UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE MEDICINA

DEPARTAMENTO DE MICROBIOLOGÍA

VARIANTES VIRALES DEL VIH-1 Y RESISTENCIAS A ANTIRRETROVIRALES EN LA COHORTE DE NIÑOS Y ADOLESCENTES INFECTADOS POR VIH-1 DE LA COMUNIDAD DE MADRID

TESIS DOCTORAL DE:

MIGUEL DE MULDER ROUGVIE

BAJO LA DIRECCIÓN DE:

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Madrid, 2013

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Variantes Virales del VIH-1 y Resistencias a Antirretrovirales en la Cohorte de Niños y Adolescentes Infectados por VIH-1 de la Comunidad de Madrid

Esta memoria ha sido presentada para optar al grado de Doctor en Microbiología Médica por la Universidad Complutense de Madrid por el licenciado:

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Vº Bº de la Directora de Tesis:

“Espero morir como he vivido, respetándome a mí mismo como condición para respetar a los demás y sin perder la idea de que el mundo debe ser otro y no esta cosa infame”

José Saramago

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ABREVIATURAS

µl	Microlitros
Ac	Anticuerpo
ADN	Ácido desoxirribonucleico
AF	Análisis filogenético
Ag	Antígeno
ARN	Ácido ribonucleico
ARNm	ARN mensajero
ARV	Antirretroviral(es)
CDC	Centro para el Control y Prevención de Enfermedades de EE.UU.
céls	Células
CEVIHP	Colaborativo Español para la Infección VIH Pediátrica
CHIPS	Cohorte Británica de Niños Infectados por VIH-1
CoRISpe	Cohorte de la Red de Investigación en SIDA pediátrico
CRF	Forma(s) recombinante(s) circulante(s)
CV	Carga viral del VIH-1
DBS	Muestras de sangre seca (*Dried blood spots*)
DHHS	Departamento de Sanidad y Servicios Sociales de EE.UU.
DT	Desviación típica
FI	Factor de impacto
HSH	Hombres que tienen sexo con hombres
IAS-USA	Sociedad Internacional sobre el SIDA de EE.UU.
IC	Intervalo de confianza
Ig	Inmunoglobulina
INT	Integrasa
IP	Inhibidores de la proteasa
ITIAN	Inhibidores de la transcriptasa inversa análogos de nucleós(t)ido
ITINAN	Inhibidores de la transcriptasa inversa no análogos de núcleos(t)ido

kb	Kilobase
LANL	Laboratorio Nacional de Los Álamos, EE.UU.
log	Logaritmo decimal
LTR	Secuencias largas repetidas
ml	Mililitros
MRA	Mutaciones de resistencia adquiridas
MRT	Mutaciones de resistencia transmitidas
NCBI	Centro Nacional de Información Biotecnológica, EE.UU.
NJ	Vecino más próximo (*Neighbour-joining*)
NS	Estadísticamente no significativo
OMS	Organización Mundial de la Salud
OPS	Organización Panamericana de la Salud
pb	Pares de bases
PCR	Reacción en cadena de la polimerasa
PENTA	Red Europea para el Tratamiento del SIDA Pediátrico
PR	Proteasa
RIS	Red de Investigación en SIDA
SIDA	Síndrome de la inmunodeficiencia adquirida
TAR	Tratamiento antirretroviral
TARGA	Tratamiento antirretroviral de gran eficacia
TI	Transcriptasa Inversa
UDI	Usuarios de drogas inyectadas
URF	Forma(s) recombinante(s) única(s)
VHB	Virus de la hepatitis B
VHC	Virus de la hepatitis C
VIH	Virus de la inmunodeficiencia humana
VIS	Virus de la inmunodeficiencia del simio

FÁRMACOS ANTIRRETROVIRALES FRENTE AL VIH

Inhibidores de la transcriptasa inversa análogos de nucleós(t)ido

ABC	Abacavir
ddI	Didanosina
FTC	Emtricitabina
3TC	Lamivudina
d4T	Estavudina
AZT, ZDV	Zidovudina
TDF, TNF	Tenofovir

Inhibidores de la transcriptasa inversa no análogos de nucleós(t)ido

EFV	Efavirenz
NVP	Nevirapina
ETR	Etravirina
RPV	Rilpivirina
DLV	Delavirdina

Inhibidores de la proteasa ("/r" indica coadministración con Ritonavir en baja dosis como potenciador farmacológico)

ATV/r	Atazanavir
DRV/r	Darunavir
FPV/r	Fosamprenavir
IDV/r	Indinavir
LPV/r	Lopinavir
NFV	Nelfinavir
SQV/r	Saquinavir
TPV/r	Tipranavir

Inhibidores de la entrada

T-20	Enfuvirtide
MVC	Maraviroc

Inhibidores de la integrasa

RAL	Raltegravir

AMINOÁCIDOS

A	Alanina
C	Cisteina
D	Ácido aspártico
E	Ácido glutámico
F	Fenilalanina
G	Glicina
H	Histidina
I	Isoleucina
K	Lisina
L	Leucina
M	Metionina
N	Asparagina
P	Prolina
Q	Glutamina
R	Arginina
S	Serina
T	Treonina
V	Valina
W	Triptófano
Y	Tirosina

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SUMMARY

The World Health Organization estimates that 2.5 million children below 15 years old were living with HIV by the end of 2012. During 2012, 350.000 children were infected with HIV and 250.000 died from AIDS related causes. In developed countries, mother-to-child transmission (MTCT) rates have decreased to 1-2%, mainly due to the expanded access to highly active antiretroviral treatment (HAART) and prevention efforts in HIV testing, prenatal care, formula feeding, elective caesarean delivery and pregnancy monitoring. However, MTCT still occurs in high-income countries, mainly due to infected immigrants from countries with a high HIV prevalence and within socially at-risk groups who have lower rates of pregnancy monitoring and HIV testing. In middle and low-income countries, the increasing access to services for preventing MTCT and the growing availability of antiretroviral therapy, have reduced the number of AIDS-related deaths and new HIV infections.

Access to treatment has dramatically changed the course infection, reducing morbidity, mortality, viraemia and increasing the life expectancy of HIV-infected children. Because of the use of HAART, an emergence of antiretroviral resistance among treated patients has been observed, mainly due to a poor adherence or sub-optimal drug dosing or regimens, limiting the therapeutic efficacy. Resistance genotyping has become a standard of care in HIV infection management. Although the clinical significance of HIV drug resistance has been well documented in the adult populations, studies on the prevalence and patterns of drug-resistance mutations in the paediatric HIV-infected populations remain limited.

HIV-1 group M is classified into 9 subtype variants (A, B, C, D, F, G, H, J, K) and multiple inter-subtype recombinants. Non-B subtypes and their recombinants are responsible for nearly 90% of the 34 millions infected subjects worldwide, being prevalent in Sub-Saharian Africa. Although they are a minority in industrialized countries, these HIV-1 strains are increasingly prevalent and rapidly spreading in Spain in new HIV-1 diagnostics among native and foreign population from endemic countries for these variants. The infection with non-B variants has epidemiological, clinical and therapeutical implications. HIV-1 diversity may confer distinct pathogenesis, impact in the disease progression and on HIV infection outcome. Furthermore, some HIV-1 variants present clade-specific substitutions in positions related to drug-resistance, which can accelerate the emergence of drug-resistant viruses, change or induce alternative pathways of resistance and influence the interpretation of genotypic resistance algorithms. Thus, the proper detection, description and monitorizing the spread of HIV-1 variants in representative cohorts is essential for keeping track of the epidemic.

