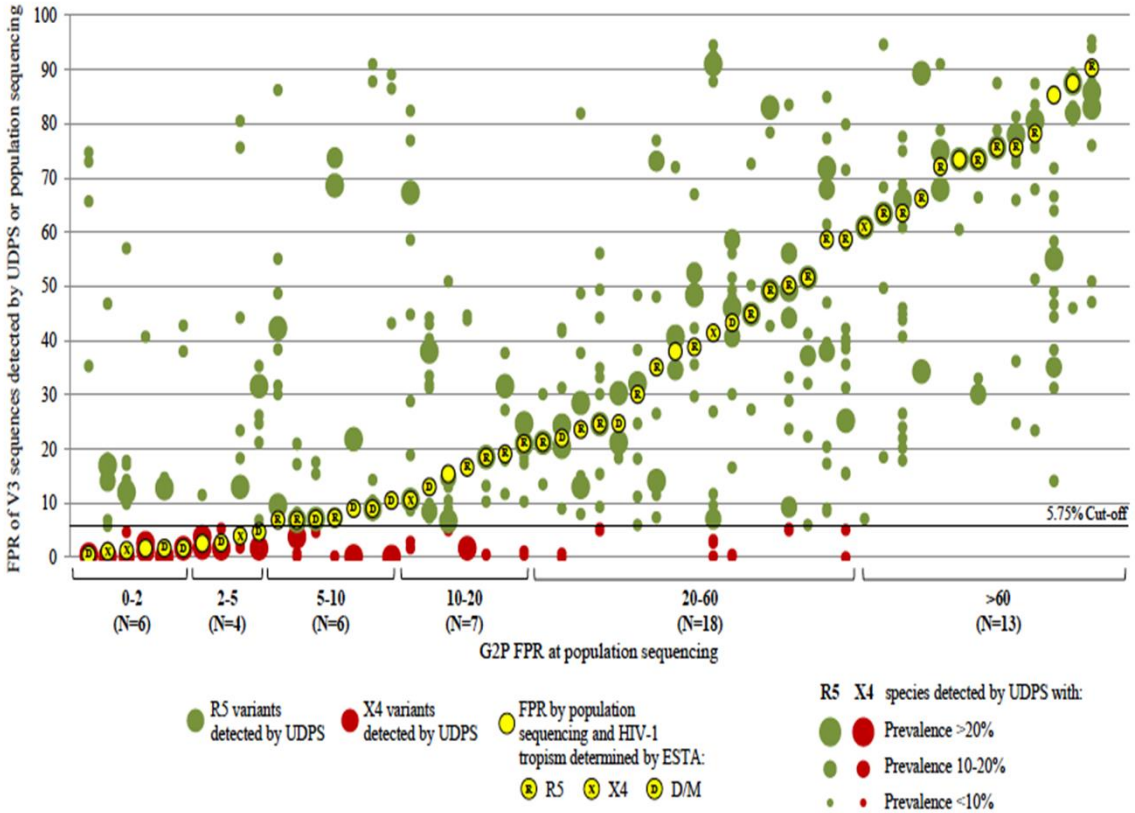


Figure 3. The graph reports the distribution of FPR values of all the V3 variants detected by UDPS in each patient according to FPR ranges at population V3 sequencing. The relative dimension of green and red dots represents the prevalence of R5 and X4 species detected by UDPS. Yellow dots represent the FPR determined by population sequencing and letters within dots indicate the phenotypic tropism determined by ESTA (R = pure CCR5 tropism, X = pure CXCR4 tropism, D= dual/mixed tropism. For blank yellow dots, ESTA result was not available. A FPR of 5.75 has been used as cut-off to infer HIV-1 co-receptor usage of V3 sequences obtained by both V3 population and ultra-deep sequencing.

