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Effect of the HIV-1 Integrase Inhibitor Raltegravir on Drug Susceptibility, Replication Capacity and Residual Viremia in HIV-infected Subjects

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Dr. Xavier Avilés Puigvert

“La ignorancia afirma
o niega rotundamente;
la Ciencia duda”
Voltaire

CONTENTS

RESUMEN

Raltegravir es el primer inhibidor de la integrasa del VIH-1 aprobado para su uso en pacientes infectados por el VIH. Sinembargo, como el resto de los inhibidores, la emergencia de mutaciones de resistencia limita su eficacia clínica.

El entendimiento de la prevalencia diferencial del ratio de mutaciones dentro de la región codificadora de la integrasa en pacientes con tratamiento TARGA (Tratamiento Antirretroviral de Gran Actividad) pero sin inhibidores de la integrasa, podría ayudar a identificar si algunas mutaciones virales o polimorfismos naturales ocurren, y si la diversidad genética del gen de la integrasa tiene implicaciones importantes en la respuesta clínica a los inhibidores de la integrasa. Por lo tanto, el primer objetivo de esta tesis doctoral fue explorar la evolución longitudinal de la región codificadora de la integrasa viral en pacientes que había recibido, durante una media de 10 años, terapia antirretroviral sin inhibidores de la integrasa. Un objetivo adicional, fue el estudio del efecto de los cambios genotípicos a la susceptibilidad al raltegravir y a la capacidad replicativa, en aquellas muestras que acumularon el mayor número de substituciones aminoacídicas durante el periodo de estudio. Nuestros resultados mostraron que las integrasas virales, después de una terapia antirretroviral prolongada, mantenían la sensibilidad genotípica y fenotípica al raltegravir. Además los cambios genotípicos a lo largo del tiempo, condujeron hacia una mejora de la capacidad replicativa de virus recombinantes para la integrasa viral, sugiriendo que el tratamiento antirretroviral prolongado no tiene coste biológico a nivel de capacidad replicativa del virus. Por lo tanto, nuestros datos apuntan que los tratamientos actuales no disminuyen la capacidad

replicativa de la integrasa viral ni influyen la susceptibilidad al raltegravir.

Hasta el momento, no existen estudios que evalúen la contribución fenotípica de mutaciones fuera de la región codificadora de la integrasa en pacientes que fracasan a tratamientos que contenían raltegravir. Las mutaciones de resistencia en la integrasa podrían jugar un papel importante en los cambios fenotípicos, tales como la capacidad replicativa y la susceptibilidad farmacológica. El segundo objetivo de esta tesis, fue comparar las contribuciones epistáticas de la integrasa, la proteasa, la transcriptasa reversa y el resto del genoma del VIH-1 en el fitness viral y la susceptibilidad al raltegravir en pacientes con y sin mutaciones de resistencia en la integrasa viral pero que fracasaban al TARGA conteniendo inhibidores de la integrasa. Nuestros resultados sugirieron una ausencia de efectos epistáticos entre los genes del VIH-1 en relación a la susceptibilidad farmacológica al raltegravir. Sinembargo, cambios genotípicos en la proteasa, la transcriptasa reversa y fuera de la polimerasa, compensaron la capacidad replicativa de virus que contenían mutaciones de resistencia en la integrasa viral. Por lo tanto, en pacientes que fracasaban a terapias que contenían inhibidores de la integrasa, la susceptibilidad al raltegravir estuvo esencialmente modulada por las mutaciones de resistencia dentro de la región codificadora de la integrasa, mientras que otros genes del VIH-1 estaban involucrados en la modulación del fitness viral.

En pacientes con TARGA que consiguen suprimir la carga viral, se ha detectado la existencia de una viremia residual con métodos ultrasensibles. Si esta viremia residual refleja una replicación viral de bajo grado, o es el resultado de una producción viral por parte de reservorios estables, no está claro. Esta pregunta tiene serias implicaciones clínicas, porque si la viremia residual refleja una replicación de bajo grado, entonces la intensificación del TARGA podría ser útil para prevenir una evolución viral con el consecuente fracaso farmacológico. Los nuevos agentes farmacológicos, como los inhibidores de la integrasa, nos proporcionan nuevas herramientas con las que poder evaluar los reservorios virales que persisten en pacientes con TARGA. Raltegravir tiene un efecto único sobre las formas de ADN viral, incrementando las formas episomales 2-LTRs cuando la replicación es inhibida por el fármaco. El tercer objetivo de esta tesis fue evaluar si la intensificación del TARGA con raltegravir era capaz de impactar los niveles de ADN viral y la activación inmune. La intensificación con raltegravir bloqueó la replicación activa y la producción de virus infecciosos en un 29 por ciento de los pacientes. Nuestro estudio también reveló la relación efecto-causa entre la replicación activa y la activación inmune, sugiriendo que bajo un TARGA supresivo, la replicación activa es una causa, en vez de una consecuencia, de la anormal activación inmune. Por lo tanto, la intensificación del TARGA perturba el reservorio viral con implicaciones importantes para las estrategias terapéuticas dirigidas a alcanzar la erradicación viral.

SUMMARY

Raltegravir is the first *in-class* HIV-1 integrase inhibitor approved for the treatment of HIV-1 infected subjects. However, like all other HIV inhibitors, the emergence of drug resistance limits its clinical efficacy.

Understanding the differential prevalence rate of mutations within the integrase coding region from Highly Active Antiretroviral Therapy (HAART)-experienced but integrase inhibitor–naïve subjects could help identify whether clinically relevant viral mutations or natural polymorphisms occur, and whether the sequence diversity of the integrase gene has important implications in the clinical response to integrase inhibitors. Therefore, the first objective of this project thesis was to explore intrasubject longitudinal evolution of the HIV-1 integrase-coding region over a median of 10 years of heavy antiretroviral therapy in integrase inhibitor–naïve subjects. An additional objective was to explore changes in phenotypic susceptibility to raltegravir and replication capacity in those samples that accumulated the highest number of amino acid substitutions during the study period. Our results showed that HIV-1 integrase from longitudinal samples did not show evidence of genotypic or phenotypic resistance to raltegravir. Long-term antiretroviral pressure did not impair the raltegravir susceptibility and appeared to drive the replication capacity of integrase-recombinant viruses towards improved phenotypes, suggesting no fitness cost associated with long-term treatment. Therefore, our data suggest that current antiretroviral regimens do not diminish the fitness of integrase or influence raltegravir efficacy.

No studies have evaluated the potential phenotypic contributions of mutations outside the integrase coding region in subjects whose raltegravir-containing regimens fail. Integrase resistance mutations could play a major role in phenotypic changes, such as replication capacity and drug susceptibility. The second objective of this thesis project was to compare the relative epistatic contributions of integrase, protease, reverse-transcriptase and the rest of the HIV-1 genome on viral fitness and susceptibility to raltegravir in subjects with and without raltegravir-resistance associated mutations and whose raltegravir-containing regimen has failed. Our results suggested an absence of epistatic effects between HIV-1 genes with regard to in vitro susceptibility to raltegravir. However, changes in protease, reverse-transcriptase and outside *Polymerase* compensated for the ex vivo replication capacity of viruses containing integrase resistance mutations. Therefore, raltegravir susceptibility was essentially driven by resistance mutations within the integrase coding region, whereas other HIV-1 genes were involved in modulating viral fitness in subjects whose raltegravir-containing regimen failed.

In subjects under a suppressive HAART, residual viremia has been detected using ultra-sensitive methods. Whether residual viremia reflects ongoing viral replication at low levels or the production of virus from stable reservoirs remains unclear. Importantly, this question has immediate clinical implications, because if residual viremia reflects ongoing viral replication, then the intensification of the current treatment might be useful in preventing viral evolution and subsequent treatment failure. New classes of antiretroviral agents, such as integrase inhibitors, provide new tools to assess the viral

reservoirs that persist in HAART-suppressed subjects. Raltegravir has a unique effect on viral DNA forms, leading to a measurable increase in 2-LTRs DNA circles when replication is inhibited. The third objective of this project thesis was to assess whether raltegravir intensification of the HAART regimen was able to impact the levels of HIV-1 DNA and immune markers in subjects with undetectable viremia measured by standard assays. Raltegravir intensification in HAART-suppressed subjects blocked active replication and production of infectious virus in 29 percent of subjects. Our study also revealed a causative relationship between active replication and immune activation, suggesting that under a suppressive HAART, active replication is a cause rather than a consequence of aberrant immune activation. Therefore, treatment intensification disturbs the latent reservoir, with important implications for therapeutic strategies aimed at achieving viral eradication.

Chapter 1

General Introduction

HUMAN IMMUNODEFICIENCY VIRUS

Human immunodeficiency virus (HIV) is a lentivirus, a member of the retrovirus family, that causes acquired immunodeficiency syndrome (AIDS) [1], a condition in humans in which the immune system begins to fail, leading to opportunistic infections. HIV was originated in non-human primates in sub-Saharan Africa and was transferred to humans late in the 19th or early in the 20th century [2]. HIV infection in humans is considered pandemic, affecting about 0.6 percent of the world's population according to the World Health Organization.

HIV preferentially infects immune system cells. The main HIV targets are CD4+ T-lymphocytes, but other cells expressing CD4+ in their surface, such as monocytes, macrophages or dendritic cells, are susceptible to infection. It has been described that some of these cells could be acting as HIV reservoirs, increasing the latency period and the development towards AIDS [3]. Productive infection with HIV-1 is associated with a progressive decline of the CD4+ T cell population and an increase in viral load (**Figure 1**)

CLASSIFICATION AND STRUCTURE

HIV is a member of the genus Lentivirus from the family of Retroviridae. Lentiviruses are transmitted as single-stranded RNA viruses. HIV is roughly spherical with a diameter of about 100-150 nm. HIV is composed of two copies of positive single-stranded RNA that codes for the virus's nine genes (*gag, pol*, *env, tat, rev, nef, vif, vpr* and *vpu*) enclosed by a conical capsid. Those nine genes encode for 19 proteins (**Figure 2**).

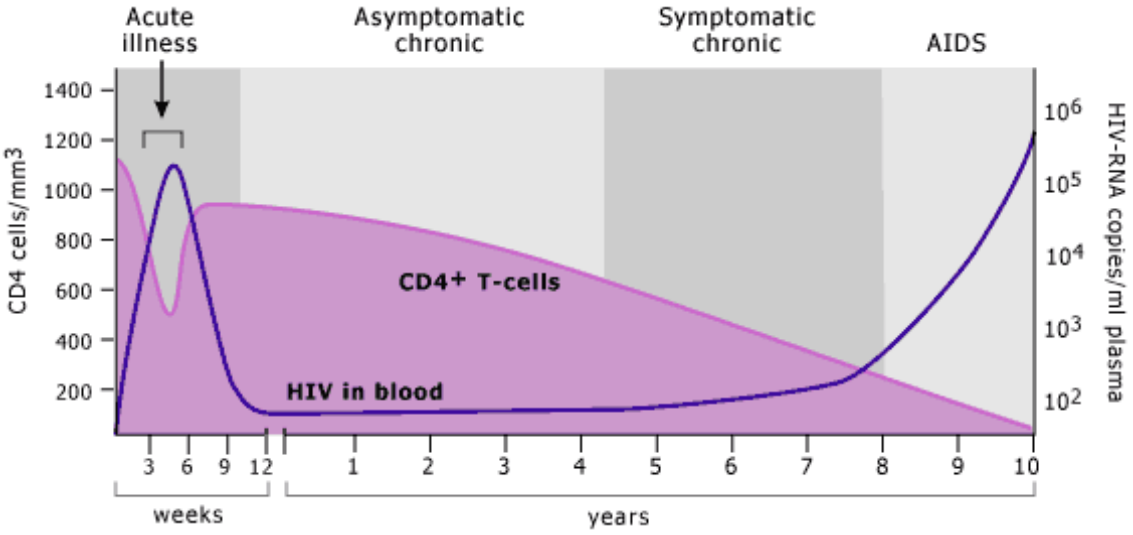


Figure 1. Course of HIV-1 infection in the absence of antiretroviral therapy. Clinically, it is characterized by an initial acute illness, which last between two and four weeks. It is a period of rapid viral replication during which a pronounced increase in viral load occurs. At the same time, the number of CD4+ T cells decrease dramatically. After this phase, symptoms disappear, probably due to strong specific cytotoxic response. Then an equilibrium is achieved between viral replication and cellular and humoral immune response over a period of years, known as the chronic phase, where the subject remains in an state of clinically asymptomatic latency. As time goes on, the immune system is progressively deteriorated and loses the capacity to control viral replication on the onset of the AIDS phase.

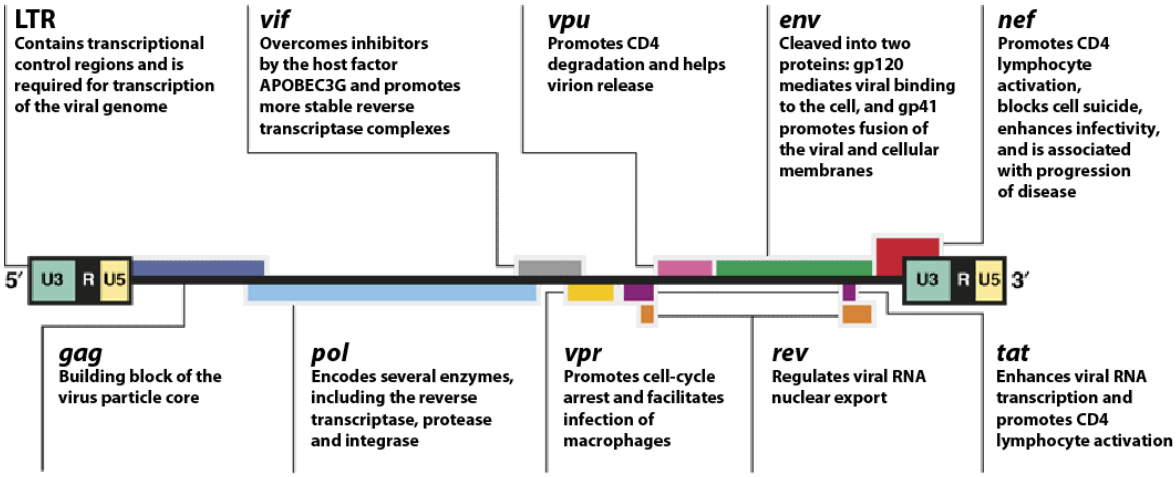


Figure 2. HIV-1 structure genome and its major functions. Adapted from http://www.gladstone.ucsf.edu/gladstone/site/publicaffairs/section.php?id=1707

- ***Gag*** codes for the *Gag* polyprotein, which is processed during maturation to MA (matrix protein, p17); CA (capsid protein, p24); SP1 (spacer peptide 1, p2); NC (nucleocapsid protein, p7); SP2, spacer peptide 2, p1) and p6.
- ***Pol (Polymerase)*** codes for the viral enzymes Reverse Transcriptase, Integrase and Protease:

  Reverse-transcriptase: also known as RNA-dependent DNA polymerase is a DNA polymerase enzyme that transcribes single-stranded RNA into double-stranded DNA. It also helps in the formation of a DNA double-helix once the RNA has been reverse transcribed into a single strand cDNA.

  Protease: Is an aspartyl-protease which is mainly responsible for proteolytic processing of the polyproteins codified by the viral genome, transforming them into mature proteins required for the assembly of the virions.

Integrase: The main function of integrase is to insert the viral DNA into the host chromosomal DNA. The integrase protein is incorporated when virions infect new cells, together with two copies of RNA and the reverse-transcriptase, in a structure called the nucleoprotein complex. Within this complex the reverse trascription of RNA is performed, forming a double-stranded DNA. The DNA remains associated with viral proteins like integrase, and cellular proteins forming the pre-integration complex which will be transported to the nucleus of infected cells, where the integration step will be performed.

- ***Env (Envelope)*** codes for gp160, the precursor to gp120 and gp41, proteins embedded in the viral envelope which enable the virus to attach to and fuse with target cells.
- ***tat, rev, vpr*** are transactivator proteins
- ***vif, nef, vpu*** are regulatory proteins

REPLICATION CYCLE OF HIV AND ANTIRETROVIRAL DRUGS

Viral entry

Entry to the cell begins through high affinity interaction between the envelope glycoprotein gp120, the host cell CD4 and a chemokine receptor (generally either CCR5 or CXCR4) on the cell surface [4]. Then gp41 allows membrane fusion and the internalization of the virus into the host cell. After HIV has bound to the target cell, the HIV RNA and various enzymes, including reverse-transcriptase, integrase and protease, are injected into the cell [4]. Entry can be blocked by entry inhibitors (**Figure 4**) (**Table 1**).

Table 1. HIV-1 Entry Inhibitors

Brand Name	Generic Name	Abbreviation	Company/Institution
Fuzeon	enfuvirtide	ENF	Trimeris/Hoffman-LaRoche
Selzentry or Celsentri	maraviroc	MVC	Pfizer
	Pro140*		Progenics
	Ibalizumab*		Tanox/Genentech
	INCB9471*		Incyte
	PF232798*		ViiV Healthcare
	SP-01A*		Samantian Pharm
	TAK652*		Tobira
	Vicriviroc*		Schering-Plough/Merck

*In development Adapted from http://www.aidsmeds.com and [5]

Transcription

Shortly after the viral capsid enters the cell, the reverse-transcriptase liberates the single-stranded (+)RNA genome from the attached viral proteins and transforms it into double-stranded DNA [6]. The synthesis of the double-stranded DNA is performed due to the ribonuclease activity of the reverse-transcriptase that degrades the viral RNA during the synthesis of cDNA, as well as DNA-dependent DNA polymerase activity that creates a sense DNA from the antisense cDNA. Together, the cDNA and its complement form a double-stranded viral DNA that is then transported into the cell nucleus (**Figure 4**). Reverse transcription can be blocked by Nucleoside Reverse-Transcriptase Inhibitors (NRTIs) (**Table 2**) and Non-Nucleoside Reverse-Transcriptase Inhibitors (NNRTIs) (**Table 3**).

Table 2. Nucleoside reverse-transcriptase inhibitors

Brand Name	Generic Name	Abbreviation	Company/Institution
Atripla	efavirenz* + tenofovir + emtricitabine	EFV + TDF + FTC	Bristol-Myers Squibb and Gilead Sciences
Combivir	zidovudine + lamivudine	AZT + 3TC	ViiV Healthcare
Emtriva	emtricitabine	FTC	Gilead Sciences
Epivir	lamivudine	3TC	ViiV Healthcare
Epzicom	abacavir + lamivudine	ABC + 3TC	ViiV Healthcare
Retrovir	zidovudine	AZT or ZDV	ViiV Healthcare
Trizivir	abacavir + zidovudine + lamivudine	ABC + AZT + 3TC	ViiV Healthcare
Truvada	tenofovir DF + emtricitabine	TDF + FTC	Gilead Sciences
Videx EC	didanosine	ddI	Bristol-Myers Squibb
Viread	tenofovir disoproxil fumarate (DF)	TDF or Bis(POC) PMPA	Gilead Sciences
Zerit	stavudine	d4T	Bristol-Myers Squibb
Ziagen	abacavir	ABC	ViiV Healthcare
Racivir*		RCV	Pharmasset
	amdoxovir*	AMDX or DAPD	RFS Pharma
	apricitabine*	ATC	Shire Pharmaceuticals
	Fosalvudine*		Heidelberg Pharmaceuticals
	elvucitabine*	Beta-L-Fd4C	Achillion

*In development

Adapted from http://www.aidsmeds.com and [5]

Table 3. Non-Nucleoside reverse-transcriptase Inhibitors

Brand Name	Generic Name	Abbreviation	Company/Institution
Atripla	efavirenz + tenofovir + emtricitabine	EFV + TDF + FTC	Bristol-Myers Squibb and Gilead Sciences
Intelence	etravirine	ETV	Tibotec
Rescriptor	delavirdine	DLV	Pfizer
Sustiva	efavirenz	EFV	Bristol-Myers Squibb
Viramune	nevirapine	NVP	Boehringer Ingelheim, Roxane Laboratories
	rilpivirine*		Tibotec
	BILR*		Boehringer Ingelheim
	Calanoline A*		Sarawac Medichem Pharm
	GSK 2248761*		ViiV Healthcare
	MIV-150*		Medivir & Chiron
	RDEA806*		Ardea
	UK435061*		ViiV Healthcare

*In development Adapted from http://www.aidsmeds.com and [5]

Integration

The integration of the viral DNA into the host cell genome is carried out by the viral enzyme integrase. Cell activation triggers the expression of the proviral genome, producing and releasing new viral particles that can infect new target cells. Integrase has been identified as the main enzyme responsible for the integration step. The integration process is performed in several steps [7]. The first step is called 3´end processing, where two nucleotides are removed from both 3´ends of the viral DNA. The second step consists of the joining of the processed DNA into the cellular genome, separated by five base pairs flanking the integration region, performing a DNA strand transfer. Integrase is thought to be responsible for both steps, except for the last

step, where cellular enzymes repair the DNA integrated. Duplication of the five base pairs flanking the integrated DNA, removal of the two unpaired nucleotides at the 5´ends, filling the single gap and a final ligation of viral DNA are functions realized by repair cellular enzymes [8]. Integration can be blocked by integrase inhibitors (**Figure 4**). There are several integrase inhibitors currently under clinical trial (**Table 4**), with raltegravir (MK-0518) becoming the first to receive FDA approval in October 2007.

Table 4. Integrase Inhibitors

Brand Name	Generic Name	Abbreviation	Company/Institution
Isentress	raltegravir	RAL	Merck & Co
	elvitegravir*		Gilead
	S/GSK1249572*		ViiV Healthcare

*In development adapted from http://www.aidsmeds.com and [5]

Concretely, the empirical formula of raltegravir is $C_{20}H_{20}FKN_6O_5$ and the molecular weight is 482.51 (PubChem substance SID 47208327). The structural formula is represented in **Figure 3**:

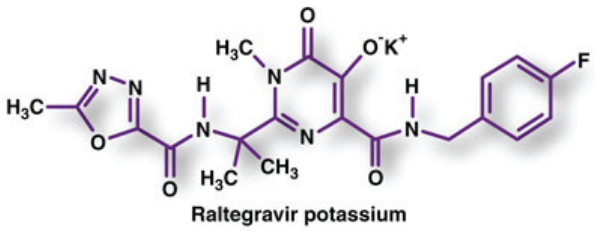


Figure 3. Structural formula of raltegravir

Raltegravir was initially approved only for use in individuals whose infection has proven resistant to other HAART drugs. However, in July 2009, the FDA granted expanded approval for raltegravir for use in all patients. Raltegravir plus optimized background therapy provided better viral suppression than optimized background therapy alone for at least 96 weeks [9-11], becoming an important new addition to antiretroviral therapy. Raltegravir is an HIV-1 strand transfer inhibitor which works by disrupting the insertion of linear cDNA into human DNA. These inhibitors only bind with high affinity to integrase when the enzyme is in a specific complex with viral DNA [12]. The inhibitor-bounded complex is not competent to bind the cellular or target DNA substrate, and the net result is selective inhibition of strand transfer. Blocking strand transfer and integration allows the viral DNA to be metabolized by cellular enzymes. Although most of the unintegrated viral DNA may be degraded, alternate metabolic pathways involving recombination and repair produce 1 and 2 long terminal repeat (1, 2-LTR) episomal HIV DNA byproducts. LTR-HIV-DNA circles have become a defining feature of integrase strand transfer inhibitors (reviewed in [13]). Therefore, an increase in episomal cDNA occurs when active replication is inhibited by this kind of integrase inhibitor [14, 15].

Transcription and Translation

The activation of the provirus occurs through the cellular replication machinery and as a result favors the production of mRNA from the virus. Viral mRNA is exported from the nucleus into the cytoplasm, where it is translated into the regulatory proteins *Tat, Rev, Vpu* and *Nef.* As the newly produced *Rev* protein accumulates in the

nucleus, it binds to viral mRNAs and allows unspliced RNAs to leave the nucleus, where they are otherwise retained until spliced [16]. At this stage, the structural proteins *Gag, Gag-Pol* and *Env* are produced from the full-length mRNA. The full-length RNA is actually the virus genome: it binds to the *Gag* protein and is packaged into new virus particles.

New virion production and budding

Assembly of new HIV-1 virons begins at the plasma membrane of the host cell. The *Env* polyprotein (gp160) is processed into the two HIV envelope glycoproteins gp41 and gp120. These are transported to the plasma membrane of the host cell where gp41 anchors the gp120 to the membrane of the infected cell. The *Gag* and *Gag-Pol* polyproteins also are associated with the plasma membrane along with the HIV genomic RNA as the forming virion begins to bud from the host cell.