The aims of this Thesis were: i) to summarize clinical, epidemiological and immunological features of the Madrid cohort of HIV-infected children and adolescents between 1993-2011; ii) to determine the prevalence of drug resistance mutations in viruses present in treatment *naïve* and treatment experienced patients and iii) to identify and characterize by phylogenetic analysis the HIV-1 infecting variant of each patient form the Madrid paediatric cohort. The Thesis was completed with the analysis and comparison of these parameters in other three paediatric cohorts from New York, Honduras and El Salvador.

Since the beginning of the epidemic in Spain, 530 patients have been registered in the Madrid cohort of HIV-infected children. By December 2011, 175 of them remained under clinical follow-up in paediatric units, 112 had been transferred to adult units, 62 had been lost to follow-up and 185 had died. Despite having a good clinical and immunological situation at sampling time, nearly all had a history of HIV-related symptoms, were heavily pretreated and referred a long history of treatment combinations. Drug resistance is a major obstacle for an effective treatment. Seven out of each ten pretreated patients from the Madrid cohort of HIV-1 infected children were infected by viruses harbouring resistance to at least one drug family. By drugs families, one third were resistant to protease inhibitors, over half of them were resistant to nucleoside reverse transcriptase inhibitors and less than one third were resistant to non-nucleoside reverse transcriptase inhibitors. Three drug family resistance was selected in one of each ten patients from the cohort. Over a 13% of all treatment naïve patients were resistant to at least one drug family. The overall prevalence of HIV-1 non-B infections among HIV-1 paediatric population from Madrid (10.9%) was similar to those previously published in other European paediatric cohorts and to those published in adult population in Spain. Nevertheless, an increasing complexity of HIV-1 infecting variants was observed during the study period. The determined prevalence of non-B infections among children from the Madrid cohort born before year 2000 was very low (4.2%), but in children born after year 2000 it reached a 39% of all new infections in the cohort.

The presented Thesis highlights the importance of an active surveillance of drug resistance prevalence among HIV-1 infected children that require a lifelong treatment and monitoring HIV-1 diversity to be aware of how this may impact on HIV infection outcome. Perinatally infected children in developed countries are reaching adulthood an therefore clinicians should consider that young adults infected during childhood do not present same clinical features as those young infected by other routes and that they require a specific clinical follow-up.

INTRODUCCIÓN

BIOLOGÍA DEL VIH

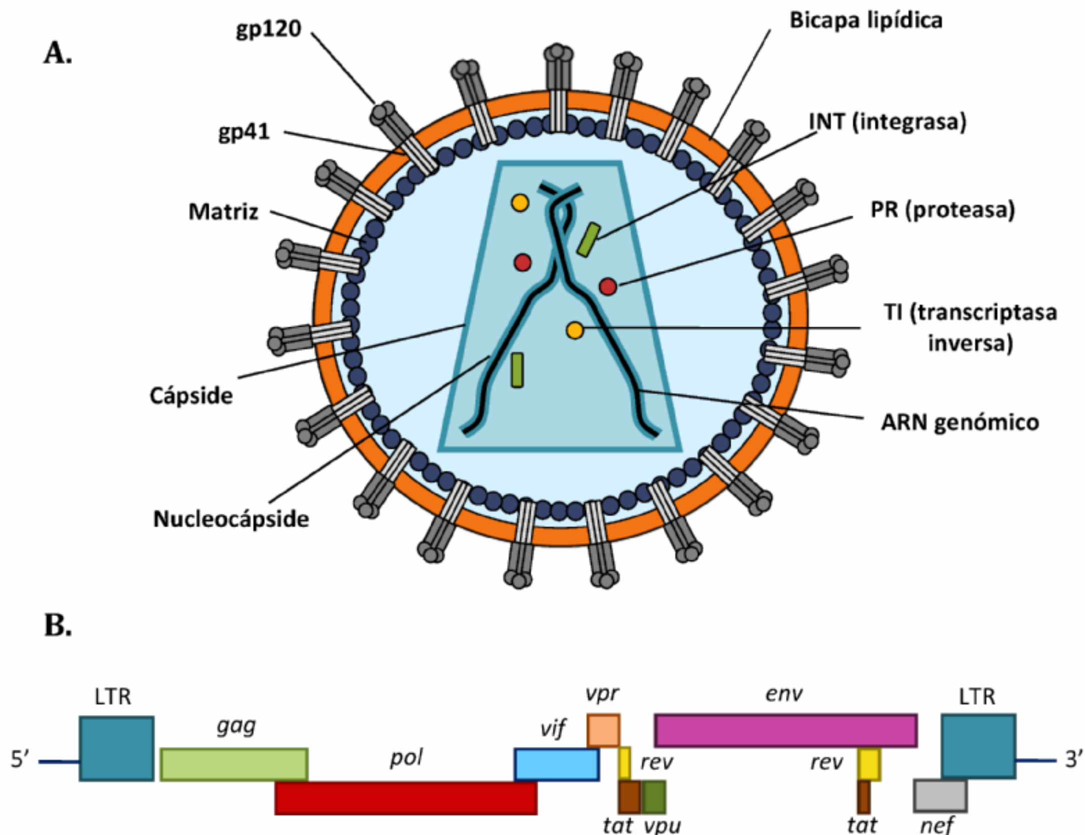
El virus de la inmunodeficiencia humana (VIH) es el agente infeccioso causante del síndrome de inmunodeficiencia adquirida (SIDA). Es un virus ARN clasificado dentro de la familia de los retrovirus humanos (*Retroviridae*) y perteneciente al género *Lentivirus*[1]. Su origen se remonta a múltiples transmisiones zoonóticas del virus de la inmunodeficiencia del simio (VIS) desde diversos primates no humanos que tuvieron lugar en África central y occidental a principios del siglo XX[2].

El VIH-1 tiene forma esférica con un diámetro de 100-120 nanómetros. El virión está constituido por una envoltura externa o bicapa lipídica tomada de la membrana de la célula humana infectada durante el proceso de salida de nuevas partículas virales por gemación. En la envoltura se encuentran la glicoproteína gp120 unida a la glicoproteína transmembrana gp41 en forma de trímeros. Bajo la envoltura se sitúa la matriz proteica y en el interior se encuentra la cápside icosaédrica. Dentro de ésta se localizan tanto las enzimas virales como el material genético del virus, constituido por dos cadenas sencillas de ARN de polaridad positiva de aproximadamente 9,8kb asociadas a las proteínas de la nucleocápside[3].

El genoma del VIH contiene tres genes principales: *gag, pol y env* que son comunes a todos los retrovirus. Adicionalmente, el VIH tiene genes encargados de codificar los componentes de la partícula vírica y de regular la expresión de los mismos. El gen *gag* codifica principalmente las proteínas estructurales que conforman la matriz, la cápside y la núcleocápside. El gen *pol* codifica a las proteínas virales proteasa (PR), transcriptasa inversa (TI) e integrasa (INT) que participan en la maduración viral, síntesis de ADN a partir del ARN del virus y en su integración en el genoma celular, respectivamente. El gen *env* codifica el precursor de las glicoproteínas de la envoltura. El VIH-1 posee además varios genes con capacidad reguladora (*tat*, *vif, vpr, vpu,* y *nef*)[4,5] esenciales para que se lleve a cabo el ciclo viral a través de las proteínas que codifican. En los extremos 5’ y 3’ del genoma se encuentran secuencias largas repetidas (LTR), que permiten la circularización e integración en el genoma celular. La estructura y organización genética del VIH se muestran en la **Figura 2.1.**