Svicher et al PLoS One 2013

Massively parallel pyrosequencing highlights minority variants in the HIV-1 env quasispecies deriving from lymphomonocyte sub-populations

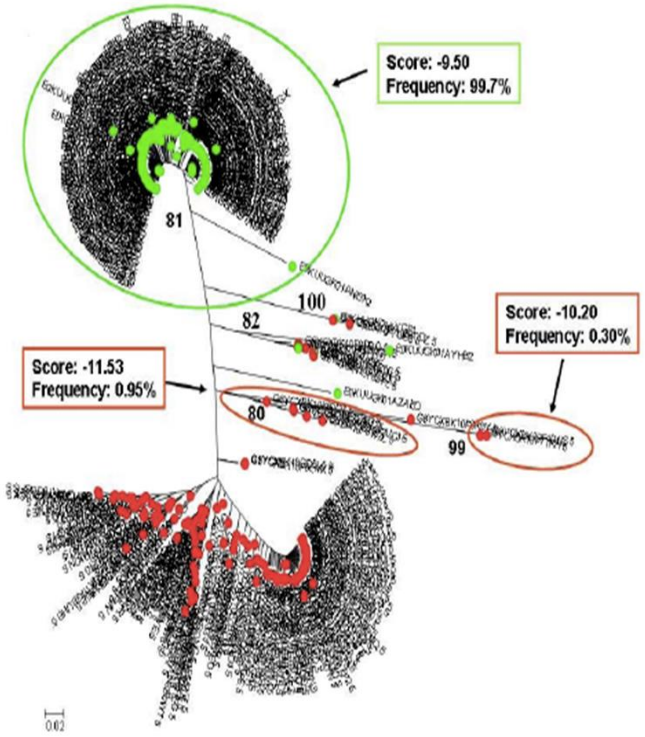


Figure 1
Individual phylogenetic tree of HIV-1 V3 amino acid sequences from Pt.1. Proviral quasispecies harboured by monocytes (red circle = CD36-provirus) and T lymphocytes (green circle = CD26-provirus) of Pt.1 were analyzed after long term suppression of viremia. Nucleotide sequences

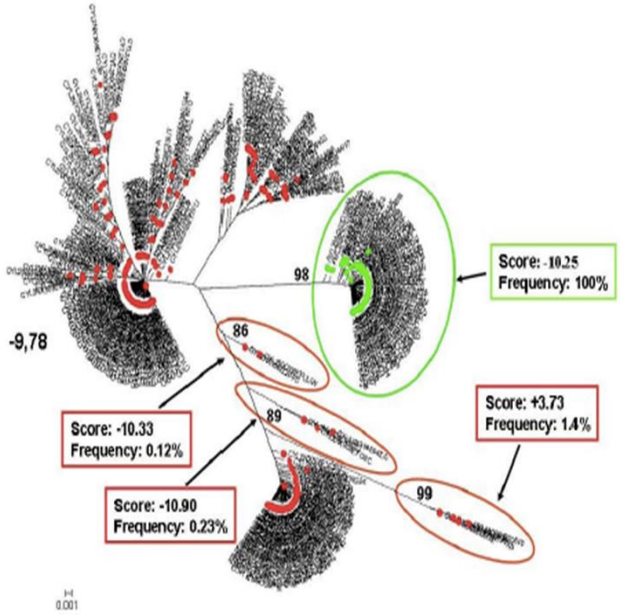


Figure 2
Individual phylogenetic tree of HIV-1 V3 amino acid sequences from Pt.2. Proviral quasispecies harboured by monocytes (red circle = CD36-provirus) and T lymphocytes (green circle = CD26-provirus) of Pt.2 were analyzed, as in Fig. 1.

Rozera et al Retrovirology 2009

The evaluation of viral resistance in HIV can be an excellent and exportable model

also for other important viruses

such as Hepatitic C Viruses

Università di Roma Tor Vergata

15th European Meeting on HIV & Hepatitis - Treatment Strategies & Antiviral Drug Resistance

Rome, Italy - June 7-9 2017

Clinical relevance of accurate HCV genotype and subtype assignment by HCV Sanger and next generation sequencing in the era of new direct acting antiviral agents