Virion maturation

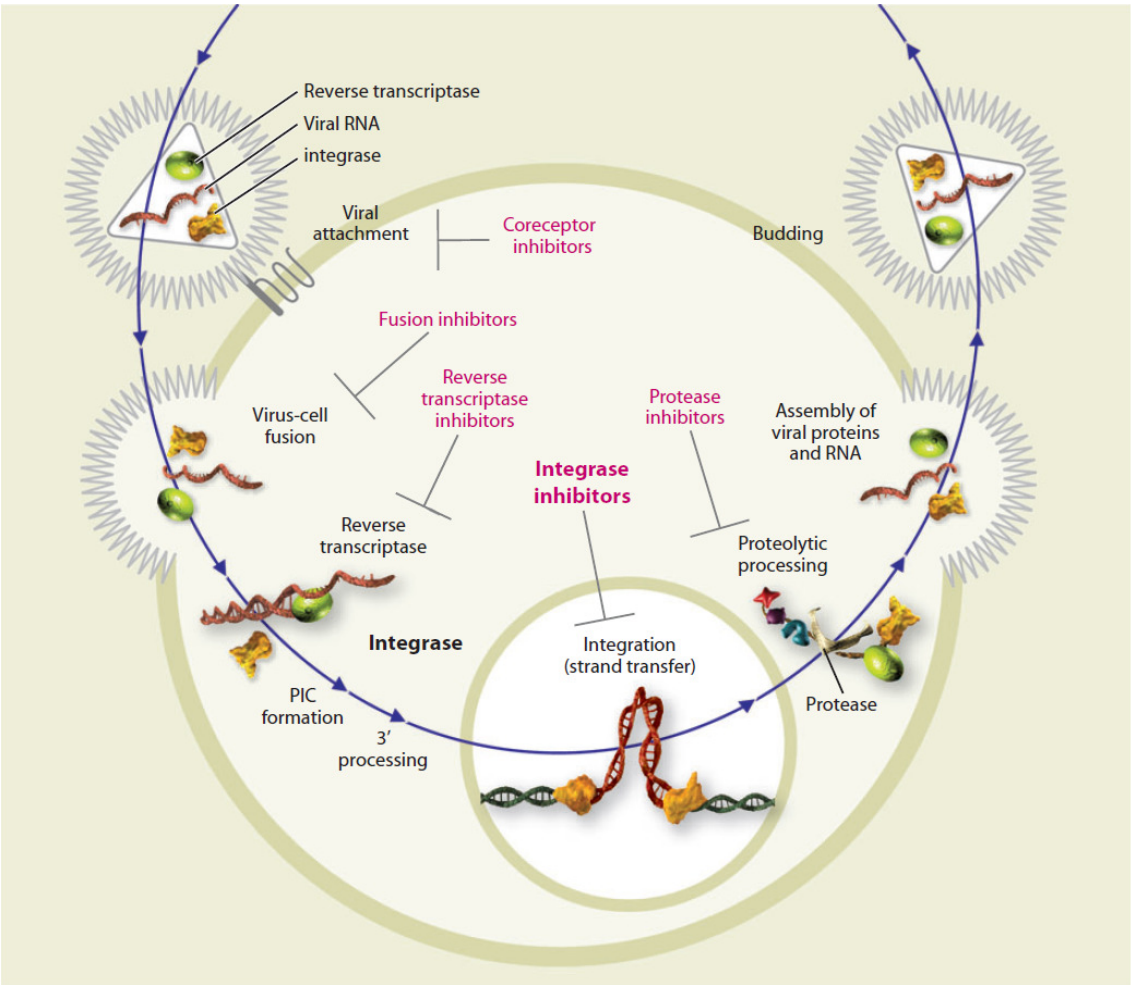
Maturation either occurs in the forming bud or in the immature virion after it buds from the host cell. During maturation, HIV proteases cleave the polyproteins into individual functional HIV proteins and enzymes. The various structural components then assemble to produce a mature HIV virion. The mature virus is then able to infect another cell. This cleavage step can be inhibited by protease inhibitors (**Table 5**).

Table 5. Protease Inhibitors

Brand Name	Generic Name	Abbreviation	Company/Institution
Aptivus	tipranavir	TPV	Boehringer Ingelheim
Crixivan	indinavir	IDV	Merck & Co
Invirase	saquinavir	SQV	Roche Laboratories
Kaletra	lopinavir + ritonavir	LPV/r	Abbott Laboratories
Lexiva	fosamprenavir	FPV	ViiV Healthcare
Norvir	ritonavir	RTV	Abbott Laboratories
Prezista	darunavir	DRV	Tibotec
Reyataz	atazanavir	ATV	Bristol-Myers Squibb
Viracept	nelfinavir	NFV	Pfizer, Roche Laboratories

Adapted from http://www.aidsmeds.com and [5]

Figur4. Schematic representation of the HIV life cycle and antiretroviral drug targets



Adapted from [13]

HIV binds to CD4 cell surface molecules; entry into the cell also requires binding to the co-receptors CXCR4 and CCR5. This step can be inhibited by fusion/entry inhibitors. Then, HIV is uncoated inside the cell and the reverse-transcriptase copies genomic RNA into DNA. This step can be blocked by reverse-transcriptase inhibitors. Viral DNA can integrate into DNA and become a part of the cellular genome. This step makes the infection irreversible: integrase inhibitors are designed to block this step of infection. Finally, the virus uses cellular machinery to synthesize viral proteins. Several of these are long amino acid chains which must be cleaved by a specific viral protease before new viral particles can become active. Protease inhibitors block viral maturation at this step.

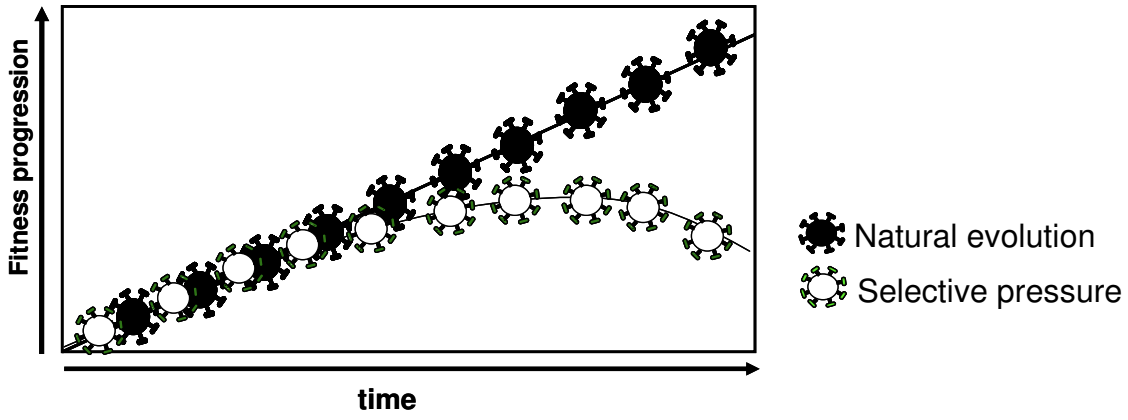
HIV-1 QUASISPECIES AND VIRAL FITNESS

The rapid turnover, high-mutation rate and the high frequency of recombination result in a diverse viral population. HIV-1 reverse-transcriptase has no proofreading ability, and its error rate has been estimated to be between 10^{-4} and 10^{-5} mutations per nucleotide and replication cycle (reviewed in [17, 18]). If approximately 10^9 to 10^{10} viral particles are produced each day in an infected person, then these particles must be the product of at least 10^7 to 10^8 replication cycles. Given the length of the HIV-1 genome (approximately 10,000 nucleotides), it is likely that every possible single-point mutation will occur at least once each day in an infected individual [19]. The degree of potential genetic change drives the diversification of HIV-1 in response to the selective pressure of host immune responses or antiretroviral therapy. When we talk about divergent, rapidly changing HIV-1 variants, we frequently use the term 'quasispecies' [20] to designate closely related but distinguishable genomes that undergo continuous genetic variation, competition and selection.

Drugs targeting HIV protease, reverse-transcriptase, envelope and integrase are included in HAART regimens, which help reduce the morbidity and mortality of HIV-infected subjects. However, the emergence of resistant viruses is a significant obstacle to effective long-term management of HIV infection and AIDS [18]. The evolutionary pathways leading to resistance have been widely studied for many antiretroviral drugs. In general, the evolution of drug resistance is characterized by severe in vitro fitness losses when the drug is not present (**Figure 5**), which can be partially overcome by compensatory mutations or other adaptive changes that restore

replication capacity. Fitness is a complex parameter aimed at describing the replication adaptability of an organism to its environment.

Figure 5. HIV-1 Fitness evolution under natural and different selective pressures



HIV fitness has been correlated with viral diversity and disease progression, therefore natural evolution trends to increase fitness overtime [21]. However, it has been described that a selective pressure, such as antiretroviral therapy, selects for viral variants with impaired fitness.

FITNESS AND DEVELOPMENT OF RESISTANCE MUTATIONS

Fitness and development of protease resistance mutations

Primary resistance mutations to protease inhibitors usually involve amino acid substitutions at positions located at the substrate/inhibitor binding site (reviewed in [18]). Examples include D30N, G48V, I50V, V82A or I84V. Often, these amino acid changes have a deleterious effect on the replication kinetics of HIV-1 [22-24]. The

effects caused by drug resistance mutations can be mitigated by other amino acid replacements. For example, multidrug-resistant virus arising during prolonged therapy with indinavir contained the substitutions M46I, L63P, V82T and I84V in the protease-coding region [25, 26]. Crystallographic studies of the mutant enzyme revealed that the substitutions at codons 82 and 84 were critical for the acquisition of resistance, while the amino acid changes at codons 46 and 63, which are away from the inhibitor binding site, appear as compensatory mutations [27, 28]. Overall, resistance mutations to protease inhibitors results in the fitness loss of the new resistance variants, although compensatory mutations have been described to restore the replication capacity of highly resistance virus.

Fitness and development of reverse-transcriptase resistance mutations

It is widely accepted that, in general terms, mutations conferring resistance to reverse-transcriptase inhibitors do not reduce fitness to the same extent as those conferring resistance to protease inhibitors (reviewed in [17]). The K103N is probably the predominant mutation observed in subjects receiving NNRTIs. It emerges easily, partly because it results from a single nucleotide substitution. Available data indicate that K103N had a little or moderate effect on HIV-1 replication capacity [29, 30], despite its significant influence on resistance. In vivo dynamics data indicate that HIV-1 variants carrying K103N can persist over long periods following the discontinuation of NNRTI. This emphasizes that the K103N mutation has little impact on viral fitness in vivo [31]. In contrast, other mutations confer reduced replication capacity. Therefore, the nevirapine resistance mutation V106A has

been consistently reported to severely affect viral fitness, while substitution V106M/I did so only moderately [32, 33]. However, recovery of viral fitness by these viruses was attributed to the presence of substitution L74V, which probably increases protein stability [34]. In general, at all clinically relevant levels of drug exposure, NNRTI-resistant HIV exhibits higher replication capacity than the reference virus [35].

In addition, a number of mutations associated with resistance to NRTIs have been shown to decrease viral fitness. The most extensively studied are M184V and M184I in the reverse-transcriptase. These mutations develop quickly after initiation of therapy with lamivudine (which has a low-genetic barrier). The low replication efficiency of M184V and M184I containing isolates has been attributed to their diminishing effect on reverse-transcriptase processivity [36]. However, the reduction in fitness could be smaller, depending on the viral genetic background [26]. Mutations in genomic regions outside the reverse-transcriptase may compensate for reductions in replication capacity conferred by resistance mutations [37].

Fitness and development of integrase resistance mutations

At present, the most studied integrase inhibitor has been raltegravir. HIV resistance to raltegravir is a consequence of mutations located close to the integrase active site. Resistance to raltegravir has been associated with three genetic pathways defined by the mutations Y143R/C, Q148H/R/K and N155H [38, 39]. The N155H pathway has been described as being initiated earlier, followed by the emergence and further dominance of viral genomes carrying mutations of the Q148R/H/K or of the Y143R/C pathways. Moreover, raltegravir

resistance mutations are suspected of reducing replication capacity relative to the wild type, suggesting an association between drug resistance mutations, fitness cost and decreased enzymatic efficiency of integrase[40]. It has been shown that the appearance of additional mutations leads to greater phenotypic resistance. The most studied compensatory mutations has been the G140S substitution, which is found in the integrase coding region harboring Q148H, has a compensatory effect on replication capacity and also decreases susceptibility to raltegravir [40]. One unique property of HIV resistance to raltegravir is that these different resistance pathways are mutually exclusive and appear to evolve separately in distinct viral genomes [39].

DYNAMICS OF PERSISTENT VIREMIA

Despite the success of HAART in controlling HIV infection and reducing HIV-associated mortality, current drug regimens are unable to completely eradicate HIV infection. Current HAART is able to suppress viremia to below the limit of detection of available clinical assays [41]. However, residual low level viremia has been detected with ultrasensitive assays which are able to measure down to 1 copy HIV-1 RNA/ml plasma [42, 43]. Two main hypotheses have been postulated in order to explain the HIV persistence: one view is that viral persistence is mediated by a long-lived population of latently infected CD4+ T lymphocytes which are able to release viral particles, and an alternate view is that antiviral therapy is not completely suppressive, with low-level HIV replication persisting in T cells in the blood or in the lymphatic tissue compartment. Therefore, whether

residual viremia reflects ongoing viral replication at low levels or the production of virus from stable and/or latent reservoirs without additional cycles of replication remains unclear.

Mechanisms of viral latency

Many hypotheses have been proposed to explain the molecular mechanism of HIV latency (reviewed in [44, 45]). Two mainly different forms of latency have been observed in resting CD4+ T-lymphocytes: *pre-integration* and *post-integration* latency.

Pre-integration latency is a consequence of HIV fusion with non-dividing resting lymphocytes, therefore the viral life cycle is blocked before the integration of the HIV DNA. This blockage leads to an accumulation of both linear and circular forms of unintegrated viral dsDNA within the resting CD4+ T cells. It has been showed that the half-life of the unintegrated viral DNA is very short (approximately 1 day) [46], thus this pre-integration latency does not appear to contribute to the long-term viral persistence observed after prolonged periods of HAART. However, the block in the integration of the unintegrated HIV DNA integration can be revoked if activation of infected cells takes place with viral integration and productive infection as a possible consequence [47, 48].

Post-integration latency results when infected active CD4+ T-lymphocytes revert to a resting memory state once infection has taken place. Resting CD4+ T cell stability and their long half-lives represent major obstacles to HIV-1 eradication [49, 50]. This latency has been documented in different groups of subjects as being more relevant for HIV-1 persistance over time [49-51]. However, in subjects under a suppressive HAART regimen, resting CD4+ T cells are unable to

produce detectable levels of residual viremia in the absence of activating stimuli [52] and are not recognized by the immune system or antiretroviral compounds. Many different mechanisms have been proposed to establish and maintain this post-integration latency in HIV-1 infection. Some of the more important ones are thought to be viral integration sites, chromatin environment, lack of key transcription factors, impaired viral activator Tat and RNA interference (reviewed in [45]).

Ongoing viral replication

Several authors have provided evidence for ongoing viral replication, reporting the evolution of the HIV-1 *in-vivo* despite suppressive HAART [53-55]. Conversely, longitudinal studies of subjects highly suppressed by HAART have shown a lack of evolutionary change [56]. In addition, evidence for decay of the latent reservoir in subjects under suppressive HAART have been reported by cell culture and mathematical models, providing evidence of insignificant influence of ongoing replication [57-59]. However, some authors have provided evidence that residual viremia could come from both latently infected cells and continuous ongoing viral replication [60-62]. Overall, interpretations of the results have been controversial. Importantly, this question has immediate clinical implications, because if residual viremia reflects ongoing viral replication, then the intensification of the current treatment will be useful for preventing viral evolution and the subsequent treatment failure. In terms of HIV-1 eradication, the effectiveness of intensification strategies could impact on the duration of the HIV-1 eradication effort, meaning that new strategies will be needed. However, little is known about whether such

intensification can perturb the viral reservoirs that persist despite HAART [63, 64].

THERAPEUTIC STRATEGIES FOR VIRAL ERADICATION

Treatment intensification

The aim of the HAART intensification is to archive a complete suppression of residual viremia. The inhibitors of the CCR5 cellular co-receptor and HIV-1 integrase have been newly incorporated to the HAART, thus the availability of new antiretroviral classes provides an opportunity to intensify antiretroviral suppression. At the moment, treatment intensification studies do not reduce persistent low level viremia [63, 65]. However, the effect of treatment intensification on different cellular and molecular markers still needs to be elucidated.

Immunosuppressants

The addition of immunosuppressants to HAART combinations has been proposed to decrease the activation of CD4+ T cells and reduce their susceptibility to viral infection and replication [44]. Corticosteroids, mycophenolic acid, cyclosporine A, hydroxyurea and thalidomide have been assayed in several clinical trials. These compounds directly decrease the activity of transcription factors such as NF-κB or NFAT, protect the cell from apoptosis or reduce the production of pro-inflammatory cytokines such as tumour necrosis factor, thereby reducing immune stimuli, HIV-1 replication and cell destruction. Some benefit from their application has been observed in small trials, but drug withdrawal causes viral load to return to basal

levels. However, their efficacy in certain situations needs to be elucidated in controlled clinical trials.

Reactivation of the latent reservoir

The combination of HAART with new therapeutic agents that can reactivate latent reservoirs would lead to HIV-1 eradication through the induction of the expression of the integrated HIV genome, making it vulnerable to immune-mediated killing and antiretroviral therapy (reviewed in [44, 45]). A number of methods have been proposed for the reactivation of latently infected cells. Interestingly, IL-7 can reactivate HIV-1 in latently infected cells in vitro through the induction of the Janus kinase–signal transducer and activator of transcription (JAK–STAT) signalling pathway, making this cytokine an attractive candidate for future studies. In addition, Prostratin has been shown to increase HIV-1 transcription in latently infected T cells and down regulates HIV-1 receptors, which has the additional advantage of decreasing the risk of re-infection, making it a future candidate for clinical development. Finally, histone deacetylase (HDAC) inhibitors, which promote latency by regulating genome structure and transcriptional activity, have proved effective in-vitro.

HYPOTHESIS AND OBJECTIVES

Understanding the differential prevalence rate of mutations within the integrase coding region of HAART-experienced and integrase inhibitor–naïve subjects may help identify whether clinically relevant viral mutations or natural polymorphisms occur, and whether the sequence diversity of the integrase gene has important implications on the clinical response to integrase inhibitors. In addition, no studies have evaluated the potential phenotypic contributions of mutations outside the integrase coding region in subjects whose raltegravir-containing regimens fail. Therefore, integrase natural polymorphism and resistance mutations could play a major role on phenotypic changes, such as replication capacity and drug susceptibility in subjects taking a raltegravir-containing regimen and in subjects on raltegravir-sparing regimens. Therefore, we pursued the following objectives:

Objective 1: To explore intrasubject longitudinal evolution of the HIV-1 integrase-coding region over a median of 10 years of heavy antiretroviral therapy in integrase inhibitor-naïve subjects. Also to explore changes in phenotypic susceptibility to raltegravir and replication capacity in those samples that accumulated the highest number of amino acid substitutions during the study period. This objective will be addressed in Chapter 3, entitled "Raltegravir susceptibility and the fitness progression of HIV-1 integrase in subjects on long-term integrase-sparing antiretroviral therapy".

Objective 2: To compare the relative epistatic contributions of integrase, protease-reverse-transcriptase and the rest of the HIV-1 genome on viral fitness and susceptibility to raltegravir in subjects with and without raltegravir-resistance associated mutations and whose raltegravir-containing regimen has failed. This objective will be addressed in Chapter 4, entitled "The HIV-1 integrase genotype strongly predicts raltegravir susceptibility but not viral fitness of primary viral isolates".

Whether residual viremia reflects ongoing viral replication at low levels or the production of virus from stable reservoirs remains unclear. Importantly, this question has immediate clinical implications, because if residual viremia reflects ongoing viral replication, then the intensification of the current treatment will be useful for preventing viral evolution and subsequent treatment failure. New classes of antiretroviral agents against viral integrase and coreceptors increase the treatment options for HIV-1 infected individuals and provide new tools for assessing the viral reservoirs that persist in HAART-suppressed subjects. Raltegravir provided better viral suppression than optimized background therapy alone for at least 96 weeks. Accordingly, the intensification of HAART with raltegravir could have beneficial effects on the speed of elimination of the HIV-1 reservoir. This could offer further insight into the mechanisms of viral latency and hidden or cryptic viral replication. We thus pursued the following objective:

Objective 3: To asses if raltegravir intensification of the HAART regimen is can impact the levels of HIV-1 DNA and immune markers in subjects with undetectable viremia for at least one year. This objective will be addressed in Chapter 5, entitled "HIV-1 replication and immune dynamics are affected by raltegravir intensification of HAART-suppressed subjects".

Chapter 2

Materials and Methods

STUDY SUBJECTS

Subjects included in "Raltegravir susceptibility and the fitness progression of HIV-1 integrase in subjects on long-term integrase-sparing antiretroviral therapy" study

We selected 45 highly antiretroviral-experienced HIV-1–infected subjects with longitudinal samples separated by a median of 10 years (range, 4-13) and for whom the first sample was taken either before they started antiretroviral treatment or early after they started monotherapy/bitherapy. Demographic, antiretroviral treatment, CD4 cell counts, and viral load data were available. A median of 3 plasma samples (range, 2-4) per subject were taken between 1993 and 2007. All sequences were subtyped using the REGA HIV-1 automated subtyping tool [66]. In order to asses intra-subject phenotypic changes, we chose the 5 subjects whose HIV-1 integrase sequences accumulated between 3 and 14 amino acid substitution differences during the study period. All participants provided informed consent approved by the Germans Trias i Pujol Hospital Review Board.

Subjects included in "The HIV-1 integrase genotype strongly predicts Raltegravir susceptibility but not viral fitness of primary virus isolates" study

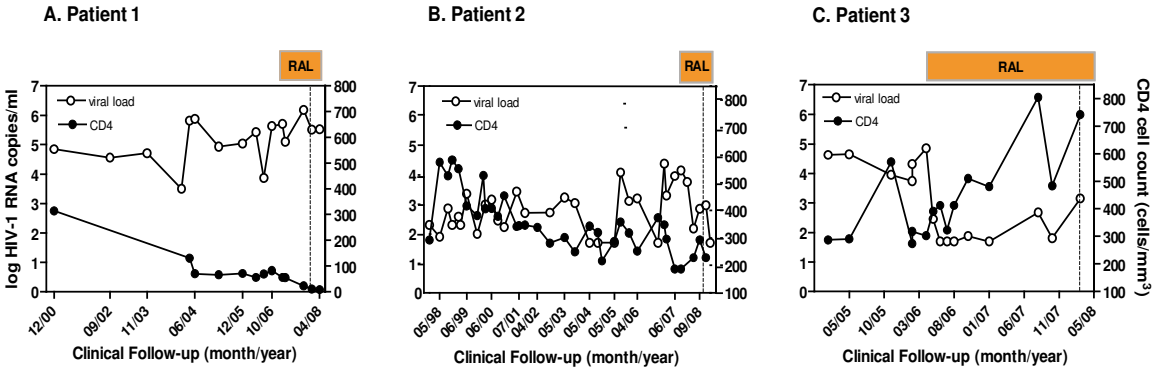
To evaluate the relative contribution of protease-reverse-transcriptase, integrase and the rest of the HIV-1 genome to viral fitness and susceptibility to raltegravir, HIV-1 integrase resistance genotyping was performed in plasma samples from all the subjects failing a raltegravir-containing regimen at initiation of the study (6 out of 90 subjects). Virological failure was defined by 2 consecutive plasma

viral load measures above the limit of detection of current assays (50 copies HIV-1 RNA/mL plasma). Two subjects with raltegravir-associated mutations were directly included in the study. The remaining four subjects did not present raltegravir resistance mutations, and one was selected as a representative case of this group. Selected subject 1 harbored the most frequent raltegravir resistance pathway in the integrase coding region: G140S+Q148H+S230N [67]. Subject 2 harbored the Y143R+G163R raltegravir resistance mutations and subject 3 lacked any evidence of genotypic resistance in integrase, although treatment with raltegravir had failed. Plasma levels of raltegravir were determined to ensure that all three subjects had taken raltegravir during the study period (Delphic Laboratories). Data on treatment with raltegravir, $CD4^+$ T cell count and plasma viral load were available for all subjects (**Figure 6**). All participants provided informed consent approved by the Germans Trias i Pujol Hospital Review Board.

Subjects included in "HIV-1 replication and immune dynamics are affected by raltegravir intensification of HAART-suppressed subjects" study

We enrolled 69 HIV-seropositive subjects on suppressive HAART for at least one year in this open-label and not placebo-controlled study. HAART regimens included two nucleoside reverse-transcriptase inhibitors and either a protease inhibitor (lopinavir or atazanavir) or a non-nucleoside reverse-transcriptase inhibitor (efavirenz or nevirapine). We randomly assigned 45 subjects to a treatment group intensifying HAART with raltegravir (Merck Sharp & Dohme) for 48 weeks and 24 subjects to a control arm.

Figure 6. Immunological and virological outcome of antiretroviral therapy



Immunological and virological outcome of antiretroviral therapy. Orange boxes represent the period of treatment with raltegravir. Dotted lines represent the time point of virological failure at which plasma HIV-1 RNA was amplified and cloned within recombinant vectors.

One individual in the control group was lost to follow-up before study initiation. A second individual also in this group, with two positive viral load measurements, was excluded for further analysis. We stratified raltegravir-treated subjects in a post hoc analysis into 2-LTR+ and 2-LTR− subgroups according to the detection of episomal cDNA. All participants provided informed consent approved by the Germans Trias i Pujol, the Clinic de Barcelona and the Santa Creu i Sant Pau Hospital Review Board.

POPULATION-BASED SEQUENCING OF THE HIV-1

Viral RNA was extracted from plasma samples using the QIAamp Viral RNA kit (Qiagen) and the full-length coding region of interest was amplified. For integrase sequencing, the RT-PCR (SuperScript One-Step reverse-transcriptase-PCR kit, Invitrogen) was performed using primers EinteF and EinteR (nucleotides 2997-3019 and 5554-5574 of

the HIV_{HXB2} numbering system respectively). A nested PCR (Platinum® Taq DNA Polymerase High Fidelity, Invitrogen) was then carried out with primers NinteF and NinteR (nucleotides 3111-3134 and 5251-5270, respectively). Population-based sequencing was carried out with primers NinteF (nucleotides 4540-4560), NinteR (4142-4165), and 1seqF, 2seqF, 3seqF (4744-4764). For sequencing of the full-length HIV-1 *polymerase* coding region the RT-PCR was performed using the primers 1417U23 and 5464L27 (nucleotides 1417-1440 and 5437-5464). Three different nested PCRs were subsequently carried out to amplify integrase, protease-reverse-transcriptase and *polymerase* respectively. The nested and the sequencing PCRs for the integrase coding region were carried out with the same primers described above. The nested PCR to amplify the protease and reverse-transcriptase coding region was carried out with the primers 1811U25 and 4335L25 (nucleotides 1811-1836 and 4310-4335). Population-based sequencing was carried out with primers 5'prot2, 3'prot2, 2772U22 and 3300U35 (nucleotides 2136-2163, 2621-2650, 2772-2794, 3300-3335 respectively). The nested PCR to amplify the *polymerase* coding region was carried out with the primers 2084U26 and 5456L26 (nucleotides 2084-2110 and 5430-5465). Population-based sequencing was carried out with primers 5'prot2, 3'prot2, 2772U22, 3300U35, 1seqF, 5116L26 and 4150U20 (nucleotides 2136-2163, 2621-2650, 2772-2794, 3300-3335, 4540-4560, 5090-5116 and 4150-4170 respectively). Population-based sequencing of integrase, protease and reverse-transcriptase was performed using Big-Dye Terminator Cycle Sequencing and the ABI 3100 sequence analyzer (Applied Biosystems). All sequences were assembled, aligned and

edited using the Sequencher (GeneCodes, v.4.6) and the Bioedit software.