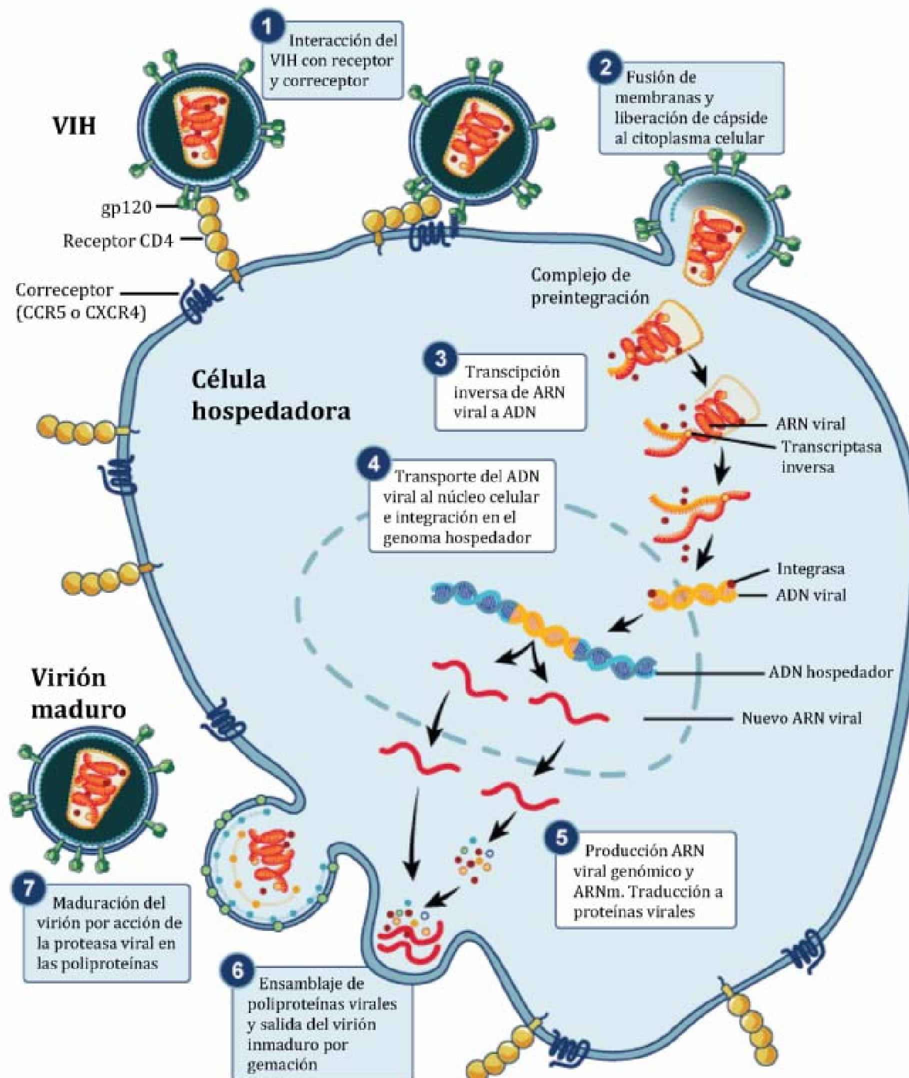
El ciclo infectivo y de replicación se puede resumir en las siguientes etapas (**Figura 2.2**). 1) Interacción entre el virión y su célula diana (linfocitos T CD4+ principalmente) por medio de la glicoproteína viral gp120, del receptor celular CD4, y de otras proteínas de membrana celulares que actúan como correceptores de virus, entre las que destacan CCR5 y CXCR4.

Figura 2.1. Estructura del virión (A) y genoma del VIH (B).



2) Fusión de la envuelta del virión con la membrana celular, con la consiguiente liberación en el citoplasma celular de la cápside viral. Una vez internalizada la partícula viral, la cápside se desensambla y libera el genoma viral. 3) Síntesis de ADN a partir del ARN viral o transcripción inversa, realizado por la TI. Una vez sintetizado, el ADN proviral se acopla a una serie de factores celulares y virales formando el complejo de preintegración. 4) Este complejo es transportado al núcleo, donde mediante la acción de la INT viral y las secuencias LTR se integra en el genoma celular, constituyendo la forma proviral del VIH. Una vez integrado, el VIH puede permanecer latente, replicarse de forma controlada o experimentar una replicación masiva, con el consiguiente efecto citopático sobre la célula infectada. 5) La replicación del VIH comienza mediante la transcripción del ADN proviral utilizando la maquinaria celular, produciendo ARN genómico viral y ARNm viral. Una vez en el citoplasma, el ARNm proporciona la información para la síntesis de proteínas virales. La traducción genera poliproteínas o precursores proteicos que deben ser procesadas en fragmentos funcionales. 6) Las proteínas virales procesadas son ensambladas y se produce el proceso de gemación a través de la membrana celular. 7) El último paso es la maduración por la acción de la proteasa viral que procesa las poliproteínas precursoras para formar el virión infectivo[6].

Figura 2.2. Ciclo replicativo del VIH.



Adaptado del *National Institutes of Allergy and Infectious Diseases*, EE.UU. (http://www.niaid.nih.gov/topics/HIVAIDS/Understanding/Biology/pages/hivreplicationcycle.aspx).

DIVERSIDAD GENÉTICA DEL VIH

Una de las principales características del VIH-1 es su gran heterogeneidad genética, debida a la combinación de su alta tasa de replicación y sus elevadas tasas de mutación y recombinación. Se calcula que se producen y destruyen entre 10^{10} y 10^{12} nuevos viriones cada 2,5 días, que es el tiempo transcurrido entre generaciones virales[7]. Mientras tanto, por cada ciclo replicativo se producen, aproximadamente, 0,3 sustituciones nucleotídicas y de 2 a 3 eventos de recombinación en cada cadena de ARN viral[8,9]. Las mutaciones, incluyendo inserciones y deleciones, permanecen en el genoma debido a que la TI carece de actividad

correctora de errores, siendo incapaz de eliminar los nucleótidos erróneamente incorporados durante la transcripción inversa. Todo ello resulta en la excepcional diversidad que se observa tanto en cada paciente como a nivel poblacional.

Dentro de un individuo infectado el virus se organiza como una población altamente heterogénea de distintas variantes con genomas íntimamente relacionados pero no idénticos. Esta estructura poblacional, llamada "cuasiespecie viral", se caracteriza por un proceso constante de generación de mutantes, por la competición entre ellos y por la acción de eventos de selección positiva y/o negativa en las variantes generadas. Estos procesos tienen como resultado la dominancia del genoma o genomas virales con mayor eficacia biológica o *fitness* (variantes mayoritarias) rodeado de un amplio espectro de mutantes (variantes minoritarias) con un extenso rango de fenotipos[10]. En teoría, todas estas variantes circulantes pueden integrarse en forma de provirus en las células y así estar representadas en el reservorio de linfocitos latentemente infectados. Si el medio en el que replican cambiase, por ejemplo por la respuesta inmunitaria o por la exposición a fármacos antirretrovirales (ARV), la selección positiva seleccionaría la variante de la cuasiespecie que llevara aquellos cambios que supusieran una ventaja competitiva en ese ambiente replicativo.

