Universitat Autònoma de Barcelona

Next-Generation Virus Genotyping for HIV-1 Surveillance and Clinical Management

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El doctor **Roger Paredes Deiros**, investigador principal de l'Institut de Recerca de la SIDA (IrsiCaixa), de l'Hospital Universitari Germans Trias i Pujol,

Certifica:

Que el treball experimental realitzat i la redacció de la memòria de la Tesi Doctoral titulada **"Next-Generation Virus Genotyping for HIV-1 Surveillance and Clinical Management"** han estat realitzats per la Maria Casadellà Fontdevila sota la seva direcció, i considera que és apta per a ser presentada per a optar al grau de Doctora en Bioquímica, Biologia Molecular i Biomedicina per la Universitat Autònoma de Barcelona.

I per tal que en quedi constància, signa aquest document a Badalona, a 5 de setembre de 2016.

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Certifica:

Que el treball experimental realitzat i la redacció de la memòria de la Tesi Doctoral titulada **"Next-Generation Virus Genotyping for HIV-1 Surveillance and Clinical Management"** han estat realitzats per la Maria Casadellà Fontdevila sota la seva direcció, i considera que és apta per a ser presentada per a optar al grau de Doctora en Bioquímica, Biologia Molecular i Biomedicina per la Universitat Autònoma de Barcelona.

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Dr. Jaume Farrés Vicén

Als meus pares

A la Laia

A en Pere

Abbreviations

/r	Ritonavir-boosted
3TC	Lamivudine
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral treatment
ARTHIVdb	Antiretroviral-specific human immunodeficiency virus data base
ARV	Antiretroviral
ASPCR	Allele-specific PCR
ATV	Atazanavir
AVA	Amplicon variant analyser
AZT	Zidovudine
CCR5	C-C chemokine receptor type 5
CI	Confidence interval
CXCR4	C-X-C chemokine receptor type 4
DNA	Deoxyribonucleic acid
DRM	Drug resistance mutation
DRMV	Drug resistance minority variant
EFV	Efavirenz
ESTA	Enhanced sensitivity Trofile assay
FTC	Emtricitabine
G2P	Geno2Pheno
GSS	Genotypic sensitivity score
HCV	Hepatitis C virus
HIV-1	Human immunodeficiency virus- type 1
HIVdb	Human immunodeficiency virus data base
HR	Hazard ratio
HVB	Hepatitis B virus
I	Intermediate resistance

IAS-USA	International AIDS society- United States of America
IDV	Indinavir
IN	Integrase
InSTI	Integrase strand transfer inhibitor
IQR	Inter-quartile range
IVDU	Intravenous drug user
LFDRM	Low frequency drug resistance mutation
LMIC	Low and middle income countries
LPV	Lopinavir
MSM	Men who have sex with men
NGS	Next generation sequencing
NNRTI	Nonnucleoside analogue reverse transcriptase inhibitor
NRTI	Nucleoside and nucleotide analogue reverse transcriptase inhibitor
NVP	Nevirapine
PCR	Polymerase chain reaction
pegIFN	Pegilated interferon
PI	Protease inhibitor
PR	Protease
QC	Quality control
R	Resistant
RAL	Raltegravir
RBV	Ribavirin
RNA	Ribonucleic acid
RPV	Rilpivirine
RT	Retrotranscriptase
S	Susceptible
sdNVP	Single dose nevirapine
TDF	Tenofovir
TDR	Transmitted drug resistance
VF	Virological failure
VL	Viral load
WHO	World Health Organisation
WT	Wild type

ZDV	Zidovudine

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Summary / Resum

Summary

Although HIV-1 infection cannot be cured, antiretroviral therapy is able to persistently block virus replication, limiting the damage on the immune system. This can prevent and/or revert immune deterioration in most subjects, prolonging life expectancy and increasing its quality. Furthermore, antiretroviral treatment is the most effective tool available to prevent onward HIV-1 transmission. One of the most critical issues in HIV-1 treatment is resistance mutations. HIV-1 is distributed in quasispecies, which allows the virus to rapidly adapt and escape from adverse drug or immune pressure. It also implies that potentially relevant low-frequency mutants exist, which may go unnoticed by conventional genotypic assays. In other words, clinicians might miss important information to optimize antiretroviral treatment choices.

By using ultrasensitive sequencing techniques we have demonstrated that low frequency drug resistance mutations may be clinically important in certain patients and when prescribing particular antiretroviral regimens. We found that pre-existing low frequency drug resistance mutations are clinically relevant in subjects who present late to clinics with advanced immune suppression, who start treatment with non-analogue reverse transcriptase inhibitors, as its presence is associated with higher risk of virological failure. We have also observed that the presence of these minority mutations may be important for HIV-1 infected people living in low and middle income countries, where patients are often given first-line tenofovir containing regimens with no virological monitoring. We found that the prevalence of tenofovir resistance after virological failure is high, and absence of genotyping could compromise tenofovir use as second-line regimen. Therefore, surveillance of tenofovir resistance should be a priority in treated and naïve populations, at least by population sequencing. Another important message is that, to date, transmission of integrase strand transfer inhibitors (INSTIs) resistance in Europe remains at negligible levels. Now that they are frequently being prescribed in resource rich settings, this could lead to the emergence of transmitted resistance mutations and the need for integrase genotyping in the coming years.

Regarding the analysis of the presence of the resistance mutation S282T in HIV-1/HCV co-infected patients, we did not detect it, and globally it has only been observed in few patients, with reversion to wild-type viruses within several weeks. Also, prescribing new direct antiviral agents result in HCV cure in the majority of patients, with no need to test for drug resistance mutations before administration.

In general, detection of minority drug resistance may help to avoid virological failures and to prevent the transmission of drug resistant HIV.

Resum

Tot i que la infecció pel virus de la immunodeficiència humana de tipus 1 (VIH-1) roman sense cura, la teràpia antiretroviral és capaç de bloquejar de manera persistent la replicació viral, limitant el dany al sistema immunitari. Aquest fet pot prevenir i/o revertir el deteriorament immunitari en la majoria de pacients, prolongant la seva esperança de vida i incrementant la seva qualitat. A més, el tractament antiretroviral és la eina disponible més eficaç per prevenir la posterior transmissió del virus. Una de les qüestions més crítiques en el tractament del VIH-1 son les mutacions de resistència. El VIH-1 es distribueix en quasispecies, fet que permet al virus adaptar-se i escapar ràpidament de l'efecte dels fàrmacs o de la pressió immunitària. També implica que mutacions minoritàries que poden ser potencialment rellevants també son presents, i poden passar desapercebudes pels assajos genotípics convencionals. En altres paraules, els metges poden perdre informació important necessària per optimitzar les opcions de tractament antiretroviral.

Mitjançant tècniques de seqüenciació massiva hem demostrat que les mutacions minoritàries de resistència als antiretrovirals poden ser clínicament importants en alguns pacients i quan es prescriuen règims antiretrovirals concrets. Vam trobar que les mutacions de resistència minoritàries pre-existents son clínicament rellevants en pacients que es presenten tard a la clínica amb una supressió immunològica avançada que comencen tractament amb inhibidors de la transcriptasa reversa no anàlegs de nucleòsids. La seva presència s'associa amb més risc de fracàs virològic. També hem observat que la presència d'aquestes mutacions minoritàries pot ser important pels individus infectats per VIH-1 que viuen en regions en vies de desenvolupament, on els pacients sovint son tractats amb règims de primera línia que contenen tenofovir sense un posterior monitoreig de la càrrega viral. Vam trobar que la prevalença de mutacions de resistència a tenofovir després d'un fracàs virològic és alta, i l'absència de genotipat podria comprometre la utilització del tenofovir com a règim de segona línia. Per tant, la vigilància de les resistències a tenofovir hauria de ser una prioritat tant en pacients naïve a tractament com en pacients experimentats, com a mínim mitjançant seqüenciació poblacional. Un altre

missatge important és que fins a dia d'avui la transmissió de mutacions de resistència als inhibidors de la integrasa a Europa roman a nivells mínims. Ara que s'han convertit en una família de tractament que es prescriu freqüentment en països desenvolupats, podria portar a l'emergència de mutacions de resistència transmeses i a una necessitat de genotipar per les mutacions a integrasa en els propers anys.

Pel que fa a l'anàlisi de la presència de la mutació de resistència S282T en pacients co-infectats per VIH-1/hepatitis C, no la vam detectar en cap cas, i globalment s'ha vist en molt pocs pacients, amb una reversió a forma *wild-type* passades unes setmanes. A més, amb els nous règims anomenats agents antivirals directes els pacients es curen en la majoria de casos, sense necessitat de testar les mutacions de resistència abans d'administrar-los.

En general, la detecció de resistències minoritàries als fàrmacs pot ajudar a evitar fracassos virològics i prevenir la transmissió de variants resistents de VIH.

Resumen

Aunque la infección por el virus de la inmunodeficiencia humana de tipo 1 (VIH-1) permanece sin curación, la terapia antirretroviral es capaz de bloquear de manera persistente la replicación viral, limitando el daño al sistema inmunitario. Este hecho puede prevenir y/o revertir el deterioro inmunitario en la mayoría de pacientes, prolongando su esperanza de vida e incrementando su calidad. Además, el tratamiento antirretroviral es la herramienta disponible más eficaz para prevenir la posterior transmisión del virus. Una de las cuestiones más críticas en el tratamiento del VIH-1 son las mutaciones de resistencia. El VIH-1 se distribuye en quasispecies, lo que permite al virus adaptarse y escapar rápidamente del efecto de los fármacos o de la presión inmunitaria. También implica que pueden existir mutaciones minoritarias potencialmente relevantes, pueden pasar desapercibidas por los ensayos genotípicos convencionales. En otras palabras, los médicos pueden perder información importante necesaria para optimizar las opciones de tratamiento antirretroviral.

Mediante técnicas de secuenciación masiva hemos demostrado que las mutaciones minoritarias de resistencia a los antirretrovirales pueden ser clínicamente importantes en algunos pacientes y cuando se prescriben regímenes antirretrovirales concretos. Encontramos que las mutaciones de resistencia minoritarias pre-existentes son clínicamente relevantes en pacientes que se presentan tarde en la clínica con una supresión inmunológica avanzada que comienzan tratamiento con inhibidores de la transcriptasa reversa no análogos de nucleósidos. Su presencia se asocia con mayor riesgo de fracaso virológico. También hemos observado que la presencia de estas mutaciones minoritarias puede ser importante para los individuos infectados por VIH-1 que viven en regiones en vías de desarrollo, donde los pacientes a menudo son tratados con regímenes de primera línea que contienen tenofovir sin un posterior monitoreo de la carga viral. Encontramos que la prevalencia de mutaciones de resistencia a tenofovir tras un fracaso virológico es alta, y la ausencia de genotipado podría comprometer la utilización del tenofovir como régimen de segunda línea. Por

tanto, la vigilancia de las resistencias a tenofovir debería ser una prioridad tanto en pacientes naïve a tratamiento como en pacientes experimentados, como mínimo mediante secuenciación poblacional. Otro mensaje importante es que hasta el día de hoy la transmisión de mutaciones de resistencia a los inhibidores de la integrasa en Europa permanece a niveles mínimos. Ahora que se han convertido en una familia de tratamiento que se prescribe frecuentemente en países desarrollados, podría llevar a la emergencia de mutaciones de resistencia transmitidas y una necesidad de genotipar por las mutaciones en integrasa en los próximos años.

En cuanto al análisis de la presencia de la mutación de resistencia S282T en pacientes co-infectados por VIH-1/hepatitis C, no la detectamos en ningún caso, y globalmente se ha visto en muy pocos pacientes, con una reversióna la forma *wild-type* pasadas unas semanas. Además, con los nuevos regímenes, llamados agentes antivirales directos, los pacientes se curan en la mayoría de casos, sin necesidad de testar las mutaciones de resistencia a los fármacos antes de administrarlos.

En general, la detección de resistencias minoritarias a los tratamientos puede ayudar a evitar fracasos virológicos y prevenir la transmisión de variantes resistentes de VIH-1.

Preface

Human Immunodeficiency Virus type 1 (HIV-1) and Hepatitis C Virus (HCV) infections are two of the most important diseases ever afflicting humanity.

In 2015, 36.7 million people were living with HIV worldwide, with 2.1 million newly infected and 1.1 million related deaths globally. HIV-1 gradually damages the immune system. Without treatment, a person with HIV-1 is at high risk of developing serious clinical complications and death. Fortunately, the number of HIV-1-infected persons receiving antiretroviral treatment is rising every year, from 10.7 million on 2010 to 17 million on 2015. Current treatments work by reducing the viral load and preserving and/or restoring the immune system. However, the virus is highly genetically polymorphic and easily adaptable to the selective pressure of treatments, what can lead to a virological failure and a rebound in the viral load.

There are ambitious plans to reduce HIV prevalence and new infections: UNAIDS has the defined global target of reducing new HIV infections to fewer than 500.000 worldwide by 2020, although it has been stalled in the recent years and we are still far from accomplishing it. Also, 90-90-90 plan has the objective by 2020 of achieving 90% of all people living with HIV knowing their HIV status, 90% of all HIV diagnosed people receiving ART and 90% of all people on ART having viral suppression. Much remains to be done to bring these percentages to reality.

Hepatitis C virus is a globally prevalent pathogen and the causal agent of persistent liver infections in most infected patients. Globally, between 130 and 150 million people worldwide have chronic HCV infection. It has a high genetic variability, with seven genotypes and more than 50 subtypes, with different prevalence and distribution worldwide. As with HIV, HCV has a highly effective but low fidelity replication, and this combined with its small genome and large population size allows the rapid evolution of the virus. There is currently no vaccine for HCV infection, but new direct antiviral agents can cure approximately 90% of infected subjects, thereby reducing the risk of death from liver cancer and cirrhosis. However, access to diagnosis and treatment is low and limited.

Both HIV-1 and HCV adopt a quasispecies distribution in their human hosts and both can overcome the effect of therapy through the selection and accumulation of resistance mutations in their genomes. Due to the quasispecies distribution of these two viruses, sometimes such drug-resistant mutations occur in low-frequency variants that might be missed by standard Sanger sequencing assays. Next-generation sequencing techniques allow detecting such minority viruses with higher sensitivity. However, the clinical value of that is under discussion. This thesis explores the value of next-generation sequencing for clinical management and HIV-1 (Chapters 1 to 3) and HCV drug resistance surveillance (Chapter 4).

This thesis has been presented as a compendium of publications in peer-reviewed scientific journals. All studies were performed in IrsiCaixa, in collaboration with different national and international laboratories for sample collection and data interpretation. The introduction has been submitted as a review to *Virus Research* It provides a background in human immunodeficiency virus type 1 (HIV-1) deep sequencing strategies for the clinical management of HIV-1 infection. Chapter 1 is a paper published in AIDS on May 2015, in collaboration with Hospital Clínic de Barcelona, addressing the clinical impact of ultrasensitive sequencing in late presenters. In chapter 2, we collaborated with laboratories all over Europe involved in the the SPREAD programme of the European Society for Antiviral Research to address the prevalence of transmitted integrase strand-transfer inhibitor resistance mutations throughout Europe. The study was published on July 2015 in the Journal of Antimicrobial Chemotherapy. This work was recently cited in the 2016 IAS-USA antiretroviral guidelines for treatment and prevention of HIV infection in adults. Chapter 3 was done in collaboration with the University of KwaZulu-Natal in Durban, Harvard Medical School in Boston and Emory University in Atlanta and was published in AIDS on January 2016. This work described a 70% prevalence of tenofovir resistance in subjects developing virological failure to tenofovir regimens in South Africa. International bodies such as the WHO HIV Drug resistance group are using such information to elaborate policies to end the HIV pandemic globally.

Chapter 4 is a collaboration with “HIV and HCV Genetic and phenotypic Variability” group in IrsiCaixa, in which we studied the presence of S282T mutation in HIV/HCV co-infected subjects. It was published in the Journal of Clinical Virology on September 2013 and is an example of how deep sequencing can also be applied to the study of antiviral resistance in other viruses.

Introduction

Deep Sequencing for HIV-1 Clinical Management

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1. Introduction

Human immunodeficiency virus type 1 (HIV-1) infection affects more than 35 million people globally and causes nearly 1.5 million deaths every year [1,2]. More than half of the people in need worldwide are already receiving antiretroviral treatment (ART). If the other half could be treated, the HIV-1 pandemic could potentially be ended. The emerging HIV-1 resistance epidemic[3], however, poses a major threat to achieving that goal.

Although HIV-1 infection cannot be cured [4], antiretroviral therapy (ART) is able to persistently block HIV-1 replication, limiting HIV-1's damage on the immune system [5]. This prevents and/or reverts immune deterioration in most subjects, prolonging life expectancy and increasing quality of life [6–10]. Furthermore, ART is the most effective tool available to prevent onward HIV-1 transmission [11] and one of the most cost-effective interventions in medicine [12,13].

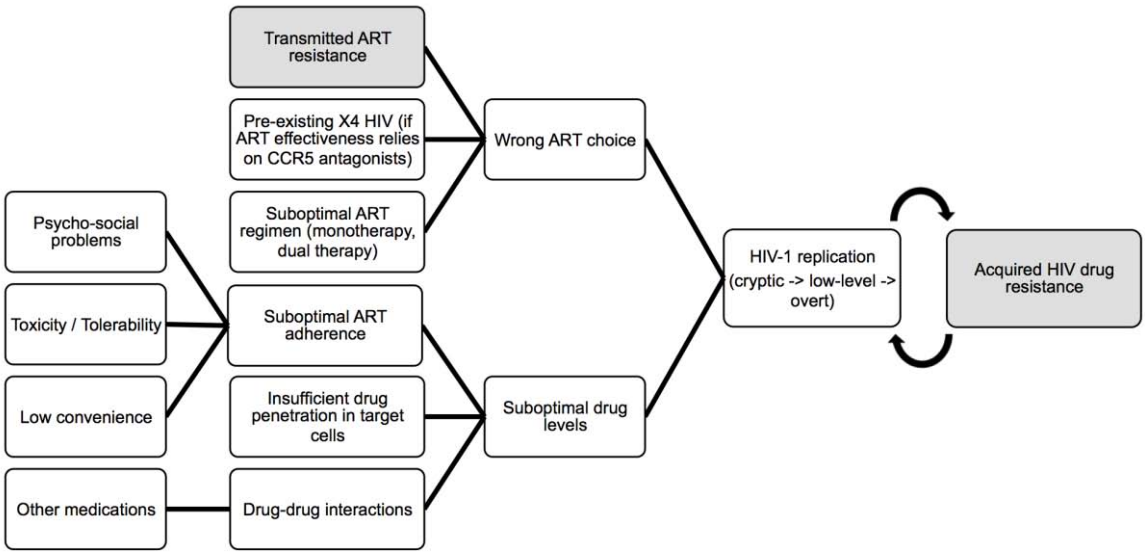
However, ART pressure might not be sufficient to block HIV-1 replication in a number of situations; i.e., if suboptimal ART is prescribed, there is pre-existing HIV-1 drug-resistance, drug penetration in target cells is insufficient, patient's treatment adherence is incomplete, or when drug-drug interactions decrease ART levels or increase toxicity [14]. Lack of suppression of viral replication in the presence of ART, even at cryptic levels, allows HIV-1 resistance to evolve with further accumulation of drug resistance mutations and ultimately leads to overt virological failure [15]. **(Figure 1)**

In each individual, HIV-1 is structured following a quasispecies distribution [16–18], i.e., a swarm of highly-related but genotypically different viral variants. Such distribution is consequence of a high virus replication rate in the absence of ART ($10^{9\text{-}14}$ new virions created per day) combined with high mutation and recombination rates (1 mutation and 2-3 recombination events, respectively, per each new virion)[19–23] due to the lack of proofreading activity of HIV-1's polymerase [24].