CALCULATION OF THE EVOLUTION RATE

Codon-aligned sequences were submitted to the SNAP server for dN/dS analysis [68, 69]. The evolution rate was calculated as follows:

$$\frac{\sum_{0}^{n}\left(\frac{S_t - S0/m}{t_t - t_0}\right)}{n} x100$$

being S_t - S_0 the number of amino acid substitutions between the last and first sequence, m the number of amino acid positions, t_t - t_0 the time (in years) between the last and first sequence, and n the number of subjects analyzed.

CONSTRUCTION OF CLONING VECTORS

Construction of HIV-1 integrase-defective hemiplasmid vector (pJM30Δintegrase)

We constructed an HIV-1 integrase-defective hemiplasmid vector (pJM30**Δ**integrase) (**Figure 7**) to create labelled recombinant HIV-1 integrase viruses. The p83-2 plasmid (containing the 5'-half-HIV-1 genome plasmid) [70] was used to remove the restriction site *KpnI* at position 3831 by site-directed mutagenesis. After verification of the sequence integrity of the modified genomic region, we subcloned the HIV-1 fragment *AgeI* (3486) - *KpnI* (4158) into the original p83-2 plasmid to eliminate secondary mutations within the mutagenized plasmid. The integrase-coding region between the restriction site *KpnI*

at position 4158 and the restriction site *NdeI* at position 5123 was then replaced by a polylinker.

Construction of HIV-1 integrase-defective plasmid vector (pNL4-3Δintegrase)

We constructed a cloning vector consisting of HIV-1 integrase–defective plasmid (pNL4-3**Δ**integrase) (**Figure 8**). Briefly, The pJM30Δintegrase (containing the 5'-half-HIV-1 genome plasmid without integrase) was used to obtain the fragment from AgeI (3486) to EcoRI (4808). We then subcloned the pJM30Δintegrase fragment AgeI (3486) to EcoRI (4808) into the original pNL4-3.

Figure 7. Integrase-defective hemiplasmid vector (pJM30**Δ**integrase)

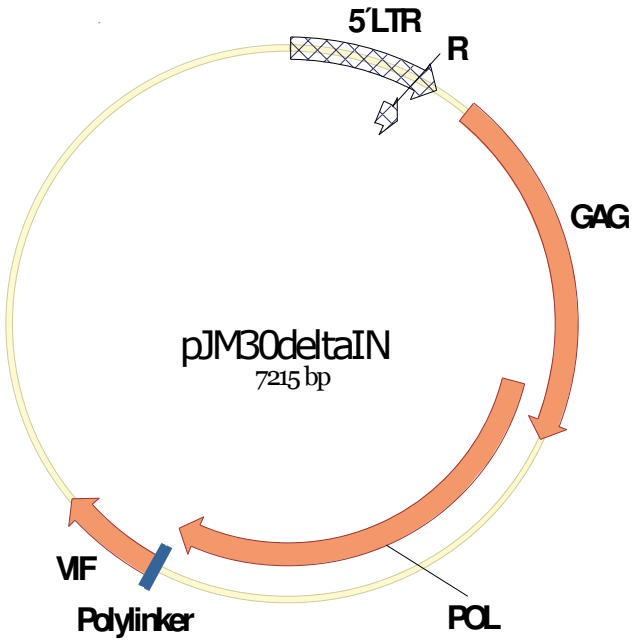
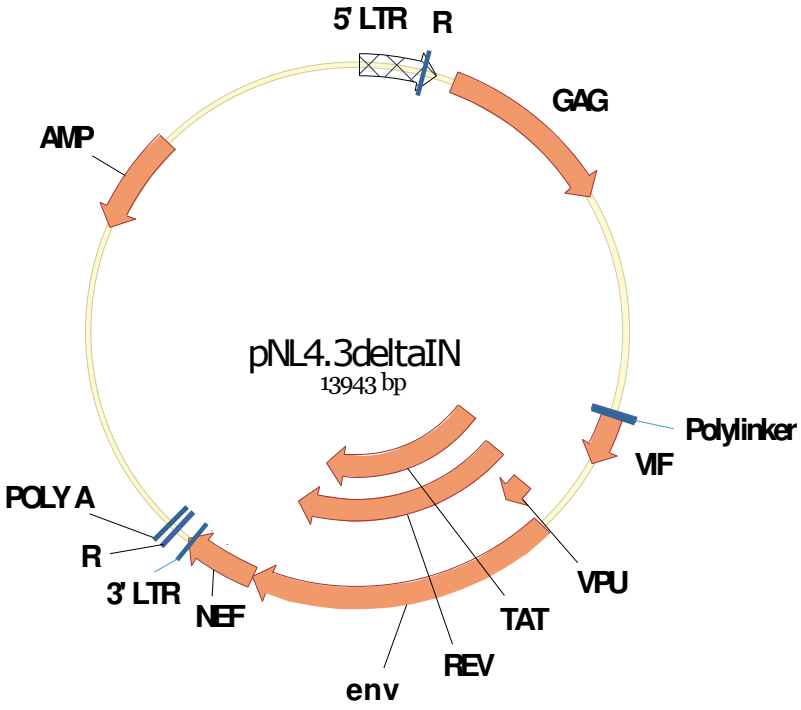


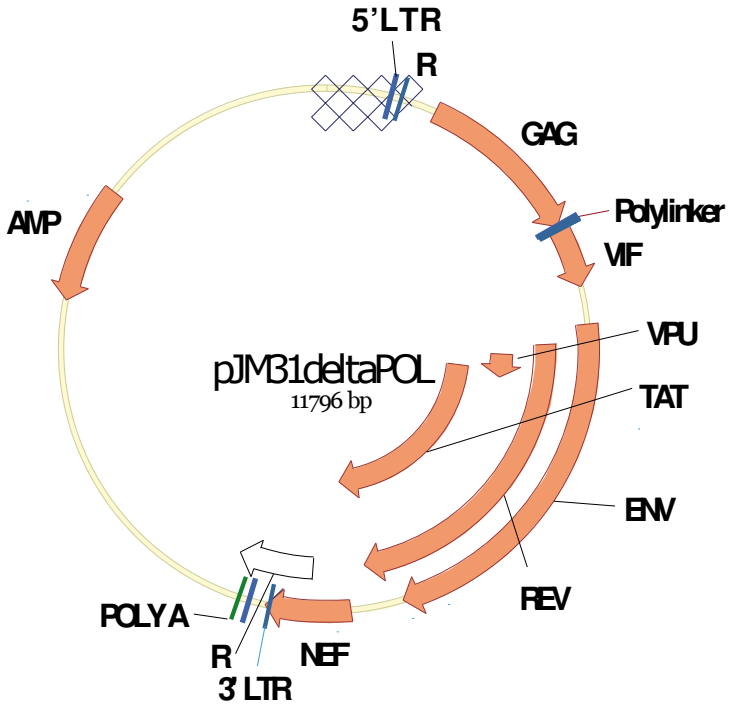
Figure 8. Integrase-defective plasmid vector (pNL4.3**Δ**integrase)



Construction of HIV-1 polymerase-defective plasmid vector (pJM31ΔPolymerase)

We constructed an HIV-1 *polymerase*-defective plasmid (pJM31ΔPolymerase) (**Figure 9**). This plasmid was derived from pJM30ΔPolymerase, which in turn was created by deleting the protease and reverse-transcriptase coding regions using a reverse amplification of pJM30Δintegrase. To obtain pJM31ΔPolymerase, pJM30ΔPolymerase was cut with ApaI (2011) and EcoRI (2665), and subcloned into pJM31Δprotease-reverse-transcriptase [71].

Figure 9. Polymerase-defective plasmid vector (pJM31ΔPolymerase)



GENERATION OF RECOMBINANT VIRUSES

Generation of labelled integrase-recombinant viruses

To evaluate raltegravir susceptibility and the fitness progression of HIV-1 integrase in subjects on long-term integrase-sparing antiretroviral therapy, we constructed integrase-recombinant viruses from 5 selected subjects whose HIV-1 integrase sequences accumulated between 3 and 14 amino acid substitution differences during the study period. Two integrase-recombinant viruses were constructed for each selected subject. These contained integrase from the first sample, obtained either before antiretroviral treatment was started or early after monotherapy/bitherapy (indicated as "first") or

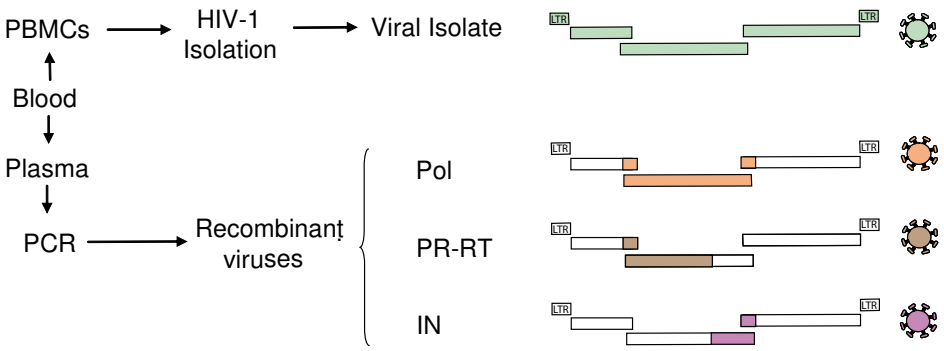
integrase from the last sample, obtained after as many as 13 years of multiple antiretroviral regimens (indicated as “last”). PCR-amplified fragments of the HIV-1 integrase generated using plasma HIV-1 RNA from infected subjects were digested with *KpnI* and *NdeI*. The resulting 863-bp fragment was then ligated into a *KpnI/NdeI* pre-digested pJM30Δintegrase. After transformation of *E. coli*-competent cells, individual recombinant clones were obtained and their integrase genotypes were verified by DNA sequencing. For each subject and sample, a representative clone of the previously obtained HIV-1 integrase population-based sequence was chosen to generate the integrase-recombinant virus. Infectious HIV-1 molecular clones were generated by electroporation of MT-4 cells and co-transfecting the proviral 5’-half-genome plasmid (pJM30Δintegrase reconstructed with subject-derived HIV-1 integrase) and the 3’-half-genome plasmid (p83-10), previously cut with *EcoRI*. Two different p83.10 plasmids (p83.10-GFP, carrying the reporter gene of the green fluorescence protein, and p83.10-RFP, carrying the reporter gene of the DsRed fluorescence protein) [72] were used to identify viruses from the first and last samples, during competition assays. Titration of recombinant viruses was performed using TZM-bl cells, which contain a luciferase gene under control of the HIV-1 promoter [73].

Generation of recombinant viruses

In order to compare the relative contribution of integrase and protease-reverse-transcriptase to viral fitness and drug susceptibility, we constructed three different recombinant viruses for each subject (**Figure 10**). PCR-amplified fragments containing the HIV-1 integrase generated from plasma HIV-1 RNA from infected subjects were

cotransfected with the linearized pNL4-3**Δ**integrase to generate integrase-recombinant viruses in MT4 cells [71]. PCR-amplified fragments of the HIV-1 *polymerase* generated from plasma HIV-1 RNA from infected subjects were cotransfected with the linearized pJM31ΔPolymerase to generate *polymerase*-recombinant viruses in MT4 cells. Finally, we created protease-reverse-transcriptase recombinant viruses using the vector pJM31Δprotease-reverse-transcriptase as described elsewhere [71]. All recombinant viruses were sequenced to ensure that amino acid changes were preserved after generation of recombinant forms. Recombinant viruses were titrated using TZM-bl cells, which contain a luciferase gene under the control of the HIV-1 promoter [73].

Figure 10. Cloning strategy to generate recombinant virus



Cloning strategy to generate recombinant virus. HIV-1 was isolated from the subjects' peripheral blood mononuclear cells (PBMC). PCR-amplified fragments of the HIV-1 integrase, protease-reverse-transcriptase and polymerase generated from plasma HIV-1 RNA of infected subjects were cotransfected with the linearized deleted vectors pNL4.3Δintegrase, pJM31Δprotease-reverse-transcriptase and pJM31ΔPolymerase to generate recombinant viruses in MT4 cells. All recombinant viruses were sequenced to ensure that amino acid changes were preserved after generation of recombinant constructs.

Generation of integrase-recombinant viruses as controls

Two additional recombinant viruses were constructed as a control. One was an HIV-1 integrase-recombinant virus from a subject whose raltegravir-containing therapy had failed and who harboured integrase resistance mutations G140S and Q148H. The second was constructed using site-directed mutagenesis by introducing substitution T66I within the integrase of the plasmid p83.2. T66I integrase mutation confers resistance to the integrase inhibitors from the diketo acid drug family [74]. The mutagenesis procedure was performed with the kit GeneTailor Site-Directed Mutagenesis System (Invitrogen), following the manufacturer's instructions. Oligos sequences are; MutaIN4403F: 5´-AGG AAT ATG GCA GCT AGA TTG TAT ACA TTT AGA AG-3´ and MutaIN4393R: 5´TAC AAT CTA GCT GCC ATA TTC CTG GGC TAC AGT-3´. A fragment corresponding from KpnI to NdeI restriction sites were re-cloned within p83.2, previously cut by the mentioned enzymes, to avoid putative modifications introduced by the Taq High Fidelity Polymerase (Invitrogen) during the PCR procedures. The final construct was corroborated by sequencing.

RECOVERY OF PRIMARY VIRUS ISOLATES

In order to compare the relative contribution of the HIV-1 full length genome to viral fitness and drug susceptibility, HIV-1 was isolated from the subjects' peripheral blood mononuclear cells (PBMC) according to a standard protocol [75]. Briefly, PBMCs from two healthy donors were mixed and cultured at 10^6 cells/mL in R20 medium (RPMI 1640 medium supplemented with 20% fetal calf serum [FCS]), and stimulated with 10 U/mL of interleukin 2 (IL-2) and 3 μg/mL of

phytohemaglutinin (PHA). After 3 days of culture, PBMCs were added at a 1:1 ratio and the coculture was maintained up to 21 days in R20 medium with 20 U/mL of IL-2, by performing one fresh-medium feeding and one fresh-cell feeding once a week. Virus production was monitored using an HIV-1 p24 antigen enzyme-linked immunosorbent assay (Perkin Elmer) in the supernatant of the cell cultures, and supernatants were collected and filtered using a 0.45-µm filter when a concentration of 50 ng/mL of p24 was reached. After recovery of the primary virus isolates, the *polymerase* reading frame was sequenced to ensure that all the genotypes were identical in virus recovered from PBMCs, recombinant viruses, and plasma population-based genotyping.

RALTEGRAVIR SUSCEPTIBILITY ASSAY

200 tissue culture infective doses ($TCID_{50}$) of each viral stock were used to infect 10,000 TZM-bl cells (multiplicity of infection [MOI] = 0.02) in quadruplicate in 96-well optical-bottom plates containing 32.5 µg/mL diethylaminoethyl-dextran and 4-fold serial dilutions ranging from 0.06 nM to 4 mM of raltegravir. Replication was monitored by measuring luciferase expression in the infected target cells 48 hours after infection using the Bright-Glo Luciferase Assay (Promega, Barcelona, Spain). Percent inhibition was determined by calculating the difference in relative light units (RLU) between test wells and negative control wells, dividing this result by the difference in RLUs between positive control wells (without drug) and negative control wells, subtracting from 1, and multiplying by 100. Inhibition curves were defined using a sigmoid dose-response curve with a variable slope. The fold-change in drug susceptibility was determined

by dividing the IC_{50} for every sample virus by the IC_{50} for the raltegravir-sensitive virus.

GROWTH RATE ASSAY

PBMCs from healthy donors were cultured in RPMI 1640 medium supplemented with 20% fetal calf serum (FCS) and stimulated with 10 U/mL of interleukin 2 (IL-2) and 3 µg/mL of phytohemaglutinin (PHA) for 3 days before infection. A total of 5 x 10^6 PHA-stimulated PBMCs were infected with 10,000 $TCDI_{50}$ (MOI = 0.002) of each viral stock in 1 mL of final volume for 2 hours at 37ºC. The cells were then washed twice with phosphate-buffered saline and cultured at 10^6 cells/mL in T25 flasks for 10 days. Each day, 250 µL of supernatant were collected and 300 µL of fresh RPMI supplemented with 20% FCS and IL-2 were added. Growth kinetics of the viruses were monitored using a HIV-1 p24 antigen enzyme-linked immunosorbent assay (Perkin Elmer) in the supernatant of samples collected from cell cultures. Growth kinetics based on p24 antigen production were analyzed by fitting the log-transformed p24 data into a linear model. The growth rate experiments were performed in duplicate.

COMPETITION ASSAY

To evaluate the fitness progression of HIV-1 integrase in subjects on long-term integrase-sparing antiretroviral therapy, the fitness of recombinant viruses, which carry the integrase from viral isolates from the “first” and “last” samples of the same subject, was determined by viral competition after dual infection of MT-4 cell cultures. The “first”

and “last” HIV-1 integrase sequences were linked to GFP or RFP reporter genes respectively. Competition between the viruses generated from each subject was induced with unequal proportions (20:80, 50:50, and 80:20 of the “first” and “last” viruses according to titration in TZM-bl cells). A total of 1 x 10^6 MT4 cells were cultured in RPMI 1640 supplemented with 10% FCS, 50 mg/mL of penicillin, and 50 mg/mL of streptomycin, before being infected with a total of 20,000 $TCID_{50}$ (MOI = 0.02) in a final volume of 1 mL for 2 h at 37ºC. Cells were washed twice with phosphate buffered saline and cultured at 2 x 10^5 cells/mL in 6-well tissue culture plates. At days 7, 12, 17, and 21, 1 x 10^6 fresh cells were reinfected by inoculating 100 µl of supernatant from the culture containing the competed viruses. Cells obtained at each passage were centrifuged, genomic DNA was extracted, and a multiplex PCR reaction was performed with fluorescently labelled primers specific for the GFP or RFP reporter genes. A total of 0.5 µL of product amplification was mixed with 20 µL of formamide and 1.5 µL of the molecular size marker, labelled with carboxytetramethyl rhodamine (Applied Biosystems). The mixture was denatured at 95ºC for 5 minutes and separated by capillary electrophoresis before being analyzed in a 310 Genetic Analyzer (Applied Biosystems). Samples were analyzed using GeneScan software (Applied Biosystems). Two peaks (97 bp and 102 bp of the GFP and RFP genes, respectively) were clearly differentiated in the chromatogram. The area of each peak, divided by the total area of both peaks, gave the relative proportion of virus variants. To ensure that both genes were amplified with the same efficiency by the multiplex PCR reaction, a standard curve was constructed using a gradient of different concentrations of p83.10-GFP and p83.10-RFP plasmids. The correlation coefficient between

percentages of plasmid input and the percentage of gene measured by GeneScan analysis was calculated. The concentrations tested ranged from 0% to 100% of the sample input. The correlation coefficients were 0.999 for both genes.

NUCLEIC ACID PURIFICATION FOR QUANTIFICATION OF HIV-1 DNA FORMS

HIV-1 DNA purification was isolated as previously described [76] with some modifications. Briefly, a median of 60 x 10^6 PBMCs were purified at week 0, 2, 4, 12 and 24 by Ficoll centrifugation and cell pellets were resuspended in 350µl of P1 buffer (Qiaprep miniprep kit, Qiagen). Then, 250µl of cell resuspensions were used for extrachromosomal HIV-1 DNA extraction (Qiaprep miniprep kit, Qiagen) using the modification for the isolation of low-copy-number plasmids, and total cellular DNA was purified from 100µl of cell resuspensions with a standard protocol (QIAamp DNA Blood Kit, Qiagen).

QUANTIFICATION OF HIV-1 2–LONG TERMINAL REPEAT CIRCLES

Extrachromosomal DNA was isolated at weeks 0, 2, 4, 12 and 24 post-intensification. A single-step, real-time PCR was used to quantify 2-LTR circles in a 50µl PCR reaction mix containing 25µl of TaqMan® Universal PCR Master Mix (Applied Biosystems), 20µl of extrachromosomal HIV-1 DNA and primers and probe that span the 2-LTR circle junction (**Figrue 11a**). The forward primer was 5’ CTA ACT AGG GAA CCC ACT GCT 3’ and the reverse primer 5´GTA GTT CTG CCA

ATC AGG GAA G 3´. The fluorescence taqman probe was 5´AGC CTC AAT AAA GCT TGC CTT GAG TGC 3´. Amplification reactions were performed with an Applied Biosystems 7000 Real-time PCR system. The thermocycling conditions were: 95ºC 10 min, 50 cycles at 95ºC 15 sec and 60ºC 1 min, and a final cycle of 72ºC 5 min. Copy number estimates of 2-LTR circles were performed in duplicate and determined by extrapolation from a standard curve generated with a plasmid that harbors the sequence of the 2-LTR junction and CCR5 gene. 2-LTR copy number was calculated relative to CCR5 gene copy number as determined from the chromosomal fraction. Samples corresponding to the same subject were evaluated in the same real time plate to minimize inter-experiment variation.

QUANTIFICATION OF TOTAL HIV-1 DNA

Chromosomal DNA was extracted at weeks 0, 2, 4, 12 and 24 after initiation of intensification. A single-step real-time PCR was used to quantify total HIV-1 DNA in a 50µl PCR reaction mix containing 25µl of TaqMan® Universal PCR Master Mix (Applied Biosystems), 20µl of chromosomal HIV-1 DNA and primers and probe that anneal in the 5' and 3' end of the R and U5 region of the LTR respectively, as has previously been described [77] (**Figure 11b**) using a forward primer; 5' GG CTA ACT AGG GAA CCC ACT G 3' and a reverse primer; 5´GCT AGA GAT TTT CCA CAC TGA CTA A 3´. The fluorescence taqman probe was 5´GGA TCT CTA GTT ACC AGA GTC A 3´. Amplification reactions were performed with an Applied Biosystems 7000 Real-time PCR system. The thermocycling conditions were: 95ºC 10 min, 50 cycles at 95ºC 15 sec and 60ºC 1 min and a final cycle of 72ºC 5 min.

Amplification and calculation of copy number was determined as for 2-LTR DNA.

QUANTIFICATION OF INTEGRATED HIV-1 DNA

Chromosomal DNA was extracted at weeks 0, 2, 4, 12 and 24 after intensification. A two-step real-time PCR was used to quantitate integrated HIV-1 DNA using Alu-LTR primers as has previously been described [77] (**Figure 11c**). Briefly, in the first round of PCR only 12 cycles of amplification was performed, integrated HIV-1 sequences were amplified with two outward-facing Alu primers together with an HIV-1 LTR specific primer extended with a lambda phage-specific heel sequence in a 20µl reaction mixture. In a second round of PCR, a lambda-specific primer and an LTR primer was used on 1/10th of the first-round PCR product in a 50µl mixture PCR reaction. The forward primer was 5' ATG CCA CGT AAG CGA AAC T 3' and the reverse primer 5´GCT AGA GAT TTT CCA CAC TGA CTA A 3´. The fluorescence taqman probe was 5´GGA TCT CTA GTT ACC AGA GTC A 3´. Amplification and calculation of copy number was determined as for 2-LTR DNA.

SINGLE-COPY ASSAY FOR HIV-1 RNA CONCENTRATIONS IN PLASMA

We used a real-time RT–initiated PCR assay that quantifies HIV-1 RNA concentrations down to one copy per ml of plasma on all samples at baseline and at week 12 as previously described [43]. Briefly, HIV-1 RNA extraction was performed from 7 ml of subject plasmas to which 200ul of RCAS viral stock was added at the start of the assay in order to monitor the RNA extraction efficiency of the RT-PCR reaction. An HIV-1

sample result is rejected if the RCAS internal control values measures <15,000 copies/reaction, approximately 50% of RCAS input. A two-step, RT-PCR was used to measure both HIV-1 and RCAS with standard curves, primers and probes previously described [43]. Amplification reactions were performed with an Applied Biosystems 7000 Real-time PCR system.

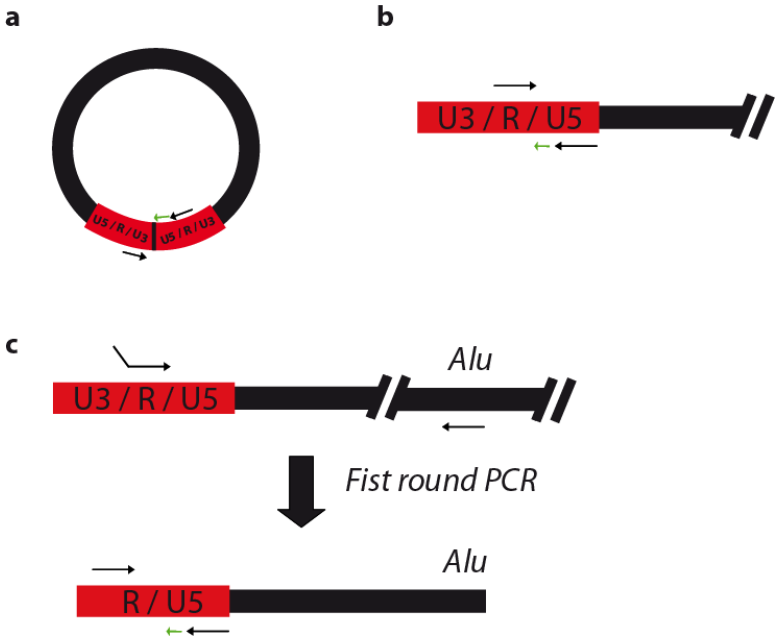
ANALYSIS OF LYMPHOCYTE SUBSETS AND IMMUNE ACTIVATION

Different combinations of antibodies were used to characterize different lymphocyte subsets: combination #1 evaluated naïve/memory subsets and contained CD45RA-FITC, CD31-PE, CD38-PerCP, CD3-APC-Cy7, CD4-APC and CD8-PE-Cy7; combination #2 evaluated the activation of CD8 T cells and contained HLA-DR-FITC, PD-1-PE, CD38-PerCP, CD45RO-APC, CD3-APC-Cy7 and CD8-PE-Cy7. All antibodies were obtained from Becton Dickinson. 20µl of fresh whole blood was incubated with antibodies for 15 min at room temperature. Acquisition of flow cytometry data was performed on an LSRII flow cytometer (Becton Dickinson). At least 30,000 lymphocytes were collected for each sample. Analyses were performed with FlowJo software (Tree Star Inc.).