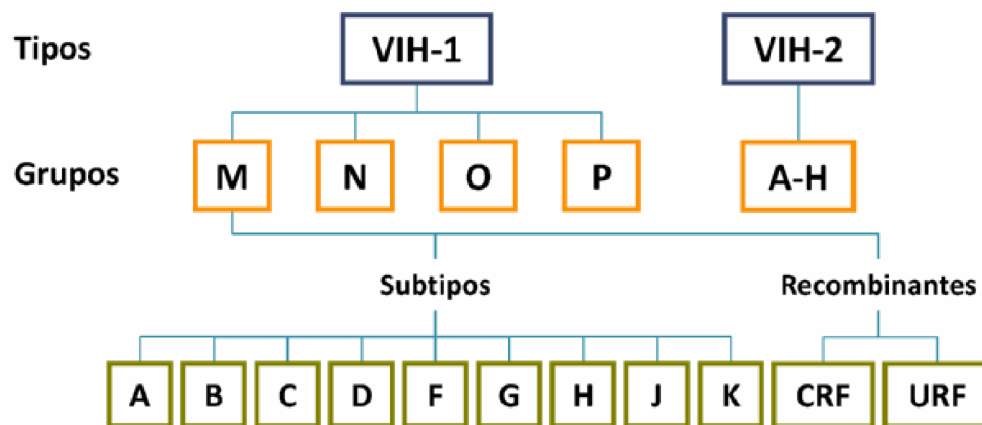
VARIANTES GENÉTICAS DEL VIH

A nivel global, la variabilidad del virus se traduce en la aparición de numerosas cepas (**Figura 2.3**), que son el objeto de estudio de la epidemiología molecular. Basándose en su homología genética, el VIH se clasifica en dos tipos: 1 y 2[11,12]. El VIH-1 es el más extendido y es el responsable de la mayor parte de los casos de infección por VIH en el mundo. El VIH-2, identificado en 1986[12], es más cercano filogenéticamente al VIS que al VIH-1 y parece ser menos patogénico y menos transmisible.

Basándose en su homología genética el VIH-1 ha sido clasificado en cuatro grandes grupos: grupo M (*main* o principal), grupo O (*outlier* o externo), grupo N (no-M, no-O) y el grupo P. Los grupos O, N y P presentan baja prevalencia y se encuentran restringidos a África subsahariana occidental y central o a casos aislados originarios de esa región. Solo el VIH-1 grupo M se ha expandido desde África, el origen de la pandemia, al resto de continentes. La distribución geográfica de los subtipos del grupo M es muy heterogénea y, poco a poco, los límites geográficos entre subtipos van desapareciendo. Los virus del grupo M, responsables del 97% de los cerca de 34 millones de infecciones estimadas a nivel mundial[13], han sido subdivididos

en nueve subtipos denominados por letras (A, B, C, D, F, G, H, J, K)[11] así como en recombinantes entre ellos. Los virus recombinantes o mosaico del VIH-1 llevan fragmentos genómicos de distintos subtipos de los viriones parentales. Estas formas recombinantes pueden ser circulantes (CRF, *circulating recombinant forms*) o únicas (URF, *unique recombinant forms*). Las CRF son recombinantes entre subtipos con secuencia completa y puntos de recombinación comunes y que han sido reconocidos en más de tres individuos infectados no relacionados epidemiológicamente. Hasta el momento se han descrito 58 CRF[14], cada uno designado por un número identificativo y por los subtipos genéticos que están presentes en su genoma. Los URF son recombinantes intersubtipo y se han encontrado en individuos aislados o en grupos de personas infectadas relacionadas epidemiológicamente, pero no comparten los puntos de recombinación entre subtipos observados en los CRF conocidos, lo que no permite agruparlos con ellos.

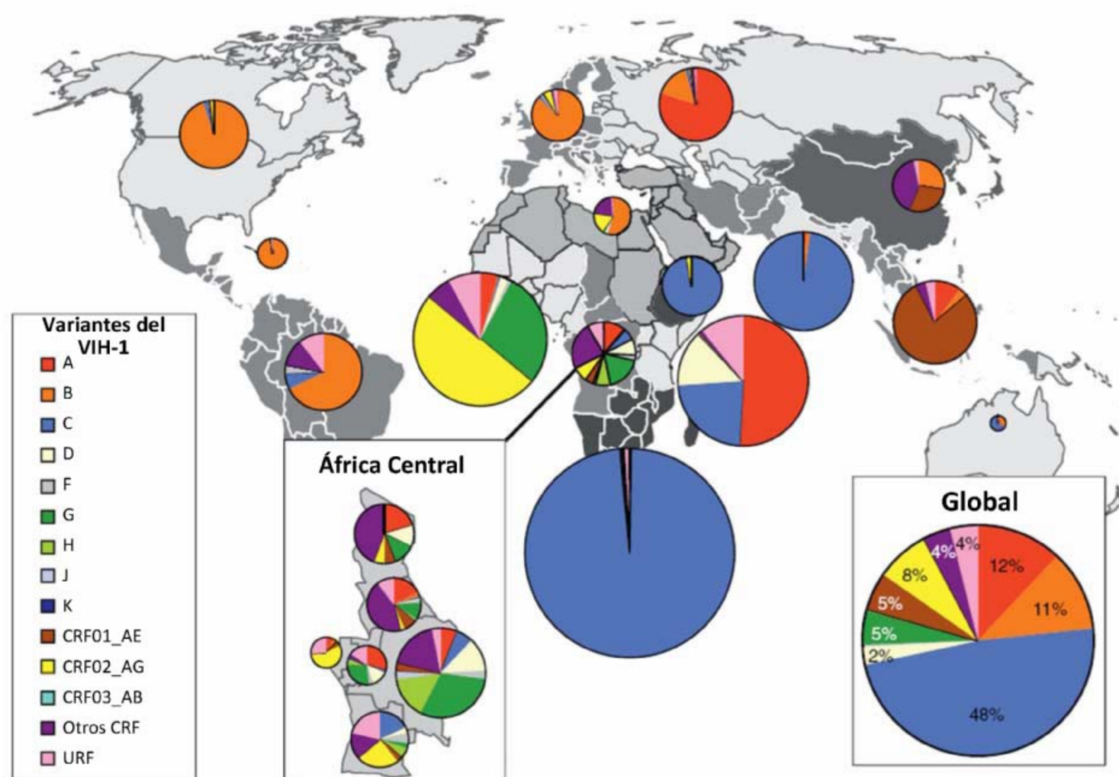
Figura 2.3. Esquema de la clasificación de las variantes del VIH.



La distribución mundial de las distintas variantes dentro del grupo M del VIH-1 es heterogénea[15] (**Figura 2.4**). El subtipo B del grupo M del VIH-1, que representa únicamente el 11% de las infecciones en el mundo, es el predominante en Europa occidental, Estados Unidos de América (EE.UU.) y Canadá. El subtipo B es el más estudiado y utilizado como modelo para el diseño y desarrollo de fármacos ARV, vacunas, ensayos diagnósticos y gran parte de la investigación básica en el VIH-1. Al resto de subtipos y formas recombinantes del grupo M del VIH-1 se les denomina “variantes no-B” del VIH-1. Las variantes no-B son mayoritarias globalmente y responsables del 89% de los 34 millones de infectados a nivel mundial. Así, en África central, por ser el epicentro de la pandemia, coexisten un gran número de variantes del VIH-1. La forma recombinante CRF02_AG es la más frecuente en África occidental. El subtipo C predomina en África del sur y oriental y también en la India. El subtipo A, en los países de la

antigua Unión Soviética y en ciertas regiones de África oriental. En el sudeste asiático la variante predominante es el recombinante CRF01_AE.

Figura 2.4. Distribución mundial de los subtipos y algunas formas recombinantes del VIH-1 entre 2004 y 2007.



Adaptado de Hemelaar *et al.*, 2011.