The quasispecies distribution allows HIV-1 to rapidly adapt and escape from adverse drug or immune pressure. It also implies that potentially relevant low-frequency mutants exist, which might not be detected by less sensitive HIV-1 genotypic assays. In other words, clinicians might miss important information to optimize ART choices. Novel point-mutation assays and next-generation sequencing (NGS) techniques increase the sensitivity of genotyping from 20% to approximately 1% of mutants in the quasispecies [25,26]. Similarly, NGS approaches provide improved sensitivity to detect CXCR4-using or X4 HIV, which has implications for immune recovery, clinical progression and virological response to CCR5 antagonists [27,28].

In an era when ART is indicated for anyone living with HIV-1 anywhere in the World, HIV-1 genotyping remains key for both clinical management [29–31] and public health surveillance, and will play an essential role to ending the HIV-1 pandemic in the coming decades [32].

Figure 1. Clinical pathways to HIV-1 drug resistance



2. Sequencing strategies

2.1. Sanger sequencing

For many years, virus population sequencing using Sanger's technique [33] has been the gold standard for HIV-1 drug resistance testing, both for research and clinical routine. Sanger sequencing is feasible for most laboratories with basic molecular biology equipment, is straightforward to perform and generally affordable, particularly with home-brew methods. It is easily scalable to a few dozen tests per week using a single PCR instrument and a single technician with part-time dedication, which fits many small or mid-scale HIV-1 laboratories. Sanger-based genotyping has been extensively validated in clinical trials and is supported by equally validated and often publicly available laboratory protocols and interpretation algorithms and rules that can be retrieved automatically [34,35]. This allows standardized reporting of resistance testing results to clinicians, researchers and public health officials, which has been instrumental in the past to ensure its acceptability among HIV-1 caregivers and policymakers.
However, due to its intrinsic sequencing chemistry, Sanger sequencing can only provide a consensus sequence of the whole quasispecies in each HIV-1-infected individual, being able to detect only those nucleotides present in at least 10-20% of the virus population. There is solid evidence that, at least in some cases, low-frequency genotypic information missed by Sanger sequencing might impact ART efficacy and could be important to improve HIV-1 resistance surveillance [36,37]. The advent and rapid technical evolution of NGS platforms, coupled with rapid reductions in costs, simplification of laboratory procedures, improvements in turnaround time to results and testing scalability, as well as the development of automated bioinformatic pipelines are gradually increasing NGS use in HIV-1 diagnostics.

2.2. Next Generation Sequencing

All NGS techniques perform parallel sequencing of hundreds of thousands to millions of individual DNA molecules, enabling the quantification of different viral variants from the same sample at clonal level with higher sensitivity than Sanger sequencing and at cheaper cost per base [37–40]. Technically, the field is rapidly

evolving and different NGS platforms have become available. Their main characteristics are summarized in **Table 1**.

All NGS platforms available to date require reverse transcription and PCR amplification before HIV-1 sequencing. This limits the lower sensitivity threshold to the intrinsic error rate of the reverse transcriptase, i.e., 10^{-4} or 1 error per every 10^4 nucleotides copied. Therefore, even in the presence of high HIV-1 RNA levels, it is unrealistic to expect any reliable detection of HIV-1 variants below 0.5 to 1% in the virus population. Also, true assay sensitivity of any ultrasensitive genotyping method depends on the number of RNA molecules in the original sample. The RNA copy in the assay depends on the plasma HIV-1 RNA concentration, the volume of plasma used and the efficiency of the RNA extraction process (discussed in [41]). The efficiency of the reverse transcriptase step also determines the starting copy number, since NGS platforms sequence DNA and not RNA molecules. As a rule of thumb, reliable detection of variants at 1% frequency will require HIV-1 RNA levels of at least 1000 copies/mL.

Although mutant detection above 1% frequency is generally robust and reliable, linear quantification of mutants in the 1% to 100% range is often affected by biases during library preparation due to the presence of resistance mutations or polymorphisms in primer binding sites, PCR-founding effects or random resampling of input DNA molecules. Primer ID partially avoids PCR resampling bias by including a random sequence tag in the first primer so that every template receives a unique ID [42–44]. Sequences obtained with this strategy can then be identified, the initial copy number can be quantified and the error and bias can be corrected to a great extent.

As NGS is approaching the clinic, a number of challenges must be overcome before it becomes generally available for routine diagnostics **(Table 2)**. Accessibility to NGS testing is improving for HIV-1 clinicians, with companies like Monogram Biosciences (San Francisco, California) already using NGS for proviral DNA HIV-1 genotyping (Genosure Archive Assay). Hands-on time and platform costs for laboratories processing NGS samples are decreasing. The cost per megabase of raw data of DNA sequence decreased 370-fold from US$5.200

Table 1. Characteristics of the main NGS technologies.

Number of single reads, cost per Gb and instrument costs is approximate and based on current literature; costs may vary between countries, regions and even laboratories. PE: paired-end; SE: single end; Tb: terabyte Gb: gigabyte; Mb: megabyte; bp: base-pairs; NA: not available; B: billion; M: million; K: thousand. (Sources: Allseq INC., n.d.; Goodwin et al., 2016; Metzker, 2010, and personal experience)

Company	Platform	Library Amplification	Sequencing principle	Systems	Time per run	Total Output / run	Read Length	Number of single reads	Cost per Gb (US$)	Instrument price (US$)	Error type (%)	Advantages	Disadvantages	Currently available
Roche	454	Emulsion PCR on microbeads	Pyrosequencing	454 GS Junior	10 h	35Mb	Up to 600 bp	70K - 0.1 M	40K	125K	Indel, 1%	- Long read length - High experience in HIV-1 testing	- Homopolymer errors affecting key DRMs (e.g., K65R, K103N) - Long hands-on time	No
				454 GS FLX Titanium XLR70	10h	450Mb	Up to 600 bp	1M	15.5K	NA	Indel, 1%			
				454 GS FLX Titanium XL+	23 h	700 Mb	Up to 1000 bp	1M	9.5K	450K	Indel, 1%			
Thermo Fisher	SOLID	Emulsion PCR on microbeads (PCR on FlowChip for 5500W models)	Sequencing by ligation	Solid 5500 Wildfire	6 days	80-160 Gb	50-75 bp (SE)	700 M	130	251K	AT bias, ≤0.1%	- Two-base encoding corrects for intrinsic error	- Long run time	Yes
				Solid 5500 XL	10 days	160-320 Gb	50-75 bp (SE)	1.4 B	70	NA	AT bias, ≤0.1%			
	Ion Torrent	Emulsion PCR on microbeads	Semiconductor-based sequencing by synthesis	Ion PGM 314	3.7-23h	30-100Mb	200-400 bp (SE)	Up to 0.6M	25-3500	50K	Indel, 1%	- Less expensive equipment	- Homopolymer errors affecting key DRMs (e.g., K65R, K103N) - Equivalent to 454	Yes
				Ion PGM316	3-5h	300Mb-1Gb	200-400 bp (SE)	2-3M	700-1000	50K	Indel, 1%			
				Ion PGM318	4-7h	600Mb-2Gb	200-400 bp (SE)	4-5.5M	450-800	50K	Indel, 1%			
				Ion Proton	2-4h	10Gb	Up to 200 bp (SE)	60-80M	80	224K	Indel, 1%			
				Ion S5	2.5-4h	600Mb-15Gb	200-400 bp (SE)	3-80M	300-2400	65K	Indel, 1%			
Illumina		PCR amplification of adapter-ligated fragments	Sequencing by synthesis. Bridge-PCR on flow cell surface.	MiSeq (v2. or v.3)	4-56h	15Gb	300bp (PE)	12-50M	110-1000	99K	Substitution, 0.1%	- High sequence yield - Streamlined library preparation - Low input DNA concentration required - High experience in HIV-1 testing	- Substitution errors might induce false mutations if not properly corrected - Short sequence size that complicates haplotype reconstruction	Yes
				NextSeq 500 High	11-29h	129Gb	75bp (SE)-150bp (PE)	400M (SE) – 800M (PE)	33-43	250K	Substitution, <1%			
				NextSeq 500 Mid	15-26h	16-40Gb	75bp (SE)-150bp (PE)	Up to 260M (PE)	40-42	250K	Substitution, <1%			
				HiSeq 2500 v2. Rapid	7-60h	9-150Gb	36 (SE) – 250bp (PE)	600M	40-230	690K	Substitution, 0.1%			

			HiSeq 2500 v3	2-11 days	47-300Gb	36 (SE) – 100bp (PE)	1.5B (SE) - 3B (PE)	45-180	690K	Substituti on, 0.1%			
			HiSeq 2500 v4	29h-6 days	64-500Gb	36 (SE) – 125bp (PE)	2B (SE)-4B (PE)	30-150	690K	Substituti on, 0.1%			
			HiSeq 3000/4000	1-3.5 days	105-750Gb	2 x 150bp	60B	22-50	740-900K	Substituti on, 0.1%			
Pacific Biosciences	dsDNA template capped by hairpin loops (SMRTbell™)	Single molecule real-time long reads sequencing by synthesis	RSII	4h	500Mb-1Gb	20kb	55K	1000	695K	13% single pass; indel	- Long read length - Platform-specific software tools available - PCR-free experiments avoid amplification bias -Good performance in high GC content regions -High quality consensus reads	- High input DNA concentration required - Low yield at high accuracy - Higher error rate (insertions) for individual reads - Expensive equipment - Limited experience in HIV-1 testing	Yes
			Sequel	Up to 240 minutes	5Gb-10Gb	8-12kb	350K	NA	350K	NA			
Oxford Nanopore Technologies	No pre-amplification steps required	Real time sequencing	MK 1 MinION	Up to 48h	Up to 1.5Gb	>100K	>100K	750	1K	Indel, 12%	- Long read length - Portable (MinION) - Single-molecule - Low-cost - Streamlined sample preparation - PCR-free experiments avoid amplification bias	- Performance still being tested and optimized - Substitutions and indels (high error rate yet) -No experience in HIV-1 testing	Yes
			PromethION	NA	Up to 4Tb	NA	NA	NA	75K	NA			
BGI	Template enrichment in solution	Combinatorial probe-anchor ligation or combinatorial probe-anchor synthesis	BGISEQ 500 FCS	24h	8-40Gb	50 (SE) - 100bp (PE)	NA	NA	200K	AT bias, ≤0.1%	- Two flow cells can be run simultaneously with different conditions - Short hands-on time	- Only commercialized in China - Performance still being tested and optimized - No experience in HIV-1 testing	Yes
			BGISEQ 500 FCL		40-200Gb			NA	250K	AT bias, ≤0.1%			

in 2001, to US$0.014 in 2015 [46]. Equipment and maintenance costs, however, remain unaffordable for many low-income countries. The most important limitation, however, is the lack of automated, validated and robust but simplified bioinformatic analyses coupled with HIV-1 resistance interpretations to enable NGS use and interpretation by laboratory technicians, but even this is improving rapidly.

Table 2. Ideal requirements of NGS platforms for routine HIV-1 diagnostics

Need	Target/s
Increased automation	• Highly automated sample preparation • Time from sample to sequencing library completed: ≤ 2 days • Time from sample to HIV-1 resistance report produced: ≤ 1 week • Complete procedure, from sample to report, doable by one laboratory technician with part-time dedication
Increased flexibility and scalability	• Cost-effective from at least 10-20 samples/week to be useful for small clinical laboratories • Scalable to >100 samples/week to be useful for large reference laboratories
Ensured technical robustness	• Results should be highly reproducible between and within tests and be resilient to variations during library preparation • Appropriate positive and negative controls included • QA/QC panels periodically tested
Automated bioinformatics	• Robust and comprehensive bioinformatic analyses doable by a part-time dedicated technician with no knowledge in bioinformatics • Output: HIV-1 resistance interpretation reports
Clinical value ensured	• Clinical guidelines to transfer results to clinical practice.
Decreased costs	• Final genotyping test per <30 euros for LMICs, including bioinformatic analysis and resistance reports • Reduction in upfront and maintenance costs
Decreased work load and complexity in the laboratory	• Complete procedure, from sample to report, doable by one laboratory technician with part-time dedication
Turn-around time to results	• <15 days, ideally <1 week

3. Clinical Value of NGS

3.1. Treatment-naïve subjects

Ultrasensitive HIV-1 genotyping detects 1.5 to 3-fold more DRMs than Sanger sequencing in all clinical settings, also in ARV-naïve individuals [47–50]. Low-frequency drug-resistant mutants (LFDRM) may be acquired through person-to-person transmission [51,52] or be generated *de novo* through error-prone replication[53]. The two most important questions for clinicians are, however, if the detection of LFDRMs is technically robust and, therefore, they can rely that any mutation reported is not false, and to what extent the LFDRMs detected impact the efficacy of ART.

Many factors affect the clinical relevance of LFDRMs; the most essential ones can be summarized with the following relationship:

$$Clinical\ relevance \approx \frac{prevalence\ in\ human\ populations, mutant\ frequency\ in\ the\ individual}{genetic\ barrier\ to\ resistance\ of\ the\ ART\ chosen}$$

The **prevalence of DRMs in ARV-naïve subjects** is directly proportional to (a) the time since the introduction of the different ARV drug/s and their relative use in the patient's population; (b) the number of subjects developing virological failure to those drugs in the population, and (c) the frequency of the DRMs in subjects with virological failure, from whom mutations might be transmitted to ART-naive patients. Conversely, the fitness cost conferred by the mutation/s to the virus is inversely related to the pre-existence of TDR mutants.

The most clinically-relevant DRMs in ART-naïve subjects will be those more likely to pre-exist before ART and more likely to reduce the virological efficacy of regimens, i.e., those affecting drugs with low genetic barrier to resistance and with high prevalence among ART-naïve subjects **(Table 3)**. It is important to note that the same principles apply to both Sanger and NGS. The latter simply detects more mutants because it has greater technical sensitivity.

Table 3. Factors affecting the clinical relevance of pre-existing drug resistance mutants in ART-naïve subjects

		Factors influencing the prevalence of DRMs in ART-naïve subjects					**Prevalence of DRMs in ART-naïve subjects**	**Genetic barrier to resistance**	**Clinical relevance of DRMs in ART naïve subjects**
		Time since introduction of the drugs (in RLS)	Relative use in RRS (in RLS)	Frequency of mutations in VF	Amount of VFs in RRS (in RLS)	Fitness cost			
NRTI		+++ (+++)	+++ (+++)	++	+ (+++)	++	++	+/++	**++**
NNRTI									
	EFV, NVP	+++ (+++)	+++ (+++)	+++	+ (+++)	+	+++	+	**+++**
	ETR, RPV	++ (0)	+ (0)	+++	+ (0)	+	++	++	**++**
PI/r		+++ (+)	++ (+)	+	++ (+)	+++	+	+++	**0**
INSTI									
	RAL, ELV	++(0)	+++ (0)	++	+ (0)	+	0	+	**0**
	DTG	+ (0)	++ (0)	+	+ (0)	+	0	++	**0**

The main factors influencing the prevalence of DRMs in ART-naïve subjects are the time since the drugs were introduced, their relative use in the patient's population, the amount of DRMs that are selected when the drugs fail (e.g., high for lamivudine or raltegravir and low for boosted PIs), the amount of VFs in the patient's population and the fitness cost conferred by the DRMs to the virus. Regardless of their frequency in the patient's virus population, DRMs will be clinically relevant in ART-naïve subjects if they are likely to be detected and affect drugs with low or intermediate genetic barrier. For example, PI DRMs are not relevant in ART-naïve subjects because their prevalence is low and boosted PIs have a high genetic barrier to resistance. Conversely, INSTI resistance mutations are not relevant in ART-naïve subjects today because their prevalence remains negligible. However, given the low/intermediate genetic barrier to resistance of INSTIs, detecting INSTI-resistant mutations would become highly clinically relevant if their prevalence in ART-naïve increased. It is important to note that the same rules apply for both majority and minority DRMs.

+, low; ++, intermediate; +++, high; 0, not significant; RRS, resource-rich settings; RLS, resource-limited settings; DRM, drug-resistance mutation; VF, virological failure; ART, antiretroviral therapy; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; PI/r ritonavir-boosted protease inhibitors; INSTI, integrase strand transfer inhibitors.

The most frequently observed mutations in ART-naïve subjects are NRTI-related, particularly TAMs [54–56], and NNRTI resistance mutations [48,57]. These drug classes have been around for long time, have been used extensively, have been prescribed to more people prone to develop virological failure and in less optimal conditions including sequential mono or dual therapy, and are often associated to emergent resistance when virological failure occurs. In comparison, primary PI-resistance mutations are rare in ART naïve subjects. They are rarely selected during virological failure when the PI is pharmacologically boosted and usually confer significant fitness costs to the virus, being less easy to transmit.

Transmission of INSTI resistance remains anecdotal [58–61]. This family was introduced more recently, drugs were generally prescribed in better conditions (e.g., alongside other 2 active drugs in the regimen), are very well tolerated and have a simple posology, which improves ART adherence and limits the incidence of virological failure. Whereas first-generation INSTIs have low genetic barrier to resistance, new INSTIs with higher genetic barrier like dolutegravir are increasingly being prescribed, being less likely to develop resistance upon virological failure.

The **level at which each drug resistance mutation is present** in the virus population is also important. Studies have shown a dose-dependent association between the level of LFDRMs and the risk of virological failure to first-line NNRTI therapy [48,57,62]. It has been suggested that the mutational load (i.e., the mutant frequency multiplied by the total HIV-1 RNA levels) might predict virological failure with higher accuracy than the mutant frequency alone [63,64]. For example, a K103N mutant present in 1% of viruses would have a greater impact on the efficacy of first-line EFV-based therapy if the subject had a viral load of 100.000 copies/mL than 1000 copies/mL, because the amount of K103N mutants would be 1000 copies/mL in the first case and only 10 copies/mL in the second one. However, studies to date have not been able to find a consistent cut-off in either mutant frequency of mutational load that might identify subjects at higher risk of developing virological failure with sufficient accuracy.

Finally, the **availability of ART alternatives** is another essential factor to determine the need and clinical utility of HIV-1 resistance testing. Resistance testing is not needed if ART is given under a strict public health approach that includes one predefined 1st-line and one predefined 2nd-line ART combination.

The main studies addressing the impact of LFDRMs on the efficacy of ART in both treatment-naïve and -experienced subjects are summarized in **Table 4.**

3.1.1. Non-Nucleoside Reverse Transcriptase Inhibitors

Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) have been the preferred drugs for ARV-naïve subjects for many years, and remain the preferred choice for first-line ART in low and middle-income countries [65]. The Achilles' heel of first-generation NNRTIs (efavirenz and nevirapine) is their low genetic barrier to resistance, i.e., single mutations, which can often be transmitted, significantly impair their efficacy. Pre-existing NNRTI resistance has important economic implications in LMICs, because virological failure to NNRTI-based ART often leads to resistance to the NRTI backbone and switching to significantly more expensive PI-based regimens.

In a systematic review and pooled analysis of published studies mostly using allele-specific PCR (ASPCR) [57], detection of pre-existing low-frequency NNRTI-resistant mutants was associated with a dose-dependent 2,5 to 3 fold increased risk of virological failure to first-line NNRTI-based ART. Using the most sensitive ASPCR assay, 11 subjects had to be tested to avoid one virological failure. In further analyses [66] the presence of minority NNRTI resistance mutations and NNRTI adherence were both found to be independent predictors of virologic failure, but also modified each other's effects on virologic failure. As expected, the effect of LFDRMs was most prominent at higher levels of medication adherence.