SOLUBLE CD14 (SCD14)

The concentration of sCD14 was measured by ELISA (Diaclone) using 1/50 plasma dilutions at baseline and week 24. All cases were run in duplicate.

Figure 11. Primers and probes used to amplify different HIV-1 DNA forms.



(a) Primers and probe that span the 2-LTR circle junction for amplification of episomal HIV-1 DNA. **(b)** Primers and probe that anneal in the 5' and 3' end of the R and U5 region of the LTR respectively for amplification of total HIV-1 DNA. **(c)** Primers and probe used for amplification of integrated HIV-1 DNA. In the first round of PCR two outward-facing Alu primers together with an HIV-1 LTR specific primer extended with a lambda phage-specific heel. In a second round of PCR, a lambda-specific primer and an LTR primer was used.

STATISTICAL ANALYSIS

To compare means between first and last samples of integrase recombinant virus in drug susceptibility and growth rate assays we applied the Student's *T* test. To compare means between different recombinant viral constructs in drug susceptibility and growth rate assays, we applied the *t* test. A *P* value <.05 indicated statistical significance.

We used the Mann-Whitney U test to compare medians between the control and intensification arm (or between the 2-LTR+ and 2-LTR− subgroup) and the signed rank test (paired test) to compare longitudinal changes. To adjust for the presence of 2-LTR circles at baseline, we also used analysis of variance models for comparisons between groups and linear mixed models to study longitudinal changes within groups. Because the ultrasensitive viral load and 2-LTR circles presented either multiple detection limits or a high percentage of values below them, we used regression order statistics methods to calculate summary statistics, and we performed comparisons between groups by the Peto-Prentice test. We analyzed differences in proportions between groups through the Pearson's chi square, considering the continuity correction or the Fisher's exact test, as appropriate. We analyzed longitudinal differences in 2-LTR circles and ultrasensitive viral load within groups by the Peto-Prentice-Wilcoxon test for paired data. We computed correlation between total or integrated HIV-1 DNA and immune activation parameters with the Spearman's rho coefficient. We performed statistical analyses with SAS 9.1 and the R package. We generated graphics were generated with GraphPad Prism 5.0 software.

Chapter 3. Results I

Raltegravir Susceptibility and the Fitness Progression of HIV-1 Integrase in Subjects on Long-Term Integrase-Sparing Antiretroviral Therapy

INTRODUCTION

Most drugs approved during the last twenty years to inhibit the replication of HIV-1 have targeted viral protease and reverse-transcriptase enzymes. Inhibitors of the HIV-1 integrase enzyme have only recently been used in HAART, with promising results. The integrase of HIV-1 is responsible for the integration of proviral DNA into the host genome [78, 79], an indispensable step in productive HIV-1 infection of human cells. The protein HIV-1 integrase is 288 amino acids long and folds into three functional domains: (1) an N-terminal zinc finger (amino acids 1 to 50) that contains an HHCC motif [80, 81]; (2) the central catalytic core domain (amino acids 51 to 212) that contains the DDE triad (Asp64, Asp116, and Glu152) and requires a divalent metal cation for activity; and (3) a C-terminal DNA-binding domain (amino acids 213 to 288). To be fully functional in vivo, integrase also requires cellular co-factors, of which the most widely described is the human lens epithelium-derived growth factor (LEDGF/p75), which is required during chromosomal tethering [82]. The integrase is encoded at the 3'end of the *polymerase* gene, in-frame with the protease and reverse-transcriptase coding regions. Thus, the three major drug-targeted HIV-1 enzymes (protease, reverse-transcriptase, and integrase) mature from the same large Gag-Pol precursor polyprotein ($Pr160^{Gag\text{-}Pol}$). Moreover, the preintegration complex includes reverse-transcriptase, integrase, and viral DNA [83], with some evidence suggesting that integrase interacts with reverse-transcriptase [84-87].

Many compounds with very diverse chemical structures have been reported to inhibit integrase activity [79], but development of integrase inhibitors has been plagued by difficulties in identifying

compounds that target integrase [88]. The only integrase inhibitor currently approved for clinical use is raltegravir, however, S/GSK 1249572 and elvitegravir, are investigational new integrase inhibitors which are in clinical development. Raltegravir blocks the integration of HIV-1 cDNA through the inhibition of DNA strand transfer. A 96-week analysis showed that raltegravir plus optimized background therapy had a greater antiretroviral efficacy in subjects with triple-class resistance whose antiretroviral therapy failed than in those taking placebo plus optimized background therapy [11, 89, 90]. The potential toxicity of raltegravir is thought to be low, as there is no human homologue of HIV-1 integrase [91]. However, as with all other antiretrovirals, resistance has been shown to occur with integrase inhibitors [92-94], Thus, HIV-1 variants with raltegravir or elvitegravir resistance mutations have been induced in vitro using increasing concentrations of these drugs [95, 96], and have also been identified in vivo in most virologic failures with raltegravir- or elvitegravir-containing regimens [95],[97]. Moreover, raltegravir resistance mutations are suspected of reducing replication capacity relative to the wild-type, suggesting an association between drug resistance mutations, fitness cost, and decreased enzymatic efficiency of integrase [95].

Understanding of the differential prevalence rate of mutations in viral strains from HAART-experienced integrase inhibitor–naïve subjects may help to identify whether clinically relevant viral mutations or natural polymorphisms occur and whether the sequence diversity of the integrase gene has important implications in the clinical response to integrase inhibitors. In this study, we explore intrasubject longitudinal evolution of the HIV-1 clade B integrase-

coding region over a median of 10 years of heavy antiretroviral therapy in integrase inhibitor–naïve subjects. We also explore changes in phenotypic susceptibility to raltegravir and replication capacity in those samples that accumulated the highest number of amino acid substitutions during the study period.

RESULTS

Subjects characteristics

The samples included in this study were taken from 45 highly antiretroviral-experienced HIV-1–infected subjects (89% men, median age 45 years) with longitudinal samples separated by a median of 10 years (range, 4-13) and for whom the first sample was taken either before they started antiretroviral treatment or early after they started monotherapy/bitherapy. These subjects were highly antiretroviral-experienced and had been exposed to a median of 13 antiretroviral drugs with a median of 6 NRTIs, 2 NNRTIs, and 5 protease inhibitors. Moreover, 40% (18/45) had also been treated with the fusion inhibitor enfuvirtide, while none had received integrase inhibitors. At the first time-point, 4 out of 45 subjects (9%) were naïve, 22% were taking monotherapy [AZT 18% (8/45), ddI 4% (2/45)], and 42% were on dual therapy [AZT/ddC 29% (13/45), AZT/ddI 9% (4/45), AZT/3TC 2% (1/45), 3TC/d4T 2% (1/45)]. The median plasma viral load in these initial samples was 4.84 log10 copies/mL and the median CD4 count was 250 cells/mm3. After a median of 10 years of multiple antiretroviral combination regimens, the mean change in HIV-1 RNA was -0.65 log10 copies/mL and the CD4 cell count increased by only 15 cells/mm3, reflecting multidrug treatment failure. Subtype

determination showed that all subjects were infected with HIV-1 subtype B variants.

The integrase gene remains highly conserved after long-term antiretroviral therapy

The integrase gene (867 bases) was amplified and sequenced using 123 plasma samples from 45 subjects. Analysis of all the amino acid sequences revealed that the integrase of HIV-1 subtype B is a conserved protein with 42% (121/288) of the amino acid residues achieving ≥ 97% conservation and 25% (73/288) completely conserved. The HHCC zinc-binding site, the DDE catalytic triad, and the LEDGF/p75 integrase binding domain between residues 128-132 and 161-173 were all ≥ 97% conserved. No deletions were seen in the integrase sequence, while only two of the 123 integrase sequences (both from the same subject) had an insertion at position 193. Of the 64 amino acid substitutions previously associated with resistance to integrase inhibitors [98], we found 15 in our samples (S17T, V72I, L74I, L101I, K111T, S119R, S119G, T124N, V151I, M154I, K156N, V165I, V201I, I203M, and D232N) (**Table 6**). However, no mutations associated with resistance to raltegravir (N155H, Q148H/R/K, G140S/A, Y143R) [9] or elvitegravir (T66I, E92Q, Q146P, S147G) [96] were detected. The evolution rate of HIV-1 integrase during the study period was 0.06% amino acid substitutions per year. When we analysed codon-specific ratios of nonsynonymous-to-synonymous substitution rates (dN/dS), we observed that the integrase was constrained, with a low dN/dS value (0.15), suggesting that there is no positive selection acting on the integrase during antiretroviral

treatment targeting protease and reverse-transcriptase. Nevertheless, comparison of the first and last sequences of each subject revealed a slight increase in the prevalence of some of the changes associated with resistance to integrase inhibitors (**Table 6**).

Longitudinal samples taken from subjects treated with integrase inhibitor-sparing regimens show no evidence of phenotypic resistance to raltegravir

The IC_{50} of raltegravir was measured in five selected subjects whose HIV-1 integrase sequences accumulated between 3 and 14 amino acid substitutions (**Tables 7, 8 and 9**). None of the integrase-recombinant viruses derived from subjects A1 to A5, either before or after several years of antiretroviral therapy, showed a significant decrease in raltegravir susceptibility, with a fold change less than 3 as compared to HIV-1_{NL4-3} in all cases (**Figure 12**). In addition, the fold change on raltegravir susceptibility was not different between integrase-recombinant viruses derived from the first time-point samples (mean fold change = 1.88; 95% CI, 1.22 - 2.54) or after several years of antiretroviral therapy (mean fold change = 1.75; 95% CI, 1.55 - 1.94). Conversely, an integrase-recombinant virus derived from a subject failing a raltegravir-containing regimen (subject B) and harbouring resistance mutations G140S and Q148H (**Table 7, 8 and 9**) had a 23.2-fold increase (95% CI, 7.3 - 74.3) in the IC_{50} of raltegravir (**Figure 12**). However, one HIV-1_{NL4-3} sample (sample C) containing the T66I substitution, which confers resistance to integrase inhibitors from the diketo acid drug family [74], retained full susceptibility to raltegravir (fold change in IC_{50} = 1.1; 95% CI, 0.8 - 1.5) (**Figure 12**).

	N-Terminal	Catalytic Core Domain												
	17	72	74	101	111	112	119	124	151	154	156	165	201	203
HIV-1HXB2	S	V	L	L	K	T	S	T	V	M	K	V	V	I
Changes associated with resistance to INI	T	I	M/I/A	I	T	I	G/R	N	I	I	N	I	I	M
Frequency at the first time-point	S(78) N(9) C(7) T(2) S/T(2) S/N(2)	V(38) I(53) T(2) V/I(7)	L(98) I(2)	L(58) I(38) L/I(4)	K(85) T(7) Q(2) R(2) T/A(4)	T(91) A(7) V(2)	S(72) P(14) G(8) R(4) T(2)	T(67) A(24) N(9)	V(98) V/I(2)	M(96) I(2) L(2)	K(96) N(4)	V(91) I(7) V/I(2)	V(64) I(29) V/I(7)	I(89) M(9) I/M(2
Frequency at the last time-point	S(75) N(12) C(7) T(2) S/N(4)	V(25) *I(66) T(2) V/I(7)	L(98) I(2)	L(46) *I(52) L/I(2)	K(83) *T(13) Q(2) R(2)	T(89) V(7) *T/I(2) *V/I(2)	S(72) P(17) G(4) *R(5) T(2)	T(67) A(22) *N(11)	V(96) *I(2) V/I(2)	M(94) *I(4) L(2)	K(96) N(4)	V(89) *I(11)	V(62) *I(31) V/I(7)	I(87) *M(13

Table 6. Evolution in frequency of changes at codons previously associated with resistance to different integrase inhibitors

Table 7. Amino acid substitutions within the HIV-1 integrase (N-Terminal Domain)

	N-Terminal Domain														
Virus	3	6	7	11	13	14	17	20	25	28	31	32	39	45	50
HIV-$1_{NL4,3}$	D	D	K	E	E	K	S	R	D	L	V	V	S	L	M
LONG-TERM HAART-EXPERIENCED PATIENTS															
A1 first	-	T	D	D	-	-	-	-	-	-	I	-	-	-	-
A1 last	-	S	E	-	-	-	-	-	-	-	I	-	N	-	-
A2 first	-	-	R	D	D	-	-	-	-	M	-	I	R	-	-
A2 last	-	-	-	D	D	-	-	-	-	M	I	I	R	-	-
A3 first	-	-	-	D	-	-	C	-	-	-	-	-	R	-	I
A3 last	E	-	-	D	D	-	C	-	-	-	I	-	R	-	-
A4 first	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-
A4 last	-	-	-	-	-	-	-	K	-	-	I	-	-	-	-
A5 first	-	-	-	D	-	-	-	K	-	-	I	-	-	-	-
A5 last	-	-	-	D	-	-	-	-	E	-	I	-	-	-	-
RALTEGRAVIR-EXPERIENCED PATIENT															
B first	-	-	-	D	-	R	-	-	-	-	I	-	C	-	I
B last	-	-	-	D	-	R	-	-	-	-	I	-	C	-	I
SITE-DIRECTED MUTANT															
T66I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

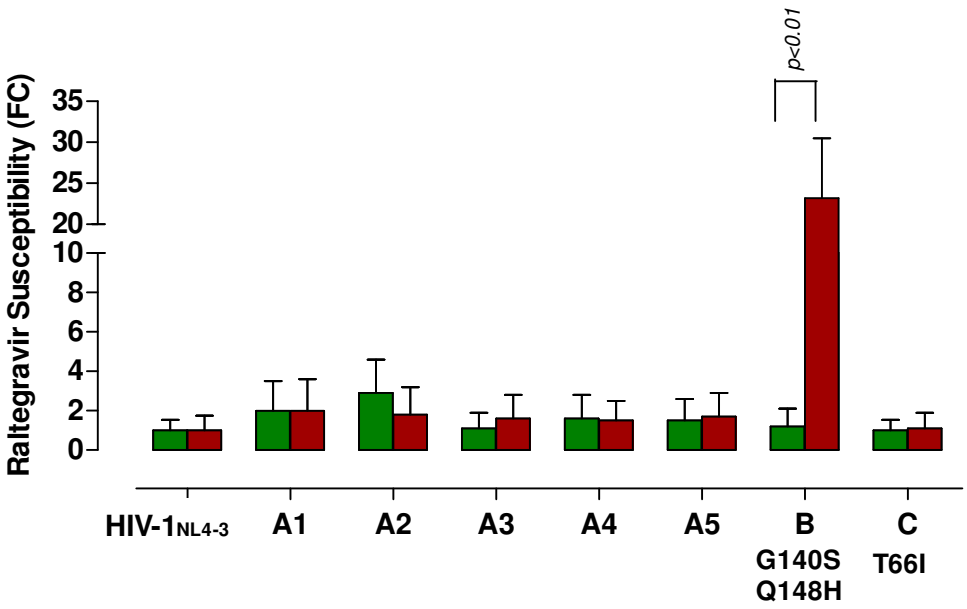
Virus	Catalytic Core Domain																											
	66	72	84	101	111	112	113	119	122	124	125	140	148	151	163	165	172	181	192	Inser	193	193	201	205	206	210	212
HIV-1$_{NL4,3}$	T	V	I	L	K	T	V	S	T	T	T	G	Q	I	G	V	L	F	G	~		G	V	A	T	T	E
LONG-TERM HAART-EXPERIENCED PATIENTS																											
A1 first	-	I	-	I	-	-	I	P	I	-	A	-	-	V	T	-	-	-	-	~		D	I	-	-	-	-
A1 last	-	I	M	I	-	-	I	P	-	-	A	-	-	V	-	I	-	-	V	G		N	I	-	S	-	-
A2 first	-	I	-	-	-	-	I	-	-	-	-	-	-	V	-	-	-	-	-	~		-	I	-	-	-	-
A2 last	-	I	-	-	-	-	I	-	-	-	-	-	-	V	-	-	-	-	-	~		-	I	-	-	-	-
A3 first	-	-	-	-	A	-	I	-	-	-	-	-	-	V	-	-	-	-	-	~		-	-	-	S	-	-
A3 last	-	I	-	I	T	-	I	-	-	-	-	-	-	V	-	-	-	-	-	~		-	-	-	S	-	-
A4 first	-	I	-	I	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	~		-	-	-	-	-	-
A4 last	-	I	-	I	Q	-	-	-	-	-	-	-	-	V	-	-	-	-	-	~		-	-	-	S	I	-
A5 first	-	I	-	I	T	-	I	-	-	A	-	-	-	V	-	-	-	-	-	~		-	I	-	-	-	-
A5 last	-	I	-	I	T	-	I	P	-	A	-	-	-	V	-	-	-	L	-	~		-	I	-	-	-	A
RALTEGRAVIR-EXPERIENCED PATIENT																											
B first	-	I	-	I	-	-	I	-	-	-	A	-	-	-	-	-	I	-	-	~		-	I	S	-	-	-
B last	-	I	-	I	-	I	I	-	-	-	A	**S**	**H**	V	-	-	I	-	-	~		-	I	S	-	-	-
SITE-DIRECTED MUTANT																											
T66I	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	~		-	-	-	-	-	-

Table 8. Amino acid substitutions within the HIV-1 integrase (Catalytic Core Domain)

Table 9. Amino acid substitutions within the HIV-1 integrase (C-Terminal Domain)

	C-Terminal Domain											
Virus	219	220	222	227	230	234	253	254	256	265	284	286
HIV-$1_{NL4,3}$	K	I	N	Y	S	V	D	N	D	A	R	D
LONG-TERM HAART-EXPERIENCED PATIENTS												
A1 first	-	-	-	-	-	L	-	-	-	-	-	N
A1 last	-	-	-	-	-	L	-	-	N	-	-	-
A2 first	-	L	-	C	-	L	-	-	-	-	G	-
A2 last	-	L	-	-	-	L	-	-	-	-	G	-
A3 first	-	-	-	-	-	L	-	-	E	V	-	-
A3 last	-	-	-	-	-	L	-	-	E	-	-	-
A4 first	-	V	-	-	-	L	-	-	E	-	-	-
A4 last	-	-	-	-	-	L	-	-	E	-	-	-
A5 first	N	-	K	-	-	L	-	-	-	-	-	-
A5 last	N	-	K	-	-	L	-	-	-	-	-	-
RALTEGRAVIR-EXPERIENCED PATIENT												
B first	-	-	-	-	N	L	E	D	E	-	-	-
B last	-	-	-	-	N	L	E	-	E	-	-	-
SITE-DIRECTED MUTANT												
T66I	-	-	-	-	-	-	-	-	-	-	-	-

Figure 12. Raltegravir susceptibility



The fold change in raltegravir susceptibility was determined by dividing the IC50 for each integrase-recombinant virus by the IC50 for its corresponding HIV-1NL4.3 (the green bar corresponds to HIV-1NL4.3-GFP, and the red bar to HIV-1NL4.3-RFP). Samples A1 to A5. Green bars correspond to integrase-recombinant viruses from the first samples (A1first to A5first cloned into p83-10-GFP) and red bars correspond to integrase-recombinant viruses from the last samples (A1last to A5last cloned into p83-10-RFP). Sample B corresponds to integrase recombinant viruses with substitutions G140S/Q148H from a subject on a raltegravir-containing regimen. The green and red bars correspond to an integrase-recombinant virus before treatment with raltegravir (Bfirst) or after treatment failure (Blast), respectively. Sample C corresponds to an HIV harbouring the substitution T66I. The green bar is HIV-1NL4.3-GFP, and the red bar is the single mutant T66I. Statistical analyses were performed using the unpaired t test.

Long-term pressure with protease and reverse-transcriptase inhibitors do not impair the fitness of HIV-1 integrase

To measure differences in viral fitness more accurately, dual infections were induced in cell culture. The proportions of integrase-recombinant viruses derived from "first" and "last" samples were estimated over time by length polymorphism detection method. Before applying this detection system to growth competition experiments, the method was set up using different mixtures of plasmids containing either the GFP or the RFP reporter genes. The method was sensitive to at least 10% of one of the viral strains (**Figure 13**).

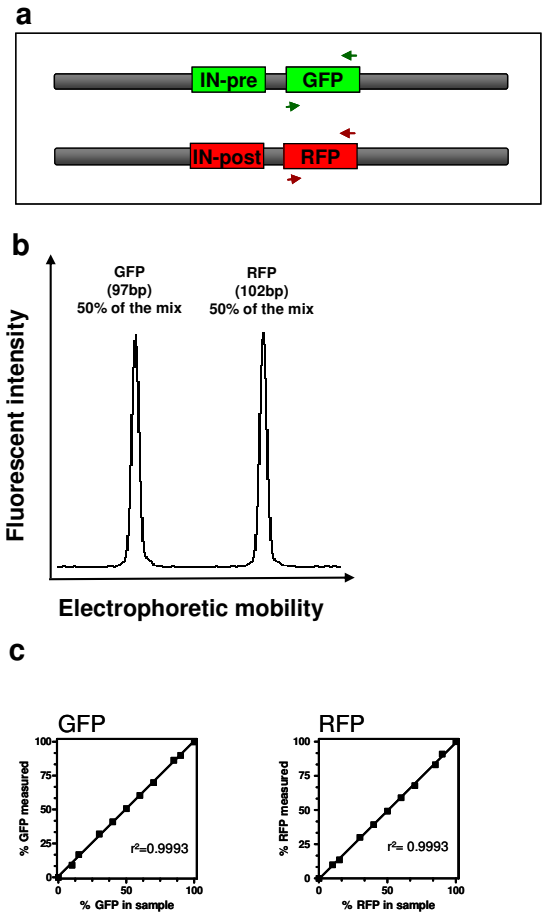
Moreover, two recombinant HIV-1_{NL4-3}, one containing the GFP reporter gene and the other containing the RFP reporter gene, were competed as controls. Results showed that no virus could outgrow the other, therefore excluding the possibility that fitness differences were affected by the reporter proteins (data not shown). Integrase-recombinant viruses from the last time-point samples (A1last, A2last, A4last, and A5last) outgrew their counterparts from the first time-point samples (A1first, A2first, A4first, and A5first) when assayed in a drug-free environment (**Figure 14**). Only integrase-recombinant viruses derived from subject A3 showed an identical replication capacity between the first- and last- samples, which is consistent with the replication kinetics described below. For each comparison, cultures were set up with three unequal proportions of the competing viruses, and all of them provided consistent results over 21 days of culture.

However, the integrase-recombinant virus derived from a subject whose raltegravir-containing regimen failed (subject B) and the HIV-

1_{NL4-3} sample containing the T66I substitution (sample C) were out-competed by their wild-type integrase-recombinant counterpart (**Figure 15a and 15b**), suggesting that selection of integrase inhibitor resistance mutations has a fitness cost for the virus in the absence of drug.