Una de las consecuencias de los movimientos poblacionales por intercambios comerciales, migración y turismo ha sido la introducción de variantes no-B del VIH-1 en países desarrollados donde la variante mayoritaria es el subtipo B. De hecho, la presencia de variantes no-B es cada vez mayor tras su introducción y posterior expansión entre pacientes inmigrantes y autóctonos. La proporción de subtipos no-B y formas recombinantes del VIH-1 entre los nuevos diagnósticos varía entre países, alcanzando incluso el 50% en Francia y Bélgica[16-20]. En España, la proporción de variantes no-B también está en alza, y hoy suponen el 10-15% de las infecciones por VIH[21-23]. Aunque la variante no-B más frecuente en España es el CRF02_AG, se han detectado casos de infección por todos los subtipos no-B del VIH-1 y múltiples formas recombinantes incluyendo recombinantes complejos[24]. De hecho recientemente se ha descrito en España que una de cada 10 nuevas infecciones en adultos se debe a variantes no-B del

VIH-1 y siete de cada 10 infecciones causadas por variantes no-B están causadas por cepas recombinantes[23].

En pacientes pediátricos la prevalencia de infección por variantes no-B del VIH-1 varía en función del país y del año de estudio. Así, en Francia se detectó un 36.4% de variantes no-B entre todos los nuevos diagnósticos de VIH en recién nacidos entre 1994 y 1996[25] y en la ciudad de Nueva York alcanzó el 16,7% entre 2001 y 2002[26]. Los primeros estudios de identificación de variantes no-B en población pediátrica infectada en Madrid se realizaron en niños de procedencia no española infectados entre 1988 y 2006[27].

DIAGNÓSTICO DE LA INFECCIÓN POR VIH EN PEDIATRÍA

La infección por VIH es asintomática o cursa con sintomatología inespecífica durante los 2-3 primeros meses de vida. Por ello, el diagnóstico precoz de la infección VIH debe basarse en pruebas de laboratorio. El diagnóstico de la infección por VIH en niños infectados por transmisión vertical menores de 18 meses requiere el uso de pruebas virológicas que detecten directamente el virus, su genoma o sus proteínas, ya que los anticuerpos específicos frente al VIH (IgG) transferidos por vía transplacentaria por su madre pueden interferir en la interpretación de las pruebas serológicas[28].

El diagnóstico de la infección por VIH-1 en los niños mayores de 18 meses se realiza mediante serología como en el adulto, mientras que en los menores de 18 meses se utilizan ensayos virológicos. Entre ellos se encuentra la amplificación del ADN proviral mediante la reacción en cadena de la polimerasa (PCR), cuya sensibilidad aumenta con la edad desde el 40% durante la primera semana de vida hasta el 96% a partir del mes de vida (con especificidad del 99%) y/o la amplificación por PCR del ARN viral libre en plasma. Esta última es la técnica que se usa en la mayoría de los centros empleando técnicas comerciales. Su sensibilidad también aumenta con la edad: 25-40% durante la primera semana, incrementando a partir de la tercera semana de vida hasta alcanzar el 90-100% a partir de los 2-3 meses[29].

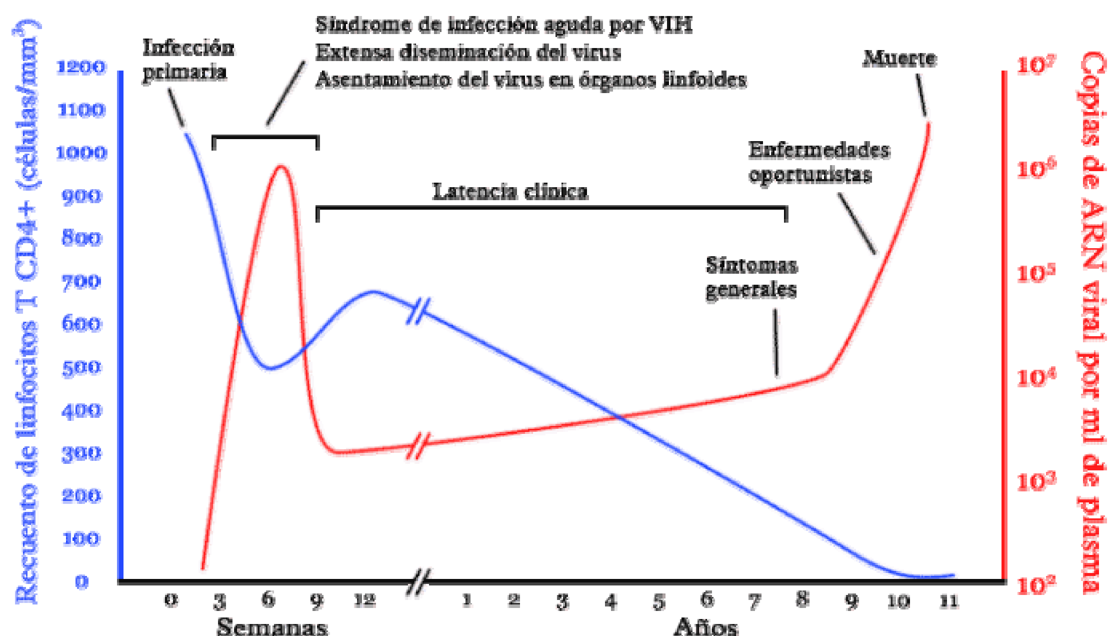
CURSO NATURAL DE LA INFECCIÓN

La infección por VIH se clasifica en diversas etapas, identificadas por un conjunto de síntomas e indicadores clínicos. La fase aguda se inicia en el momento del contagio. En un plazo de días, el VIH infecta tanto a las células expuestas como a los ganglios linfáticos. Durante ese tiempo, el VIH se multiplica dentro del organismo hasta alcanzar niveles muy

elevados. A continuación ocurre la fase crónica o de latencia clínica. En ella el portador es asintomático, ya que el sistema inmune tiene una gran capacidad para regenerar las células destruidas por el virus, aunque la infección viral termina por desgastar al sistema inmunológico.

En ausencia de tratamiento antirretroviral (TAR), la mayoría de los pacientes desarrollan SIDA en 5-10 años. A causa de esto, aumenta la carga viral (CV) y disminuye la capacidad de recuperación del sistema inmune. En la etapa crítica final de la infección por VIH o etapa SIDA el portador posee un sistema inmunológico muy debilitado y una reducida capacidad citotóxica hacia el virus. Aumentan las tasas de replicación del virus, disminuyendo la capacidad de reacción ante otros agentes causantes de enfermedades. De esta manera, el sujeto es presa potencial de numerosas infecciones oportunistas que le pueden conducir a la muerte. En ausencia de TAR, el virus se replica constantemente e infecta los linfocitos T CD4. La mayoría de los pacientes que han desarrollado SIDA no sobreviven más de 3 años sin recibir TAR. Sin embargo, incluso en esta fase crítica el VIH puede ser controlado mediante TAR. La progresión de la enfermedad puede variar debido a factores asociados al virus y a factores genéticos e inmunológicos del hospedador, existiendo progresores rápidos y lentos[28].

Figura 2.5. Curso natural de la infección por VIH.



PARTICULARIDADES DE LA INFECCION POR VIH EN PEDIATRÍA

El curso natural de la infección por VIH en edad pediátrica tiene particularidades propias[30]:

-La mayoría de niños se infectan por transmisión vertical, en una etapa de pleno desarrollo y diferenciación celular. El VIH provoca un grave trastorno de la inmunidad, que se manifiesta clínicamente por infecciones graves y por una mayor predisposición para el desarrollo de algunas neoplasias.

-En ausencia de TAR la progresión de la enfermedad es más rápida en niños infectados por transmisión vertical que en adultos, adolescentes y que en niños infectados por otras vías como la transmisión sexual o parenteral[31,32]. De hecho, entre el 15-25% de los niños con infección por VIH-1 adquirida por transmisión vertical presentan una forma de enfermedad con peor pronóstico, caracterizada por infecciones bacterianas graves y por enfermedades definitorias de SIDA antes de cumplir los 12 meses de vida, con una supervivencia inferior a 2 años[33].