M. Aragri*, V.C. Di Maio, C. Pillinini, M.C. Bellocchi, L. Carioti, E. Teti, P. Rossi, C Masetti, L. Gianserra, A. Pieri, V.P. Palitti, V. Cento, A. Bertoli, S. Barbaliscia, I. Lenci, S. Francioso, P. Cacciatore, E. Polillo, S. Landonio, M. Melis, M. Paoloni, V. Micheli, L.A. Nicolini, S. Marenco, L. Lambiase, M. Milana, I. Maida, L. Sarmati, A. Pellicelli, L. Nosotti, E. Biliotti, M. Marignani, F. Sozio, C. Minichini, D. Romagnoli, B.Bruzzone, M. Siciliano, G. D'Ettorre, M. Lichtner, C. Sarrecchia, A. Grieco, F. Morisco, C. Mastroianni, J. Vecchiet, M. Puoti, N. Caporaso, S. Bruno, A. Craxì, P. Tarquini, C. Magni, S. Babudieri, G. Taliani, V. Vullo, A. Picciotto, M. Andreoni, C. Pasquazzi, G. Parruti, M. Angelico, C.F. Perno, F. Ceccherini-Silberstein

HCV Sanger sequencing confirmed the previous genotype by commercial-assays in 89.7% of cases analysed

	Patients (N)	Patients (%)
Genotype/subtype confirmed	1627	89.7
"Problematic" genotype new assignment		

Overall, 95 out of 1813 (5.2%) HCV infected patients candidate to start a treatment containing a DAA showed a discordant genotype or subtype according to the sequencing

Discordant cases			
	Discordant genotypes	37	2.0
	Genotype 1 with discordant subtype	58	3.2
Total		**1813**	**100**

Aragri M et al., et al., 15th European Meeting on HIV & Hepatitis 2017

HCV Sanger sequencing confirmed 2/44 (4.5%) mixed cases given by commercial assays

Pre-sequencing subtype	Genotype year	NS3-NS5A-NS5B phylogenetic analysis Sanger Sequencing
1a + 1b (N=15)	2012 (N=1) Others NA	1a (N=9) 1b (N=6)
1a + 2	NA	2c
1a+3 (N=3)	2016 (N=1) Others NA	1a (N=2) **1a+3a (N=1)**
1a+4 (N=4)	NA	1a (N=2) 4d (N=1) **1a+4d (N=1)**
1b+2 (N=9)	NA	1b (N=4) 2c (N=4) 3a (N=1)
1b+3 (N=3)	2015 (N=1) Others NA	1b (N=2) 3a (N=1)
1b+4 (N=4)	NA	1b (N=2) 4d (N=2)
1+4	2006	4r
1+3+4	NA	4d
2+4 (N=3)	NA	2c (N=3)