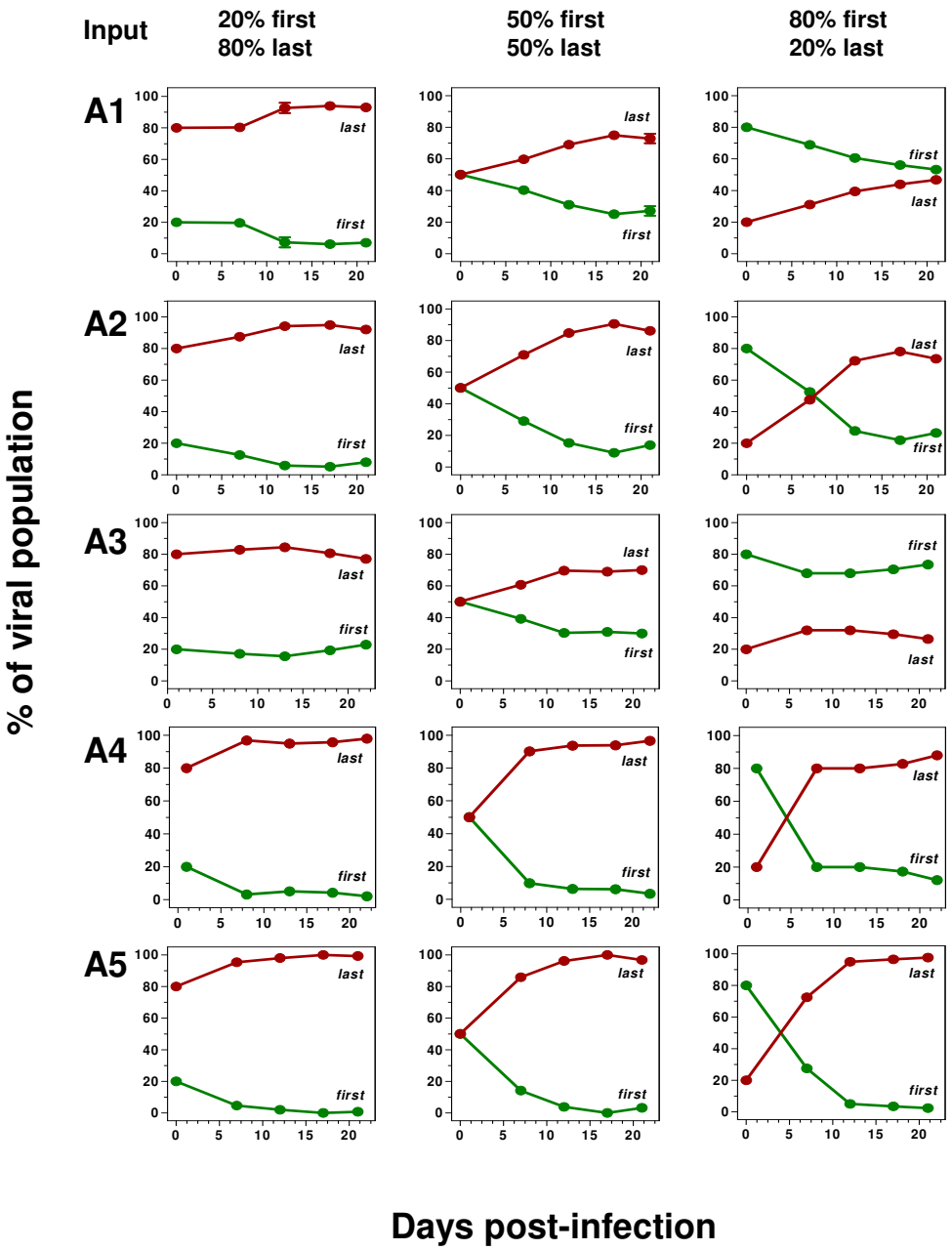
In addition, the replication capacity of these recombinant viruses in the absence of drug was also determined by the kinetics of $p24^{Gag}$ production in PBMCs. The slope of the increase in $p24^{Gag}$ for recombinant viruses was compared to the slope of the corresponding HIV-1_{NL4-3} containing either the GFP or the RFP reporter gene, and percent replication capacity was calculated (**Figure 16**). Of note, HIV-1_{NL4-3} containing either the GFP or RFP reporter gene had the same replication capacity. The growth rate of integrase-recombinant viruses derived from subject samples (A1 to A5) ranged from 5% to 117% as compared with the HIV-1_{NL4-3} replication capacity. All integrase-recombinant viruses derived from samples taken after several years of antiretroviral therapy (A1last to A5last) had higher replication capacities than their counterpart viruses derived from the first sample (A1first to A5first) (**Figure 16**). The growth kinetics of the integrase-recombinant virus derived from the subject failing a raltegravir-containing regimen (subject B) had less replication capacity as compared as its non-resistant counterpart recombinant virus, but the difference was not statistically significant (**Figure 16**). Similarly, the HIV-1 sample containing T66I substitution (sample C) had a 31% lower replication capacity than HIV-1_{NL4-3} (**Figure 16**).

Figure 13. Schematic representation of GeneScan detection for competition experiments



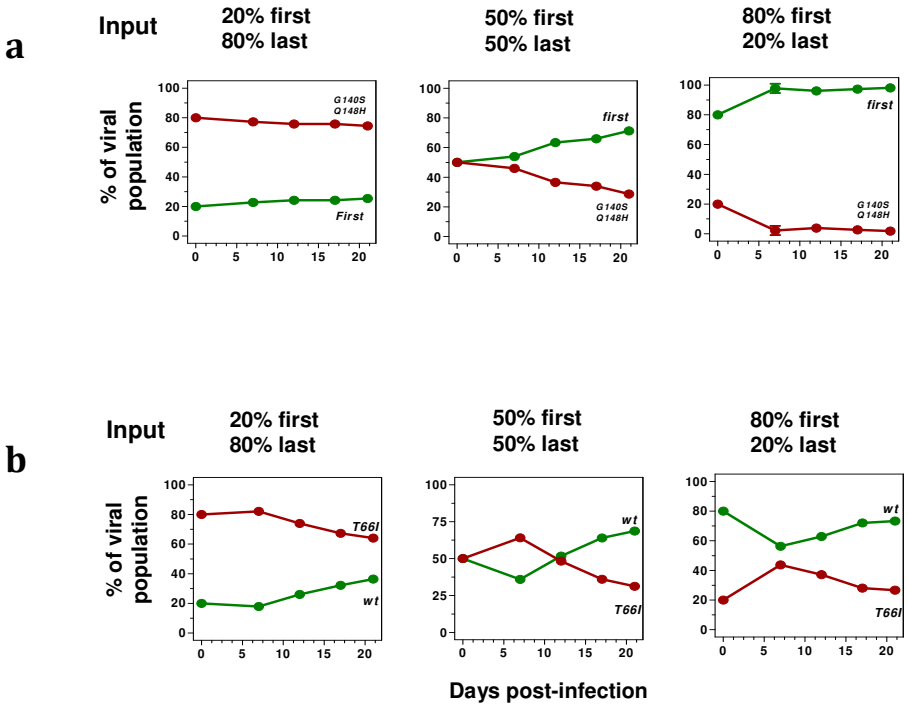
(a) Cells obtained at each re-infection were centrifuged, genomic DNA was extracted, and a multiplex PCR reaction was performed with fluorescently labelled primers specific for the GFP or RFP reporter genes. **(b)** Labeled PCR products were denatured, separated by capillary electrophoresis, and analyzed in a 310 Genetic Analyzer (Applied Biosystems), Samples were analyzed using GeneScan software. Two clearly differentiated peaks appeared in the chromatogram, corresponding to 97 bp and 102 bp of the GFP and RFP genes, respectively. **(c**) Sensitivity of the GeneScan technique. Different proportions of plasmids containing reporter genes GFP or RPF were mixed at different ratios, amplified by the PCR Multiplex system, and analyzed by GeneScan software. (GFP) Percent of GFP detection at the different ratios assessed. (RFP) Percent of RFP detection at the different ratios assessed.

Figure 14. Competitions assays in absence of drugs



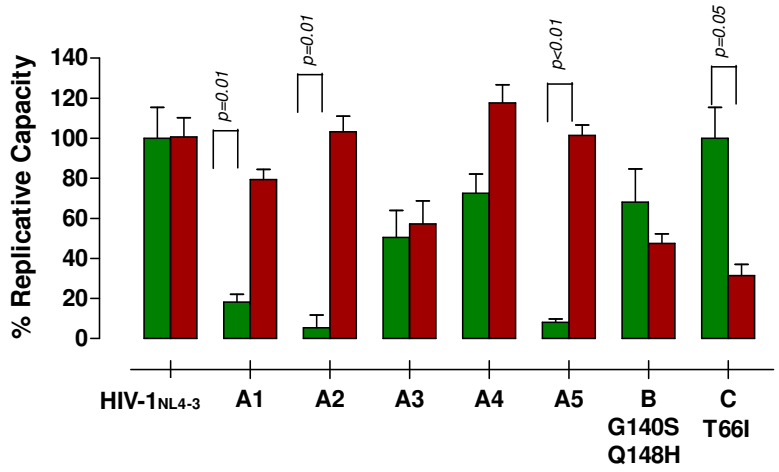
Intrasubject competition experiments of integrase-recombinant viruses from subjects A1 to A5; viruses labelled as A1first to A5first (recombinant viruses from the first time-point) are represented with green symbols and viruses labelled as A1last to A5last (recombinant viruses from the last time-point) are represented with red symbols. Error bars represent the standard errors of two independent measurements.

Figure 15. Competition assays in absence of drugs



(a) Competition of integrase recombinant viruses from a subject failing raltegravir-containing therapy; Afirst (before raltegravir treatment) is represented with green symbols and Alast (raltegravir-resistant virus with substitutions G140S/Q148H) is represented with red symbols. **(b)** Wild type virus (HIV-1NL4.3) is represented with green symbols and T66I (HIV harbouring the substitution T66I) is represented with red symbols. Error bars represent the standard errors of two independent measuremen

Figure 16. Replication capacity assays in absence of drugs



Growth kinetics based on $p24^{Gag}$ production was analyzed by fitting a linear model to the log-transformed p24 data by maximum likelihood methods. The slope of recombinant virus $p24^{Gag}$ antigen production after PBMC infection was compared to the slope of a wild type virus, and the percent of replication capacity was calculated and represented. A1 to A5, integrase-recombinant viruses from the first time-points (green bar) and last time-points (red bar). Sample B, recombinant virus before raltegravir (green bar) and recombinant virus harbouring G148H and G140S (red bar). Sample C, HIV-1NL4.3-GFP (green bar) and recombinant virus with the T66I substitution (red bar). Control viruses, HIV-1NL4.3-GFP (green bar) and HIV-1NL4.3-RFP (red bar).

Chapter 4. Results II

The HIV-1 Integrase Genotype Strongly Predicts Raltegravir Susceptibility but Not Viral Fitness of Primary Virus Isolates

INTRODUCTION

The HIV-1 genome encodes three enzymes for viral replication. These are protease, reverse-transcriptase and integrase. Currently, the most widely used drugs target viral protease and reverse-transcriptase. Inhibitors of HIV-1 integrase are a new addition to HAART [9, 10]. Raltegravir is a first-in-class integrase strand transfer inhibitor. It has been approved for the treatment of HIV-1 infection in treatment-experienced adult subjects with evidence of viral replication and HIV-1 strains that are resistant to multiple antiretroviral agents. However, in July of 2009, the FDA granted expanded approval for raltegravir for use in all patients. Viral resistance also emerges with integrase inhibitors [92-94]. Resistance to raltegravir has been associated with three genetic pathways defined by the mutations Y143R/C, Q148H/R/K and N155H [39], and the appearance of additional mutations leads to greater phenotypic resistance [95]. It has also been shown that, in some subjects, raltegravir-containing regimens fail to achieve durable viral suppression in the absence of genotypic resistance markers in integrase [99-101]. This absence of raltegravir resistance mutations has been associated with lower viral replication rates [101].

The HIV-1 envelope coding region linked to viral characteristics such as viral entry, coreceptor usage and viral transmission has been shown to play an important role in the ex vivo fitness of wild-type HIV-1 [102]. In addition, the emergence of drug-resistant viruses through the selection of drug resistance–associated mutations might also have detrimental effects on viral fitness [17]. A large body of evidence suggests that gene interactions might modulate viral replication and

drug susceptibility [103]. It has been shown that the preintegration complex includes reverse-transcriptase, integrase and viral DNA [83], and some authors suggest that integrase interacts with reverse-transcriptase [84-87, 104]. The integrase substitution V165I, previously associated with resistance to integrase inhibitors, has been shown to be positively associated with substitutions F227L and L210W in reverse-transcriptase [105]. In addition, the three major HIV-1 drug-targeted enzymes (protease, reverse-transcriptase and integrase) mature from the same large Gag-Pol polyprotein ($Pr160^{Gag-Pol}$), thus suggesting the potential for interaction between them. However, no studies evaluate the potential contribution of mutations outside integrase in subjects whose raltegravir-containing regimens fail.

This study aims to compare the relative contribution of integrase, protease-reverse-transcriptase and the rest of the HIV-1 genome on viral fitness and susceptibility to raltegravir in subjects with and without raltegravir-resistance associated mutations and whose raltegravir-containing regimen has failed.

RESULTS

Subjects characteristics

The samples included in this study were obtained from three of a total of six HIV-1–infected subjects whose raltegravir-containing regimen had failed. The remaining 3 subjects did not present any integrase resistance mutations. Subject 1 harboured G140S+Q148H+S230N (the most common genotypic resistance

pathway for raltegravir), subject 2 presented Y143R+G163R and subject 3, although presenting virological failure with a raltegravir-containing regimen, lacked all of the integrase resistance mutations described to date (**Table 10**).

Table 10. Resistance pattern of protease, reverse-transcriptase and integrase coding regions

Subject		Resistance mutations at RAL failure
1	PR	L10F, V32I, L33F, M46L, I54A, A71V, T74P, I84V, L89V, L90M
	RT	M41L, E44D, D67G, K70T, K101E, V118I, Y181C, G190A, L210W, T215Y, K219N
	IN	G140S, Q148H, S230N
2	PR	None
	RT	None
	IN	Y143R, G163R
3	PR	L10F/L, E35G/R, M46L, I54V, A71V, G73S, I84V, L90M
	RT	M41L, D67N, L74V, K101P, V179D, M184V, G190A, L210W, T215Y, K219N
	IN	None

Major and minor drug resistance mutations for all the genotypes: recombinant virus, whole virus recovered from PBMCs and plasma population based-genotyping within protease, reverse-transcriptase and integrase coding region of selected subjects according to Stanford Genotype Resistance Interpretation Algorithm (v 6.0.1)

All subjects were highly antiretroviral-experienced. The plasma viral load of selected subjects at failure was 6.2, 3.0 and 3.3 log10 HIV-1 RNA copies/mL, with a CD4+ lymphocyte count of 23, 288 and 742 cells/mm3 for subjects 1, 2 and 3, respectively (**Figure 6**). Raltegravir plasma levels, which are usually in the 0.01 to 4.71 μg/mL range, were 0.77, 4.36 and 0.11 μg/mL for subjects 1, 2 and 3, respectively [106]. At the time the sample was collected, subject 1 was taking raltegravir, abacavir, lamivudine, ritonavir, enfuvirtide, tenofovir, darunavir and zidovudine, and had ten resistance mutations associated with protease

inhibitors and nine mutations associated with reverse-transcriptase inhibitors (**Table 10**). Previous antiretroviral agents were lopinavir, tipranavir, didanosine, atazanavir, indinavir, efavirenz, stavudine, nelfinavir, nevirapine, saquinavir and zalcitabine. Subject 2 was taking raltegravir monotherapy due to voluntary discontinuation of protease and reverse-transcriptase inhibitors. At virological failure he had no primary resistance mutations related to protease or reverse-transcriptase inhibitors (**Table 10**). His previous antiretroviral agents were atazanavir, tenofovir, emtricitabine, ritonavir, saquinavir, nevirapine, lopinavir, didanosine, abacavir, stavudine and nelfinavir. Similarly, when the regimen failed, subject 3 was taking raltegravir, emtricitabine, maraviroc, ritonavir, tenofovir and tipranavir, and harbored 8 primary mutations associated with increased resistance to protease inhibitors and 11 associated with reverse-transcriptase inhibitors (**Table 10**) [107]. Previous antiretroviral agents for subject 3 were atazanavir and lopinavir.

Defects on raltegravir susceptibility are not modulated by viral genes other than the integrase

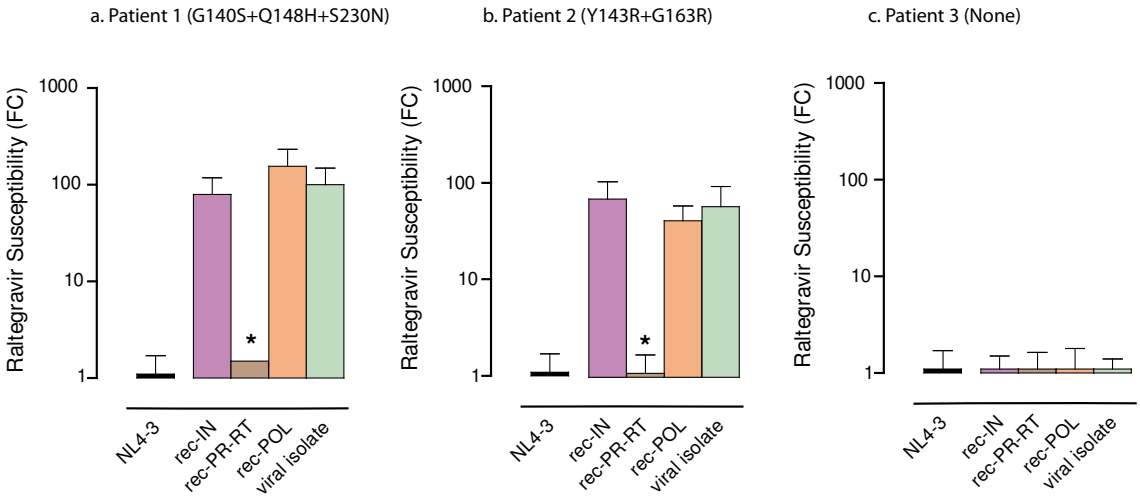
To evaluate the role of protease, reverse-transcriptase and integrase mutations in phenotypic susceptibility to the integrase inhibitor raltegravir in subjects whose regimen failed, we constructed integrase, protease-reverse-transcriptase and *polymerase*-recombinant viruses from plasma samples (**Figure 10**). The IC_{50} to raltegravir was measured in TZM-bl cell cultures. For subject 1 (G140S+Q148H+S230N), the integrase-recombinant viruses showed a marked decrease in susceptibility to raltegravir, with a fold-change of

79 (95% CI, 39–158). In addition, the *polymerase*-recombinant viruses had a fold-change of 154 (95% CI, 77–309). Nevertheless, the protease-reverse-transcriptase recombinant viruses did not show a significant decrease in susceptibility to raltegravir (**Figure 17a**). We observed that fold-change susceptibility to raltegravir in *polymerase*-recombinant viruses was higher than in integrase-recombinant viruses; however, this increase was not statistically significant and was not seen in the viral isolate. Similarly, the integrase-recombinant viruses in subject 2 (Y143R+G163R) showed a significant decrease in susceptibility to raltegravir, with a fold-change of 70 (95% CI, 34–141) compared to HIV-1_{NL4-3}. In addition, the fold-change of the *polymerase*-recombinant viruses was 41 (95% CI, 17–98), similar to that of the integrase-recombinant viruses. Nevertheless, the protease-reverse-transcriptase recombinant viruses did not show a marked decrease in susceptibility to raltegravir (**Figure 17b**). Conversely, the integrase-recombinant viruses derived from subject 3 (no evidence of resistance in the integrase) while his raltegravir-containing regimen was failing showed a fold-change of 0.7 (95% CI, 0.4–1.4) and retained full susceptibility to this drug. In addition, neither protease-reverse-transcriptase (IC50 = 1.1; 95% CI, 0.5–2.1) nor *polymerase*-recombinant viruses (IC50 = 2.1; 95% CI, 0.7–2.9) showed a decrease in susceptibility to raltegravir (**Figure 17c**).

In order to evaluate the putative role of HIV-1 genes other than integrase, protease and reverse-transcriptase in susceptibility to raltegravir, autologous replication-competent viruses were obtained from the PMBCs of each subject at virological failure. Raltegravir susceptibilities were assayed and the IC_{50} was measured in TZM-bl cell cultures. The results for subject 1 showed that the entire virus had a

fold-change increase in the IC_{50} of 99 (95% CI, 49–198), which was comparable to the values for integrase and *polymerase*-recombinant viruses from the same subject (**Figure 17**). Similarly, subject 2 showed a fold-change in IC_{50} of 58 (95% CI, 36–94), which was comparable to the values for integrase and *polymerase*-recombinant viruses from the same subject (**Figure 17b**). In contrast, subject 3 did not show any increase in resistance to raltegravir when the full-length genome was evaluated (IC_{50} = 0.7; 95% CI, 0.3–3.9) (**Figure 17c**).

Figure 17. Raltegravir Susceptibility



Fold-change in raltegravir susceptibility of the three selected subjects (**a, b** and **c**). TZM-bl cells were infected with recombinant viruses containing serial dilutions of raltegravir ranging from 0.01 nM to 5 μM of raltegravir. Replication was monitored by measuring luciferase expression in infected target cells 48 h after infection. Inhibition curves were defined using a sigmoid dose-response curve with a variable slope. The fold change in raltegravir susceptibility was determined by dividing the IC_{50} for each recombinant virus by the IC_{50} of HIV-1NL4.3. P values of <.05 indicated statistical significance and are marked by an asterisk.

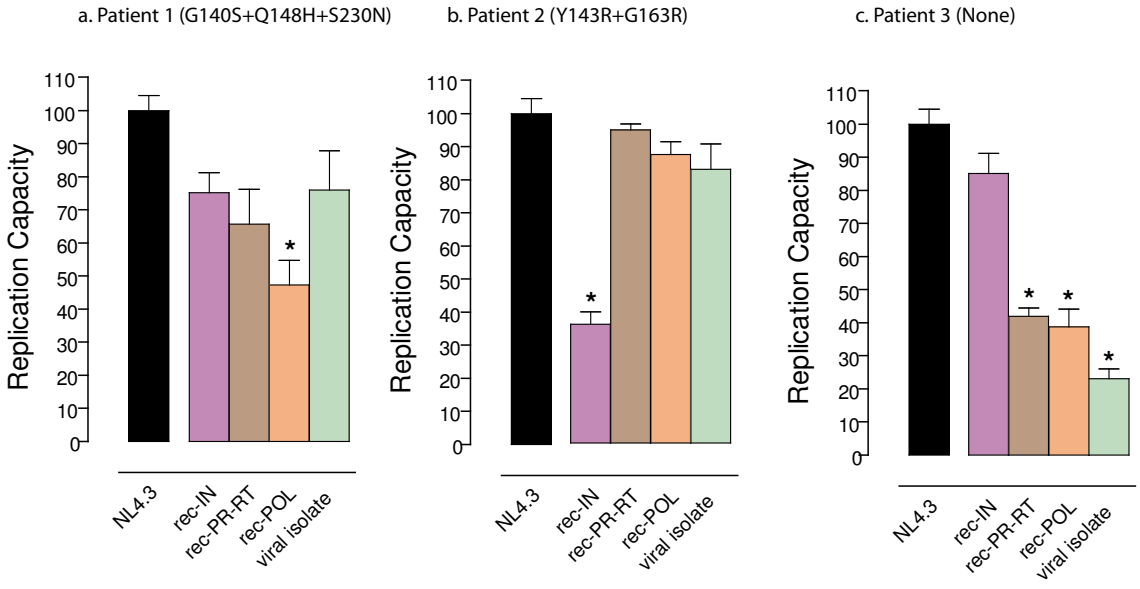
Defects on viral fitness are modulated by resistance mutations within protease, reverse-transcriptase and integrase, and it is compensated by regions outside polymerase

The replication capacity of recombinant viruses in the absence of drug was determined by the kinetics of $p24^{Gag}$ production in PBMCs. The slope of the increase in $p24^{Gag}$ for recombinant viruses was compared to that of HIV-1_{NL4-3}. The growth rate of the recombinant viruses derived from subject 1, who harbored resistance mutations within protease, reverse-transcriptase and integrase, showed a decrease in viral replication capacity dependent on resistance mutations cloned within the recombinant vectors: the replication capacity of *polymerase*-recombinant virus was 47.2% with respect to HIV-1_{NL4-3}. This was lower than that of protease-reverse-transcriptase recombinant virus (65.6%), and the replication capacity of protease-reverse-transcriptase was lower than that of integrase-recombinant virus (75.2%), thus showing a cumulative effect of resistance mutations in protease-reverse-transcriptase and integrase on viral fitness (**Figure 18a**). The same results were obtained from the recombinant viruses derived from subject 3, who harboured resistance mutations within protease and reverse-transcriptase but lacked integrase resistance mutations; therefore, the replication capacity of *polymerase*-recombinant virus was 38.7% with respect to HIV-1_{NL4-3}, that is, similar to the replication capacity of protease-reverse-transcriptase recombinant virus (41.8%) but lower than that of integrase-recombinant virus (85.2%) (**Figure 18c**). In addition, the results obtained from subject 2, who harboured resistance mutations within integrase but lacked primary protease and reverse-transcriptase resistance mutations, showed that integrase-

recombinant virus had the lowest replication capacity (35.8%). Interestingly, defects in the replication capacity of integrase-recombinant virus were compensated by polymorphisms within the protease and reverse-transcriptase coding regions, as was the case for *polymerase*-recombinant virus (87.1%) (**Figure 18b**).

The contribution of the whole HIV-1 genome to replication capacity was, however, divergent in the three subjects. Thus, viral isolates from subject 1 showed a greater *ex vivo* replication capacity (75.9%) than the recombinant virus containing *polymerase* (47.2%) from the same viral isolates, which showed a compensatory effect outside the *polymerase* coding region (**Figure 18a**). In contrast, the viral isolate from subject 2 showed a similar replication capacity to that of *polymerase*-recombinant virus, which in turn was similar to that of HIV-1_{NL4-3} (**Figure 18b**). However, viral isolates from subject 3 showed an even lower *ex vivo* replication capacity (22.9%) than their corresponding *polymerase*-recombinant viruses (38.7%) (**Figure 18c**). Of note, this subject was taking maraviroc, a CCR5 antagonist, at the time of virological failure, and mutations in the viral envelope could also have contributed to the lower replication capacity observed. Of note, all genotypes were identical in plasma HIV-1 RNA, recombinant virus, and viruses recovered from PBMCs.

Figure 18. Replication Capacity



Ex-vivo replication capacity of the three selected subjects (a, b and c). Growth kinetics based on $p24^{Gag}$ production was analyzed by fitting a linear model to the log-transformed p24 data using maximum likelihood methods. The slope of recombinant virus $p24^{Gag}$ antigen production after PBMC infection was compared to the slope of a wild type virus. P values of <.05 indicated statistical significance and are marked by an asterisk.

Chapter 5. Results III

HIV-1 Replication and Immune Dynamics are Affected by Raltegravir Intensification of HAART-Suppressed Subjects

INTRODUCTION

HAART is able to sustain suppression of plasma viremia below the limit of detection of standard assays [41]. However, viremia rapidly resumes if therapy is interrupted, suggesting that viral reservoirs persist in the face of HAART. HIV-1 persistence in HAART has been attributed to the presence of a long-lived reservoir of latently infected, memory $CD4^+$ T-cells. This model is supported by the presence of replication-competent virus in peripheral blood lymphocytes and by the lack of evolution in viral cDNA [56, 59, 108-110]. In fact, therapy intensification should have no impact on the reservoir that persists in HAART [111]. While it is generally believed that HAART stops active infection, increased immune activation persist in HAART-suppressed subjects [112]. Immune hyperactivation is a hallmark of HIV-1 infection [113], causing increased levels of proliferation and apoptosis. However, a causative link between active viral replication and immune activation has not been established. Additional studies [23, 53-55, 57, 61, 63, 76, 114, 115] suggest that low level, active or "cryptic" replication may persist in the face of suppressive HAART. In addition, residual low-level viremia has been detected with ultrasensitive assays measuring down to 1 copy of HIV-1 RNA per millilitre of plasma [42, 43]. Whether residual viremia reflects viral replication or the production of virus from stable reservoirs without additional cycles of replication is unknown. Importantly, this question has immediate clinical implication, because if residual viremia reflects ongoing viral replication, then the intensification of the current treatment will be useful for preventing viral evolution and the subsequent treatment failure. In terms of HIV-1 eradication, effectiveness of intensification

strategies could impact the length of HIV-1 eradication and on the contrary, new strategies will be needed.