-Los niños que adquieren la infección en las últimas semanas del embarazo o en el momento del parto son asintomáticos al nacer. Los recién nacidos con síntomas clínicos que sugieren infección por VIH-1 tienen un elevado riesgo (40%) de progresar a SIDA antes del año[34].

-El periodo de incubación de la infección VIH por transmisión vertical dura entre 4-5 meses de media. La sintomatología clínica es inespecífica y está asociada a una escasa ganancia de peso o detención del crecimiento[35].

-La edad media de progresión a SIDA en pacientes con infección VIH por transmisión vertical en ausencia de TAR es de 4 a 6 años, frente a los 9,8-15 años de los adultos. El riesgo de progresar a SIDA es especialmente alto durante el primer año de vida (10-30%) y disminuye a partir de entonces al 2-3 % anual[32,36,37].

-En ausencia de TAR, la mortalidad asociada al VIH es elevada durante el primer año (6-16%), decreciendo un 3,5% anual hasta los 7 años, para incrementarse de nuevo a partir de esta edad. La supervivencia media de los pacientes pediátricos que no recibieron TAR se sitúa entre los 7 y los 8 años[38,39].

-El riesgo de padecer fracaso virológico a los cinco años de comenzar el TAR es el doble en niños que en adultos[40].

PARÁMETROS PARA GUIAR EL TRATAMIENTO ANTIRRETROVIRAL

Los principales parámetros a evaluar durante el seguimiento del TAR son el número y porcentaje de linfocitos T CD4+, la CV plasmática, la detección de resistencias, la concentración plasmática de fármacos ARV, la determinación del tropismo viral, la evaluación del nivel de adherencia al TAR y la caracterización de la variante genética infectante.

Linfocitos T CD4

El principal efecto de la infección por el VIH es una progresiva pérdida del número de linfocitos T CD4 en sangre periférica y tejidos linfoides[41]. El valor predictivo de los linfocitos T CD4 es menor en recién nacidos y niños pequeños, debido al solapamiento en los primeros meses de vida entre progresores rápidos y lentos. Para monitorizar la progresión de la infección en niños menores de 6 años es más útil emplear el porcentaje de linfocitos T CD4 que su número total, ya que éste último varía en la infancia hasta los 6 años. A partir de esa edad el valor absoluto de linfocitos T CD4 es el marcador de progresión clínica más usado[42]. Trabajos recientes recomiendan utilizar el número total de linfocitos T CD4 y no su porcentaje al iniciar TAR, puesto que los últimos parecen tener menor valor pronóstico[43].

En presencia de TAR, se observa un incremento mantenido de linfocitos T CD4[44]. Estos cambios cuantitativos están acompañados de cambios cualitativos en la respuesta inmune y de una disminución del riesgo de padecer infecciones oportunistas[45,46]. La forma más práctica de valorar la restauración inmune es medir el incremento del número de linfocitos T CD4+ que ocurre desde las primeras semanas de TAR y que suele ser lento, pero constante en el tiempo. Durante el primer año de TAR debería existir un aumento mínimo de 50-100 linfocitos T CD4/mm^3, pero no es raro observar una discordancia entre las respuestas virológica e inmunológica. Así existen, pacientes que mantienen una cifra de linfocitos T CD4 estable tras la administración del TAR y otros en los que disminuye a pesar de tener una CV indetectable[47]. En población pediátrica infectada y asintomática debe medirse la cifra de linfocitos T CD4 cada 3-6 meses[48].

Monitorización de la carga viral

La determinación de la CV plasmática es un parámetro fundamental para evaluar la respuesta al TAR, cuyo objetivo es alcanzar la supresión de la replicación viral y valores indetectables de viremia (<50 copias de ARN-VIH-1/ml). Esta cifra es el límite de detección de la mayoría de las técnicas comerciales para cuantificar la viremia, si bien las más modernas

detectan hasta 20 copias/ml o incluso menos, valores con los que se asume que no se seleccionan mutaciones de resistencia[49]. Es conveniente medir la CV plasmática a las cuatro semanas de haber iniciado del TAR para comprobar la respuesta virológica y como medida indirecta de adherencia. Se debe monitorizar la CV cada 3-4 meses en todo niño infectado[28,29]. Sin embargo, la frecuencia de la determinación de la CV puede incrementarse si se observa deterioro clínico, inmunológico o virológico, o para confirmar un valor no esperado[28,29].

Debido a la alta carga viral basal en niños (de 10.000 a 10.000.000 copias de ARN-VIH-1/ml), éstos pueden tardar más de 24 semanas en alcanzar la supresión de la replicación viral en plasma tras la administración de TAR. El fracaso terapéutico se define como una respuesta inadecuada al TAR ya sea clínica, inmunológica o virológica. Se sospechará fracaso terapéutico si tras alcanzar la supresión viral la CV vuelve a ser detectable o si una vez iniciado el TAR se dan dos determinaciones consecutivas detectables. En caso de aparecer virus con mutaciones de resistencia, el fracaso terapéutico se considerará virológico. El fracaso virológico es el más precoz, y viene definido por la respuesta virológica incompleta o rebrote de la carga viral persistente o repetido después de haber conseguido una CV indetectable. Para el fracaso inmunológico se consideran 2 situaciones: la respuesta inmunológica incompleta al TAR y el descenso del recuento o porcentaje de linfocitos CD4 durante el mismo. El fracaso clínico se define como la aparición de una manifestación clínica y progresión de la enfermedad en pacientes que han recibido TAR un mínimo de seis meses

Estudio de resistencias a fármacos antirretrovirales

La detección de variantes virales resistentes a los fármacos puede realizarse mediante técnicas genotípicas o fenotípicas[50]. Los ensayos genotípicos detectan cambios específicos en la región genética viral que codifica las proteínas diana de los fármacos ARV (TI, PR, INT y proteínas de la envoltura). Las fenotípicas determinan la respuesta de la población viral mayoritaria a concentraciones crecientes de los distintos fármacos ARV.

La interpretación apropiada de los estudios genotípicos se desarrolla mediante algoritmos computarizados que requieren una actualización continuada. En Internet se puede acceder a excelentes bases de datos públicas para la interpretación de genotipo: http://engine.euresist.org/data_analysis/viral_sequence/new, http://www.geno2pheno.org, http://www.retic-ris.net/default_principal.asp?idx=&cidioma=2 y http://sierra2.stanford.edu/sierra/servlet/JSierra, entre otras. También se han identificado las

mutaciones más relevantes asociadas a resistencia para pacientes no expuestos (*naïve*)[51] y para aquellos con experiencia previa al TAR[52] (**Tabla 2.1**).

La transmisión de variantes del VIH-1 resistentes a ARV ha sido ampliamente documentada y esta transmisión se ha asociado a fracaso al TAR[53,54]. Estos mutantes resistentes pueden quedar archivados por meses o años en el ADN proviral en el genoma de la célula hospedadora y emerger al ser seleccionados por el TAR[55]. Por ello es recomendable realizar un ensayo de resistencia en todo paciente no tratado. En esta situación es preferible un ensayo de resistencia genotípico porque, además de determinar la presencia de mutaciones a ARV, nos permite identificar el subtipo viral. En un paciente que recibe TAR con fracaso virológico, la no detección de mutaciones obedece mayoritariamente a falta de adherencia al TAR. Para la determinación genotípica de resistencias fármacos antirretrovirales existen técnicas comerciales que amplifican mediante PCR el gen *pol* del VIH-1 (*ViroSeq® HIV-1 genotyping system* de *Abbott Molecular* y *TruGene® HIV-1 genotyping assay* de *Siemens Healthcare Diagnostics*). También existen métodos no comerciales de amplificación basados en PCR caseras, de menor coste.