The clinical value of low-frequency NNRTI-resistant mutants was further confirmed in a case-control study involving seven European cohorts and using

Table 4. Selected studies addressing the clinical value of ultrasensitive genotyping

Study	ART exposure	ART tested (number of subjects)	Study design	Genotyping technique	Risk of VF if LFDRM are detected
[101]	Naïve	EFV/3TC + ABC/AZT (316)	Case control	ASPCR for K103N, Y181C, M184V	• OR= 8.71 (P= 0.004) for any LFDRM
[102]	Naïve	NRTI (21) / NNRTI (14) / PI (38)	Cohort	ASPCR for K103N, M184V, L90M	• No association, but only 2 VF out of 13 evaluable subjects receiving NNRTIs
[103]	Naïve	3TC+FTC+ EFV (18)/ NVP (4)	Case control	ASPCR for K65R, K103N, Y181C, M184V, M184I	• HR=6.0 (P=0.001) → 4/4 (100%) VF if any LFDRM present vs. 3/18 (17%) VF if no LFDRM detected
[104]	Naïve	EFV/NVP (84)	Cohort	ASPCR for K103N	• No association. VF in 24% of 17 patients with and 15% of 67 patients without K103N (HR=1.75, P=0.468)
[105]	Naïve	EFV (37) / NVP (56)	Case control	ASPCR for K103N	• HR=∞, P=0.001→ 4/18 (22%) VF if K103N present vs. 0/75 (0%) VF if K103N not detected
[26]	Naïve	NNRTI (84) / PI (109) / PI+NNRTI (63)	Cohort	454	• HR= 2.73 (P=0.007) if NNRTI LFDRM present • HR= 8.4 (P=0.002) if PI LFDRM present • PIs were not boosted
[106]	Naïve	NNRTI (46)	Cohort	SNaPshot	• No association, but only 1VF
[107]	Naïve	EFV (290)	Case cohort	ASPCR for K103N, Y181C	• Y181C: HR=3.45 (P<0.001) in adherent subjects • K103N: HR=1.58 (p=0.220)
[64]	Naïve	EFV (476)	Cohort	ASPCR for K103N	• OR= 2.33 (P=0.003) if K103N detected • OR= 47.4 (P=0.001) if K103N load>2000 copies/ml
[108]	Naïve	TDF+FTC+ PI/r (81)/ NNRTI (65)	Cohort	ASPCR for K65R, K103N, M184V	• No association, LFDRM detected in 7/65 subjects initiating NNRTIs (2 K65R, 3 M184V and 1 K103N) and no VF in any of them
[57]	Naïve	NNRTI (1263)	Pooled analyses from 10 studies	ASPCR (9 studies), SNaPshot (1 study)	• HR= 2.6 [95% CI, 1.9-3.5; P<0.001] overall • Dose-effect relationship between LFDRM frequency / mutational load and VF detected
[109]	Naïve	NNRTI (208)	Cohort	ASPCR for K103N, Y181C	• No association, but only 7/183 (4%) VF overall
[48]	Naïve	NVP (42)/ EFV (218)	Case control	454	• OR=2.75 (P=0.005) if ≥1 any RTI LFDRM present • OR=2.41 (P=0.024) if ≥1 any NNRTI LFDRM present • Dose-effect relationship between LFDRM frequency / mutational load and VF confirmed
[110]	Naïve	PI/r (57)	Cohort	454	• No association • VF in only 1/14 subjects with PI TDRs • Most PI TDRs found in isolation and had low resistance algorithm scores
[28]	Naïve	PI/r (84) EFV (57)	Cohort	454	• EFV: HR=4.3 (P=0.074) • PI/r: HR=1.8 (P=0.350), even with HIVdb score≥10
[83]	ART-naïve (76) and experienced (27)	EFV (103)	Cohort	ASPCR for K103N, Y181C	• K103N >1% associated with inferior HIV-1 RNA response to EFV (change in HIV-1 RNA level, +0.5 vs. -1.1 log copies/mL, P<0.001).
[85]	ART-experienced	ETR / PI/r /RAL (101)	Cohort	454	• HR=4.6, P=0.007, if 454-GSS <3
[86]	ART-experienced	RAL(87),ETR (84), DRV (83)	Cohort	454 and Illumina	• OR=2.8 (P=0.090) if ETR LFDRM present.
[93]	ART-experienced	Maraviroc (1827)	Clinical trials	454	• Subjects with R5-HIV: VL<50 copies/mL at week 48 in 67% of maraviroc vs. 69% of EFV recipients, similar than with ESTA

(Table 4) ASPCR: allele-specific PCR; EFV: efavirenz; NVP: nevirapine; 3TC: lamivudine; FTC: emtricitabine; ABC: abacavir; ZDV: zidovudine; RAL: raltegravir; ETR: etravirine; TDF: tenofovir; DRV: darunavir; /r: ritonavir boosted; NNRTI: non-nucleoside analogue reverse transcriptase inhibitor; NRTI: nucleoside/nucleotide analogue reverse transcriptase inhibitor; PI: protease inhibitor; HR: hazard ratio; OR: odds ratio; LFDRM: low frequency drug resistance mutant; TDR, transmitted drug resistance; VF: virological failure; ART: antiretroviral treatment; ESTA, Enhanced-sensitivity Trofile® Assay (Monogram Biosciences).

centralized 454 genotyping [48]. From 260 evaluable ART-naïve subjects with no pre-existing resistance mutations by population sequencing who achieved undetectable HIV-1 RNA levels after initiating ART with two NRTIs plus one NNRTI, those subsequently developing virological failure were more than 2-fold more likely to harbour pre-existing LFDRMs than those who remained virologically suppressed for a matched duration of time. The study also confirmed a dose-effect relationship between both the frequency level and the mutational load of LFDRMs and the risk of virological failure to first-line NNRTI-based ART. The observed association was consistent regardless other factors, including the NRTI backbone started and nevirapine vs. efavirenz use.

Detection of NNRTI LFDRMs also proved to be clinically relevant in women from LMIC with prior exposure to NNRTI as regimens to prevent mother-to-child transmission (pMTCT) [67]. Women previously exposed to single dose nevirapine (sdNVP) [68,69]and with low-frequency NVP resistance mutations had increased risk for VF with NVP regimens [70]. Such increased risk, however, applied if NNRTI-based ART was started during the first 6-12 months after delivery, suggesting that the progressive decline in frequency was reducing the clinical significance of such mutants. Data on the clinical value of LFDRMs with current B+ regimens is lacking.

3.1.2. Protease Inhibitors

In contrast with NNRTIs, studies have not demonstrated any clinical value of NGS to optimize the efficacy of first-line boosted PI ART in ART-naïve subjects. A subanalysis of the Castle Study [71] comparing the efficacy of first-line atazanavir/ritonavir vs. lopinavir/ritonavir, each in combination with tenofovir-emtricitabine regimen in ARV naïve subjects, showed that although 454 sequencing increased the detection of PI-associated resistance mutations and polymorphisms by 2-3-fold, virological response to both regimens was not affected by presence of LFDRMs [72]. In a recent analysis from our own group using a stricter criterion to define PI resistance, we were also unable to detect an influence of LFDRMs on first-line PI therapy outcomes[28]. Protease inhibitors have a high genetic barrier to resistance, i.e., numerous mutations are needed to develop a substantial impact on virologic response to treatment (Wensing et al. 2015). Viruses with multiple PI-resistance mutations are rarely transmitted [74,75]. Moreover, when transmission occurs, PI-resistant viruses are usually transmitted as single clones and rarely back-mutate, because back-mutation of accessory PI mutations often leads to insurmountable fitness valleys. Compensatory fixation [76] might thus explain why transmitted PI-resistant mutants remain relatively homogeneous for prolonged periods of time. Obtaining solid evidence of an impact of LFDRMs on boosted PIs, if such impact exists, might possibly require very large studies.

3.1.3. Integrase Strand Transfer Inhibitors

Treatments including integrase strand-transfer inhibitors (InSTIs) have become the preferred choice for first-line ART in Western countries [77,78] because they are virologically non-inferior or even superior to other alternatives, are well-tolerated and can often be prescribed coformulated with 2 NRTIs as single tablet regimens (STR). Given recent agreements for cost reductions in LMICs [79] dolutegravir has also been included as a preferred drug in WHO guidelines [65]. Raltegravir and elvitegravir have low-genetic barrier to resistance but transmission of INSTI-resistant mutants remains

anecdotal [60,61]. Using highly-sensitive ASPCR testing, Charpentier et al. detected very low levels of Q148R mutants in 81% of 32 ARV-naïve subjects never exposed to INSTIs [80]. Pre-existing, spontaneously generated INSTI-resistant mutants might be selected during suboptimal INSTI therapy[81]. However, in a representative pan-European surveillance study [59] our group found no signature InSTI mutations circulating in Europe by either Sanger or Deep sequencing in ART naïve subjects in 2006-2007, before INSTIs began to be being prescribed. Similar findings have been reported from othe European cohorts [58]. In this latter study, reduced occurrence of Q148H/R/K+G140S/A was seen in non-B clades versus subtype B, and was explained by the higher genetic barrier to the G140S mutation observed in all non-B clades analyzed. As long as transmitted INSTI resistance remains clinically negligible, there might be no need to perform baseline integrase resistance testing in INSTI-naive subjects, neither by Sanger nor NGS. However, such recommendation should regularly be revised according to periodic INSTI resistance transmission surveillance.

3.2. Antiretroviral-experienced subjects

The clinical relevance of DRMs is harder to evaluate in ART-experienced subjects [25]. They are, however, our most difficult to treat patients and those who require more accurate DRM analyses to prevent additional virological failures, exhaustion of treatment options and increased mortality [82]. A number of studies have shown that detection of LFDRMs is more likely in ART-experienced subjects. For example, using ASPCR, Halvas et al [83] found more low-frequency NNRTI-resistant mutants in NNRTI-experienced subjects than in NNRTI–naïve individuals. Moreover, detection of K103N at frequencies >1% was significantly associated with reduced response to EFV-containing treatment. Similarly, Lecossier et al. [84] detected more minority K103N mutants in subjects developing virological failure to nevirapine and in patients interrupting treatment with this drug than in NNRTI-naïve subjects.

In a multicenter cohort study in 4 Spanish centers [85] genotypic sensitivity scores (GSS) calculated from 454 sequencing data predicted salvage ART

outcomes better than those calculated from Sanger sequencing. In a multivariate analysis, a GSS <3 by 454 sequencing was independently associated with more than 4-fold increased risk of VF to salvage ART, compared with a 454-GSS >3. The study was, however, retrospective, and could not fully rule out hidden bias or confounding. In addition, the ability of Sanger sequencing to predict ARV outcomes was very small. In comparison, in a sub-analysis of the TRIO study in 87, 84 and 83 ART-experienced subjects with multidrug-resistant HIV-1 initiating raltegravir, etravirine and ritonavir-boosted darunavir for the first-time [86] LFDRMs were detected in 6, 27 and 22 subjects respectively, using 454 and Illumina sequencing. However, only subjects with baseline etravirine LFDRMs showed a trend to increased risk of virological failure.

4. Viral Tropism

Tropism determination is required before maraviroc prescription, as this is a selective CCR5 co-receptor antagonist, and approximately 10-15% of treatment-naïve subjects and 50% of experienced subjects have viruses that can also use CXCR4 coreceptor [87]. A number of studies have shown an association between VF to CCR5-antagonists and the presence of minority populations of CXCR4-using viruses [88–91].

In a retrospective analysis of two clinical trials of maraviroc in treatment-experienced subjects [92], V3-loop 454 sequencing was a better predictor of maraviroc response than the first version of the Monogram's phenotypic Trofile® Assay. In a reanalysis of the MERIT trial [93] comparing 454-sequencing with the Enhanced Sensitivity Trofile Assay (ESTA), deep sequencing led to a better prediction of which subjects would have had responded to maraviroc. Deep sequencing has proved to be at least as accurate as the most sensitive phenotypic assays being more cost-effective and generally faster to perform [94]. The availability of freely available semi-automated interpretation systems of deep V3-loop sequencing such as Geno2Pheno[454] [95], has simplified the use and interpretation of NGS data.

Moreover, equivalent tropism results can be obtained with different NGS platforms[96].

5. NGS in low and middle-income countries

Antiretroviral treatment is provided in low and middle income countires (LMIC) following a public health approach, where HIV-1 drug resistance testing is only used in surveys to inform national and regional ART policy [65]. With the increased exposure to antiretroviral drugs, however, the prevalence of transmitted and acquired drug resistance is steadily increasing in adults and children [3], endangering our ability to reach the 90-90-90 WHO goals for 2020 (90% of all HIV-infected diagnosed, 90% of HIV-positive on ART, and 90% of those on ART with HIV-1 suppression)[97]. Countries and global health agencies are therefore beginning to consider HIV-1 drug resistance testing also for clinical management[98]. Next-generation sequencing platforms might be particularly suited for centralized testing of large numbers of samples given their high capacity for multiplexing, which is associated with reductions in sequencing costs.

The prevalence of transmitted NNRTI resistance is increasing in many LMIC as they progress in their population coverage, reaching 10-15% of ART-naïve subjects [3,75]. Until dolutegravir becomes available, subjects with pre-existing NNRTI resistance, even at low frequency levels, should rather start ritonavir-boosted PI ART, which is a more expensive option for LMICs. Pre-ART NGS resistance testing might be useful to accurately identify the majority of subjects in whom NNRTI ART could still be safely prescribed in this scenario.

The utility of NGS in treatment-experienced subjects is under debate. In a recent study using Illumina MiSeq sequencing in Durban, South Africa, [99] we found nearly 70% of K65R prevalence in subjects developing virological failure first-line ART containing TDF, which was missed by Sanger sequencing in 30% of the subjects. Conversely, the EARNEST trial [100]

observed that, even in the presence of high-level NRTI drug resistance, second-line regimens including boosted PI plus two NRTIs maintained better virological outcomes than PI monotherapy, suggesting that residual NRTI activity is enough to suppress HIV-1 replication when combined with boosted PIs regardless of the actual NRTI resistance profile.

A number of major challenges beyond the scope of this review remain before drug resistance testing reaches routine clinical management in low-income countries. More studies will be needed to clarify its clinical utility and cost-effectiveness for patient management. Reductions in library preparation costs will be essential to make NGS more affordable. Deployment of robust and low-cost automated bioinformatic analysis tools to LMICs will also be key for that purpose.

6. Conclusion

Next-generation sequencing is advancing our knowledge and improving our ability to diagnose and act upon HIV-1 resistance. The clinical utility of resistance testing is determined by different equally important factors, namely: the prevalence of a certain mutations or mutation patterns in a population; the frequency at which each mutant or set of mutants are present in an individual; the genetic barrier to resistance of the drug or drug combination challenged by the resistant mutant, and the availability of alternative ART options. To date, ultrasensitive genotyping has proved to improve ART outcome predictions in ARV-naïve subjects starting nevirapine or efavirenz and CCR5 antagonists, and might also be helpful to select salvage ART regimens in ART-experienced subjects. Detection of LFDRMs, by the contrary, has not provided any evident benefit relative to Sanger sequencing in ART-naïve subjects initiating ritonavir-boosted PIs. Given the current lack of transmitted INSTI-resistant mutations, HIV-1 genotyping using either Sanger or NGS techniques is not currently mandatory before initiating an INSTI. However, continued surveillance of INSTI resistance transmission is absolutely necessary because anecdotal reports show that INSTI-resistant mutants can

indeed be transmitted. If INSTI transmission increased to significant levels, then integrase genotyping would become a major clinical need. In a scenario where global HIV-1 eradication is now considered possible, making NGS accessible also to LMICs is a challenge we must address. Reductions in sequencing costs, particularly in library preparation, and accessibility to low-cost, robust but simplified automated bioinformatic analyses of NGS data will remain essential to end the HIV-1 pandemic.

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Objectives and Hypotheses

Objectives

1. To investigate if the detection of pre-existing drug resistant minority variants and/or X4 HIV-1 variants could improve the efficacy of first line combined antiretroviral therapy in late presenters with advanced disease.

2. To define the natural genotypic variation of the HIV-1 integrase gene in Europe for epidemiological surveillance of integrase strand transfer inhibitor resistance.

3. To evaluate the prevalence of tenofovir resistance mutation K65R in subjects from South Africa developing virological failure to first-line tenofovir containing regimen.

4. To investigate whether NS5B S282T resistance mutation pre-exists in treatment naïve HCV/HIV-1 co-infected subjects.

Hypotheses

1. Pre-existing minority HIV-1 resistance variants and the presence of X4 HIV-1 increase the risk of virological failure in late presenters with advanced disease.

2. Integrase polymorphisms but not integrase strand transfer inhibitor signature resistance mutations pre-existed before the introduction of InSTIs in clinical practice.

3. Sanger sequencing underestimates the prevalence of tenofovir-disyproxyl fumarate resistance after TDF failure in subtype C viruses.

4. Mutation S282T in the HCV NS5B gene pre-exists at low frequency levels.

Chapter 1

Clinical Value of Ultrasensitive HIV-1 Genotyping and Tropism Testing in Late Presenters with Advanced Disease

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Abstract

Objective: This article aims to investigate if the detection of preexisting drug-resistant minority variant (DRMV) and/or X4 HIV-1 variants could improve the efficacy of first-line combined antiretroviral therapy (ART) in late presenters.

Design: Post-hoc, combined analysis of two open-label, prospective, randomized clinical trials comparing first-line ART with efavirenz (EFV) vs. ritonavir-boosted protease inhibitors (PI/r)-based regimens in ART-naïve, HIV-1-infected patients, with $CD4^+$T-cell counts less than 100 cells/µl and wild-type HIV-1 by bulk sequencing.

Methods: Pre-ART samples were reanalyzed for the presence of DRMVs and X4 HIV-1 using 454 sequencing. Kaplan-Meier curves and Cox regression were used to evaluate the association between X4 HIV and DRMVs and risk of virological failure.

Results: From 141 evaluable patients, 57 received EFV-, and 84 received PI/r-including first-line ART. Median pre-ART $CD4^+$T-cell counts and HIV-1 RNA levels were 39 cells/mm^3 and 257,424 copies/mL, respectively; 35.5% of patients had X4 HIV variants. Detection of DRMVs leading to an ART-specific cumulative HIVdb score ≥10 increased the risk of virological failure in patients initiating EFV [Log-Rank p=0.048, HR=4.3 (95CI: 0.8, 25.0), p=0.074], but not in those starting PI/r. Presence of X4 HIV did not affect virological outcomes, but was associated with impaired $CD4^+$T-cell count recovery over 2 years [214 vs. 315 cells/µl with X4 vs. R5 HIV-1 tropism, respectively, p=0.017].

Conclusions: Accounting for pre-existing DRMVs may improve the outcomes of first-line nonnucleoside reverse transcriptase inhibitor-based ART in late presenters with advanced immune suppression. Presence of X4 HIV-1 at diagnosis predicts impaired immune restoration under ART.

Introduction

One of the main unsolved public health problems in HIV/AIDS is that, still, more than 30% of people living with HIV in Western countries are diagnosed at late stages of their infection [1], that is, when their $CD4^+$T-cell counts have already decreased to less than 200 cells/µl or have developed an AIDS-defining illness [2]. Late HIV-1 diagnosis is associated with increased mortality and morbidity [1]. Late presenters are more likely to develop antiretroviral treatment (ART) failure [3], suffer AIDS-defining illnesses, transmit HIV to other individuals and achieve suboptimal immune reconstitution with ART [4]. Up to a third of all HIV-related deaths today are estimated to be consequence of late diagnosis. Late presentation thus implies a huge economic burden to healthcare systems [5–7]. It is crucial to identify and provide late presenters with the best treatment option as early as possible.