New classes of antiretroviral agents against viral integrase and coreceptors increase the treatment options for HIV-1-infected individuals and provide new tools to assess the viral reservoirs that persist in HAART-suppressed subjects. Raltegravir, an integrase strand transfer inhibitor, has recently been approved for HIV-1-infected subjects with limited treatment options and for fist line therapy. Raltegravir plus optimized background therapy provided better viral suppression than optimized background therapy alone for at least 96 weeks [9-11]. Raltegravir blocks integration of linear viral cDNA, that is subsequently circularized by host DNA repair enzymes to form episomes containing two copies of the viral long-terminal repeat (2-LTR circles), or undergoes recombination to form a 1-LTR circle. Therefore, an increase in episomal cDNA occurs when active replication is inhibited by integrase inhibitors [14, 15] (**Figure 19**). We exploited this unique relationship between episomes and raltegravir to reveal active replication in subjects on HAART and specifically to determine whether raltegravir intensification impacted viral cDNA intermediates and immune activation parameters in HAART-suppressed subjects.

The availability of new antiretroviral classes provides an opportunity to intensify antiretroviral suppression. The inhibitors of the HIV-1 integrase have been newly incorporated to the HAART. Therefore, integrase inhibitors offer the challenge to intensify current therapies

The quantification of episomes viral cDNA are valid surrogate markers of ongoing viral replication in HIV-1 infected subjects [76,

115]. Viral episomes are formed after completion of viral cDNA synthesis and, after translocation of the viral genome to the cell nucleus, recombination events and direct ligation of the LTRs lead to the formation of episomes cDNA, containing 1- or 2LTRs circles. 2LTR circles have been postulated as a marker for ongoing viral replication since they are labile forms [76, 115-117]. Interestingly 2LTR circles has been reported to increase *in-vitro* after the addition of integrase inhibitors, since they prevent viral genomes for integration and therefore lineal forms of HIV-1 DNA are likely recircularized [14, 15].

RESULTS

Subjects characteristics

A total of 69 subjects with undetectable plasma viremia by standard assays (<50 HIV-1 RNA copies/mL) for more than one year (median=5 years) were randomized to intensify their HAART with raltegravir (n=45), or to continue their HAART (n=24) for 48 weeks. Subjects in the intensified treatment group had been exposed to a median of 7 antiretroviral drugs with a median of 4.4 NRTI, 1.1 NNRTI, and 1.5 protease inhibitors. Non-intensified subjects had been exposed to a median of 6.7 antiretroviral drugs with a median of 4.2 NRTI, 1.0 NNRTI and 1.4 protease inhibitors. None of the enrolled subjects had previously been exposed to integrase inhibitors. In the present study, only data from 24 weeks are presented. Two subjects in the control group were excluded from further analysis (see material and methods section). Subject characteristics are summarized in **Table 11.**

Table 11. Subject characteristics in the control and raltegravir-intensified groups

	Control *n* = 22 [a]	Intensification *n* = 45	*P*-value between groups [b]
Age, years, mean ± SD	44.8 ± 8.0	46.1 ± 8.8	0.569
Females, *n* (%)[c]	6 (27.3)	6 (9.0)	*0.187*
ART			
Protease inhibitor-containing regimen, *n* (%)[c]	8 (36.4)	14 (31.1)	*0.878*
Number of exposed ART drugs, mean ± SD	6.7 ± 0.65	7.0 ± 0.38	*0.662*
Previous salvage regimen, n (%)	3 (14)	10 (22)	*0.744*
Pre-HAART Therapy, n (%)	7 (32)	13 (29)	*1*
Time from diagnosis (years), mean ± SD	11.0 ± 5.1	11.0 ± 6.5	*0.795*
Time with suppressive ART (years), mean ± SD	4.5 ± 3.1	5.0 ± 3.0	*0.646*
Total HIV-1 DNA			
Baseline, (copies per million PBMCs), median [IQR]	14.1 [3.1–61.3]	10.3 [4.5–38.3]	*0.713*
Week 24, (copies per million PBMCs), median [IQR]	41.7 [2.2–124.8]	9.6 [0.75–66.0]	*0.183*
P-value within group (baseline *vs.* week 24)[d]	***0.010***	*0.315*	
Integrated HIV-1 DNA			
Baseline, (copies per million PBMCs), median [IQR]	1.9 [0–41.7]	0 [0–7.4]	*0.229*
Week 24, (copies per million PBMCs), median [IQR]	2.3 [0–87.1]	0 [35.6–104.3]	*0.097*
P-value within group (baseline *vs.* week 24)[d]	*0.579*	*0.322*	
CD4$^+$ T-cell			
CD4 at baseline, Absolute (cells μl^{-1}), median [IQR]	503 [371–600]	530 [434–786]	*0.333*
CD4 at week 24, Absolute (cells μl^{-1}), median [IQR]	482 [374–636]	614 [486–745]	*0.072*
P-value within group (baseline vs. week 24)[d]	*0.103*	*0.138*	
$CD4^+CD45RA^-$ at baseline, (%)[e], median [IQR]	65.9 [63.6–74.2]	68.6 [43.0–80.2]	*0.943*
$CD4^+CD45RA^-$ at week24, (%)[e], median [IQR]	69.0 [60.7–81.6]	76.1 [59.0–82.4]	*0.713*
P-value within group (baseline vs. week 24)[d]	*0.787*	*0.155*	
$CD4^+CD45RA^-CD38^+$ at baseline, (%)[e], median [IQR]	26.8 [23.9–31.7]	26.2 [19.9–35.1]	*0.910*
$CD4^+CD45RA^-CD38^+$ at week24, (%)[e], median [IQR]	34.3 [29.7–37.6]	30.4 [23.85–34.7]	*0.237*
P-value within group (baseline vs. week 24)[d]	***0.008***	*0.360*	
$CD8^+$ T-cell			
CD8 at baseline, Absolute (cells μl^{-1})[e], median [IQR]	722 [470–1051]	660 [467–961]	*0.631*
CD8 at week 24, Absolute (cells μl^{-1})[e], median [IQR]	830 [590–958]	658 [498–1017]	*0.402*
P-value within group (baseline vs. week 24)[d]	*0.344*	*0.163*	
$CD8^+CD45RO^+CD38^+$ at baseline, (%)[e], median [IQR]	14.9 [9.9–18.8]	15.6 [10.7–21.0]	*0.867*
$CD8^+CD45RO^+CD38^+$ at week24, (%)[e], median [IQR]	16.7 [12.0–22.9]	11.9 [6.5–15.3]	*0.071*
P-value within group (baseline vs. week 24)[d]	*1*	*0.241*	
$CD8^+$HLA-$DR^+CD45RO^+$ at baseline, (%)[e], median [IQR]	19.5 [11.1–25.7]	16.5 [8.9–24.6]	*0.502*
$CD8^+$HLA-$DR^+CD45RO^+$ at week24, (%)[e], median [IQR]	20.1 [10.4–25.8]	14.7 [8.5–23.8]	*0.293*
P-value within group (baseline vs. week 24)[d]	*0.898*	*0.984*	
$CD8^+$HLA-DR^+CD38^+ at baseline, (%)[e], median [IQR]	16.1 [11.9–21.1]	11.7 [9.4–27.4]	*0.690*
$CD8^+$HLA-DR^+CD38^+ at week24, (%)[e], median [IQR]	20.4 [18.7–28.7]	14.6 [10.0–25.9]	*0.065*
P-value within group (baseline vs. week 24)[d]	***0.041***	*0.641*	
Ultrasensitive plasma viral load			
Baseline (copies ml^{-1}), median [IQR]	0.5 [0.4–0.6]	0.5 [0.4–0.6]	*0.334*[f]
Week 12 (copies ml^{-1}), median [IQR]	0.4 [0.32–0.5]	0.4 [0.39–0.5]	*0.721*[f]
P-value within group (baseline vs. week 12)[g]	*0.102*	*0.523*	
Soluble CD14			
Baseline ($\mu g\ ml^{-1}$), median [IQR]	7.7 [5.9–8.8]	7.8 [6.6–9.2]	*0.479*
Week 24 ($\mu g\ ml^{-1}$), median [IQR]	7.1 [6.3–8.7]	8.3 [6.9–9.7]	*0.136*
P-value within group (baseline vs. week 24)[d]	*0.571*	*0.213*	

In addition, subject characteristics of subgroups after the post-hoc analysis between 2-LTR+ and 2-LTR- subjects are summarized in **Table 12**.

Table 12. Subject characteristics between the 2-LTR^{-} and 2-LTR^{+} subgroups

	Intensification n=45		*P-value between groups*[a]
	2LTR^{-} n = 32	2LTR^{+} n = 13	
Age, years, mean±SD	47.6 ± 9.2	43.7 ± 7.4	*0.633*
Females, (%)[b]	18.7	0	*0.16*
ART			
PI-containing regimen at intensification, n (%)[b]	6 (18.7)	8 (61.5)	*0.011*
Number of exposed ART drugs, mean±SD	7.1 ± 0.5	6.8 ± 0.7	*0.747*
Previous salvage regimens, n (%)[b]	8 (25)	2 (15)	*0.710*
Pre-HAART Therapy, n (%)[b]	10 (31)	3 (23)	*1*
Time from diagnosis, (years), mean±SD	12.3 ± 5.1	10.1 ± 6.1	*0.249*
Time with suppressive ART, (years), mean±SD	5.2 ± 2.9	3.5 ± 2.5	*0.075*
Total HIV-1 DNA			
Baseline, (copies per million PBMCs), median [IQR]	9.4 [2.6-36.6]	22.6 [8.4-55.0]	*0.278*
Week 24, (copies per million PBMCs), median [IQR]	9.62 [0.8-35.1]	35.8 [0.4-139.4]	*0.518*
P-value within group (baseline *vs.* week 24)[c]	*0.08*	*0.557*	
Integrated HIV-1 DNA			
Baseline, (copies per million PBMCs), median [IQR]	0 [0-7.4]	0.04 [0-8.9]	*0.856*
Week 24, (copies per million PBMCs), median [IQR]	0 [0-5.7]	0 [0-1.5]	*0.541*
P-value within group (baseline *vs.* week 24)[c]	*0.468*	*0.578*	
CD4^{+} T-cell			
CD4 at baseline, Absolute (cells μl^{-1}), median [IQR]	532 [434-746]	520 [434-803]	*0.802*
CD4 at week 24, Absolute (cells μl^{-1}), median [IQR]	614 [462-763]	590 [540-739]	*0.709*
P-value within group (baseline vs. week 24)[c]	*0.161*	*0.085*	
CD8^{+} T-cell			
CD8 at baseline, Absolute (cells μl^{-1}), median [IQR]	642 [476-867]	732 [450-962]	*0.831*
CD8 at week 24, Absolute (cells μl^{-1}), median [IQR]	593 [487-1029]	744 [600-952]	*0.309*
P-value within group (baseline vs. week 24)[c]	*0.136*	*0.252*	
Ultrasensitive Viral Load			
Baseline (copies ml^{-1}), median [IQR]	0.6 [0.6-0.6]	0.5 [0.4-0.5]	*0.353*
Week 12[d], (copies ml^{-1}), median [IQR]	0.4 [0.4-0.5]	0.4 [0.4-0.5]	*0.977*
P-value within group (baseline vs. week 12)[c]	*0.895*	*0.201*	
Soluble CD14			
Baseline (μg ml^{-1}), median [IQR]	7.5 [6.3-8.6]	8.9 [7.3-10.5]	*0.064*
Week 24 (μg ml^{-1}), median [IQR]	8.0 [6.7-9.2]	9.5 [8.3-10.4]	*0.027*
P-value within group (baseline *vs.* week 24)[c]	*0.533*	*0.210*	

PI, protease inhibitors; ART, antiretroviral therapy; SCA, Single copy assay; Viral load (copies RNA/ml plasma); [a] *P*-value between groups: U Mann Whitney test; [b]Pearson's chi square; [c]*P*-value within groups: signed rank test (paired data); [d]Peto-Prentice test; PI were lopinavir or atazanavir, and NNRTI were efavirenz or nevirapine

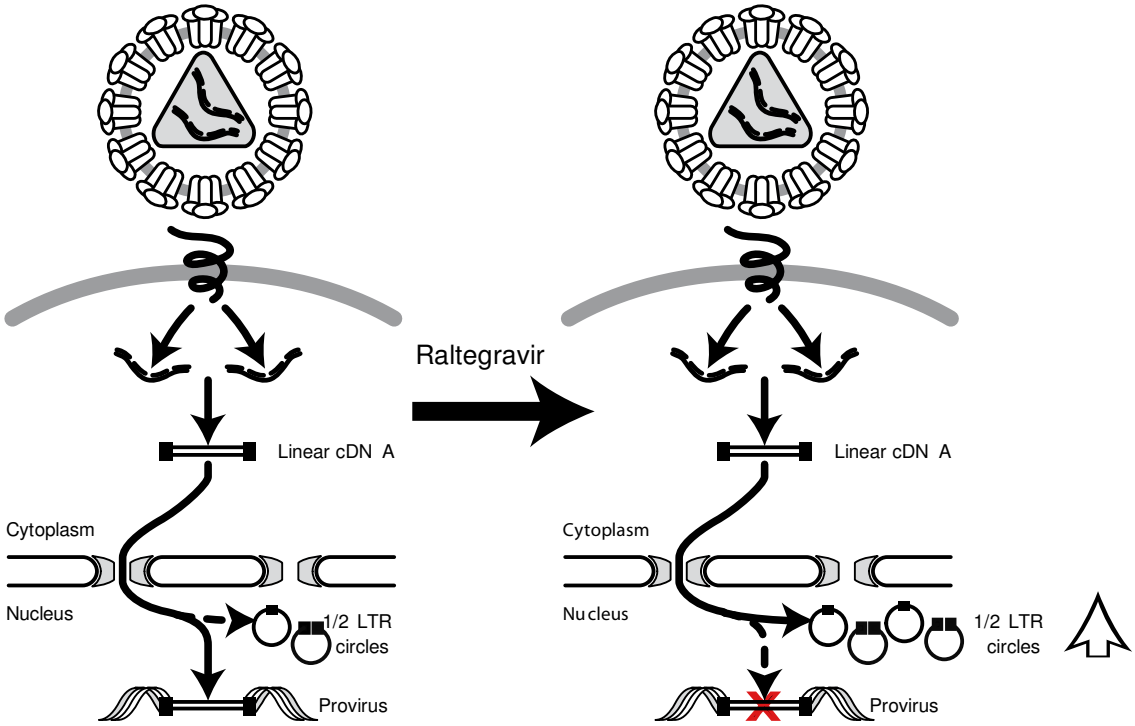
Raltegravir intensification results in a specific and transient increase in 2-LTRs circles in a large percentage of subjects

In the formation of 2-LTR circles, U3 and U5 sequences within the 5' and 3' LTRs ligate to form a unique U3-U5 circle junction that is not represented in other forms of viral cDNA. Therefore, 2-LTR circles were specifically measured by real-time PCR with primers flanking the 2-LTR circle junction. During the study, 2-LTR circles were detectable at one or more time points in 29% (13/45) of the subjects intensified with raltegravir but in only 5% (1/22) of the control group (p=0.025). Within the treatment intensification group (n=45) there was a significant and transient increase in 2-LTR circles at weeks 2 and 4 compared to baseline (p=0.036 and p=0.023 respectively), and a subsequent significant decrease afterwards (p=0.024) (**Figure 20a and 20b**).

Longitudinal changes in the 13 subjects of the intensification group with detectable 2-LTR circles at one or more time points (subsequently referred to as 2-LTR+) are depicted in **Figure 21.**

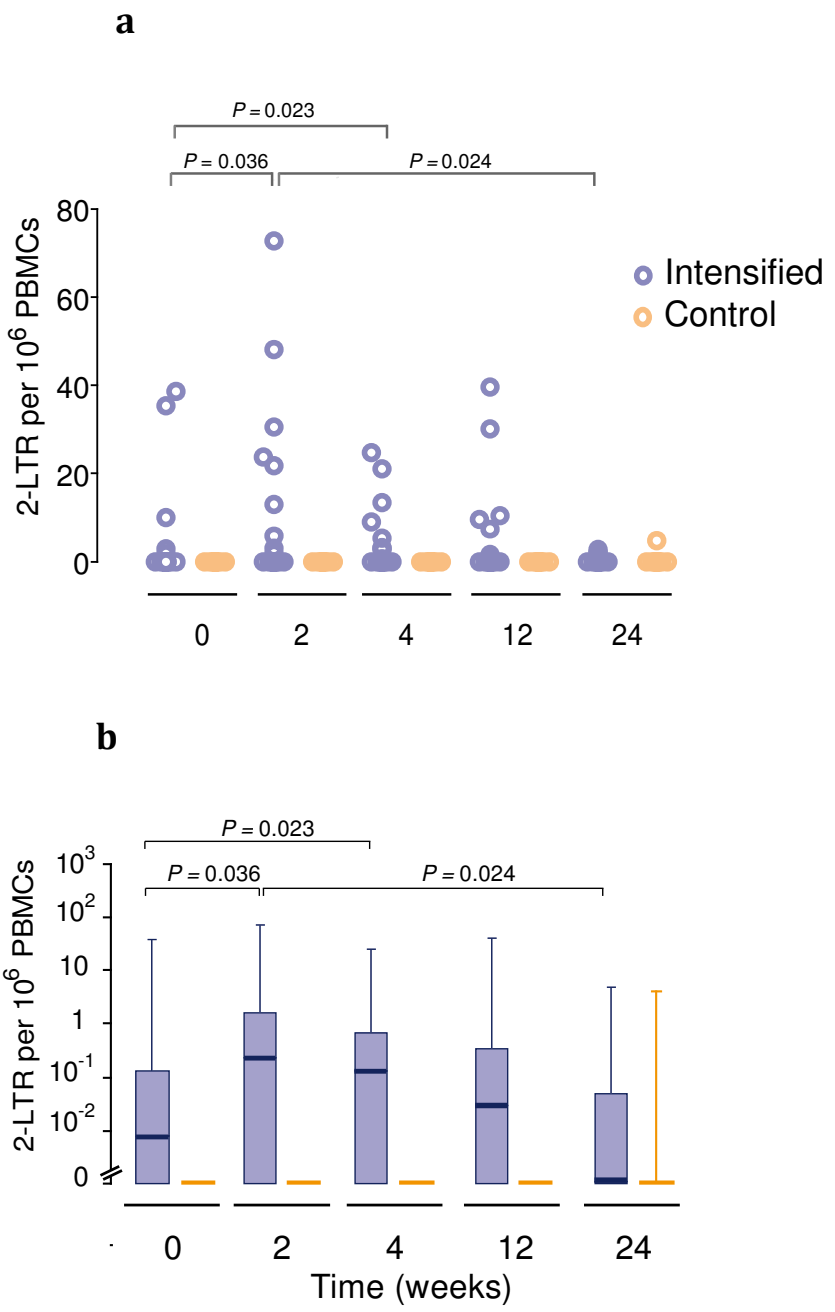
2-LTR circle dynamics was consistent in these subjects with an increase at week 2 and a subsequent decrease thereafter. Of the 13 subjects in which 2LTR circles were impacted by raltegravir, five were 2LTR+ at baseline. Those five subjects did not bias the analysis since the increases in 2LTR circles at weeks 2 and 4 were still significant if they were excluded (p-values 0.046 and 0.008 respectively) **Table 13**.

Figure 19. Impact of Raltegravir on HIV-1 DNA forms



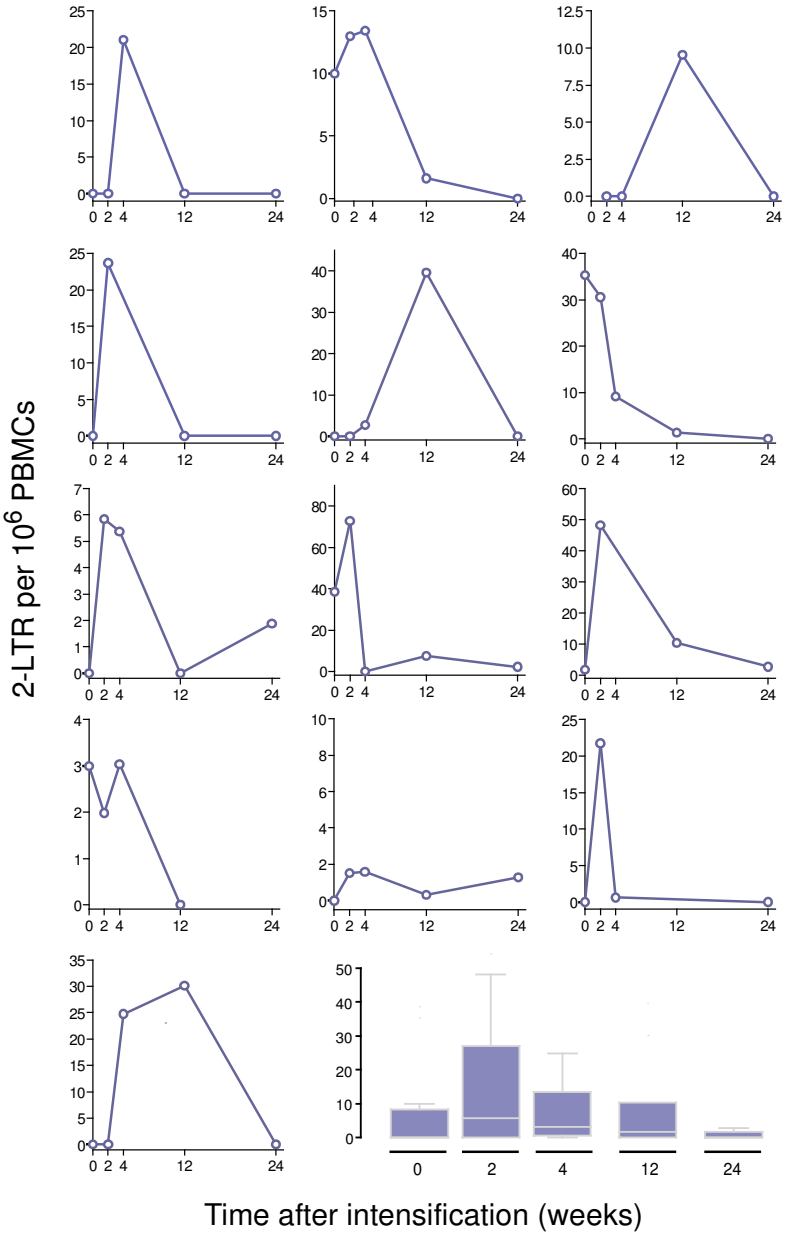
Raltegravir blocks integration to promote episome formation. In the presence of raltegravir, integration of linear viral cDNA is blocked and subsequently is circularized by host DNA repair enzymes to form episomes containing 2 copies of the viral long terminal repeat (2-LTR circles) or undergoes recombination to form a 1-LTR circle. Therefore, an increase in episomal cDNA occurs when active replication is inhibited by raltegravir

Figure 20. Impact of raltegravir intensification on episomal HIV-1 DNA (2-LTR circles) in HAART-suppressed subjects



(**a**) Changes in episomal HIV-1 DNA (2-LTR circles) between groups during the study period. Data are absolute numbers. (**b**) Data are median, 25 and 75 percentiles calculated by ROS method. Blue boxes represent the intensified arm (n = 45) while the control arm (n = 22) is represented by orange boxes. P-values (Peto-Prentice-Wilcoxon test, paired data) are indicated.

Figure 21. Individual pattern of 2-LTRs dynamics



Individual longitudinal changes in 2-LTR levels during therapy intensification with raltegravir. The amount of episomal HIV-1 DNA (2-LTR circles) was measured at weeks 0, 2, 4, 12 and 24 after initiation of the study. The individual data from the 13 subjects included in the intensified 2-LTR+ subgroup are shown. The last graphic summarizes the overall dynamics of the changes in the amount of detectable 2-LTR circles in the intensified 2-LTR+ subgroup (n = 13) during therapy intensification with raltegravir. Data are median, 25 and 75 percentiles.

Table 13. P-values (Peto-Prentice-Wilcoxon test, paired data) corresponding to longitudinal changes in 2-LTR circles in the intensification group.