Concentraciones plasmáticas de fármacos antirretrovirales

La monitorización terapéutica de fármacos ARV consiste en la determinación de sus niveles terapéuticos. Es una estrategia por la cual la dosis de un fármaco se modifica en función de su concentración plasmática con el objetivo de mantenerla dentro de unos límites terapéuticos previamente definidos, mejorando su eficacia terapéutica y evitando su toxicidad[56,57]. La monitorización de las concentraciones plasmáticas de los fármacos ARV permite optimizar el TAR y ajustar las dosis administradas para obtener la máxima eficacia con la mínima de toxicidad. Las dosis de fármacos ARV deben ser ajustadas a la edad y grado de maduración de los pacientes, debido a la diferente farmacocinética en función de éstos parámetros. Por lo general las dosis que requieren los pacientes pediátricos son más elevadas, en proporción al peso o superficie corporal, que las establecidas para los adultos, excepto en el periodo neonatal, en el que la información sobre la eficacia del TAR es menor, sobre todo en prematuros.

La monitorización de los niveles plasmáticos de fármacos ARV permite monitorizar la adherencia, evaluar la respuesta terapéutica, evitar procesos de toxicidad por sobredosificación, evitar la selección de resistencias por concentraciones subóptimas, optimizar las dosis de fármacos de acuerdo al perfil genotípico de la cepa infectante y prevenir

Tabla 2.1. Listados de mutaciones de resistencia a antirretrovirales en pacientes *naïve* (OMS, 2009) y pretratados (IAS-USA, 2011).

Listado OMS actualización 2009[51]			Listado IAS-USA actualización 2011[52]			
IP	ITIAN	ITINAN	IP *primarias*	IP *secundarias*	ITIAN	ITINAN
L23I	M41L	L100I	D30N	L10V/I/R/F/C	M41L	V90I
L24I	K65R	K101E/P	V32I	V11I	A62V	A98G
D30N	D67N/G/E	K103N/S	M46I/L	G16E	K65R	L100I
V32I	T69D/ins	V106M/A	G48V	K20R/M/I/T/V	D67N	K101E/H/P
M46I/L	K70R/E	V179F	I47V/A	L24I	T69ins	K103N
I47V/A	L74V/I	Y181C/I/V	I50L/V	L33I/V/F	K70R/E	V106M/A/I
G48V/M	V75M/T/A/S	Y188L/H/C	I54M/L	E34Q	L74V	V108I
I50V/L	F77L	G190A/S/E	Q58E	M36I/L/V	V75I	E138A/G/K
F53L/Y	Y115F	P225H	T74P	K43T	F77L	V179D/F/T
I54V/L/M/A/T/S	F116Y	M230L	L76V	F53L/Y	Y115F	Y181C/I/V
G73S/T/C/A	Q151M		V82A/T/F/S/L	I54V/T/A/S	F116Y	Y188C/L/H
L76V	M184V/I		N83D	D60E	Q151M	G190S/A
V82A/T/F/S/C/M/L	L210W		I84V	I62V	M184V/I	P225H
N83D	T215Y/F/rev		N88S	L63P	L210W	M230L
I84V/A/C	K219Q/E/N/R		L90M	I64L/M/V	T215Y/F/rev	
I85V				H69K/R	K219Q/E	
N88D/S				A71V/I/T/L		
L90M				G73C/S/T/A		
				V77I		
				V82I		
				N83D		
				I85V		
				N88D		
				L89M/V/I		
				I93L/M		

En azul, sustituciones sólo presentes en uno de los listados.

interacciones entre fármacos. Además permite identificar aquellos niños que por su perfil farmacocinético podrían beneficiarse de una simplificación del TAR. Por último, ayuda a determinar la concentración adecuada en fármacos nuevos o no aprobados para su uso pediátrico[58-60].

Determinación del HLA-B*5701

Se ha demostrado que los pacientes con antígeno leucocitario humano (HLA) B*5701 tienen un mayor riesgo de presentar una hipersensibilidad al TAR con Abacavir (ABC), fármaco inhibidor de la TI viral. Debido a que la prevalencia de B*5701 varía en la población, es importante realizar un cribado en pacientes infectados por el VIH que van a iniciar TAR con este fármaco[(61,62)]. La determinación de este genotipo es clave en la decisión del TAR a instaurar en el niño infectado. Ello justifica el realizar un ensayo farmacogenético para prevenir la toxicidad del efecto de ABC en niños con HLA-B*5701[63,64]. La clínica de la reacción de hipersensibilidad es inespecífica, ocurre generalmente dentro de las primeras 6 semanas de iniciar el TAR y obliga a retirar definitivamente el fármaco sin posibilidad de reintroducirlo.

Determinación del tropismo viral

CCR5 y CXCR4 son dos correceptores de quimiocinas presentes en células inmunitarias necesarios para la entrada del VIH en la célula. El uso de uno u otro de estos correceptores por parte del virus define su tropismo. De esta manera hay virus R5, virus X4, o virus mixtos R5X4 que pueden usar ambos correceptores. Actualmente existe una nueva generación de fármacos que son antagonistas de CCR5 y que presentan una actividad anti-VIH específica y potente[65-67] pero no aportan ningún beneficio en aquellos con tropismo X4. La determinación del tropismo viral previo al inicio del TAR con antagonistas de CCR5 es clave para la administración de este tipo de fármacos. Para ello se necesita hacer un estudio fenotípico o genotípico del tropismo viral en los pacientes candidatos a tomar un fármaco antagonista de CCR5. El ensayo fenotípico *TROFILE®* de *Monogram Biosciences* es el único validado clínicamente para identificar el tropismo por el correceptor CCR5 en pacientes infectados por el VIH[68,69]. Sin embargo, existen otros métodos genotípicos, aún no comercializados, con los que también se determina el tropismo viral.

Determinación de la adherencia al tratamiento

Se entiende por adherencia al TAR a la capacidad del paciente para implicarse correctamente en la elección, inicio y cumplimiento del mismo, a fin de conseguir una adecuada supresión de la replicación viral. El control virológico depende de múltiples factores,

pero la adherencia incorrecta es la principal causa de fracaso terapéutico, relacionándose con una mala respuesta virológica, una peor reconstitución inmune y un mayor riesgo de mortalidad[70]. Por tanto, es muy importante que los pacientes sean conscientes de su enfermedad, entiendan claramente el objetivo del TAR, participen en la decisión de iniciarlo, se sientan capaces de cumplir dicho tratamiento y comprendan la enorme importancia que tiene una toma continuada y correcta de la medicación[71]. En el contexto de la infección pediátrica se debe incidir aún más en un correcto cumplimiento en la toma de medicación tanto en niños como en adolescentes. Esta atención especial debe ser llevada a cabo por padres y/o tutores, siendo reforzada por el clínico[72].

Identificación de la variante viral infectante

La correcta identificación de subtipos y formas recombinantes del VIH-1 es de gran relevancia para entender problemas potenciales en el diagnóstico genético y serológico[73], en el uso de terapia antirretroviral[74], en la cuantificación de la viremia[75-77], en la interpretación genotípica de las mutaciones de resistencia[78-80] y para comprender la prognosis de la enfermedad[81].

Las técnicas diagnósticas, ya sean directas o indirectas, pueden verse afectadas por la variabilidad viral[73]. La detección directa mediante PCR requiere de sondas u oligonucleótidos específicos para amplificar la región diana del virus, que al estar diseñados especialmente para detectar cepas del subtipo B, pueden ser responsables de falsos negativos en ensayo de diagnóstico cuando se trata de detectar subtipos no-B o recombinantes genéticamente diferentes de la cepa B. La variabilidad genética del VIH también puede ser la responsable de fallos en la cuantificación del VIH que presentan las técnicas comerciales al determinar la CV en un paciente infectado por variantes no-B[75-77].