Studies in patients with advanced HIV disease have generally compared the efficacy of different ART combinations in virological [8], immunological and inflammatory [9,10] outcomes, but none has addressed whether HIV-1 tropism or the presence of drug-resistant minority variants (DRMV), which are not routinely evaluated, could be relevant factors influencing treatment outcomes. In previous studies, first-line efavirenz (EFV)-based ART achieved non-inferiority [12] or even superiority [8] in terms of virological efficacy than protease inhibitor (PI)-based therapy, being a suitable treatment option for ART-naïve patients with advanced disease. However, the efficacy of non-nucleoside reverse transcriptase inhibitors (NNRTIs) may be impaired by the presence of minority NNRTI-resistant variants, which could be missed by current Sanger sequencing genotyping approaches [11]. Minority PI-resistant variants have not been shown to affect the efficacy of first-line PI therapy [12,13], but studies are scarce, generally underpowered, and often mixed naturally occurring protease polymorphisms with drug-resistant mutations when defining minority variants, thus diluting the effect of resistance mutations having an impact on PI treatment outcomes.

HIV-1 tropism might also influence ART outcomes. In a subanalysis of 428 patients of the ArTEN study [14], a prospective, randomized, open-label, noninferiority trial

that compared nevirapine (NVP) vs. atazanavir/ritonavir (ATV/r), each combined with fixed-dose coformulated tenofovir/emtricitabine (TDF/FTC) in antiretroviral-naïve patients with HIV-1 infection, HIV-1 tropism was an independent predictor of virological failure to first-line ART, particularly at week 24 post ART initiation and in non-B subtypes. It is well known that patients with low $CD4^+$T-cell counts, like the ones evaluated in this study, are more likely to present CXCR4-using viruses [15]. The ArTEN tropism results, however, have not been confirmed in independent analyses.

Here, we investigated if baseline HIV-1 viral tropism and DRMV testing using next-generation sequencing were associated with virological and immunological outcomes of first-line ART in patients initiating therapy with less than 100 $CD4^+$T-cells/µl in two randomized clinical trials comparing EFV vs. ritonavir-boosted protease inhibitors (PI/r) based regimens [16].

Methods

Study design

This was a retrospective analysis of baseline clinical samples collected in two prospective, multicentric, open-label, randomized clinical trials comparing the efficacy of first-line ART with EFV vs. indinavir/ritonavir (IDV/r) combined with coformulated zidovudine along with lamivudine (Combivir; GlaxoSmithKline) (ADVANZ study[16]), and EFV vs. atazanavir/ritonavir (ATV/r) vs. lopinavir/ritonavir (LPV/r), all combined with coformulated tenofovir plus emtricitabine (Truvada; Gilead Sciences Inc.) (ADVANZ-3 study) [9]. Both studies were approved by the ethics committees of the participating hospitals and the Spanish Agency for Medicines and Healthcare Products and conducted in compliance with the Declaration of Helsinki, good clinical practice guidelines, and local regulations. Patients were adequately informed about the study objectives and signed a written

informed consent form before enrolment. All patients were ART-naïve, had $CD4^+$ T-cell counts less than 100 cells/μl and were infected with wild-type HIV-1 according to bulk sequencing at study entry. Patients were recruited from six Spanish HIV clinical centers between November 2001 and January 2003 (ADVANZ study), and from five centers between September 2007 and February 2010 (ADVANZ-3 study), and were followed during three and two years, respectively. To homogenize our dataset, we only considered data from the first two years of follow up of both studies. Virological failure was defined as two consecutive HIV-1 RNA determinations of at least 200 copies/ml or one HIV-1 RNA measurement of at least 1000 copies/ml at or after month 6 of ART. Stored pre-ART samples were reanalyzed for the presence of DRMVs and HIV-1 tropism using 454 sequencing as previously reported (Supplementary methods).[17,18]

Tropism

Geno2Pheno[454] was used to evaluate tropism directly from raw 454 .sff files. Tropism was defined using the criteria established in the previous MERIT, MOTIVATE 1 and 2 and the A4001029 trials of maraviroc in ART-naïve and experienced patients [18], that is, X4 HIV was defined as the presence of at least 2% of variants with a Geno2pheno false positive rate 3.75% or less. This definition also includes dual/mixed tropic viruses, as sequencing techniques do not differentiate between them.

HIV-1 resistance

Sequences were analyzed using the Roche/454 proprietary Amplicon Variant Analyzer (AVA) software (v2.8.0) as described previously [17]. A coverage of at least 500 reads per position was required for further analysis to ensure a minimum opportunity of detecting a low-frequency variant; codons with less than 500 reads were considered “low-covered” and treated as wild-type. According to the previous strand-dependent sequencing error patterns and negative control testing results (sequencing of a pNL43 DNA clone), only variants with frequency values on forward and reverse reads within a one log ratio and an overall frequency greater than 0.5% were utilized for downstream analysis. Amplicons with less than a 10% of the

expected length were discarded and not considered for further analyses (see [17] for a detailed description of the 454 sequencing and analysis methods, including error controls).

Drug susceptibility was analyzed using Stanford HIVdb (v6.3.1; Stanford University, California, USA). Patients with ART resistance mutations present in at least 20% of their viral population were excluded from downstream analyses. The remaining individuals were classified according to whether mutations detected impacted ART susceptibility or not. Such impact on ART susceptibility was defined as a cumulative ART-specific Stanford HIVdb score ($_{ART}$HIVdb score) of at least 10 points. For example, a subject with 3 mutants detected in whom each mutant was associated with a HIVdb score of 5 to any of the antiretroviral drugs initiated, would be categorized as having an $_{ART}$HIVdb score of at least 10 (5+5+5=15). This procedure ensured that only mutants with some impact on drug susceptibility were considered in the treatment outcomes analysis. It also allowed us to evaluate NNRTI- and PI-resistant variants together.

Statistical Analysis

Baseline subject's characteristics were described overall and according to virological outcome. P-values were obtained with the χ^2 or Fisher tests for categorical data, and the Mann-Whitney rank sum test for continuous data. Survival analyses including Kaplan-Meyer curves and Cox proportional hazards models were used to estimate differences in risk of virological failure by baseline HIV tropism and $_{ART}$HIVdb score. Separate models were constructed: one taking into account all patients, another one considering only those on EFV and a third one including patients receiving PI/r therapy exclusively. Separate multivariate Cox proportional hazards models were constructed for all patients, EFV-treated patients and PI/r-treated patients, using covariates that achieved a p-value <0.1 in each corresponding univariate analysis, in addition to the $_{ART}$HIVdb score. Sensitivity analyses using other definitions, such as presence of at least one IAS-USA 2013 NRTI and/or NNRTI mutation or an $_{ART}$HIVdb score cut-off of 5 were also performed. Finally, changes in $CD4^+$T-cell counts through year 2 of follow-up were compared according to $_{ART}$HIVdb score and HIV tropism, either including all patients

or after excluding individuals developing virological failure. Findings were confirmed with a linear mixed models analysis of the slope of $CD4^+$ gains over the study. Statistical analyses and graphs were performed using GraphPad Prism v5.0 (GraphPad Software Inc., La Jolla, California, USA), SigmaPlot v12.5 (SyStat Software Inc., San Jose, California, USA), and R.

Results

Patient's Characteristics

Out of 148 patients included in the parental ADVANZ (n=61) and ADVANZ-3 (n=87) studies, three were excluded from our analysis because of lack of follow-up data. Four additional individuals were excluded because of the existence of primary ART resistance when we considered mutations detected at frequencies of at least 20% of the virus population. This left 141 evaluable patients, who were mostly men (79%), presented with AIDS-defining diseases (54.6%), and had had sex with other men (44%) **(Table 1)**. At the time of ART initiation, the study participants were 39 years-old, had 37 $CD4^+$T-cells/µl and 257,424 HIV-1 RNA copies/mL (all median values); 8.5% were coinfected with the hepatitis B virus and 17.7% with the hepatitis C virus. Fifty-seven (40.4%) patients initiated EFV-based and 84 (59.6%) PI/r-based therapy. There were 29/141 (20.6%) patients who developed virological failure overall, 7/54 (12.3%) in the EFV arm and 22/84 (26.2%) in the PI/r arm.

Virological outcomes by tropism

HIV-1 tropism was evaluable in 139 of 141 (98.6%) patients; in the remaining two (1.4%) individuals the V3 loop could not be PCR-amplified. All patients were infected with subtype B HIV-1. Using the criteria by Swenson, et al.[18], HIV-1 was R5 in 89 (63.1%) patients and X4 in 50 (35.5%). A similar proportion of X4 HIV was observed among patients developing virological failure or not during follow-up (34.5% vs. 35.7%, respectively, p=0.755). There were no differences in time to virological failure by HIV tropism in the Kaplan-Meier analysis (log-rank test=0.756, Figure 1a).

Virological outcomes by HIV-1 resistance

Eighteen out of 141 (12.8%) samples did not amplify, leaving 123 (87.2%) evaluable patients. Using 454 sequencing, 19/123 (15.45%) patients had resistance mutations leading to an ARTHIVdb score of at least 10 **(Table 2)**. Patients with an ARTHIVdb score of at least 10 were more frequent among those developing virological failure than in those who did not (20.7% vs. 11.6%, respectively), although such difference was not statistically significant (p=0.365). In the Kaplan Meier analyses, patients with an ARTHIVdb score of at least 10 achieved virological failure earlier than those with a lower ARTHIVdb score (Figures 1b, c and d). Differences were statistically significant in the analysis of patients initiating EFV (log-rank test p=0.048), but not when considering all patients (log-rank test p=0.116) or those initiating PI/r (log-rank test p=0.494). Similar results were found in sensitivity analyses using other definitions, such as the presence of at least one IAS-USA RTI or NNRTI resistance mutation or an ARTHIVdb score cut-off of 5 (not shown).

Cox Proportional Hazards Models of Risk of Virological Failure

Overall, baseline factors significantly associated with virological failure in the univariate Cox proportional hazards model **(Table 3)** were: age below 35 years [hazard ratio (HR)=2.1, 95% confidence interval (CI): 1.0, 4.3, P=0.051] and being on the PI/r group relative to EFV [HR=2.2, 95% CI: 0.9, 5.1, P=0.072]. Factors associated with virological failure in patients receiving EFV were: having an ARTHIVdb score of at least 10 [HR=4.0, 95% CI: 0.86, 20.0, P=0.077] and having HCV co-infection [HR=4.6, 95% CI: 0.9, 23.0, P=0.061]. The only factor associated with virological failure in patients receiving PI/r was age below 35 years [HR=2.3, 95% CI: 1.0, 5.3, P=0.051]. In the multivariate Cox proportional hazards analyses, the only factor remaining independently associated with virological failure was having an ARTHIVdb score ≥10 [HR=4.3, 95% CI: 0.8, 25.0, P=0.074] in patients initiating EFV. Analyses considering HIV-1 RNA and CD4+ counts as continuous variables showed the same results (not shown).

CD4 evolution by tropism and drug resistance

Overall, median $CD4^+$T-cell counts increased from 39 (21, 63) cells/μl at baseline to 209 (120, 323) cells/μl at year 1 and 280 (160, 427) cells/μl at year 2. Pre-existing drug-resistance was not associated with different baseline $CD4^+$T-cells counts or to different $CD4^+$T-cell increases during follow-up. In contrast, patients with baseline X4 HIV [18] had significantly lower $CD4^+$T-cell counts than those infected with an R5 HIV at baseline [30 (14, 51) vs. 43 (26, 66) cells/μl, p= 0.012], after one year of ART [176 (96, 266) vs. 251 (145, 365) cells/μl, p= 0.023] and after 2 years of ART [214 (127, 316) vs. 315 (176, 461) cells/μl, p= 0.017] [median (IQR) values]. A linear mixed model confirmed the presence of significant differences in $CD4^+$T-cell slopes after ART initiation between patients with X4 and R5 HIV (7.1 vs. 9.4 cells/μl per month, p= 0.004) (Figure 2). Differences between X4 and R5 HIV-1-infected patients remained significant after excluding individuals developing virological failure (data not shown).

Table 1. Subject's characteristics at ART initiation

Characteristics	Total (n=141)	Virological Failure (n=29)	No Virological Failure (n=112)	p-value
Age, median (IQR) (Years)	39 (33, 47)	35	40.5	0.065
Gender, n (%)				0.429
Men	112 (79%)	21	91	
HIV exposure group, n (%)				0.475
MSMs	62 (44%)	9 (31%)	53 (47.3%)	
Heterosexual	60 (42.6%)	15 (51.8%)	45 (40.2%)	
IVDU	15 (10.6%)	4 (13.8%)	11 (9.8%)	
Unknown	4 (2.8%)	1 (3.4%)	3 (2.7%)	
RNA HIV-1, median (IQR) (copies/mm^3)	257,424 (66,400; 500,001)	330,000 (58,159; 671,264)	235,153 (66,393; 500,000)	0.277
CD4$^+$ T cell counts, median (IQR) (cells/mm^3)	38.5 (20; 60)	41 (21.5; 61)	37 (19; 60)	0.622
Treatment, n (%)				0.193
EFV	57 (40.4%)	7 (24.1%)	50 (44.6%)	
ATV/r	28 (19.9%)	7 (24.1%)	21 (18.8%)	
IDV/r	27 (19.1%)	6 (20.7%)	21 (18.8%)	
LPV/r	29 (20.6%)	9 (31.1%)	20 (17.8%)	
HBV coinfection, n (%)				0.716
Positive	12 (8.5%)	2 (6.9%)	10 (8.9%)	
Negative	127 (90.1%)	27 (93.1%)	100 (89.3%)	
Unknown	2 (1.4%)	0	2 (1.8%)	
HCV coinfection, n (%)				0.877
Positive	25 (17.7%)	6 (20.7%)	19 (17%)	
Negative	111 (78.8%)	22(75.9%)	89 (79.5%)	
Unknown	2 (1.4%)	1 (3.4%)	4 (3.5%)	
$_{ART}$HIVdb score				0.365
<10	103 (73%)	20 (69%)	84 (75%)	
≥10	20 (14.2%)	6 (20.7%)	13 (11.6%)	
Non amplifiable	18 (12.8%)	3 (10.3%)	15 (13.4%)	
Tropism, n (%) *(≥2% HIV with G2P ≤3.5%)* [18]				0.755
CCR5	89 (63.1%)	19 (65.5%)	70 (62.5%)	
CXCR4	50 (35.5%)	10 (34.5%)	40 (35.7%)	
Non amplifiable	2 (1.4%)	0	2 (1.8%)	
AIDS at baseline				0.888
AIDS	77 (54.6%)	15 (51.8%)	62 (55.4%)	
No AIDS	64 (45.4%)	14 (48.2%)	50 (44.6%)	

Abbreviations: MSM: men who have sex with men; IVDU: intravenous drug user; EFV: efavirenz; ATV/r: atazanavir/ritonavir; IDV/r: indinavir/ritonavir; LPV/r: lopinavir/ritonavir; HBV: hepatitis B virus; HCV: hepatitis C virus.

Table 2. Patients with mutations associated with an $_{ART}$HIVdb score ≥ 10.

Subject ID	HIV-1 RNA (copies/ml)	Treatment arm	Treatment	Mutations found		
				Mutations impacting ART susceptibility (% in the virus population)	**Mutations with no impact in ART susceptibility (% in the virus population)**	**$_{ART}$HIVdb score**
1	1,000,000	EFV	EFV,AZT,3TC	RT: M184I (1.2) PR: none	RT: V106I (1.4); T69S (10.7); L210M (23.7). PR: A71V (99.8); I13V (3.4); M36I (6.0); I62V (26.8); L63P (99.5); I93L (99.9)	60
2	406,000	EFV	EFV,AZT,3TC	RT: V179D (1.0); K103R (6.5) PR: none	RT: none PR: L10I (1.0); V77I (77.3); I93L (85.3); L63P (100)	30
3	330,000	EFV	EFV,AZT,3TC	RT: Y181C (0.8) PR: none	RT: none PR: M36I (3.0); I64L (94.8); L63P (100)	30
4	44,714	EFV	EFV,TDF,FTC	RT: V179D (99.7); L210W (5.6) PR: none	RT: T69S (2.5) PR: V77I (100)	25
5	261,000	EFV	EFV,AZT,3TC	RT: A98G (2.2); E138Q (36.5) PR: none	RT: none PR: L10V (6.1); I64L (99.8); L63P (100)	20
6	124,000	EFV	EFV,TDF,FTC	RT: M41L (0.5) PR: none	RT: T69S (1.3); V179I (0.8) PR: N83D (1.6); D60E (98.6); M36I (100)	15
7	1,600,000	EFV	EFV,AZT,3TC	RT: V179D (19.9) PR: none	RT: V106I (0.6) PR: M36I (7.4); L10F (99.8)	10
8	186,762	EFV	EFV,TDF,FTC	RT: V108I (30.3); K219Q (28.0) PR: none	RT: K101R (14.9) PR: A71T (99.1); M36I (99.2); I62V (64.3); L63P (35.5); I93L (99.9); D60E (100)	10
9	88,000	EFV	EFV,TDF,FTC	RT: V179D (22.4) PR: none	RT: none PR: I50V (0.7); I13V (99.5); M36I (5.9); I64V (99.5); V77I (85.1)	10
10	114,911	EFV	EFV,TDF,FTC	RT: E138G (5.9) PR: none	RT: T69N (2.4); V179I (99.9) PR: none	10
11	292,328	PI/r	IDV/r,AZT,3TC	RT: D67G (1.0); T69S (1.2); T69N (78.8); T215I (2.4) PR: none	RT: V106I (73.1); V179D (15.5); L210S (0.7) PR: M36I (92.9); I62V (0.7); I64V (24.7)	55
12	327,800	PI/r	ATV/r,TDF,FTC	RT: none PR: V32I (0.8); M46I (0.5)	RT: none PR: V77I (22.3); L63P (100)	25
13	371,000	PI/r	IDV/r,AZT,3TC	RT: E44D (17.0); V118I (3.7) PR: none	RT: none PR: L33I (44.2); L33V (54.9); K20R (2.2); M36I (0.5); L63P (99.3); I64L (0.7); I93L (99.3)	20
14	151,200	PI/r	ATV/r, TDF, FTC	RT: M41L (6.2) PR: none	RT: L210M (99.3); V106I (90.7) PR: I62V (100); L63P (100); I13V (99.8); M36L (98.6); V77I (27.4); M36I (13.3); L10I (12.2)	15
15	25,490	PI/r	LPV/r,TDF,FTC	RT: none PR: M46I (0.8)	RT: V106I (27.0) PR: G16E (98.9); I64V (99.8); V77I (99.8)	10
16	52,625	PI/r	ATV/r,TDF,FTC	RT: none PR: M46I (12.2)	L63P (85.5); I64V (14.3); V77I (22.8); V82I (0.5); I93L (99.4); V106I (9.8)	10
17	593,179	PI/r	ATV/r,TDF,FTC	RT: none PR: G73S (1.2)	RT: A98S (99.2) PR: I13V (96.5); G16E (12.0); I62V (99.6); L63P (83.7); I64V (99.8); I85V (2.5); D60E (100); V77I (100)	10
18	170,000	PI/r	ATV/r,TDF,FTC	RT: none PR: M46I (0.6)	RT: V106I (8.0); V75L (2.8) PR: L33I (0.6); L33V (99.3); I62V (33.0); I64V (100)	10
19	628,320	PI/r	LPV/r,TDF,FTC	RT: none PR: L33F (7.5); L33V (6.7)	RT: L210F (1.9); E138A (100) PR: L10V (3.3); I13V (1.1); K20M (38.5); M36I (89.8); I64V (99.5)	10

Abbreviations: EFV, efavirenz; PI/r, protease inhibitor/ritonavir; ATV/r, atazanavir/ritonavir; LPV/r, lopinavir/ritonavir; IDV/r, indinavir/ritonavir, TDF, tenofovir; FTC, emtricitabine; AZT, zidovudine; 3TC, lamivudine; RT, retrotranscriptase; PR: protease