	Week 2	Week 4	Week 12	Week 24
Including the 5 patients with detectable 2LTR circles at baseline	0.036	0.023	0.129	0.558
Excluding the 5 patients with detectable 2LTR circles at baseline	0.046	0.008	0.083	0.157

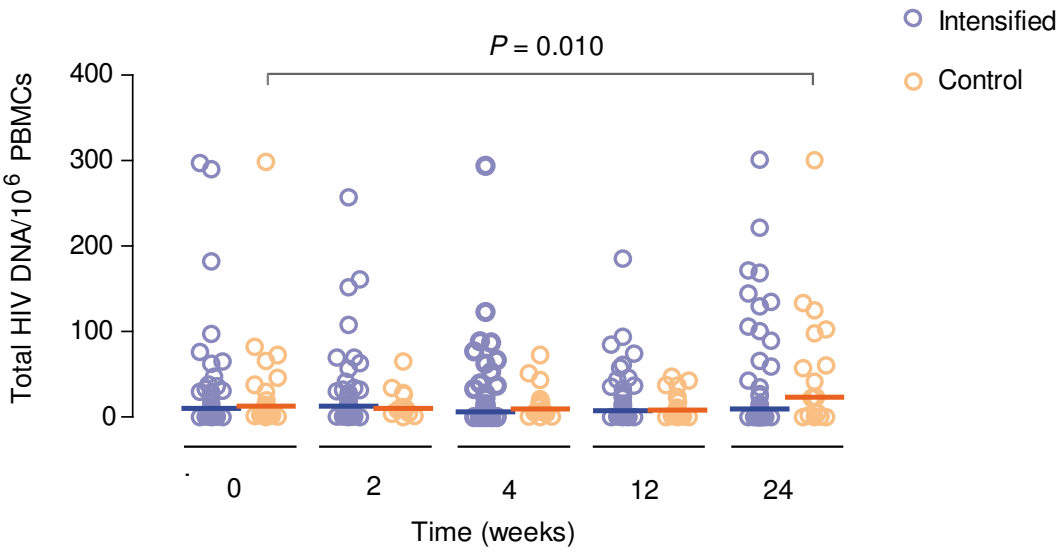
In addition, we were interested in evaluating why raltegravir only impacted 2-LTR circle dynamics in some (29%) of the intensified subjects. Therefore, we conducted a post-hoc analysis to assess whether the 2-LTR+ subjects had different baseline characteristics to the 2-LTR- subjects. A description of baseline characteristics of these two subgroups is shown in **Table 12**. When stratified by their background regimen into those on protease inhibitor or on NNRT inhibitor-containing regimens, increases in 2-LTR circles were mainly observed in subjects who intensified their protease inhibitor-containing regimen (p=0.011, **Table 12**).

Total and integrated HIV-1 DNA remain stable during raltegravir intensification

Total viral DNA forms (comprising unintegrated and integrated viral cDNA) were assayed using internal LTR primers. Total HIV-1 DNA was amplified from 100% (67/67) of the subjects at any time point in the study and no longitudinal changes were observed in total HIV-1 DNA in the intensified arm (p=0.315). An increase in total HIV-1 DNA

was observed at week 24 in the control arm (p=0.010) (**Figure 22**), suggesting some loss of viral control in this group.

Figure 22. Changes in total HIV-1 DNA after Raltegravir intensification



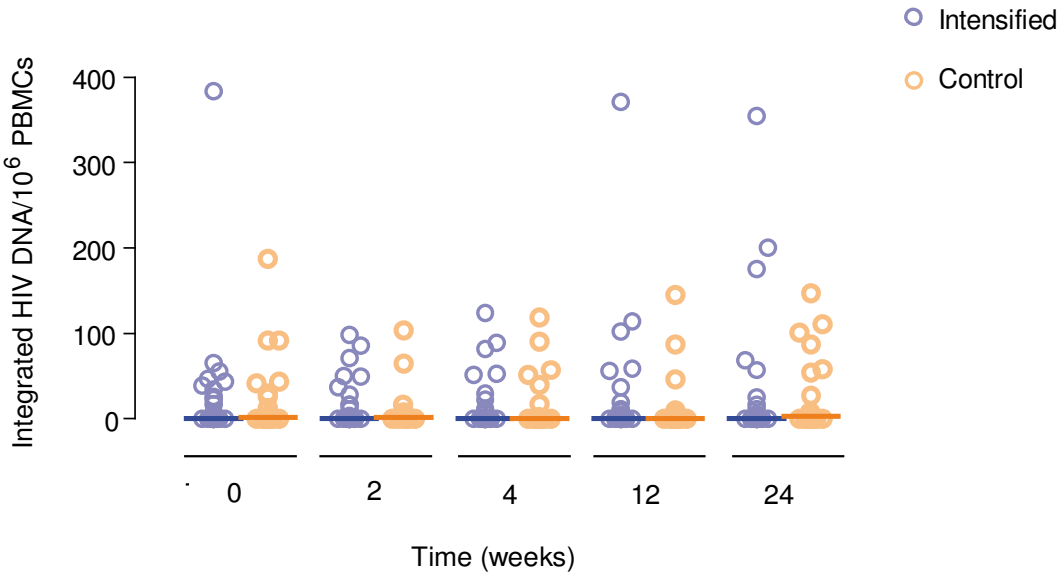
Changes in total HIV-1 DNA during 24 weeks of intensification. Blue symbols represent the intensified arm (n = 45) while the control arm is represented by orange symbols (n = 22). P-value (signed rank test, paired data) is indicated.

Integrated proviral DNA was measured using LTR-Alu primers. Integrated HIV-1 DNA was amplified from 87% (39/45) of the intensified subjects and from 86% (19/22) of the subjects randomized to the control arm at any time point after initiation of the study (p=0.252). No longitudinal changes were observed in integrated HIV-1 DNA in any of the groups during 24 weeks (control arm: p=0.579; intensification arm: p=0.322) and no differences were observed between the groups at any time interval (**Figure 23**). Of note, we found

a longitudinal correlation between total and integrated HIV-1 DNA (control arm: rho=0.45; intensification arm: rho=0.40, $p<0.001$, data not shown), although none of these parameters correlated with 2-LTR circle levels. Finally, no longitudinal changes were observed in either integrated or total HIV-1 DNA when the 2-LTR+ and 2-LTR− subgroups were compared.

Therefore, despite the effect of raltegravir on 2-LTR circles in the intensified group, total and integrated HIV-1 DNA levels remained stable throughout the period of analysis. This supports the notion that 2-LTR circles represent a minor fraction of the total cDNA species in the infected cell and further, that the majority of integrated DNA is archival and non-dynamic.

Figure 23. Changes in integrated HIV-1 DNA after Raltegravir intensification

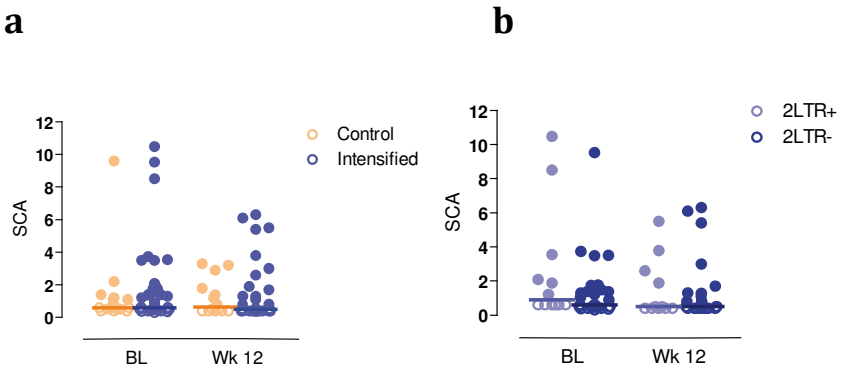


Changes in integrated HIV-1 DNA during 24 weeks of intensification. Blue symbols represent the intensified arm (n = 45) and orange symbols represent the control arm (n = 22).

Raltegravir intensification does not reduce residual viremia

A single-copy assay that quantifies HIV-1 RNA concentrations down to 1 copy per ml of plasma was performed on all baseline and week 12 samples[43]. No significant differences were found between or within the control and the intensification groups (**Figure 24a, Table 11**). Similarly, there were no differences between the 2-LTR+ and 2-LTR− subgroups (**Figure 24b, Table 12**). In summary, the virologic impact of raltegravir intensification was only revealed by analysis of 2-LTR circle dynamics.

Figure 24. Ultrasensitive viral load after raltegravir intensification



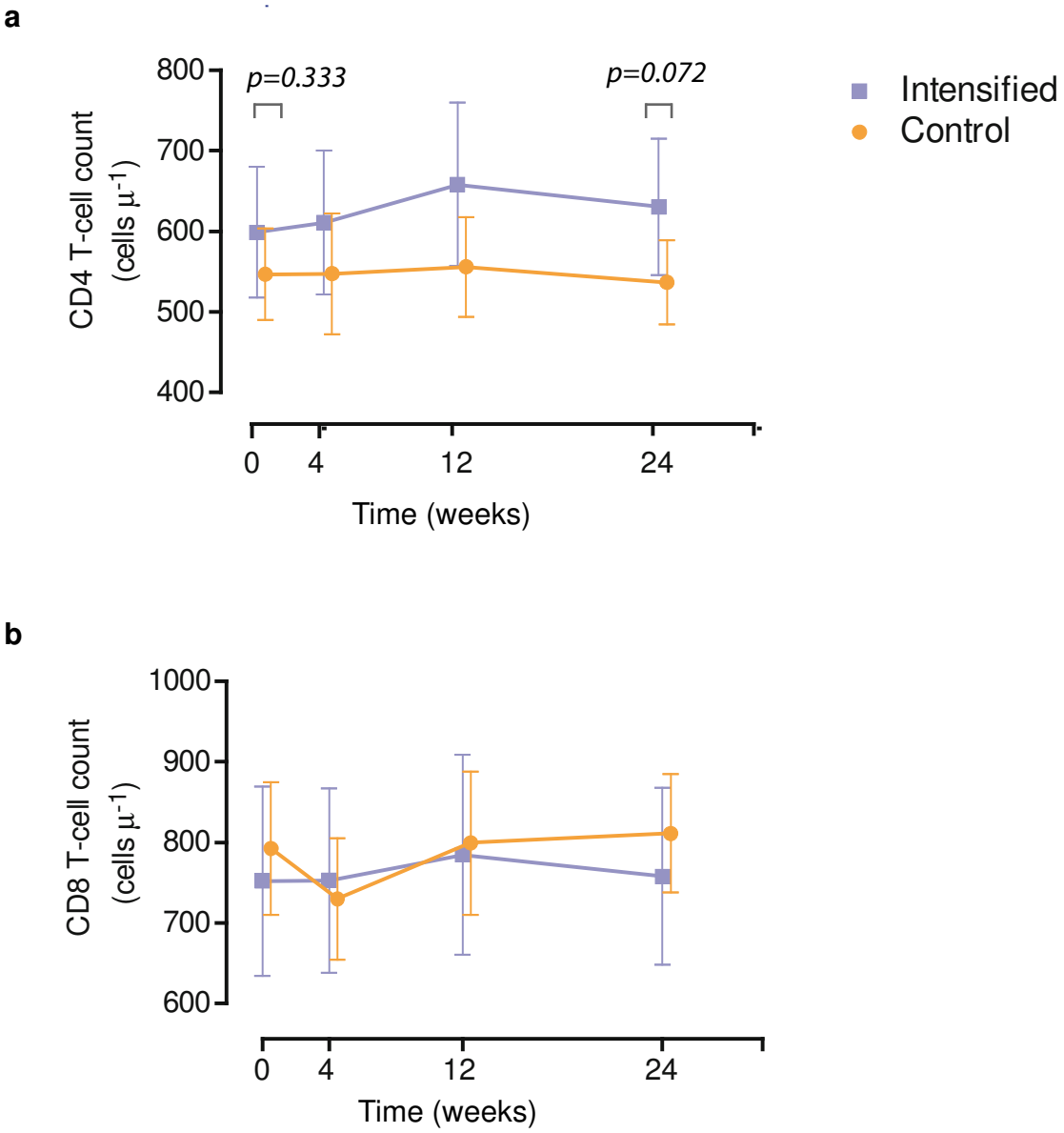
Changes in ultrasensitive viral load (SCA) during 12 weeks of intensification. Data represent median and absolute number. P values were calculated with Peto-Prentice test.

Raltegravir intensification reduces immune activation in 2LTR+ subgroup of subjects

Both the intensified and the control groups had stable $CD4^+$ and $CD8^+$ T-cell counts during the study with only a trend towards an increase in absolute $CD4^+$ T-cell counts in the intensification arm (P = 0.072, **Figure 25**) that was not confirmed by the percentage of $CD4^+$ T-cells (P = 0.947). In addition, both arms showed no major differences in $CD4^+CD45RA^-$ or activated $CD8^+$ T-cells during the study (**Table 11** and **Figure 26**). However, increases in the percentage of $CD4^+CD45RA^-CD38^+$ (P = 0.008) and $CD8^+HLA\text{-}DR^+CD38^+$ cells (P = 0.041) in the control arm were observed after 24 weeks. Although no rebound in plasma viremia or 2-LTR circles in peripheral blood mononuclear cells was evident in the control group after 24 weeks, it is possible that

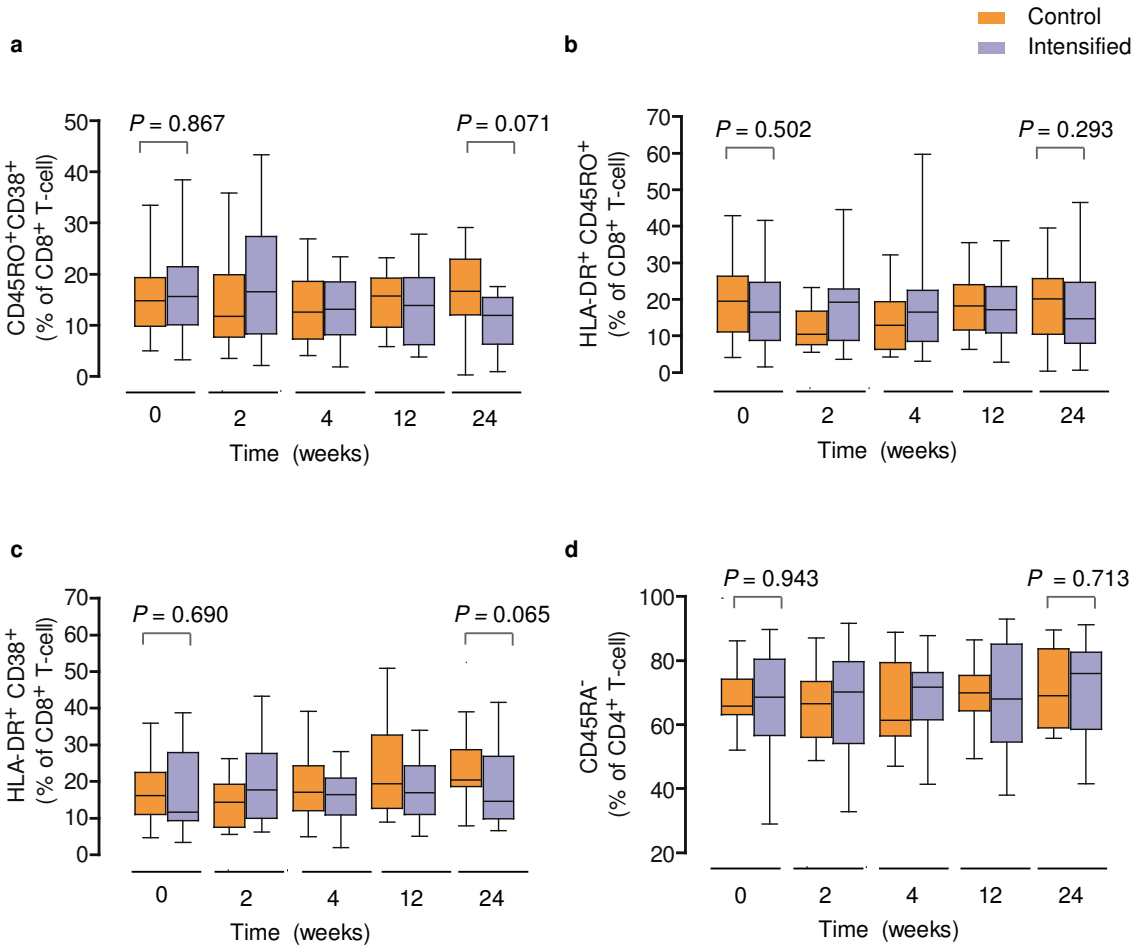
compartmentalized viral replication in the tissues contributed to the increase in T-cell activation levels and in proviral DNA.

Figure 25. Evolution of CD4 and CD8 T-cell counts



Time course evolution of the (**a**) CD4+ and (**b**) CD8+ T-cell counts was assessed in fresh blood samples by multicolour flow cytometry at weeks 0, 4, 12 and 24. Blue lines represent the intensified arm (n = 45) while the control arm (n = 22) is represented by orange lines. Mean values ± SEM. P-values (U Mann Whitney test between groups) are indicated.

Figure 26. Time course evolution of activated CD8+ T-cells and CD45RA- CD4+ T-cells between groups



Phenotypic analysis of lymphocytes subsets in intensified and control arms was assessed in fresh blood samples by multicolour flow cytometry at weeks 0, 2, 4, 12 and 24. CD8+ T-cell activation, measured as the percentage of (**a**) CD8+CD45RO+CD38+, (**b**) CD8+HLA-DR+CD45RO+, and (**c**) CD8+HLA-DR+CD38+; (**d**) Time course of the percentage of CD4+CD45RA- T-cells. Median, 25 and 75 percentiles. Blue boxes represent the intensified arm (n = 34) while the control arm (n = 18) is represented by orange boxes. P-values (U Mann Whitney test between groups; and signed rank test, paired data, within groups) are indicated.

To examine the relationship between immune activation and the observed increases in 2-LTR DNA forms, activation markers were compared between the 2-LTR^{+} and 2-LTR^{-} subgroups of intensified subjects in a post-hoc analysis. At baseline, the 2-LTR^{+} subgroup

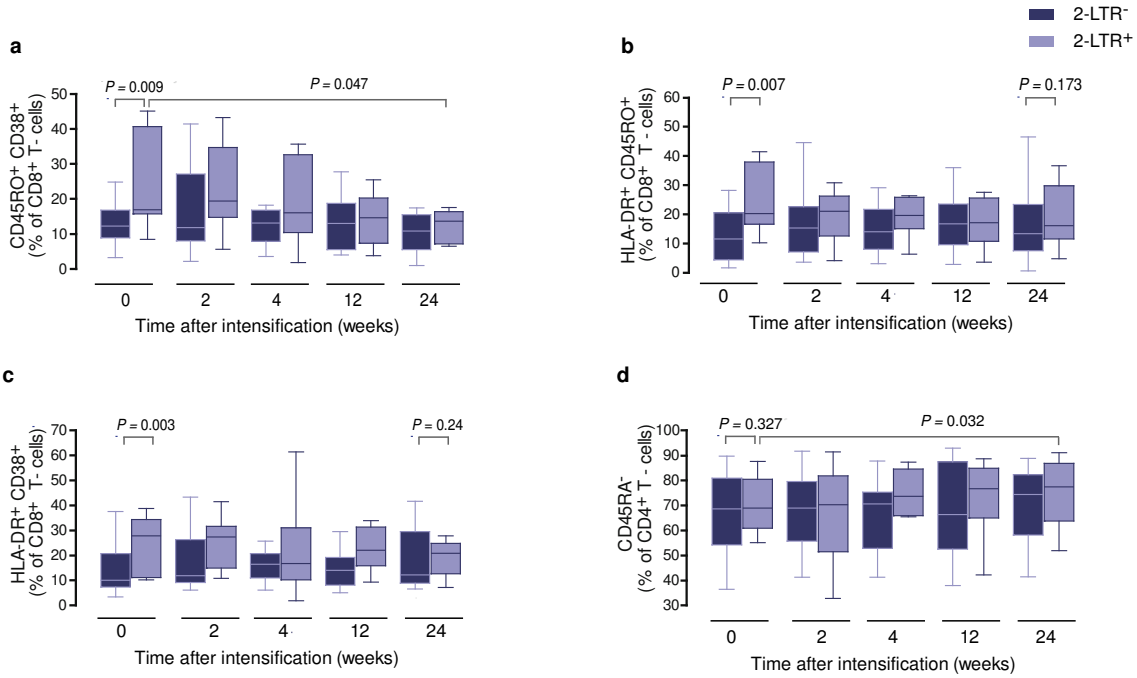
exhibited higher percentages of activated $CD8^+$ T-cells than 2-LTR^- subjects: $CD8^+CD45RO^+CD38^+$ (P = 0.009; **Figure 27a**), $CD8^+$HLA-$DR^+CD45RO^+$ (P = 0.007; **Figure 27b**), and $CD8^+$HLA-DR^+CD38^+ (P = 0.003; **Figure 27c**). Similarly, there was a higher percentage of $CD4^+CD45RA^-CD38^+$ T-cells (P = 0.019) and there was a trend towards higher concentration of plasma soluble CD14 (P = 0.064) —a surrogate marker of bacterial translocation [118]— in the 2-LTR^+ subgroup at baseline (**Table 12**). There was no correlation between immune activation and total or integrated HIV-1 DNA at baseline.

Longitudinal analysis showed a significant reduction in $CD8^+$ T-cell activation markers in the 2-LTR^+ subgroup that was particularly evident in $CD8^+CD45RO^+CD38^+$ T-cells (P = 0.047, **Figure 27a**). Similarly, the levels of $CD8^+$HLA-$DR^+CD45RO^+$ and $CD8^+$HLA-DR^+CD38^+ activation markers in the 2-LTR^+ subgroup were reduced overtime to those in the 2-LTR^- subgroup (**Figure 27b and 27c**). In contrast, a higher percentage of $CD4^+CD45RA^-CD38^+$ T-cells (P = 0.017) was maintained in the 2-LTR^+ subgroup at week 24. Interestingly, changes in $CD8^+$ T-cell activation have been linked to changes in HIV-1 replication [113], while $CD4^+$ T-cell activation appears to be more dependent on homeostatic responses and bacterial translocation [119]. Finally, there was also a trend toward a greater increase in absolute $CD4^+$ T-cell counts in the 2-LTR^+ subgroup at week 24 (P = 0.085; signed rank test), with a higher increase in the percentage of $CD4^+CD45RA^-$ T-cells (P = 0.032, **Figure 27d**).

To examine whether the five subjects with 2-LTR circles at baseline might have biased the analysis, we used regression models adjusting for the presence of 2-LTR circles at baseline (**Table 14–15**). These results strength the statistical conclusions drawn from the main

analysis and indicate that raltegravir is more likely to impact immune activation in subjects showing altered 2-LTR circle dynamics. This supports the conclusion that residual viral replication, as revealed by 2-LTR circle levels, drives immune activation and that raltegravir intensification can reduce the extent of immune activation by suppressing residual viral replication.

Figure 27. Time course evolution of activated CD8+ T-cells and CD45RA- CD4+ T-cells between subgroups



Phenotypic analysis of lymphocytes subsets in intensified after stratification on the basis of 2-LTR positivity (2-LTR$^+$) and negativity (2-LTR$^-$) was assessed in fresh blood samples by multicolour flow cytometry at weeks 0, 2, 4, 12 and 24. CD8$^+$ T-cell activation, measured as the percentage of (**a**) CD8$^+$CD45RO$^+$CD38$^+$, (**b**) CD8$^+$HLA-DR$^+$CD45RO$^+$, and (**c**) CD8$^+$HLA$^-$DR$^+$CD38$^+$; (**d**) Time course of the percentage of CD4$^+$CD45RA$^-$ T-cells. Median, 25 and 75 percentile. Blue light boxes represent the 2-LTR$^+$ subgroup (n = 12) while the 2-LTR$^-$ subgroup is represented by blue dark boxes (n = 24). One-sided P-values (Mann Whitney U test between groups; and signed rank test, paired data, within groups) are indicated.

Table 14. Regression mixed models adjusting by the presence of episomal HIV-1 DNA at baseline and comparing activation markers within the:

a. 2-LTR+ subgroup between baseline and week 24

	Unadjusted		Adjusted	
	slope	*P*-value	slope	*P*-value
$CD45RO^+CD38^+$ (% of $CD8^+$ T-cells)	-0.28	0.053	-0.29	0.047
HLA-$DR^+CD45RO^+$ (% of $CD8^+$ T-cells)	-0.01	0.664	-0.06	0.660
HLA-DR^+CD38^+ (% of $CD8^+$ T-cells)	-0.11	0.457	-0.11	0.437
$CD45RA^-$ (% of $CD4^+$ T-cells)	0.36	0.005	0.36	0.005

b. Intensification group between baseline and week 24

	Unadjusted		Adjusted	
	slope	*P*-value	slope	*P*-value
$CD45RO^+CD38^+$ (% of $CD8^+$ T-cells)	-0.14	0.074	-0.14	0.074
HLA-$DR^+CD45RO^+$ (% of $CD8^+$ T-cells)	0.06	0.417	0.006	0.407
HLA-DR^+CD38^+ (% of $CD8^+$ T-cells)	0.02	0.807	0.02	0.802
$CD45RA^-$ (% of $CD4^+$ T-cells)	0.20	0.009	0.20	0.009

Table 15. ANOVA models adjusting by the presence of episomal HIV-1 DNA at baseline comparing activation markers between:

a. 2-LTR+ and 2-LTR- subgroups at baseline and week 24

	Baseline		Week 24	
	Unadjusted *P*-value	Adjusted *P*-value	Unadjusted *P*-value	Adjusted *P*-value
$CD45RO^+CD38^+$ (% of $CD8^+$ T-cells)	0.005	0.006	0.802	0.806
HLA-$DR^+CD45RO^+$ (% of $CD8^+$ T-cells)	0.005	0.005	0.591	0.598
HLA-DR^+CD38^+ (% of $CD8^+$ T-cells)	0.009	0.005	0.838	0.841
$CD45RA^-$ (% of $CD4^+$ T-cells)	0.800	0.801	0.431	0.438

b. Intensification and Control groups at baseline and week 24

	Baseline		Week 24	
	Unadjusted *P*-value	Adjusted *P*-value	Unadjusted *P*-value	Adjusted *P*-value
$CD45RO^+CD38^+$ (% of $CD8^+$ T-cells)	0.673	0.676	0.207	0.646
HLA-$DR^+CD45RO^+$ (% of $CD8^+$ T-cells)	0.599	0.601	0.548	0.553
HLA-DR^+CD38^+ (% of $CD8^+$ T-cells)	0.926	0.923	0.074	0.077
$CD45RA^-$ (% of $CD4^+$ T-cells)	0.897	0.897	0.797	0.799

Chapter 6

Discussion

GENERAL OVERVIEW

This thesis has addressed different questions regarding the use of raltegravir in the setting of antiretroviral therapy-experienced subjects, and also how HIV-gene interactions are able to modulate the phenotypic characteristics of raltegravir resistance virus that re-emerge after treatment failure. In addition, we have investigated how raltegravir is able to perturb the latent reservoirs that persist despite a suppressive HAART.