NA= Not Available

Aragri M et al., et al., 15th European Meeting on HIV & Hepatitis 2017

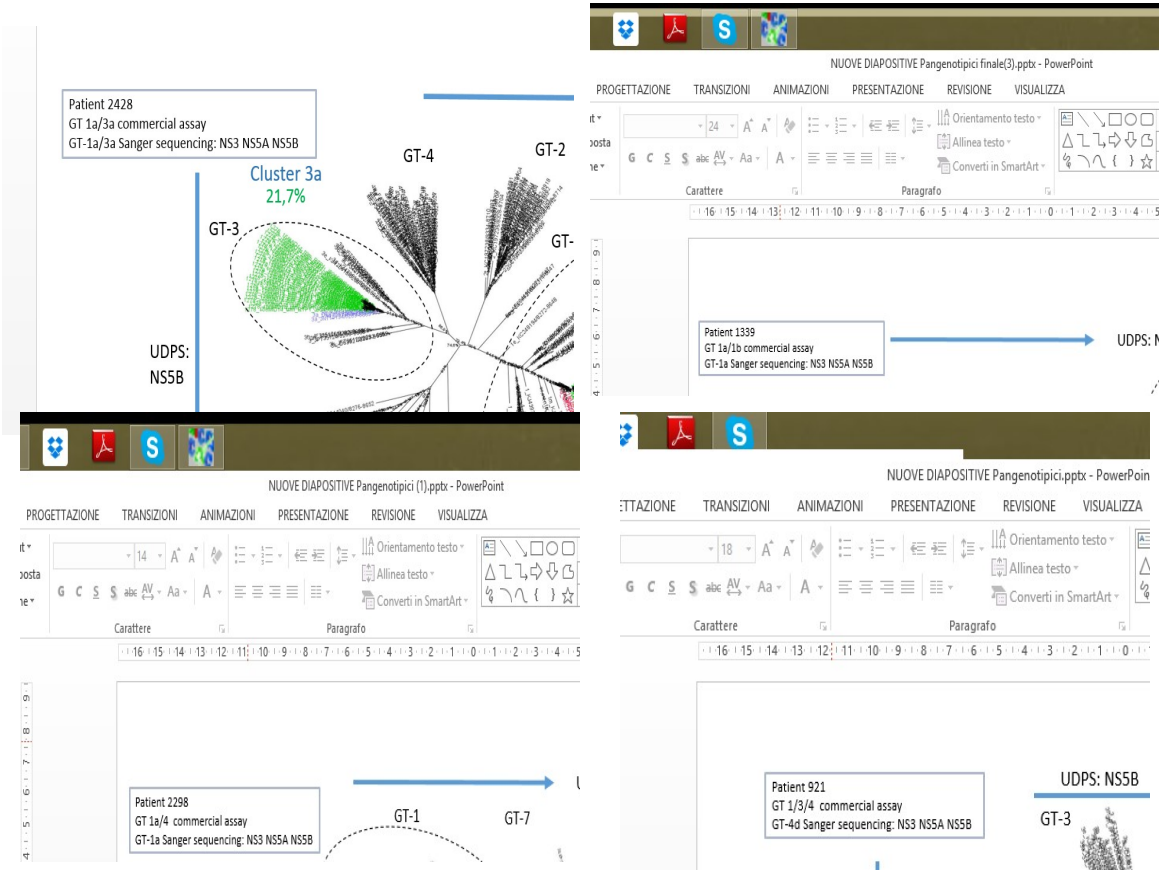
But with the UDPS in NS5B-region….. Among 19/44 patients with presumed mixed infection 4 mixed infections were confirmed

Pre-sequencing GT	ID	Genotype assay	NS3-NS5A-NS5B phylogenetic analysis Sanger Sequencing	NS5B phylogenetic analysis UDPS
1a + 3	2428	Abbott (2016)	1a/3a	1a (78.3%) + 3a (21.7%)
1a + 1b	1339	Innolipa (2007)	1a	1a (88.4%) + 1b (11.6%)
1a + 4	2298	NA	1a	1a (16.9%) + 4d (83.1%)
1 + 3 + 4	921	NA	4d	1a (12%) + 4d (88%)

NA= Not Available

Aragri M et al., et al., 15th European Meeting on HIV & Hepatitis 2017

4 cases of patients infected by “mixed” HCV infection identified by UDPS



Aragri M et al., et al., 15th European Meeting on HIV & Hepatitis 2017

Conclusions

- ✓To date, availability of high and dedicated bioinformatic servers for analysis of the NGS data has greatly accelerated the use in clinical virology.
- ✓NGS has been successfully used in monitoring of antiviral drug resistance, as well as in investigation of viral evolution, diversity and quasispecies, evaluation of human virome, virus discovery allowing investigation of previously inaccessible aspects of viral dynamics, evolution and pathogenesis with an exceptional resolution.
- ✓The advantage of the NGS platforms is their ability to characterize hundreds of different quasispecies simultaneously. This is not possible using conventional approaches.
- ✓HIV drug resistance genotyping is a clinically important tool to detect the emergence of viral resistance and maximize the benefit of current treatment options.
- ✓NGS can improve sensitivity of drug resistance genotyping while also reducing costs.

Challenges in NGS

- ✓The major barriers to their wide application for an use in routine are the initial cost of set-up, turnaround time, requirement of powerful computational facilities (especially in the past) along with the requirement of highly skilled and expert people.

- ✓It is very hard use them in resource-limited countries

- ✓The utility of NGS platforms for their extremely high sensitivity also makes them prone to unintentional contamination