Table 3. Cox proportional hazard models of risk of virological failure

		All patients						EFV arm						PI/r- arm					
		Univariate			Multivariate			Univariate			Multivariate			Univariate			Multivariate		
		HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value
Age	≥35	1	–	–	1	–	–	1	–	–	–	–	–	1	–	–	1	–	–
	<35	2.1	1; 4.3	0.051	0.6	0.3; 1.4	0.257	0.7	0.1; 5.7	0.734	–	–	–	2.3	1; 5.3	0.051	0.5	0.2; 1.3	0.190
Sex	Male	1	–	–	–	–	–	1	–	–	–	–	–	1	–	–	–	–	–
	Female	1.6	0.7; 3.7	0.229	–	–	–	2.0	0.4; 8.9	0.363	–	–	–	2.1	0.7; 5.5	0.169	–	–	–
Risk group	Heterosexual	1	–	–	–	–	–	1	–	–	–	–	–	1	–	–	–	–	–
	MSM	0.5	0.2; 1.2	0.127	–	–	–	0.5	0.1; 5.8	0.567	–	–	–	0.5	0.2; 1.2	0.112	–	–	–
	IVDU	0.9	0.3; 2.9	0.963	–	–	–	4.2	0.7; 25.5	0.113	–	–	–	0.3	0.1; 2.5	0.291	–	–	–
	Unknown	1.2	0.1; 8.8	0.883	––	–	–	10.2	0.9; 112.2	0.058	–	–	–	–	–	–	–	–	–
HIV-1 RNA (copies/ml)	<100,000	1	–	–	–	–	–	1	–	–	–	–	–	1	–	–	–	–	–
	≥100,000	1.1	0.5; 2.4	0.871	–	–	–	1.3	0.2; 6.5	0.775	–	–	–	1.0	0.4; 2.4	0.998	–	–	–
$CD4^+$ T-cell counts at baseline (cells/μl)[a]	≥50	1	–	–	–	–	–	1	–	–	–	–	–	1	–	–	–	–	–
	<50	1.1	0.5; 2.3	0.869	–	–	–	0.6	0.1; 2.5	0.462	–	–	–	1.3	0.5; 3.1	0.624	–	–	–
Treatment group	EFV	1	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	PI/r	2.2	0.9; 5.1	0.072	1.8	0.7; 4.5	0.189	–	–	–	–	–	–	–	–	–	–	–	–
HCV coinfection	No	1	–	–	–	–	–	1	–	–	1	–	–	1	–	–	–	–	–
	Yes	1.2	0.5; 2.9	0.691	–	–	–	4.6	0.9; 23.0	0.061	3.6	0.7; 18.3	0.111	0.7	0.2; 2.2	0.504	–	–	–
HBV coinfection	No	1	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–
	Yes	0.7	0.2; 3.0	0.657	–	–	–	–	–	–	–	–	–	0.8	0.2; 3.4	0.752	–	–	–
HIVdb score	<10	1	–	–	1	–	–	1	–	–	1	–	–	1	–	–	1	–	–
	≥10	2.0	0.8; 5.0	0.136	2.0	0.8; 5.0	0.147	4.0	0.8; 20.0	0.077	4.3	0.8; 25.0	0.074	2.0	0.6; 6	0.221	1.8	0.5; 5.2	0.350
Tropism [18]	CCR5	1	–	–	–	–	–	1	–	–	–	–	–	1	–	–	–	–	–
	CXCR4	0.8	0.4; 1.9	0.764	–	–	–	1.5	0.3; 8.6	0.600	–	–	–	0.7	0.2; 1.8	0.493	–	–	–

*Abbreviations: MSM, men who have sex with men; IVDU, intravenous drug user; EFV, efavirenz; PI/r, protease inhibitor ritonavir; BL, baseline. *same results were obtained for continuous variable analyses.*

Figure 1. Risk of virological failure by presence of drug-resistant minority variant or HIV-1 tropism. *Kaplan–Meier curves of time to virological failure. Symbols represent censored events. (a) Time to virological failure by HIV-1 tropism considering all patients. (b) Survival analyses by DRMV considering all patients, grouped by ARTHIVdb score 10 or <10. (c) Survival analyses by DRMV considering only PI/r-treated patients. (d) Survival analyses by DRMV considering only EFV-treated patients. DRMV, drug-resistant minority variant; EFV, efavirenz; PI/r, ritonavir-boosted protease inhibitor.*

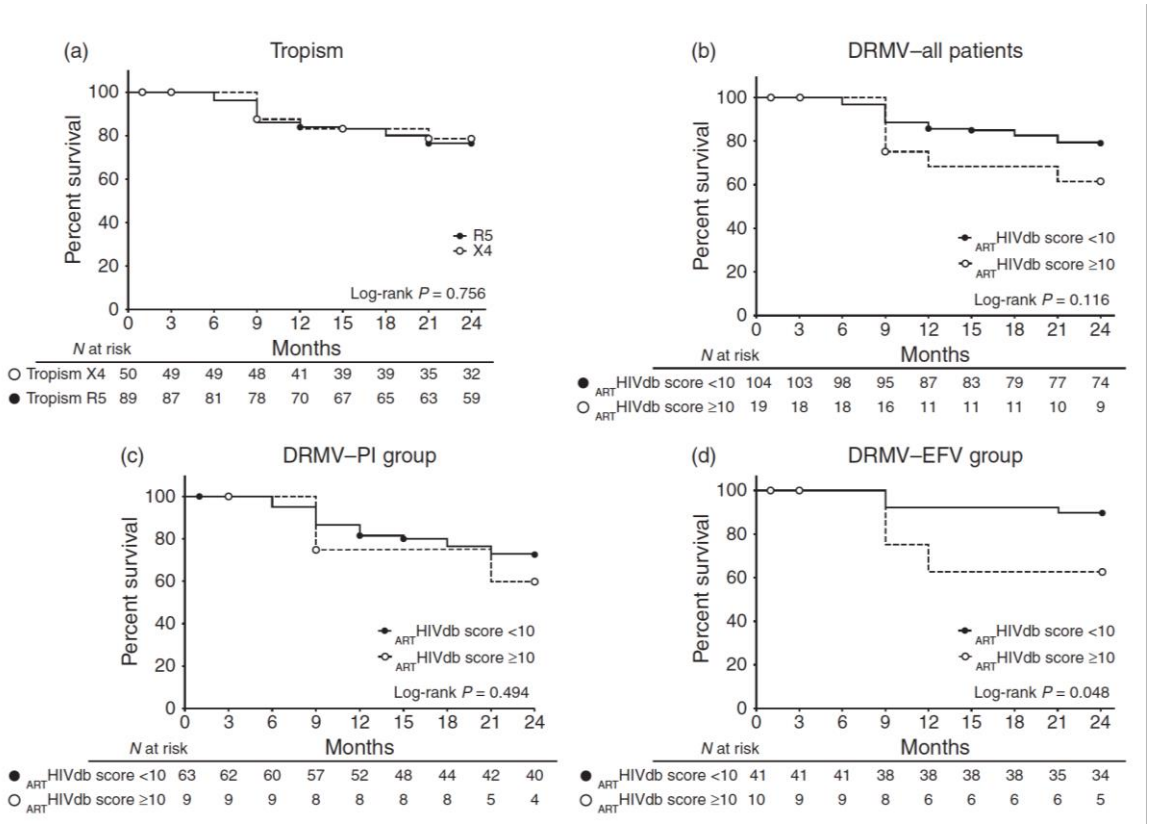
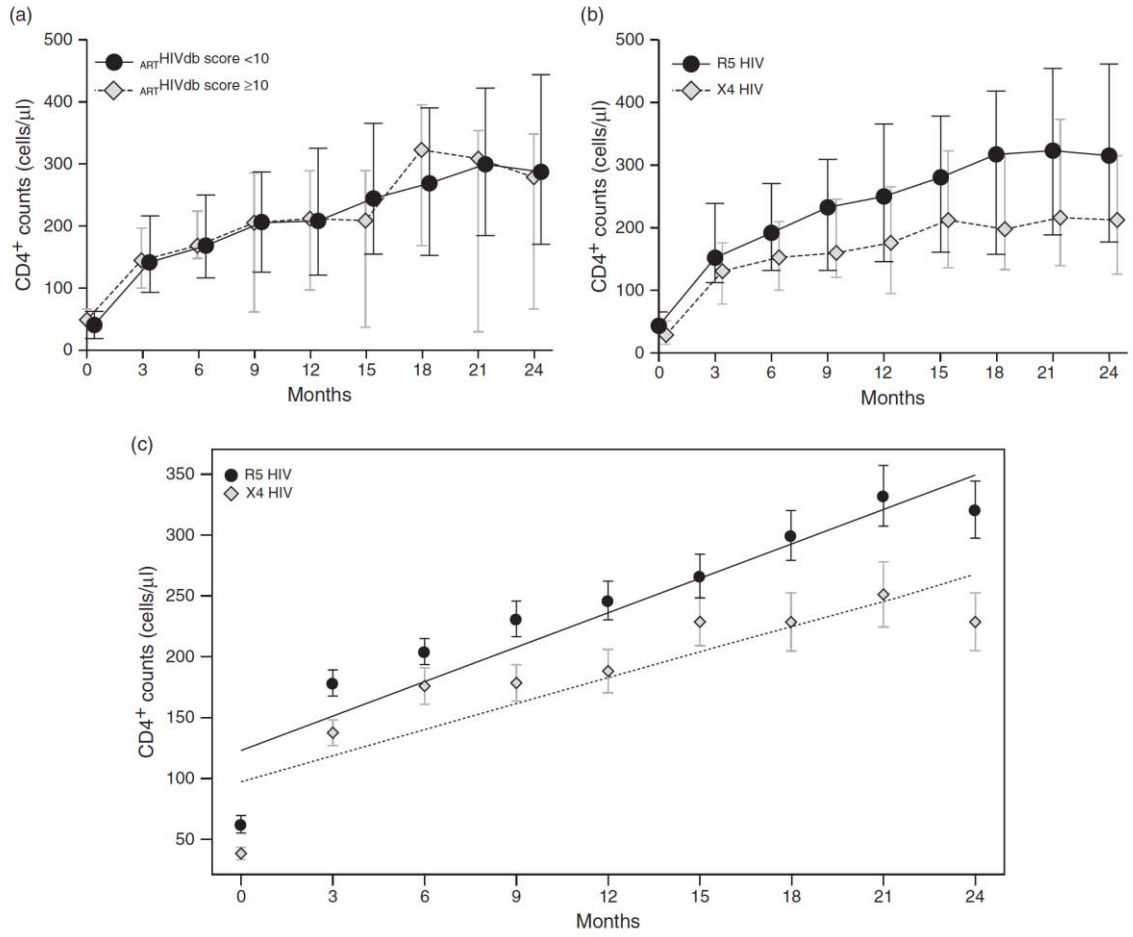


Figure 2. CD4⁺ T-cell evolution by baseline drug-resistant minority variant or HIV-1 tropism. *(a) Median (interquartile range) CD4⁺ T-cell counts by DRMV considering all patients, grouped by ARTHIVdb score ≥10 or <10. (b) Median (interquartile range) CD4⁺ Tcell counts by HIV-1 tropism (*P<0.017 at month 24). (c) CD4⁺ T-cell slopes after ART initiation by HIV-1 tropism (P<0.004). ART, antiretroviral therapy; DRMV, drug-resistant minority variant.*



Discussion

Choosing the right first-line ART is of critical importance in patients with advanced HIV disease because the short-term risk of severe clinical complications in the event of ART failure is very high [1]. In previous head-to-head comparisons, first-line EFV-based regimens showed equal immunological efficacy [12] but better virological outcomes [16] than PI/r-based regimens in individuals with very low $CD4^+$T-cell counts. Our study showed, in addition, that pre-existing DRMVs increase the risk of virological failure of first-line NNRTI-based ART in this population. Also, although pre-treatment HIV-1 tropism did not affect virological outcomes, X4 HIV-1 was associated with impaired immune reconstitution through the first 2 years of ART.

Our study is coherent with previous reports showing that pre-existing DRMVs doubles the risk of virological failure to first-line ART including NNRTIs [19–21]. In fact, the 2-year rates of virological failure of EFV therapy in patients with no DRMVs were almost negligible. Even with the arrival of new, potent and well-tolerated integrase inhibitors like dolutegravir, EFV will remain a potent, simple and affordable treatment alternative for years, more so in resource-limited settings. Our study shows that ultrasensitive genotyping is helpful to identify in which late presenters NNRTI-based ART is unlikely to fail.

The lack of association of DRMVs with virological outcomes of PI/r-including ART is consistent with previous studies [12,13,22–24]. Data on the clinical value of DRMVs on first-line PI/r ART is scarce, and definitions of PI resistance used in previous studies often included naturally occurring polymorphisms, which have little or no effect on PI susceptibility in the absence of major mutations. By using an $_{ART}$HIVdb score, we only considered mutations reducing the virus susceptibility to the ART initiated, including resistance mutations to the NRTI background, and not only PI resistance mutations. Even with this approach, however, we were unable to demonstrate an effect of DRMVs on the outcomes of first-line PI/r therapy. Further analyses (not shown) were not able to demonstrate difference in virological outcomes by NRTI backbone (zidovudine/lamivudine vs. TDF/FTC).

HIV-1 tropism did not affect virological outcomes of first-line ART. However, patients with an X4 HIV-1 had lower $CD4^+$T-cell counts at HIV-1 diagnosis, and more importantly, achieved significantly lower increases in $CD4^+$T-cell counts through the first two years of ART. Our findings contrast with those from the ArTEN trial [14], where pre-existing X4 HIV-1 was independently associated with virological failure to first-line ART including NVP or ATV/r, particularly in subtype B viruses, but did not influence $CD4^+$T-cell count recovery. In a retrospective evaluation of a clinical cohort in London [15], patients with X4 HIV also had lower $CD4^+$T-cell counts than those with R5 HIV in the absence of ART. However, increases in $CD4^+$ T-cell counts were of similar magnitude once ART was initiated. The lack of association between tropism and virological failure is plausible, as, with the exception of CCR5 and CXCR4 antagonists, antiretroviral drug-mediated HIV-1 inhibition does not depend on HIV-1 tropism.

The impaired immune recovery in patients with X4 HIV-1 is possibly associated with the advanced degree of immune deterioration in our cohort, with median 37 $CD4^+$T-cell counts/µl at study entry. Thymic $CD34^+$ cells are more easily infected by X4 HIV than R5 HIV, which could explain an increased depletion of thymic production of naïve T-cells in late presenters with X4 HIV. Although detailed immunological studies are ongoing, from a clinical perspective our findings indicate that HIV-1 tropism is a marker for impaired immune reconstitution in late presenters and baseline determination of HIV-1 tropism should be included in clinical practice. This important finding should be confirmed in further studies that should also clarify whether X4 HIV-1 could be associated with increased rates of AIDS-defining events or death in late presenters after cART initiation.

To our knowledge, this is the first clinical study evaluating the role of ultrasensitive HIV-1 genotyping in individuals with advanced HIV disease. Some strengths of the study include the prospective, randomized nature of the parent trials, which minimizes selection biases and allows a comprehensive and high quality monitoring of clinical and laboratory data; the high median HIV-1 RNA levels at the time of genotyping, which ensures adequate sampling of the viral population to detect minority variants with high sensitivity; the prolonged prospective follow-up, which

allows capturing events up to 2 years; the very low $CD4^+$T-cell counts at study entry, which allows studying the impact of ultrasensitive genotyping in a population with the greatest need of accurate first-line ART prescription; and the simultaneous evaluation of HIV-1 resistance and tropism on virological outcomes of first-line ART.

A number of limitations are also evident. The main weakness of the study is its small sample size, particularly when EFV and PI/r arms are analyzed separately, which limits the statistical power to detect differences. Also, with the exception of ATV/r, two protease inhibitors (indinavir/ritonavir and lopinavir/ritonavir) used in this study are no longer recommended for first-line ART. However, the principle of investigating the role of DRMVs in patients receiving PI/r remains valid in this setting. Formal adherence or ethnicity information data were not available to this analysis. Previous studies in ART-naive patients showed that both the presence of DRMVs and suboptimal adherence were independent risk factors for virological failure to first-line NNRTI ART but also potentiated each other's effects on virologic failure [25]. Also, the effect of DRMVs was found to be independent of the ethnicity in previous studies [21]. The current study was performed in a Spanish population almost exclusively conformed by Caucasian patients infected with a subtype B virus, so it is unlikely that ethnicity could confound our findings. Study participants had been infected and without treatment for a long time, and it is possible that they have had lost detectability of minority drug resistant variants, even with ultradeep sequencing techniques.

Previous studies have shown that ultrasensitive HIV-1 genotyping using modern next-generation sequencing can provide clinical benefits to ART-naïve [19,20] and –experienced [17] HIV-1-infected patients by improving the sensitivity of drug resistance and tropism detection with high diagnostic robustness. Our study shows that the efficacy of first-line ART in late presenters can be improved by using ultrasensitive HIV-1 genotyping to evaluate the presence of drug-resistant HIV-1 before treatment initiation. Detection of X4 HIV-1, in contrast, identifies patients more likely to have impaired immune reconstitution under first-line ART, who might benefit of additional immune-boosting approaches. Results from this study likely apply to other next-generation platforms, which have demonstrated technical equivalence to 454 sequencing [26] and are already providing advantages in

throughput, workload and cost. Although more data are needed, ultrasensitive HIV-1 genotyping might be a valuable tool in the clinical management of late presenters with advanced disease.

Supplementary methods

RNA extraction

Viral RNA was extracted from 500µl of plasma using the robot Versant kPCR Molecular System, by Siemens.

454 sequencing

Protease, first part of reverse transcriptase and V3-loop were retrotranscribed from RNA to DNA and subsequently amplified by one-step RT-PCR using *SuperScript® III One-Step RT-PCR System with Platinum® Taq High Fidelity* (Life Technologies, Paisley, UK). RT-PCR conditions were 30 min. at 52ºC for retrotranscription step, first denaturalization step of 2 min. at 94ºC, 20 cycles of 2 min. at 94ºC, 30 sec. at 50ºC and 1 min. 30 sec at 68ºC, and a final polymerization step of 5 min. at 68ºC. Each region was amplified independently using the following primers:

Region amplified	Direction	Sequence 5'- 3'	Position (HXB2)
Protease	Forward	GAGCTTCAGGTTTGGGGA	2172→2189
	Reverse	GGGCCTGAAAATCCATACAAT	2700←2720
Reverse transcriptase	Forward	GGAAGAAATCTGTTGACTCAG	2508→2528
	Reverse	GAAGCAGAGCTAGAACTGG	3441←3459
V3-loop	Forward	CAGTACAATGTACACATGGAA	6955→6975
	Reverse	CCCATGCAGAATAAAACAAATTAT	7472←7495

Amplicon libraries were generated by Nested PCR from one-step RT-PCR products. Nested PCR conditions were a first denaturalization step of 2 min. at 94ºC, 20 cycles of 2 min. at 94ºC, 30 sec. at 50ºC and 45 sec. at 68ºC, and a final polymerization step of 3 min. at 68ºC using Platinum® Taq DNA Polymerase High Fidelity (Life Technologies, Paisley, UK). 454 Fusion primers incorporated adapters A and B, and also the identifiers required for parallel sample sequencing. 7 overlapping amplicons were designed to cover the two proteins previously amplified: 2 for protease (P) and 5 for reverse transcriptase (RT). One separate amplicon was designed to cover V3-loop (V3). In cases when one amplicon failed,

adjacent amplicons were doubled with the aim to get the same sequencing coverage in the region. When the protein was not completely covered by the amplicons, that subject was classified as non-amplifiable, and not included for subsequent analyses.