Raltegravir has been approved for treatment of drug-experienced HIV-1 infected subjects, therefore in Chapter 3 we elucidate whether long-term HAART targeting protease and reverse-transcriptase was able to influence the evolution of the integrase, and the fitness and raltegravir susceptibility. Throughout the investigation, we observed that HIV-1 integrase from longitudinal samples taken from subjects treated with integrase inhibitor–sparing regimens showed no evidence of genotypic or phenotypic resistance to raltegravir. Additionally, long-term pressure with protease and reverse-transcriptase inhibitors did not impair the fitness evolution of HIV-1 integrase. Therefore, our data suggested that current antiretroviral regimens do not diminish the fitness of integrase or influence raltegravir efficacy.

Furthermore, the three major HIV-1 drug-targeted enzymes – protease, reverse-transcriptase and integrase – mature from the same polyprotein, suggesting the potential for interaction between them. Through Chapter 4 we aimed to shed light on the relative contribution of protease-reverse-transcriptase, integrase and the rest of the HIV-1 genome to viral fitness and susceptibility to raltegravir. In this chapter

we observed that susceptibility to raltegravir was driven by resistance mutations in integrase, whereas other viral genes were involved in restoring defects on viral fitness, suggesting the existence of epistatic effects on replication capacity.

Finally, we assessed in Chapter 5 whether active replication could be driving immune activation in HAART-suppressed subjects, and we examined the impact of intensification with raltegravir on viral cDNA and immune activation parameters. The results in Chapter 5 showed that raltegravir intensification resulted in a specific and transient increase in 2-LTR circle DNA in a significant percentage of subjects. Interestingly, subjects who were 2-LTR+ showed higher levels of immune activation at baseline and a subsequent normalization after intensification. This ability of raltegravir intensification to perturb the reservoir has implications for therapeutic strategies aimed at achieving viral eradication.

In the following, each results chapter will be discussed in order to hone the conclusions from this work.

DISCUSSION CHAPTER 3. RALTEGRAVIR SUSCEPTIBILITY AND FITNESS PROGRESSION OF HIV-1 INTEGRASE IN SUBJECTS ON LONG-TERM INTEGRASE-SPARING ANTIRETROVIRAL THERAPY

A number of studies have identified the presence and frequency of polymorphisms in the HIV-1 integrase gene of treatment-naïve subjects [120-124], However, no longitudinal studies of integrase evolution in treatment-experienced subjects are available, and the impact of these natural changes on phenotypic susceptibility to integrase inhibitors and on viral replication has not been fully characterized. Moreover, a stable HAART regimen with inadequate viral suppression has been reported to induce genetic evolution within the protease and reverse-transcriptase coding regions [125-127], Therefore our main objective in Chapter 3 was to evaluate the longitudinal evolution of the integrase gene over 10 years of infection in heavily antiretroviral-experienced subjects, and the impact of such change on phenotypic susceptibility to raltegravir and on replication capacity. Our results show that although the integrase gene is highly conserved, a genetic evolution may be seen in integrase of the HIV-1 subtype B obtained from subjects exposed to long-term antiretroviral treatment. We found substitutions that have been associated with resistance to different families of integrase inhibitors and a slight increase in the prevalence of some of these changes—concentrated in the central domain of the integrase—during long-term antiretroviral treatment. These data suggest that integrase evolves during long-term treatment and that it potentially modifies integrase activity and has an impact on the phenotype and the replication capacity of the virus. To evaluate this impact, we selected samples with the greatest

accumulation of genotypic changes (3 to 14 nonsynonymous substitutions). Our data showed no significant increases in phenotypic susceptibility to raltegravir in recombinant viruses from subjects who had accumulated a high number of substitutions in the integrase gene during the study period, suggesting that integrase changes occurring during several antiretroviral regimens will not diminish the virologic response to this inhibitor. This is relevant, because the subjects selected in this study are one of the main target populations for raltegravir.

Our data support previous reports that naturally occurring integrase polymorphisms had no major effects on susceptibility to some integrase inhibitors, such as L-chicoric acids [124] and diketo acids L-731,988 and L-870,810 [128], although the impact of integrase polymorphisms on susceptibility has been controversial. Some substitutions, such as the V151I mutation, found in some of our subjects, have been reported to reduce susceptibility to both L-870,810 and elvitegravir [96]. Furthermore, viruses with integrase mutations G140S and Q148H, selected in a subject failing a raltegravir-containing regimen, had a reduced in vitro phenotypic susceptibility to raltegravir. This profile has been reported to induce a 7- to 8-fold change in raltegravir resistance due to its location near the catalytic core domain of the enzyme [129]. The single T66I mutant was also shown to retain full susceptibility to raltegravir. This mutation was included because it had been reported to induce cross-resistance to the integrase-inhibiting diketo acids L-708,906, L-731,988, GS-9137, and S-1360 [130, 131].

Mutations within integrase may have a dramatic effect on both enzyme function and viral replication [132, 133]. However, natural

variability in viral integrase from HIV-infected individuals has been reported to have no effect on growth kinetics, and only minor differences were seen in 3'-end processing, suggesting that in vivo mutability might be restricted by function [124]. This study shows that evolution of HIV integrase resulted in an improved in vitro replication capacity and that enhanced fitness was not restricted by the number of amino acid changes accumulated over time. The observation that some viruses had remarkably low replication capacities is consistent with previous studies on recombinant HIV-1 viral isolates [127]. Although the specific location of certain amino acid substitutions is relevant for HIV integrase function [68, 133], none of the amino acid changes shown are in specific enzyme domains or positions previously reported as relevant for integrase activity. These results could reflect the improved fitness of sequential viral isolates from HIV-1-infected subjects during disease progression [21]. Furthermore, in the case of the integrase gene, accumulation of changes during infection would improve enzyme activity.

Viruses selected in vitro to increasing raltegravir concentrations, containing any of the leading mutations to the raltegravir resistance pathways (Q148H or N155H), have shown severe fitness defects [95]. However, occurrence of the secondary mutation G140S within the Q148H pathway has been reported to increase resistance and moderate replication defects associated with selection of Q148H [95]. This observation may explain the limited fitness impairment shown in integrase-recombinant viruses from a subject failing a raltegravir-containing regimen with both Q148H and G140S mutations. Furthermore, and in agreement with our data, viruses with the T66I substitution have shown reduced replication fitness as compared to

wild-type viruses [74, 93]. This mutation, located in the catalytic core, could impair both 3' processing and DNA strand-transfer activities of integrase [130], thus helping to explain viral fitness impairment. All these data are consistent with the general idea that resistance mutations may impair viral fitness. In particular, they could explain the maintained replication capacity between the first and last samples from subject A3, since this was the only subject in whom three changes previously associated with resistance to integrase inhibitors (V72I, L101I, and A111T) that did not appear in the first time-point were found in the last time-point.

The slight increase in the prevalence of changes associated with integrase inhibitors seen in our study could support previous reports suggesting some genotypic and phenotypic interactions between the integrase and reverse-transcriptase enzymes[85] [85, 134, 135]. Thus, the integrase substitution V165I, previously associated with resistance to integrase inhibitors, has been shown to be positively associated with substitutions F227L and L210W in reverse-transcriptase. Mutations M154I and V165I may be more common in subjects whose HAART fails [134].

In conclusion of chapter 3, the presence of natural polymorphisms in HIV-1 integrase and the low rate of evolution during long-term combination antiretroviral treatment do not seem to adversely affect in vitro raltegravir susceptibility, and appear to drive the replication capacity of integrase-recombinant viruses towards improved phenotypes in most subjects, suggesting no fitness cost associated with long-term treatment.

DISCUSSION CHAPTER 4. THE HIV-1 INTEGRASE GENOTYPE STRONGLY PREDICTS RALTEGRAVIR SUSCEPTIBILITY BUT NOT VIRAL FITNESS OF PRIMARY VIRAL ISOLATES

Preliminary studies have helped identify the pathways involved in the appearance of resistance mutations [136]. Nevertheless, studies of the potential interaction between the HIV-1 genes modulating drug resistance to integrase inhibitors and viral fitness remain scarce. In addition, phenotypic testing has proved that clade-specific integrase polymorphisms did not contribute to reduced susceptibility to integrase inhibitors [137]. However, there are no studies on gene interactions in subjects whose raltegravir-containing regimens fail. We aimed to evaluate in Chapter 4 the effects of mutations within protease, reverse-transcriptase and integrase on raltegravir susceptibility and replication capacity in subjects whose raltegravir regimen fails. A number of studies have shown that HIV drug susceptibility can be modulated by mutations outside the target gene; it is known that integrase interacts with reverse-transcriptase, and that protease inhibitor susceptibility could be modulated by reverse-transcriptase mutations and existing connections between genes coded by the Gag-Pol polyprotein [37, 83, 85, 87]. The data presented in Chapter 4 suggest that in vitro susceptibility to raltegravir is strictly modulated in the integrase coding region and that resistance mutations and polymorphisms in the protease and reverse-transcriptase coding regions do not strongly affect raltegravir susceptibility. Our data also suggest that in subjects with no resistance mutations and whose raltegravir regimen fails, the failure is not due to

protease and reverse-transcriptase amino acid changes that could modify resistance to raltegravir.

We also wanted to determine whether other genes outside the *polymerase* reading frame were able to interfere with susceptibility. The results of in vitro phenotypic susceptibility assays using primary virus isolates showed that only those from subjects 1 and 2 showed a marked decrease in susceptibility compared to the wild-type HIV-1_{NL4-3}, and the fold-change was similar to that of integrase-recombinant viruses. In addition, coding regions other than those of integrase have limited impact on susceptibility to raltegravir. This is important, because it allows us to rule out the possibility that changes in the integrase substrate, ie, long terminal repeats (LTR), are responsible for virological failure in subjects who do not present genotypic resistance in the integrase coding region, although critical regions have been reported for integrase-end DNA interaction [84]. This data is consistent with the results of studies evaluating the role of polymorphisms in the LTRs in resistance to the investigational new drug, elvitegravir, which is an integrase inhibitor [138].

As for the viral replication capacity of recombinant viruses, we observed that defects in viral fitness are modulated by resistance mutations within protease, reverse-transcriptase and integrase, and that a cumulative effect exists between all three genes. This is consistent with previous data [17, 139]. It is noteworthy that the mutation G140S, which is found in the integrase coding region harboring Q148H, has been shown to have a compensatory effect on replication capacity and also to decrease susceptibility to raltegravir [40]. integrase-recombinant viruses harboring substitutions at positions G140S+Q148H+S230N did not show a marked decrease in

replication capacity, an observation that is consistent with the results of previous studies [140]. We show that there was a marked contribution from protease or reverse-transcriptase polymorphisms to compensate for integrase-associated viral replication defects. In addition, although the emergence of protease-reverse-transcriptase drug-resistant viruses has detrimental effects on viral fitness [17], we show that impairment of protease-reverse-transcriptase resistance mutations in viral fitness can be compensated by polymorphisms in other HIV-1 genes outside the drug-targeted enzymes. However, integrase polymorphisms were not able to compensate defects in replication capacity caused by protease-reverse-transcriptase resistance mutations. Overall, our data reveal an epistatic effect between HIV-1 genes in terms of ex vivo fitness; therefore, evaluation of viral fitness will be more representative if primary virus isolates rather than recombinant fragments are used.

There are several possible explanations why the regimen taken by subject 3 in Chapter 4 (no genotypic resistance in integrase) was unsuccessful despite the subject's low viral load. Productive replication in the absence of integrase function might be mediated by the illegitimate integration of HIV-1 into host chromosomes through cellular DNA recombination enzymes [141, 142]. Viral proteins such as Vpr have been shown to mediate transcription from unintegrated HIV-1, and this may also contribute to ongoing viral replication in vivo [143, 144]. Furthermore, selective drug pressures within the microenvironments of different anatomic compartments have led to the emergence of dominant quasispecies with different resistance levels [145, 146]. Therefore, raltegravir-susceptible viruses can be found in plasma if they are from the product of independent HIV-

integrase integration events or they are from a sanctuary site where raltegravir is unable to penetrate, thus enabling wild-type viruses to be released despite high levels of raltegravir in plasma. Both options may explain the low level of virological failure in subjects with no evidence of resistance mutations and a failed raltegravir regimen. Moreover, the low ex vivo fitness of viral isolates from subject 3 might explain his reduced plasma viral load. Thus, it has been suggested that subjects lacking genotypic resistance in integrase presented a low level of virological failure [101].

From a practical perspective, these data show that the use of integrase recombinant viruses will predict in vitro raltegravir susceptibility, and such phenotype will not differ from using *polymerase*-recombinant virus or primary isolates. However, the assessment of viral fitness will significantly vary among recombinant viruses containing different HIV-1 coding regions and/or viral isolates. From the clinical standpoint it is important to emphasize that any raltegravir-containing salvage regimens should also include as many other active drugs as possible to maximize their efficacy.

In summary, these results suggest an absence of epistatic effects between HIV-1 genes with regard to in vitro susceptibility to raltegravir. However, changes in protease-reverse-transcriptase and outside the *polymerase* coding region compensated the ex vivo replication capacity of viruses containing integrase resistance mutations. Therefore, raltegravir susceptibility might essentially be driven by resistance mutations within the integrase coding region, whereas other HIV-1 genes are involved in modulating viral fitness in subjects whose raltegravir-containing regimen fails.

DISCUSSION CHAPTER 5. HIV-1 REPLICATION AND IMMUNE DYNAMICS ARE AFFECTED BY RALTEGRAVIR INTENSIFICATION OF HAART-SUPPRESSED SUBJECTS

Whether residual viremia reflects ongoing viral replication at low levels or it reflects production of virus from stable reservoirs remains unclear. Importantly, this question has immediate clinical implication, because if residual viremia reflects ongoing viral replication, then the intensification of the current treatment will be useful for preventing viral evolution and the subsequent treatment failure. The integrase inhibitor raltegravir provide an opportunity to intensify current HAART because has provided better viral suppression than optimized background therapy. Then, intensification of the HAART with raltegravir could provide beneficial effects on the speed of elimination of the HIV-1 reservoir. We assessed in Chapter 5 if raltegravir intensification of HAART regimen was able to impact the levels of HIV-1 DNA and immune markers in subjects with undetectable viremia for at least 1 year.

We were able to detect a transient increase in 2-LTR circles at one or more time points in 29% of the subjects given raltegravir but only 5% of the controls. However, total and integrated HIV-1 DNA remained stable over time. Therefore, the virological impact of raltegravir intensification was only revealed by analysis of 2-LTR circle dynamics.

In addition, immune hyperactivation is a hallmark of HIV-1 infection [113], causing increased proliferation and apoptosis. Suppressive HAART reduce immune activation [147] but does not normalize it [148]. However, a causative link between active viral

replication and immune activation has not been previously established. Both the intensified and the control group had stable CD4+ and CD8+ T cell counts during the study. However, when we examined the relationship between immune activation and the observed increased in 2-LTR DNA forms, and we compared activation marker expression between 2-LTR^{+} and 2-LTR^{-} subgroups, we observed that the 2-LTR^{+} subgroup had higher percentages in activated CD8+ T cells than did 2-LTR^{-} subjects. Similarly, there was a higher concentration of plasma soluble CD14. In addition, longitudinal analysis showed a significant reduction in CD8+ T cell activation markers in the 2-LTR^{+} subgroup.

Overall, in this study raltegravir intensification revealed the presence of active replication in a relevant percentage (29%) of subjects on suppressive HAART. Raltegravir specifically prevents integration of linear viral cDNA to promote an increase in episomal cDNA formation. Since linear cDNA is a product of reverse transcription, the increase in episomal cDNA following raltegravir intensification requires the presence of infectious virus and also requires *de novo* infection and reverse transcription: which together, strongly suggest that active viral replication persists in HAART. This, together with the observation that, in subjects on sub-optimal antiviral regimens, 2-LTR circles undergo rapid evolution [115] indicates that episomes are labile *in vivo* and as such, are surrogate markers for ongoing infection. The fact that the increase in episomal cDNA was transient further suggests that raltegravir blocked active replication and production of infectious virions (**Figure 28**). However, other factors, such as CTL-targeting of cells containing transcriptionally active episomal HIV-1 cDNA, action of cell nucleases, or 2-LTR dilution due to cell proliferation might also play a role. Raltegravir

intensification did not alter levels of total or integrated viral cDNA. This is consistent with previous observations that, in contrast to episomal cDNA, most proviral DNA is archival and non-dynamic [115].

Although all subjects had undetectable plasma viremia by standard assays, the increase in 2-LTR circles was observed mainly in those intensified subjects on protease inhibitor-containing regimens. It is possible that the presence of three reverse-transcriptase inhibitors in protease inhibitor-sparing regimens reduce the probability of formation of the linear cDNA precursor to episomal cDNA. It is also possible that active replication occurs in anatomic compartments that are less accessible to protease inhibitors or that pharmacodynamic variability of protease inhibitors contributes to this observation. Furthermore, an inability to detect episomal cDNA in about 70% of the subjects in this study suggests that current HAART can suppress active replication in most individuals. This agrees with previous studies where there was no evidence for viral evolution in subjects on HAART [56, 108, 110].

Our study also reveals a causative relationship between active replication and immune activation in $CD8^+$ T-cells. The observed normalization of immune activation in subjects showing detectable increases in 2-LTRs after raltegravir intensification suggests that in HAART, active replication is a cause rather than a consequence of aberrant immune activation.

The extent of immune activation and plasma lipopolysaccharide levels are predictive of $CD4^+$ T-cell evolution in HIV infection [112, 149, 150]. Therefore, full suppression of viral replication may facilitate immune reconstitution [148] by reducing $CD8^+$ T-cell activation and increasing $CD4^+$ T-cell survival. Our observation that raltegravir can

impact both 2-LTR circle dynamics and immune activation indicates that these parameters could prove valuable in assessing the extent to which residual replication occurs in aviremic subjects on HAART. However, we cannot exclude the possibility that there is a spectrum of residual replication that at the lower range might include even individuals with undetectable 2-LTR circles.

Our study asks to what extent active replication contributes to viral persistence in HAART. For example, the longevity of the latent reservoir may be partly attributable to continual replenishment by virus produced by active replication. It could be argued that, in the presence of HAART, there is not a complete life cycle within individual, infected cells (i.e. a cell gets infected but does not make particles) and that the infectious particles are being made by a chronically infected cell that is simply manufacturing virions. However, even in this scenario, conditions would exist for sequence evolution and for viral reservoir replenishment. Therefore, intensification regimens that prevent active replication may truncate this replenishment and accelerate the decay of the reservoirs that persist in HAART.

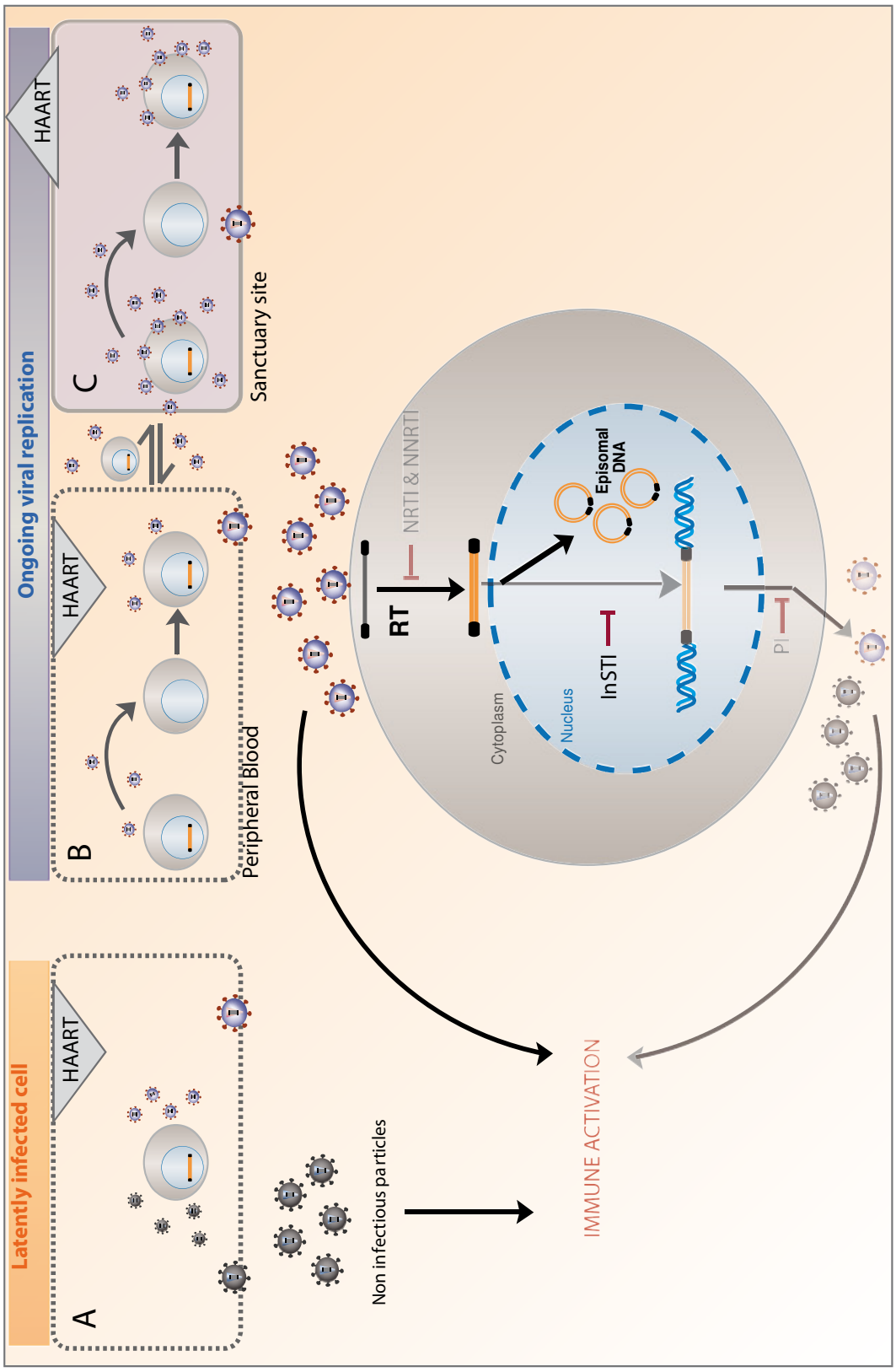


Figure 28. Impact of therapy intensification on active and latent viral reservoirs in HAART and relationship to immune activation

Figure 28. Impact of therapy intensification on active and latent viral reservoirs in HAART and relationship to immune activation. Scenario A, HAART stops viral replication but virus particles released from latently infected cells as infectious HIV particles in NNRTI-based regimens (non-infectious in protease inhibitor-based regimens) are unable to generate episomes or drive immune activation. Scenario B, assumes active replication as a consequence of incompletely suppressive HAART. Upon Raltegravir intensification, viral replication and immune activation are reduced. Scenario C, viral replication is maintained at sanctuary sites where there is incomplete drug penetration. Raltegravir penetrates this compartment and truncates ongoing replication.

Chapter 7

Conclusions

Objetive 1

To explore intrasubject longitudinal evolution of the HIV-1 integrase-coding region over a median of 10 years of heavy antiretroviral therapy in integrase inhibitor–naïve subjects. Also to explore changes in phenotypic susceptibility to raltegravir and replication capacity in those samples that accumulated the highest number of amino acid substitutions during the study period.

1.1 Although the HIV integrase is a highly conserved protein, we observe a slight genetic evolution within the integrase in subjects exposed to long-term integrase-sparing antiretroviral therapy.

1.2 Genotypic changes in the integrase, in absence of specific pharmacological pressure, do not restrict ex vivo fitness evolution of integrase-recombinant viruses. This observation is consistent with the natural evolution of viral populations that replicate in a non-selective environment.

1.3 There is no significant increase in phenotypic susceptibility to raltegravir in recombinant viruses from those subjects with the highest evolution rate in the integrase. Therefore, is unlikely that changes occurring in integrase might contribute to a poor virological response to raltegravir.

1.4 None of the amino acid substitutions which impact fitness occurs in relevant positions for integrase activity, suggesting that integrase could play an important role in viral fitness.

Objetive 2

To compare the relative contribution of integrase, protease-reverse-transcriptase and the rest of the HIV-1 genome on viral fitness and susceptibility to raltegravir in subjects with and without raltegravir-resistance associated mutations and whose raltegravir-containing regimen has failed.

2.1 There is an absence of epistatic effects between protease, reverse-transcriptase and integrase coding regions regarding in-vitro raltegravir susceptibility of whole and recombinant viruses. Therefore, raltegravir susceptibility is basically directed by resistance mutations within integrase coding region

2.2 Changes in protease-reverse-transcriptase and outside the *polymerase* coding region compensated the ex vivo replication capacity of viruses containing integrase resistance mutations

2.3 It seems to exist epistatic effects between HIV-1 genes in terms of ex vivo fitness; therefore, evaluation of viral fitness will be more representative if primary virus isolates rather than recombinant fragments are use

Objetive 3

To asses if raltegravir intensification of HAART regimen is able to impact the levels of HIV-1 DNA and immune markers in HAART-suppressed subjects with undetectable viremia.

3.1 The transient increase of 2-LTR circles suggest that raltegravir blocked active replication and production of infectious virus in 29% of subjects on a suppressive HAART.

3.2 Total and integrated HIV-1 DNA levels remained stable during intensification, indicating that most HIV-1 DNA is archival and nondynamic.

3.3 2-LTR circles are labile in vivo and, as such, are surrogate markers for ongoing infection.

3.4 2-LTR^{+} positive subjects showed higher levels of immune activation at baseline and a subsequent normalization after intensification.

3.2 Active replication was driven immune activation during a suppressive HAART. Therefore, active replication is cause rather than a consequence of aberrant immune activation.

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