Region amplified	Direction	Sequence 5'- 3'	Position (HXB2)
P 1	Forward	AACTCCCTCTCAGAAGCAG	2199→2217
	Reverse	ATTAAAGCCAGGAATGGATGG	2582←2602
P 2	Forward	TATCCTTTAGCTTCCCTC	2237→2256
	Reverse	GTTAAACAATGGCCATTGACAG	2610←2631
RT 1	Forward	TTAAATTTTCCCATTAGTCCTATTGA	2541→2566
	Reverse	ACTGGATGTGGGTGATGC	2873←2890
RT 2	Forward	AAAAGCATTAGTAGAAATTTGTACAG	2642→2667
	Reverse	ATACTGCATTTACCATACCTAGT	2929←2951
RT 3	Forward	CAGAGAACTTAATAAGAGAACTC	2780→2802
	Reverse	AGAGGAACTGAGACAACATC	3155←3174
RT 4	Forward	TCAATTAGGAATACCACATCC	2819→2839
	Reverse	TATGAACTCCATCCTGATAAATG	3243←3265
RT 5	Forward	CCAGCAATATTCCAAAGTAGC	3018→3038
	Reverse	GGAACCAAAGCACTAACAGAA	3402←3422
V3	Forward	TGGCAGTCTAGCAGAAGAAG	7010→7029
	Reverse	CCTCAGGAGGGGACCCAG	7315←7332

Nested PCR products were purified using AMPure XP beads (Beckman Coulter, Inc, Brea, CA). Concentration and quality of purified PCR products were inspected using fluorimetry (Quant-iT™ PicoGreen® dsDNA Assay Kit, Life Technologies, Paisley, UK) and spectrophotometry (Agilent DNA 1000 Kit, Agilent Technologies, Foster City, CA), respectively. Equimolar amplicon pools were merged to perform emPCR as in: *Margulies M et al, Nature 2005 Sep 15;437*, adding a ratio 1:1 between molecules and beads. Genome Sequencher FLX (454 Life Sciences/Roche) was the platform used in 454 sequencing.

Sequences were analysed using Amplicon Variant Analyzer (AVA) software (Roche/454, v2.7). Sequences were demultiplexed using both 5' and 3' multiple identifier (MID) barcodes. Before any further processing, sequences were screened for the presence of potentially contaminating pNL4.3 sequences using a homology filter. If potentially contaminated sequences were found, they were discarded to create a decontaminated sequence dataset for downstream analysis. Amplicon Variant Analyzer was then used to call resistant variants, based on the consensus alignment information for each sample. A variant list containing all drug resistant mutations reported in the Stanford HIVdb was used. According to sequencing strand-dependent error patterns and negative control testing results, sequencing of a pNL4.3 DNA clone, only those variants showing frequency values on forward and reverse reads within a 1 log ratio and an overall frequency greater than 0.5% were used for downstream analysis. Resistance profiles for each sample were created using the Sierra interface of Stanford HIVdb. Coverage of at least 500 reads per each position was required for further analysis to ensure a minimum detection of low-frequency variants. Codons with lower coverage were considered wild-type.

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Conflict of interest
We have read the journal's policy and have the following conflicts: DP has received honoraria and speakers' fees from Abbott, Bristol-Myers Squibb, Boehringer Ingelheim, Gilead, GlaxoSmithKline, Janssen-Cilag, Merck, Pfizer, Janssen and ViiV Healthcare. PD has received advisorty, consultatnt and/or lecture fees and has been a data safetu monitoring board member for Abbot, Boehringer-Ingelheim, Bristol-Myers Squibb, Gilead Sciences, Janssen, Merck Sharp & Dohme, ViiV and Ferrer. BC has been a consultant on advisory boards or participated in speakers' bureaus or conducted clinical trials with Boehringer-Ingelheim, Abbott, GlaxoSmithKline, Gilead, Janssen, Merck, Shionogi and ViiV. JMG has received research funding, consultancy fees or lecture sponsorship, or served on advisory boards, from Abbott, Boehringer-Ingelheim, Bristol-Myers Squibb, Gilead Sciences, Janssen, Merck Sharp & Dohme. JMM has received research funding, consultancy fees or lecture sponsorship, or served on advisory boards, with Cubist Pharmaceuticals, Novartis, Abbot Laboratories, Bristol-Myers Squibb, Gilead Sciences, Merck, Pfizer, Theravance, Boehringer-Ingelheim, GlaxoSmithKline, Janssen-Cilag, Dohme, Roche, Schering-Plough and ViiV Healthcare. RP has received consulting fees from Pfizer and grant support from Pfizer, Roche Diagnostics, Siemens, Merck and Boehringer-Ingelheim. All other authors report no potential conflicts.

Appendix 1
The ADVANZ and ADVANZ-3 Investigators (in alphabetical order) are: ***Advanz investigators:*** Hospital Bellvitge, Barcelona (Elena Ferrer, Daniel Podzamczer); Hospital Clínic, Barcelona (Fernando Agüero, Teresa Gallart, Felipe García, José M Gatell, Cristina Gil, Montserrat Loncá, Christian Manzardo, Josep Mallolas, José M. Miró, Montserrat Plana, Omar Sued, Tomás Pumarola, Laura Zamora); Hospital Donostia, San Sebastian (Julio Arrizalaga, Begoña Jimeno); Hospital La Paz, Madrid (Jose R. Arribas, Alicia Lorenzo); Hospital Sant Pau, Barcelona (Pere Domingo, Mónica Fuster); Hospital Universitari Vall d'Hebron, Barcelona (Carlos Azuaje, Esteban Ribera, Sara Villar). ***Advanz-3 investigators****:* Hospital de Bellvitge-IDIBELL, University of Barcelona, Barcelona (Elena Ferrer, Daniel Podzamczer, Nerea Rozas); Hospital Clínic - IDIBAPS, University of Barcelona, Barcelona (Fernando Agüero, Alberto C Guardo, Teresa Gallart, Felipe García, José M Gatell, Montserrat Loncà, Francisco Lozano, Christian Manzardo, Josep Mallolas, José M. Miró, Montserrat Plana, Laura Zamora); Hospital Universitari Germans Trias i Pujol, Badalona (Isabel Bravo, Bonaventura Clotet, Roger Paredes, Joan Romeu); Hospital Universitari de la Santa Creu i Sant Pau, Barcelona (Pere Domingo, Montserrat Fuster, Mar Gutiérrez, Gracia Mateo, Jessica Muñoz); and, Hospital Universitari Vall d'Hebron, Barcelona (Esteban Ribera, Marjorie Diaz, Adrian Curran).

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Chapter 2

Primary Resistance to Integrase Strand-Transfer Inhibitors in Europe

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Synopsis

Objective: The objective of this study was to define the natural genotypic variation of HIV-1 integrase gene across Europe for epidemiologic surveillance of integrase strand-transfer inhibitor (InSTI) resistance.

Methods: This was a multicentre, cross-sectional study within the European SPREAD HIV resistance surveillance programme. A representative set of 300 samples was selected from 1950 naive HIV-positive subjects newly diagnosed in 2006/2007. The prevalence of InSTI resistance was evaluated using quality-controlled baseline population sequencing of integrase. Signature raltegravir, elvitegravir and dolutegravir resistance mutations were defined according to the IAS-USA 2014 list. In addition, all IN substitutions relative to HXB2 were identified, including those with a Stanford HIVdb score ≥10 to at least one InSTI. To rule out circulation of minority InSTI-resistant HIV, 65 samples were selected for 454 integrase sequencing.

Results: For the population sequencing analysis, 278 samples were retrieved and successfully analysed. No signature resistance mutations to any of the InSTIs were detected. Eleven (4%) subjects had mutations on resistance-associated positions with a HIVdb score ≥10. Of the 56 samples analysed with 454 sequencing, no InSTI signature mutations were detected, whereas IN substitutions with HIVdb score ≥10 were found in 8 (14.3%) individuals.

Conclusions: No signature InSTI-resistant variants were circulating in Europe before the introduction of InSTIs. However, polymorphisms contributing to InSTI resistance were not rare. As InSTI use becomes more widespread, continuous surveillance of primary InSTI resistance is warranted. These data will be key to model the kinetics of InSTI resistance transmission in Europe in the coming years.

Introduction

HIV integrase is a key enzyme for retroviral replication and one of the main targets of modern HIV therapy.[1,2] Integrase strand transfer inhibitors (InSTIs) reached clinical practice in Europe in 2007-2008, after proving their efficacy in antiretroviral treatment-naïve and -experienced subjects.[3–6] Virological failure (VF) to the first-generation InSTIs raltegravir and elvitegravir is associated with development of resistance through three mutually exclusive pathways characterized by one signature resistance mutation in the catalytic domain of the enzyme, i.e., Y143R/C, N155H or Q148K/R/H, alongside accessory mutations that improve viral fitness or further reduce InSTI susceptibility.[7–10] The resistance profile of the second-generation InSTI dolutegravir is being defined, as few subjects developed VF in clinical trials and data from routine care is still scarce. Viruses with the Q148K/R/H plus at least one additional mutation, however, may also affect susceptibility to dolutegravir.[11,12] Based on the low genetic barrier of first generation InSTIs and with the increasing use of them in the clinical practice, surveillance of transmitted InSTI-resistant HIV will be a key to optimize InSTI efficacy. Primary InSTI resistance is still rare. However, it has begun to be reported[13,14] and will likely increase in the coming years. In addition, up to 34% of published sequences, 56% of those obtained from recent HIV infection,[15] contain polymorphisms in IN,[16] which modulate InSTI resistance in particular to raltegravir and elvitegravir and are frequently observed in InSTI VF. In this study we performed a systematic, representative description of the natural sequence variation of the IN gene across Europe, before InSTI drugs were commercially available. We also aimed to clarify the chances that spontaneously generated InSTI-resistant mutants could be circulating as minority species and be missed by routine population sequencing approaches.

Methods

A sample of 300 subjects was randomly selected from 1950 individuals enrolled in the European SPREAD programme in 2006/2007, before InSTIs were introduced into routine clinical care in Europe. SPREAD is a prospective HIV-1 resistance surveillance programme that collects representative data on the spread of HIV-1 resistance among newly diagnosed patients from all risk groups in Europe. Ethical requirements were fulfilled according to the procedure described in the ethic committee contract. Additionally, a written informed consent was obtained for each patient. Population sequencing of plasma HIV-1 was performed (ViroSeq® HIV-1 Genotyping System, Abbott; Trugene ® HIV-1 Genotyping Kit, Siemens, or in-house methods) in laboratories that successfully participated in the SPREAD quality control (QC) programme for population sequencing. Samples from laboratories who did not participate in the IN sequencing QC programme or did not successfully meet the QC criteria were tested by one of the qualified laboratories that passed the QC programme within the SPREAD network using the abovementioned commercial or in-house Sanger sequencing methods. HIV subtypes were determined using Rega Subtyping Tool vs.2 based on *pol* sequence data.[17] To screen for circulating low-frequency InSTI-resistant mutants, ultradeep IN sequencing was additionally attempted in 65 subjects randomly selected from those included in the population sequencing analyses, using a 454 FLX Genome Sequencer using the Titanium chemistry and a 1% threshold for mutant detection. Sequences were analysed using the Roches's proprietary Amplicon Variant Analyser software (v2.7). Sample contamination was ruled out by similarity analysis both against a pNL4.3 reference sequence and by per-amplicon phylogenetic analysis of all sequences >1% within an ultradeep sequencing (UDS) run. Given the lack of a widely accepted list of IN mutations for surveillance, we first listed all substitutions relative to the reference sequence HXB2 (GenBank: K03455) according to their frequency in the patient population. We then evaluated which substitutions achieved a HIVdb score≥10 to at least one InSTI, representing substitutions with potential impact on InSTI susceptibility. We also listed IN mutations included in the IAS-USA list (July 2014 update). "Signature mutations" were: Y143R/C/H, N155H or Q148K/R/H

for raltegravir; T66I, E92Q, F121Y, S147G, Q148R and N155H for elvitegravir, and G140S and Q148H for dolutegravir.

Results

Samples were retrieved and successfully analysed by population sequencing in 278 out of the 300 (92.7%) subjects selected. The prevalence of transmitted drug resistance mutations found by Sanger sequencing for PIs, NRTIs and NNRTIs was 2,5%, 9,71% and 7,91% respectively. No signature InSTI mutations were detected. By contrast, we observed IN associated mutations with HIVdb score≥10 in 11 (3.9%) patients **(Table 1, Supplementary Table S1).** Samples unsuccessfully processed had a median (IQR) number of copies/mL of 57.000 (13 212; 374 154). The HIV subtype from these samples was mainly subtype B (n=12; 54,55%) and subtype G (n=5; 22,72%).

454 data was obtained from 56/65 (86.1%) subjects. Most of them (85.2%) were infected with a subtype B HIV-1. Fifty of them (89.3%) had wild type protease (PR), reverse transcriptase (RT) and IN by Sanger sequencing, whereas 6 (10.7%) had transmitted resistance to at least 2 antiretroviral drug classes. The median (interquartile range) coverage was 4593 (3066-6598) reads per substitution found. Again, no InSTI signature mutations were detected. However, 8/56 subjects (14.3%) had IN substitutions with a HIVdb score≥10 **(Table S2)**. Of these, mutation E157Q was found in 5 (8.9%) individuals, in 2 of them as a low-frequency variant. The following mutations were found in one subject each: H51Y, G163R, both as low-frequency variants (1.9; 2.6% in the virus population each); G163K as major variant (100% in the virus population); E157Q was detected at a 22.1% frequency in a subject with transmitted Q58E mutations in protease and D67N and K219Q mutations in RT. No IN substitutions with a HIVdb score ≥10 were detected in the remaining 5 subjects with transmitted dual-class resistance.

Table 1) Subject's characteristics and sequencing results summary

	N (%)
All subjects	278 (100)
Male	231 (83.0)
Continent of origin	
Western Europe	180 (64.7)
Eastern Europe	48 (17.2)
Sub-Saharan Africa	20 (7.2)
Latin America	16 (5.7)
Others	14 (5.2)
CDC class	
A	230 (82.7)
B	21 (7.5)
C	22 (8.0)
Unknown	5 (1.8)
Median CD4+ T count (cells/µl)	411
Route of transmission	
MSM/bisexual	180 (64.8)
Heterosexual	61 (21.9)
IVDU	5 (1.8)
Other	32 (11.5)
Viral Subtype	
B	186 (67.0)
C	15 (5.4)
A	11 (4.0)
F	12 (4.3)
G	6 (2.1)
D	1 (0.3)
Unknown	47 (16.9)
Summary Sanger Sequencing	
IAS-USA Integrase Mutations	5 (1.8) [74M (2); 97A (2); 138A]
HIVdb score ≥10	11 (4.0)
Summary 454 Sequencing (n=56 subjects)	
IAS-USA Integrase Mutations	0
HIVdb score ≥10	8 (14.3)

Discussion

No signature InSTI-resistance mutations were circulating in Europe before InSTIs introduction, although potentially relevant polymorphisms could be observed. This study also indicated a limited utility of ultrasensitive genotyping for surveillance of InSTI-resistant minority variants at present, which might change if the burden of transmitted InSTI resistance increases. Elvitegravir and raltegravir have a low genetic barrier to resistance and extensive overlap in their drug resistance profiles.[18] Raltegravir is often prescribed as salvage therapy to subjects with multi drug-resistant HIV who could select for InSTI resistance, which could be transmitted to newly infected subjects. Dolutegravir has a higher genetic barrier to resistance than elvitegravir and raltegravir, but its long-term potency might be reduced in the presence of Q148R/H/K + 1 or 2 additional mutations. As dolutegravir is also often prescribed as salvage ART, dolutegravir resistance might also evolve in European populations in the coming years. Continued surveillance of InSTI resistance in Europe is thus warranted, including periodic re-evaluations of the usefulness of ultra sensitive genotyping technologies, which nowadays allow faster monitoring of transmitted resistance, particularly with large sample sets.

Substitutions detected with at least some presumed impact on ART susceptibility (i.e., having an HIVdb score ≥10) were E157Q, G163R/K, L74M, T97A, E138A, S153F and R263K. E157Q is a polymorphic accessory mutation weakly selected in patients receiving raltegravir and selected *in vitro* by elvitegravir. G163R/K are non-polymorphic mutations in all subtypes except F, often selected in patients receiving raltegravir. However, their effect on InSTIs has not been yet well studied. L74M is a polymorphic accessory mutation selected in patients receiving raltegravir, elvitegravir, and dolutegravir, which does not reduce InSTI susceptibility unless in is found in combination with other InSTI-resistance mutations. T97A is a polymorphic accessory mutation selected by raltegravir and elvitegravir that occurs in 1% to 5% of viruses from untreated persons. Combined with Y143C/R it markedly reduces raltegravir susceptibility, although it has minimal effect alone. E138A is a non-polymorphic accessory

resistance mutations usually occurring in combination with Q148 mutations, selected in patients receiving raltegravir, elvitegravir, and dolutegravir. It is associated with >100-fold reduction in raltegravir and elvitegravir susceptibility and up to 10-fold reduced dolutegravir susceptibility in combination with Q148. S153F is selected *in vitro* by dolutegravir, and it is a rare non-polymorphic mutation, reducing raltegravir and dolutegravir susceptibility by 2-fold and elvitegravir's by 4-fold. R263K is a non-polymorphic mutation selected in patients receiving raltegravir and dolutegravir and *in vitro* by elvitegravir and dolutegravir, reducing raltegravir, dolutegravir and elvitegravir susceptibility about 2-fold, 2-fold, and 3 to 5-fold, respectively.[19,20]

Therefore, as long as transmitted InSTI resistance remains at negligible levels, there is no clinical need to perform IN genotyping before initiating InSTI therapy. However, continued surveillance is key to inform clinicians and policymakers about when baseline genotyping should be systematically recommended. It is essential to perform IN gene genotyping in subjects failing InSTI therapy, as new InSTIs with alternative resistance profiles are under development and subjects should not be kept on failing InSTI regimens.

In conclusion, no signature InSTI-resistant variants were circulating in Europe before introducing InSTIs. However, polymorphisms that could contribute to InSTI resistance were not rare. As InSTI use becomes more widespread, continuous surveillance of primary InSTI resistance is warranted. This study provides an extensive assessment of primary InSTI resistance based on a representative sample of the European epidemic and is a robust baseline comparator for future InSTI surveillance, which will be a key to model the kinetics of InSTI resistance patterns of transmission in Europe in the coming years.

Supplementary information

Supplementary Table S1. Substitutions relative to HXB2 detected by Sanger sequencing

Nº of subjects (% out of 278 subjects evaluated)	Substitutions with HIVdb score≥10	Substitutions with no presumed impact on ART susceptibility,
277 (99.6)		R127K
273 (98.2)		G123S
261 (93.9)		N232D
256 (92.1)		D10E
165 (59.4)		V72I
151 (54.3)		L101I
119 (42.8)		V201I
106 (38.1)		T125A
94 (33.8)		A124T
84 (30.2)		V31I
78 (28.1)		E11D
72 (25.9)		L234I
60 (21.6)		T122I
57 (20.5)		T112V
51 (18.3)		D256E, T124N
47 (16.9)		M50I, S119P
46 (16.5)		K14R
44 (15.8)		I135V, T206S
37 (13.3)		S283G
35 (12.6)		K136Q
31 (11.2)		G134N, S17N
29 (10.4)		D25E, I113V
28 (10.1)		S39C
26 (9.4)		G193E
25 (9.0)		K136T
23 (8.3)		S24N
22 (7.9)		S230N
21 (7.6)		D167E, K111T
20 (7.2)		K211R, S119R
19 (6.8)		K156N, V32I
18 (6.5)		T112I, T218I
17 (6.1)		A265V, L28I, S119G
16 (5.8)		R20K, T125V
15 (5.4)		D232E, L45V, T218S
14 (5.0)		D41N, D6E, I208L, T124S
13 (4.7)		A21T, L74I, S119T, V165I
12 (4.3)		D55N, G163E, L45Q, Q216H, R269K
11 (4.0)		D278A, F100Y, I84M, T112A
10 (3.6)		K7R, L101F, S24G
9 (3.2)		A23V, K173R
8 (2.9)		I220L, K111R, P90S, S39N
7 (2.5)		E96D, I60M, K188R, K211Q, K215N, V151I
6 (2.2)		D253E, D286N, H171Q, I113L, M154I, N222H, N222K, P30A, Q177K, S255N, T112R, V176L, Y99F
5 (1.8)		D270N, D278N, G106A, G70E, I203M, I60V, I73V, K111A, K111Q, K160Q, L45I, M50T, Q221S, R284G, S195T, S255G, S57G
4 (1.4)		A49P, A91E, A91T, D207E, F181L, G134D, I182V, I217V, I84L, K103X, K136N, K136R, K219Q, K240R, K7Q, N254K, S17C, S17T, T124G, T124Q, V126F, V150A, V260I, V37I, V79I, Y227F
3 (1.1)		A205S, C43S, D278E, D279G, D288N, E10A, E212A, G163A, G163V, H78Q, I200L, I220V, I251L, K111N, K211T, L101V, M275V, Q221T, R231K, S123C, S230H, S24A, S24H, S255T, S81R, T112M, T218L, T97S,

		V126L, V165G, V259I, V281M
2 (0.7)	L74M, E157Q, G163K, T97A	A128T, A80S, C130Y, E138D, E13D, E212Q, E85G, E87V, G163T, G193R, H171L, H67X, I141V, I191M, I200M, I268L, K103I, K103R, K136E, K211N, K219E, K219N, K34R, K42Q, K42R, L172M, L234F, L234H, L234V, L28M, L63I, L68V, L74V, M154L, M178R, M50L, N254Q, N27H, Q164K, Q221H, Q44H, R187K, S123N, S195C, S283N, S57N, T122V, T125M, T125S, T93I, T93S, V79X, W61L
1 (0.4)	R263K, G163R, S153F, E138A	A128G, A133X, A169V, A205V, A21N, A23G, A23T, A276Q, A80V, A86T, A86X, A91V, C130X, C40F, C40R, C40Y, C43V, C65G, C65X, D167G, D167N, D202E, D202V, D256N, D25T, D270H, D270Y, D3E, D41Y, D55H, D64X, D6N, E10V, E11A, E138G, E170G, E198A, E198D, E198S, E35Q, E48d, E48G, E48K, E87Q, E96N, F181Y, F26V, G106R, G134R, G134S, G134X, G140W, G163Q, G189R, G193D, G197V, G272E, G52E, G59E, G94X, H16Q, H16Y, H171Y, H183I, H183L, H183N, H78L, H78P, H78X, I113M, I113R, I141H, I161L, I161N, I161S, I162Y, I191V, I203K, I203N, I204L, I204V, I220M, I251T, I267V, I72T, I73F, I73X, I89F, I89L, K103L, K111S, K111X, K136A, K136H, K156I, K159N, K159R, K160R, K160T, K215T, K240N, K42N, K46Q, K71Q, K71R, L101X, L102I, L102X, L158F, L172F, L172V, L234R, L28P, L2I, L45M, L45S, L63X, L68I, L68P, L68S, L68X, M178W, M178X, M22I, M22K, M275R, N117K, N120H, N144I, N155X, N184D, N18S, N222R, N222Y, N27G, P142A, P142S, P238S, P30S, Q146E, Q164P, Q168Y, Q216K, Q216R, Q221K, Q221P, Q221R, Q53P, Q62P, Q95N, Q95P, Q95X, Q9R, R199G, R228G, R262G, R269W, S119A, S230G, S24D, S24K, S255K, S283D, S39G, S39M, S39R, S81X, T112K, T112X, T122A, T122S, T124D, T124I, T124Y, T125P, T174K, T206A, T210I, V110I, V126A, V126I, V126M, V165A, V201E, V201X, V249L, V260A, V31M, V54I, V54X, V75L, V77A, V88I, W108X, W19Y, W61C, W61F, W61M, Y143D, Y143N, Y143T, Y143X

Supplementary Table S2. Substitutions relative to HXB2 detected by 454-sequencing

Nº of subjects (%out of 56 subjects evaluated)	Substitutions with HIVdb score≥10 [frequency of each mutant in the virus population]	Substitutions with no presumed impact on ART susceptibility
56 (100)		G123S
49 (87.5)		N232D
47 (83.9)		D10E
35 (62.5)		V72I
32 (57.1)		V201I
30 (53.5)		A124T
24 (42.8)		E11D, T125A
23 (41.0)		L101I
20 (35.7)		D256E
15 (26.8)		T122I, V31I
14 (25.0)		M154I, S119P
13 (23.2)		S230N, M50I
11 (19.6)		A124N, K14R
10 (17.8)		I113V, S39C
9 (16.0)		S283G
8 (14.3)		R20K, T112V
7 (12.5)		D6E, I135V, V32I, T112I, V37I
6 (10.7)		A21T, A23V, D167E, D41N, E157K, K156N, L45Q, S17N, S24N, G70E, S119G, V151I
5 (8.9)	E157Q[4.1; 22.1; 100; 100; 100]	I208L, I220L, K136Q, K211R, S24G, T218S, Y227F, L28I, T206S
4 (7.1)		A124S, D253E, D25E, I182V, K111T, K7E, L234I, P90S, V165I
3 (5.3)		A205S, D270E, D279G, D288N, D3N, E13D, G163E, G193E, I161T, I203M, I208M, I84M, K34R, L234V, L45V, L74I, N232E, R166K, S24D, T125V, T218I, V88I, E152K, G59E, K160Q, K215N, L101V, S119R, S119T, S17T, S255N
2 (3.6)		A21S, A23S, A265V, C56Y, D10K, D167G, D229E, D25N, D278N, D286N, D55N, D6S, E212A, E35Q, E48K, E96D, E96K, G134N, G163T, H171Q, H171Y, I84L, I84V, K103R, K111Q, K136R, K159R, K188E, K244R, K71R, L234F, L234H, M50T, M50V, N254H, N27S, Q274K, R199K, S17C, S255G, S283N, T112A, T112M, T122V, V259I, V32L, V77A, A80S, D6N, P30A, S39A
1 (1.8)	G163K[100], G163R[2.6], H51Y[1.9]	A124D, A124G, A196P, A33S, A33V, A8T, A91T, A98T, C280R, C43W, D10A, D10G, D116N, D202N, D207Y, D253H, D253Y, D256N, D278G, D288G, D3V, D41E, D41G, D41H, D41Y, D64A, D6T, D6V, E13K, E170K, E212H, E212I, E212Q, E212S, E212V, E35K, E69G, E85K, E96G, F100L, F100S, F181L, F185L, G118C, G134D, G189V, G193D, G4K, G52R, G70R, G82E, H51Q, H67R, I113M, I141L, I141T, I141V, I161V, I204L, I217V, I220V, I251L, I251M, I268V, I5M, I60M, I60V, I89L, K111N, K111R, R127K, K136E, K136N, K14E, K160E, K160G, K160R, K173R, K188R, K211Q, K211T, K219N, K240R, K273E, K273R, K46R, K71I, K7Q, L28M, L28P, L2F, L45I, M22I, M22R, M275I, M275V, M50L, N155D, N222K, N254K, N27D, N27Y, P142S, Q216H, Q216R, Q44H, Q44R, Q95P, Q9P, R107G, R166G, R187K, R231K, R269G, R269K, R284G, S153A, S230G, S24T, S283D, S39M, S39N, T112R, T115A, T122S, T125M, T125P, T174A, T206A, T210I, T93S, T97I, V110I, V126L, V150A, V176I, V176L, V201M, V249I, V281M, V31L, V37A, V54L, V75I, Y15C, Y227H, Y271C, Y99C, D3E, G4E, N222H, S255R, A128T

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Transparency declarations
The authors declare no conflict of interest

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Chapter 3

Treatment options after virological failure of first line tenofovir-based regimens in South Africa: an analysis by deep sequencing

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Summary

In a South African cohort of participants living with HIV developing virological failure on first-line TDF-based regimens, at least 70% of participants demonstrated TDF resistance according to combined Sanger and MiSeq™ genotyping. Sanger sequencing missed the K65R mutation in 30% of samples.

Unless HIV genotyping is available to closely monitor epidemiological HIV resistance to TDF, its efficacy as second line therapy will be greatly compromised.

Provision of antiretroviral therapy (ART) in resource-poor settings employing a public health approach has achieved major successes, saving thousands of lives and averting new HIV infections. Recently, ART initiation in all adults living with HIV disregarding CD4 cell count was recommended for the first time in World Health Organization (WHO) HIV treatment guidelines[1]. However, the ART arsenal available to most resource-poor settings remains limited, and treatment follow-up rarely includes virological monitoring. In this context, ARV resistance remains a major threat to the public health efforts to eradicate the HIV pandemic.

Tenofovir disoproxyl fumarate (TDF), in combination with lamivudine (3TC)/emtricitabine (FTC) and nevirapine(NVP)/efavirenz(EFV), remains an ARV of choice for first-line ART in Africa, being included in the South African national HIV/AIDS treatment plan for naïve patients[2]. Tenofovir has high antiviral potency, allows once-daily dosing (frequently co-formulated) and is well tolerated. However, its efficacy is diminished in the presence of the K65R mutation[3]. Subtype C, the most prevalent subtype in South Africa, selects for this mutation faster than other subtypes due to subtype-specific pathways [4,5]. This is an important concern because failure to TDF-containing regimens is often associated with additional resistance to nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTI and NNRTI). Therefore, patients developing virological failure to TDF might potentially loose multiple 2nd-line ART options.

Using Sanger sequencing, previous studies reported the emergence of K65R mutation in 23 to 69.7% of participants developing virological failure to 1st-line TDF regimens [6–9]. The true prevalence of TDF resistance, however, might have been underestimated by the lack of sensitivity of standard Sanger-based genotyping methods. Achieving a precise estimate of TDF resistance after virological failure of first-line TDF regimens is also key to inform public policy as to whether TDF might be reused in second-line ART or subsequent regimens. Transmission of TDF resistance might also potentially compromise the efficacy of PrEP strategies [1]

To evaluate the prevalence of TDF resistance using ultrasensitive sequencing methods, we developed a retrospective reanalysis of participants developing virological failure to TDF within a larger cohort study conducted at the McCord Hospital, Durban, South Africa. All participants developing virological failure to first-line ART including TDF+3TC plus an NNRTI received a genotypic resistance test using a validated in-house Sanger-based sequencing assay in Durban, South Africa. Plasma samples from those with no K65R mutation by Sanger sequencing were reanalysed at the irsiCaixa AIDS Research Institute in Badalona, Spain using MiSeq™ Illumina (Illumina Inc. California).

In brief, the complete *pol* gene was amplified and sequenced in a MiSeq™ platform using a Nextera-XT shotgun approach. A 1% threshold level was chosen for detection of minority variants. Resistance mutations were defined according to the IAS-USA 2013 list. Drug susceptibility results were defined according to Stanford HIV Drug Resistance database, and were classified following the susceptible-intermediate-resistant (SIR) code.

Out of 158 participants included in the McCord cohort at the time of this analysis, 88 participants (55.7%) had developed virological failure to TDF-including regimens. PCR amplification failed in 9 samples (10.2%) leaving 79 evaluable subjects.

Sanger sequencing detected K65R mutation in 47 out of 79 samples (59.5%). Deep sequencing was attempted in the remaining 32 samples. However, 5 out of 32 samples had been depleted of volume and could not be further evaluated. K65R mutation was found in 8 of the 27 samples evaluable by MiSeq™ (29.6%) at frequencies in the virus population ranging 1.3% to 32.5%. Considering Sanger and deep sequencing results together and assuming that none of the 5 subjects not evaluable by MiSeq™ had the K65R mutation, a conservative estimate of the overall prevalence of K65R mutation was 69.6%, a 10.1% increase in prevalence relative to Sanger sequencing. Prevalence was calculated using only TDF-failing and PCR-success subjects.

In addition, deep sequencing detected IAS-USA mutations missed by Sanger in 22 out of 27 subjects (81.4%) at frequencies ranging 1.1% to 35.7% in the virus population **(Table 1)**. Such additional mutations changed the predicted drug susceptibility in 15 out of 27 subjects (55.5%), mostly affecting TDF, etravirine (ETR) and rilpivirine (RPV), although the predicted susceptibility to NVP or EFV was not affected(Table 1). According to deep sequencing data, 21/27 (77.7%), 25/27 (92.6%), 13/27 (48.1%) and 15/27 (55.5%) were resistant to 3TC/FTC, NVP/EFV, ETR and RPV, respectively, whereas only 3 participants (11.1%) had intermediate resistance to AZT –only 1 (3%) by Sanger sequencing.

Our findings confirm initial estimations that TDF might loose antiviral efficacy in virtually all patients infected with a subtype C HIV developing virological failure to this drug. Thereby, unless HIV genotyping is available to ensure that HIV remains susceptible to TDF, the use of this drug will be greatly compromised in efficacy for second line therapy, and should not be prescribed except if no other treatment options are available. Continued surveillance of primary resistance in Africa is key to survey transmission of TDF-resistant mutants to newly HIV-infected patients, which could impact the efficacy of both first-line ART and PrEP [10,11]. To date, rates of virological failure to first-line TDF regimens and transmission of K65R mutants have remained low according to Sanger sequencing estimates. [12,13] The fitness cost of the K65R mutation, however, makes K65R mutants wane and thus might be missed by Sanger methods.

Another remarkable finding of our study was that, in addition to identifying K65R, additional resistance mutations detected with MiSeq™ relative to Sanger mainly affected the predicted susceptibility to the second-generation NNRTIs ETR and RPV, but did not largely influence viral susceptibility to other ARVs, including AZT. On the one hand, this suggests that ETR and RPV might not be good options for second-line ART regimens following EFV or NVP failure. On the other hand, our findings support AZT as a second-line drug in South Africa, used in combination with 3TC and LPV or other PIs[2] or even integrase inhibitors. Whereas routine drug resistance testing may help

decide which NRTIs to use in second-line therapy, the EARNEST trial recently showed that even without this information, second-line regimens including boosted PI plus two NRTIs retained better virological outcomes than PI monotherapy, even in the presence of high-level resistance to the NRTI backbone [14], suggesting residual NRTI activity may be sufficient when combined with highly potent boosted PI-based therapy.

Despite its limitations –including a small sample size, lack of adherence data and the inclusion of patients under clinical care which might not represent the general South Africa population– this study confirms the development of TDF resistance in most subjects developing TDF failure in South Africa, but also supports current public health algorithms for HIV clinical management.

Table 1. Antiretroviral drug resistance at virological failure of TDF-containing 1st-line ART by Sanger and ultrasensitive HIV genotyping

Subject ID	IAS-2013 mutations detected by Sanger sequencing	Additional Mutations by Illumina (Frequency in the virus population, %)	Changes in Drug Susceptibility with MiSeq™ compared to Sanger
1	D67N; M184I; V90I; V179E; Y181C; H221Y	G190A (4.74); K70E (4.5); P225H (8); V106I (9.4)	TDF (S→I); ETR (I→R); RPV (I→R)
2	D67N; K70E; M184V; A98G; K103N; V106M	K65R (20.3); L100I (2.1); Y181C (16,1)	TDF (I→R); ETR (I→R); RPV (I→R)
3	M184V; V106M; G190A	D67N (1.3); K103N (16,7); K65R (27.8); M184I (29.4); M230L (28.1)	TDF (S→R); RPV (I→R)
4	M184V; T215Y; V106M; Y188L	D67N (1.9); G190A (13.6); K101E (12.5)	TDF (S→I); ETR (S→I)
5	M184V; K103N; V108I	A62V (2.8); D67N (4.2); P225H (2.4)	AZT (S→I)
6	M184V; K103N; V106M	D67N (1.3); M230L (31.8)	ETR (S→I); RPV (S→I)
7	M184I; V90I; Y181C; H221Y	A98G (3.2); G190A (9.5); M184I (14.6); V179D (11.6)	ETR (I→R); RPV (I→R)
8	K70E; M184V; V90I; K103N; E138G	K65R (1.3)	TDF (I→R)
9	M184V; V106M; V108I; E138A; G190A	H221Y (1.6); K219E (2.7); K70E (8.6); L74V (3.2); V90I (8.8); Y115F (35.7)	TDF (S→I)
10	M184V; K103N; V106M	K103S (2.4)	
11	M184I; V90I; Y181C; K101E	M184V (9.9)	
12	V106M	No additional mutations found	
13	M184V; V106M; V179D	No additional mutations found	
14	K103N; P225H	V90I (1.2)	
15	No mutations found	Y188C (27.7)	
16	No mutations found	No additional mutations found	
17	Y115F; V106M; Y188C	A62V (2.4)	
18	D67G; T69N; K101E; V106M; H221Y	K65R (4.9)	TDF (S→R); AZT (S→I); 3TC (S→I); FTC (S→I)
19	M184V; A98AG; K103RST; G190A	K65R (32.5); V108I (9.8); Y181C (12.5)	TDF (S→R); ETR (S→R); RPV (I→R);
20	M184I	No additional mutations found	
21	K103N; M184V; P225H	K65R (30.5); K70E (16.4); A98G (5.8); L100I (10.8); V108I (1.5); K219Q (5.1)	TDF (S→R); ETR (S→I); RPV (I→R)
22	M184I; V106M; V179D; M230L	K65R (17.5); K70E (16.7); L74V (15.9); H221Y (4.1); F227C (29.4)	
23	M184V; V106M; G190A; F227L	Y115F (1.4)	
24	M184V; K103N; V108IV; P225HP	No additional mutations found	
25	M184V; K103N; G190A	D67N (4.6); K103S (33.1); E138G (1.2)	
26	D67N; K70E; M184V; V90IV; K101E; V106M; G190A; F227L	K103N (3.8); V179D (9.2); H221Y (1.4)	RPV (I→R)
27	M184V; V106M; V179D	A62V (12.1); K65R (11.8)	TDF (S→R)

** TDF: Tenofovir Disoproxyl Fumarate; ETR: Etravirine; RPV: rilpivirine; AZT: zidovudine; 3TC: lamivudine; FTC: emtricitabine. S=susceptible, I=intermediate, R=resistant.*

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Chapter 4

No detection of the NS5B S282T mutation in treatment-naïve genotype 1 HCV/ HIV-1 coinfected patients using deep sequencing

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Abstract

Background

The S282T mutation is the main variant described associated with resistance to nucleos(t)ide analogues hepatitis C Virus (HCV) NS5B polymerase inhibitors.

Objective

We aimed here to investigate whether this substitution pre-existed in treatment naive HCV/HIV-1 coinfected patients.

Study design

NS5B polymerase deep sequencing was performed at a median coverage per base of 4471 in 16 patient samples.

Results

No S282T variant was detected in the 16 analyzed samples.

Conclusion

This finding is in agreement with the high genetic barrier of nucleoside analogues NS5B polymerase inhibitors and the clinical efficacy of these compounds.

Background

The approval of two hepatitis C virus (HCV) protease inhibitors, telaprevir and boceprevir, in combination with pegylated interferon plus ribavirin (pegIFN/RBV) has improved sustained virological response rates in patients infected with HCV genotype 1 [1–3]. However, viral breakthroughs due to the selection of HCV variants resistant to the administered protease inhibitors have been reported[4]. Not surprisingly, minority HCV NS3 protease inhibitor resistance mutations were also reported in treatment-naive HCV genotype 1 monoinfected [5–8] and human immunodefiency virus type 1 (HIV-1) coinfected patients [9,10].

The estimated median in vivo HCV mutation rate is 2.5×10^{-5} mutations per nucleotide per genome replication [11]; this, combined with a virion production and clearance rate of 10^{12} virions per day [12], suggests that resistant HCV variants are likely to preexist and rapidly outgrow wild type virus under drug selective pressure. The two NS5B nucleos(t)ide analogues inhibitors that more quickly are advancing to the clinic, mericitabine and sofosbuvir, have been reported to select in vitro substitutions at position S282 which is in close vicinity to the enzyme's catalytic site. The S282T substitution has been reported to confer a 3- to 6-fold loss of in vitro sensitivity to mericitabine [13]. This substitution results in a moderate loss of antiviral activity but in a large reduction in replicative capacity[14]. However, no baseline population sequencing analysis with predicted resistance to NS5B nucleos(t)ides analogues have been detected *in vivo* in genotype 1 infected patients [8,15]. The S282T mutation has been found to be dominant only in a genotype 4a isolate [16]. Similarly, classical clonal analysis of the viral quasispecies indicated that mutation S282T was not present at baseline in treatment-naïve genotype 1 HCV infected patients [17]. Moreover, the S282T mutation has been extremely difficult to detect *in vivo* even in patients with failure to mericitabine or sofosbuvir [4]. It is notable that most failures were relapses after therapy; no viral breakthroughs.

Objective

To further explore the prevalence of S282T, we investigated here whether the NS5B S282T minor variant preexisted in HCV/HIV-1 coinfected patients. HIV-1 coinfected patients have both higher HCV viral and higher rates of treatment failure than HCV monoinfected patients. To this end, NS5B deep sequencing was performed in samples from treatment naïve genotype 1 HCV/HIV-1 coinfected patients.

Study design

Patients

Sixteen HCV/HIV-1 coinfected patients were studied (Table 1). All patient samples were infected with HCV of genotype 1a or 1b and were naive for pegIFNa/RBV treatment and direct acting antivirals (DAAs). The median HCV viral load was 5.88 (log10) IU/mL and the interquartile range was 5.72-6.10 (log10) IU/mL. All HIV-1-co-infected patients were receiving antiretroviral therapy.

Methods

HCV RNA was extracted from 1 mL of the plasma samples by ultracentrifugation and purified by using the QIAamp viral RNA minikit (QIAGEN) according to the manufacturer's instructions. Three independent RT-PCRs (RT-PCR One Step Superscript III/Platinum HF Taq) (Invitrogen) were carried out for each sample with oligonucleotides NS5B-F1 (5'-TCTCAGCGACGGGTCWTGGTC-3', H77 positions 7526-7546) and NS5B-R1 (5'-CCTGCAGMAAGYAGGAGTAGGC-3', H77 positions 9303-9324) using standard conditions. Amplicon libraries were generated from first-round PCR products. These amplicons incorporated adaptators A and B, and identifiers used in parallel sample sequencing needed for bidirectional 454 sequencing.

Nested PCR was performed with Platinum Taq High Fidelity (Life Technologies) and oligonucleotides NS5B-F3 (5'-TCTACCAATGTTGTGACYTGG-3', H77 positions 8305-8326) and NS5B-R3 (5'-GCATCGTGCAGTCCTGGAGC-3', H77 positions 8510-8529). The HCV 1b replicon plasmid (I389/NS3-3'/LucUbiNeo-ET) was retrotranscribed to RNA (Ambion, Life technologies) and tested in parallel with patient samples to determine the assay background. Nested PCR products were purified using AMPure Magnetic Beads (Beckman Coulter). Concentration and quality of purified PCR products was determined using fluorometry (PicoGreen, Life Technologies) and spectrophotometry (Lab on a Chip, Agilent Technologies). Equimolar amplicon pools were made to perform emPCR, adding a ratio of 1:1 between molecules and 454-beads. The sequence platform used was Genome System Junior (Life Sequencing/Roche). Amplicon Variant Analyzer (AVA software v2.6) was employed to analyze and obtain sequence alignments. Error corrected consensus sequences, as obtained from AVA, were used for amino acid variant calling. Variants were considered valid when present in both forward and reverse directions.

Results and Discussion

Deep sequencing results of the NS5B coding region were obtained for the 16 patient samples and genotype 1b replicon control. A total of 76007 sequences were obtained after quality checks. The resultant sequences were used to generate a list of mutations present in the tested samples. On average, 4471 reads were obtained per each nucleotide position of the 184 nucleotides sequenced. To establish the background noise derived from PCR amplification and 454 pyrosequencing, the genotype 1b replicon control was sequenced, with an average coverage of 1385 reads per nucleotide position. The background noise for replicon control was below 0.5% nucleotide substitutions per NS5B nucleotide position. The median Shannon Entropy was 0.01402 ± 0.00475 (SD) and interquartile range was 0.02568-0.00496. Shannon Entropy for genotype 1b replicon control was 0.00093 ± 0.00000. Patient amino acid sequences were compared with the corresponding prototype 1b (I389/NS3-3'/LucUbiNeo-ET) or

1a (H77) sequences (Fig. 1). When patient amino acid sequences were compared with the corresponding prototype sequences, 17 amino acids (27%) were altered and 13 (21%) showed mixed variants (Table 2). Interestingly, a variant S282G was detected in patient 12. However, the NS5B S282T mutation was not detected in any of the samples analyzed in our study. S282G was likely generated through a transition mutation, while S282T is generated though a transversion. Transitions are much more frequent than transversions, which provides a possible mechanism for its appearance and detection.

These findings extend and confirm previous studies which showed the low prevalence in vivo of the S282T mutation. In addition, our results may explain the absence of breakthroughs in patients failing therapy with NS5B nucleos(t)ide analogues and their high vivo barrier for the development of resistance. Remarkably, viral breakthrough has been rarely observed even in patients on nucleos(t)ide analogue monotherapy. Nevertheless, other mechanisms can not be discarded to explain the absence of breakthroughs in patients that fail therapy with NS5B nucleos(t)ide analogues because non-mutant HCV in some patients may resist the treatment. Therefore, absence of the S282T mutation may not be the only indicator of sensitivity to the therapy. In contrast, abundant NS3 protease inhibitor resistant mutants have been detected by deep sequencing in patients undergoing NS3 protease inhibitor monotherapy or in baseline treatment-naïve patient samples [18,19]. Most of samples of this study had minor NS3 protease inhibitor resistant mutants when they were analyzed by classical clonal analysis [9]. Furthermore, it is also possible to detect minority NS3 protease inhibitor resistant mutants even in acute hepatitis C patients which have less nucleotide quasispecies diversity when compared to chronic infected patients (data not shown). Pretreatment minority resistance substitutions to daclatasvir, an NS5A replication complex inhibitor, have been also described [20].This study therefore indicates the differences between the prevalence of NS5B nucleoside analogue inhibitor resistance and other HCV DAAs. A possible explanation for the low prevalence of the S282T mutation is the lethality of this substitution.

Although this study provides compelling evidence for the low prevalence of NS5B S282T mutants in naïve samples, our results may be limited by absence of samples from patients failing therapy with mericitabine or sofosbuvir. Further work should therefore include deep sequencing analysis of samples from relapsers failing therapy with nucleos(t)ide analogues inhibitors in order to evaluate whether the S282T mutation is lethal in most genotype 1 NS5B backgrounds.

Table 1. *Clinical characteristics of study patients.*

Patient	Gender	HCV RNA[a]	HCV genotype	AST[b]	ALT[b]	CD4+ cell count[c]	HIV RNA[d]
1	M	5.68	1b	40	78	630	<80
2	M	6.39	1b	76	200	272	<80
3	M	5.939	1b	28	15	307	<80
4	F	5.83	1b	58	52	350	<80
5	M	5.85	1b	89	208	785	6600
6	F	5.92	1b	53	89	342	<80
7	M	5.70	1b	51	56	247	3300
8	F	6.28	1b	33	31	400	<80
9	M	5.93	1b	65	111	308	<80
10	M	6.756	1a	110	116	5236	<80
11	M	5.85	1a	49	114	611	<80
12	F	5.505	1a	28	26	507	<80
13	M	5.92	1a	42	59	475	4900
14	F	6.50	1a	38	34	660	<80
15	M	5.67	1a	58	103	747	<80
16	M	5.74	1a	59	95	661	<80

[a](IU/mL) (log_{10}); [b](U/L) ; [c]CD4+ cell counts/mL; [d]copies/mL

Table 2. *HCV NS5B amino acid substitutions compared with prototype 1b (I389/NS3-3'/LucUbiNeo-ET) and 1a (H77) sequences.*

Patient[a]	Aminoacid position	Prototype amino acid	Aminoacid substitution	Frequency (%)[b]
1	250	R	K	99.3
	300	A	S	79.4
	300	A	T	19.8
2	250	R	K	99.4
	293	L	P	0.7
	300	A	T	100
3	254	R	K	99.9
	300	A	S	100
4	254	R	K	32.8
	260	L	F	0.6
	267	T	I	0.6
	300	A	T	99.4
5	254	R	K	100
	300	A	T	100
6	249	A	S	0.9
	281	A	T	1.0
	300	A	S	100
7	250	R	K	1.1
	300	A	S	98.6
	300	A	T	1.4
8	300	A	S	100
10	252	A	V	1.2
	300	R	Q	100
11	251	V	M	6.0
	300	R	Q	99.9
	305	A	T	0.6
12	300	R	Q	99.9
	282	S	G	0.8
	305	A	T	1.0
13	300	R	Q	100
14	252	A	V	5.0
	262	V	I	0.6
	300	R	Q	99.8
15	300	R	Q	75.4
16	300	R	Q	99.8

[a]Genotype 1b sequences were compared to prototype 1b (I389/NS3-3'/LucUbiNeo-ET) sequence and genotype 1a sequences were compared to prototype 1a (H77) sequence.

[b]Only frequencies above 0.5% are shown.

Figure 1. *Alignment of the HCV NS5B amino acid consensus sequences of the 16 study patients compared with prototype 1b (I389/NS3-3'/LucUbiNeo-ET) and 1a (H77) sequences. Dots indicate amino acid sequence identity.*

```
        246   *       260         *       280         *       300
1b.I389 : APEARQAIRSLTERLYIGGPLTNSKGQNCGYRRCRASGVLTTSCGNTLTCYLKAAAACRAAK :  62
1       : ....K.....................................................S..... :  62
2       : ....K.....................................................T..... :  62
3       : ........K.................................................S..... :  62
4       : .........................................................T..... :  62
5       : ........K.................................................T..... :  62
6       : .........................................................S..... :  62
7       : .........................................................S..... :  62
8       : .........................................................S..... :  62
9       : ............................................................... :  62
1a.H77  : D.Q..V..K.......V.......R.E.........................I..R......G :  62
10      : D.Q..V..K.......V.......R.E.........................I..Q......G :  62
11      : D.Q..V..K.......V.......R.E.........................I..Q......G :  62
12      : D.Q..V..K.......V.......R.E.........................I..Q......G :  62
13      : D.Q..V..K.......V.......R.E.........................I..Q......G :  62
14      : D.Q..V..K.......V.......R.E.........................I..Q......G :  62
15      : D.Q..V..K.......V.......R.E.........................I..Q......G :  62
16      : D.Q..V..K.......V.......R.E.........................I..Q......G :  62
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Conflicts of interest

The authors have declared that no conflicts of interests exist

Ethical approval

Ethical approval was given by committee of Hospital Universitari Germans Trias i Pujol under the reference number E0-10-008.

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Discussion

This doctoral thesis has shown that detection of low frequency drug resistance mutations (LFDRM) is clinically important in certain subjects and in particular antiretroviral regimens.

The first message to highlight is that pre-existing LFDRM are clinically relevant in late presenters with advanced immune suppression starting treatment with NNRTIs, as its presence is associated with higher risk of VF. Considering that more than 30% of HIV-1 infected people are diagnosed late, and late presenters are more likely to have clinical complications, it is important to choose the correct first-line regimen, avoiding possible VF due to LFDRM that were not detected before treatment initiation. Conversely, PI associated LFDRM did not have an evident impact on virological response in this population. This could possibly be extended to other first line boosted-PI regimens which share a high genetic barrier to resistance and low transmission rates of viruses harbouring multiple PI resistance mutations. This finding is consistent with previous studies[1] and suggests a limited value of pre-ART DR testing in patients initiating drugs with high genetic barrier. However, PIs might be worse tolerated and more expensive particularly in LMIC, which limits its use.

Our work also shows that low frequency drug resistance mutations are important in HIV-1 infected subjects living in low- and middle-income countries (LMIC), where patients are often given first-line TDF-containing regimens without virological monitoring. Given the high prevalence of TDF resistance after VF of first line ART, absence of genotyping could considerably compromise TDF use as second-line regimen. As it has recently been reported in a worldwide study [2] and also in our study in a South African cohort (chapter 3), drug resistance is present at a high proportion of patients after VF on a TDF-containing first-line regimen across LMIC. In our cohort, this prevalence was 70%, assessed by Illumina. By population sequencing, we were missing the 30% of resistances. In the global study, the prevalence of TDF resistance was 57%, even though assessed by NGS this number could be higher. Nonetheless, this means millions of people affected. Therefore, surveillance of TDF resistance mutations should be a priority both in treated and naïve populations, at least by population sequencing, and when possible, by NGS. Studies are

needed to evaluate the impact of low-frequency TDF-resistant mutants on TDF-including regimens.

Another important message is that transmission of InSTI resistance in Europe remains at negligible levels to date. Our study in chapter 2 analysed the prevalence of transmitted InSTI resistance in a European cohort before their introduction in clinical practice, in 2006. This study provides a wide and representative appraisal of primary InSTI resistance in Europe, giving a background of the baseline prevalence of these mutations, useful to track the evolution of InSTI TDR in upcoming studies.

Now InSTIs are frequently being prescribed in resource rich settings, and this could potentially lead to the emergence of transmitted resistance mutations and the need for integrase genotyping in the coming years. Therefore, it is warranted to monitor InSTI resistance prevalence periodically, because if it increases, integrase genotyping will become necessary in clinical practice.

This thesis also shows that ultrasensitive genotyping might also be a good tool to select the better rescue regimen in treatment-experienced patients. Viral tropism determination is required before maraviroc prescription, and different studies have proved an association between the presence of minority X4-viruses and virological failure in maraviroc treated subjects, which indicates that, whenever this drug is prescribed, a previous NGS genotyping would be ideal to avoid the miss-detecting of minority X4-viruses. In our study in late presenters, we did not see an association between the presence of X4 viruses and VF. Conversely, it predicted impaired immune restoration under ART. Deep sequencing has proved to be at least as accurate as the most sensitive phenotypic assays, more cost-effective and faster to perform. The availability of freely available semi-automated interpretation systems of deep V3-loop sequencing such as Geno2Pheno[454] (Max-Planck-Institut Informatik), has simplified the use and interpretation of NGS data.

Regarding HCV resistance mutation testing, it is not routinely performed, as resistance variants to nucleos(t)ide analogues have not been detected.

However, Q80K variant confers resistance to simeprevir, and it has been observed in 9-48% of naïve HCV genotype 1a-infected subjects. This mutation leads to reduced rates of sustained virologic response. Guidelines [3] recommend testing for its presence if simeprevir-containing regimes are considered. In chapter 4 in this thesis, we looked for the S282T resistant variant, which would confer resistance to the nucleotide analogue sofosbuvir, but we did not detect it. This mutation has only been observed in few patients, with reversion to wild-type viruses within several weeks.

The introduction of highly potent direct-acting antivirals (DAA) has enabled achieving high-sustained virological response rates in subjects with and without pre-existing resistance associated variants[4,5]. Simplified DAA-based and potentially interferon-free HCV therapy require low number of pills and shorter treatment duration, and has better tolerability and higher rates of cure. Interferon-free DAA regimens are now recommended, and result in HCV cure in the majority of patients[6]. Using all-oral DAA combination therapies, there are no longer differences in efficacy between monoinfected and HCV/HIV coinfected patients[7]. However, drug-drug interactions have to be checked carefully before DAA selection, and, if needed, HIV-1 antiretroviral regimens must be adapted to the respective HCV therapy, because they may share metabolic pathways via the cytochrome p450 system and drug transporters.

Future insights

It has been 20 years since combined HIV-1 ART started being prescribed to HIV-1 infected subjects, and 35 different drugs from five different families have received approval for its use. Along this time, they have turned to be increasingly more efficient, less toxic and more simplified, making them easier to take and improving adherence, achieving efficacies in viral suppression around 90% or higher in certain settings. If we have seen these huge improvements in 20 years, we could expect some new formulas able to avoid or compensate the effect of resistance mutations, which are one of the factors triggering VF. For instance, new integrase inhibitors with increasingly higher

genetic barrier to resistance are in the pipeline [8]. With no cure in sight, it remains key to develope drugs causing fewer long-term toxicities and strategies that do not require daily medication dosing.

New approaches to prevent HIV infection are also being developed and arising: besides the already known pre-exposure prophylaxis (PrEP) tenofovir (TDF) or emtricitabine (FTC) pills, there are also long-acting antiretroviral agents for prevention, including rilpivirine and cabotegravir, capable of being delivered systemically by injection or in the mucosa to achieve sustained levels of ART locally. In all these approaches, resistance mutations may have a crucial role and its surveillance is essential.

The World Health Organization is developing a global action plan on drug resistances worldwide for 2017-2021, in which they aim to establish effective systems to monitor, prevent and respond to the emergence of DRM. Some of the points of the plan are to strengthen laboratory capacities to enable global genotyping in LMIC and to make global DRM surveillance. To achieve these objectives, NGS could be an excellent tool. Lapointe, et.al.[9] and Dudley, et.al.[10] account for strategies that can be used for multiple HIV subtypes, and which demonstrated the potential for widespread individual testing and annual population surveillance in LMIC using Illumina sequencing.

In few years next generation sequencing has evolved rapidly and approaches are increasingly improving, regarding workload, costs, throughput and multiplexing. One of the big challenges is developing bioinformatics tools to easily interpret this huge amount of data arising from each run. To date, the most important limitation is the lack of automated, validated and robust but simplified bioinformatic analyses coupled with HIV-1 resistance interpretations to enable NGS use and interpretation by laboratory technicians, but even this is improving rapidly. Some initiatives are already available, such as Geno2Pheno for tropism interpretation, which can be used for free, or DeepCheck® for genotyping, that has a cost of 60$/sample approximately. In order to make them accessible in all settings, these tools should be robust, comprehensive, user-friendly and affordable in all contexts.

To definitively end the HIV pandemic we will need simpler and more affordable diagnostics, simpler and better tolerated ART, longer-lasting and more affordable regimens that avoid the need for daily dosing, worldwide availability of treatments, political leadership, system preparedness and implementation of global normative guidance.

Detection of minority drug resistance can help to reach these objectives by avoiding virological failures and preventing the transmission of drug resistant HIV.

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Conclusions

1) Accounting for preexisting drug resistant minority variants may improve the outcomes of first-line non-nucleoside reverse transcriptase inhibitor-based ART in late presenters with advanced immune suppression.

2) Presence of X4 HIV-1 at diagnosis predicts impaired immune restoration under ART in late presenters.

3) No signature InSTI-resistant variants were circulating in Europe before the introduction of InSTIs, in 2006-2007. However, polymorphisms contributing to InSTI resistance were not rare.

4) As InSTI use becomes more widespread, continuous surveillance of primary InSTI resistance is warranted. These data will be key to modelling the kinetics of InSTI resistance transmission in Europe in the coming years.

5) In South Africa, unless HIV genotyping is available to closely monitor epidemiological HIV resistance to TDF, its efficacy as second-line therapy will be greatly compromised.

6) In treatment-naïve HCV/HIV-1 infected patients, mutation S282T is unlikely to pre-exist.

Publications

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