Los subtipos no-B y recombinantes del VIH-1 poseen de manera natural un alto grado de polimorfismos en posiciones concretas de la PR, TI e INT asociados a resistencia a los ARV. La presencia de dichos polimorfismos naturales puede modular la capacidad replicativa del virus[82], conducir a una sensibilidad reducida a esos compuestos[83-85], seleccionar nuevas vías de adquisición de resistencia[78,86], o una adquisición más rápida de la misma[87]. Ello podría comprometer la eficacia terapéutica de estos fármacos en pacientes infectados con variantes no-B. Incluso la presencia de polimorfismos naturales en posiciones de resistencia puede modificar la interpretación de los algoritmos de interpretación de resistencia genotípica[80,88]. Los polimorfismos naturales en la PR podrían reducir la barrera genética para seleccionar

resistencia a ciertos fármacos ARV[89] y las afinidades de unión a los fármacos[90]. Por otro lado, algunos polimorfismos naturales en la PR podrían conferir una gran susceptibilidad a IPs en subtipos no-B del VIH-1[79,91]. El VIH-2 es presenta de manera natural resistencia a determinados fármacos[92,93].

A pesar de que las diferencias biológicas entre subtipos genéticos necesitan aún ser clarificadas, ya se ha confirmado su influencia en la progresión de la enfermedad. Así pacientes infectados por ciertos subtipos no-B progresan más rápidamente a SIDA y mueren antes que otros[81]. Por ello, la presencia de subtipos no-B del VIH-1 no sólo tiene implicaciones en el ámbito epidemiológico, sino también posiblemente importantes repercusiones clínicas y biológicas. La caracterización de la variante viral infectante debe realizarse mediante análisis filogenético (AF), ya que las técnicas de subtipaje online pueden presentar importantes limitaciones a la hora de identificar variantes distintas del subtipo B[94,95].

CLASIFICACIÓN CLÍNICA E INMUNOLÓGICA DE LA INFECCIÓN POR VIH EN PEDIATRIA

Los Centros para el Control y Prevención de Enfermedades (CDC) de EE.UU. definieron en 1994 un sistema de clasificación de la enfermedad VIH para menores de 13 años, que tiene en cuenta tanto la situación clínica como inmunológica de los niños[96] (**Tabla 2.2**). Se definen 4 categorías clínicas (**Tabla 2.3**) mutuamente excluyentes: N (para pacientes asintomáticos o con una única manifestación de las consideradas en el siguiente estadio); A para definir a los pacientes con sintomatología leve, que incluye la mayoría de manifestaciones inespecíficas de la infección VIH; B sintomatología moderada; C que define los pacientes con formas más graves de enfermedad o criterios diagnósticos de SIDA.

Los pacientes suelen progresar de una categoría a otra de forma escalonada, aunque pueden pasar directamente de categoría N o A a la C. Esta misma clasificación tiene en cuenta la situación inmunológica. Para ello establece tres categorías en función de la edad y el porcentaje y/o número absoluto de linfocitos T CD4. Por lo tanto los pacientes pediátricos se clasifican utilizando la letra que define su situación clínica y el número que determina su situación inmunológica.

Tabla 2.2. Clasificación clínico-inmunológica de los niños menores de 13 años infectados por VIH.

Categorías clínicas e inmunológicas				
Clínicas	**N** Asintomática	**A** Síntomas leves	**B** Síntomas moderados	**C** Síntomas graves
Inmunológicas				
Sin inmunoderpresión	N1	A1	B1	C1
Inmunodepresión moderada	N2	A2	B2	C2
Inmunodepresión grave	N3	A3	B3	C3

Inmunológicas	**<12 meses**	**1-5 años**	**6-12 años**
1 CD4	>1.500 céls/mm^3, >25%	>1.000 céls/mm^3, >25%	>500 céls/mm^3, >25%
2 CD4	750-1.499 céls/mm^3, 15-24%	500-999 céls/mm^3, 15-24%	200-499 céls/mm^3, 15-24%
3 CD4	<750 céls/mm^3, <15%	<500 céls/mm^3, <15%	<200 céls/mm^3, <15%

Tabla 2.3. Categorías clínicas según grado de inmunosupresión en niños menores de 13 años infectados por VIH.

Estadio I o Asintomático (O)
Asintomático
Linfadenopatía generalizada
Estadio II o Moderamadente sintomático (A)
Hepatoesplenomegalia persistente, erupción prurítica popular, infección fúngica de uñas, quielitis angular, eritema gingival lineal, verrugas extensas, molusco contagioso extenso, ulceraciones orales recurrentes, engrosamiento parotídeo no explicado, herpes zóster, infección respiratoria recurrente.
Estadio III o Enfermedad Avanzada (B)
Malnutrición moderada no explicada, diarrea persistente no explicada, fiebre persistente no explicada, candidiasis oral, leucoplaquia vellosa, tuberculosis ganglionar, tuberculosis pulmonar, neumonía bacteriana recurrente, gingivoestomatitis o parotiditis necrotizante aguda, neumonía intersticial linfoide, enfermedad pulmonar crónica, anemia (Hb<8g/dl), neutropenia (<500), trombopenia (<50.000) no explicadas.
Estadio IV o Enfermedad definitoria de SIDA (C)
Síndrome de emaciación grave (*wasting*), tuberculosis extrapulmonar, infección bacteriana grave recurrente, infección crónica por herpes simples, encefalopatía progresiva, neuropatía por VIH, miocardiopatía por VIH, sarcoma de Kaposi u otra neoplasia.

PECULIARIDADES DEL TRATAMIENTO ANTIRRETROVIRAL PARA NIÑOS Y ADOLESCENTES

Los objetivos de la TAR son conseguir una supresión completa y duradera de la replicación viral, preservar o restaurar la función del sistema inmunológico, minimizar la toxicidad a corto y largo plazo, minimizar la selección de resistencias, reducir la morbilidad y mortalidad asociada a la infección y mejorar de la calidad de vida. El TAR para población pediátrica presenta características propias, como son la menor disponibilidad de fármacos aprobados en

la edad pediátrica, el mal sabor de muchos de ellos que dificulta considerablemente su administración y las interacciones con otros fármacos. El desarrollo de nuevos compuestos ARV en niños sufre retraso respecto a los desarrollados para adultos. Los pacientes pediátricos presentan farmacocinéticas diferentes que varían con la edad. Los resultados de ensayos clínicos en adultos no son extrapolables a niños y existen diferencias en tolerancia y tolerabilidad. En niños la adherencia es más difícil de mantener y deben atravesar etapas críticas como la adolescencia. Por otro lado, dado la cronicidad del tratamiento en pacientes VIH, los niños infectados van a recibir a lo largo de su vida múltiples tratamientos debido a la aparición de efectos secundarios, toxicidad, resistencia virológica y falta de adherencia. Ello aumenta el riesgo de que sufran eventos de fracaso terapéutico virológico por la aparición de virus resistentes a los ARV.

Al normalizar la función inmune, se previenen las infecciones oportunistas y la progresión de la enfermedad. Desde que se comenzó a tratar pacientes pediátricos infectados por VIH con terapia antirretroviral de gran actividad (TARGA) se observó una mejoría clínica espectacular, aumento de peso, disminución de infecciones bacterianas, descenso de infecciones oportunistas, disminución de ingresos hospitalarios y menor progresión a SIDA y mortalidad[97-100]. Por ello ha aumentado la tasa de supervivencia de estos pacientes, que llegan a la adolescencia y a la edad adulta con una adecuada situación inmunológica y virológica[101-104].

La actividad de la TI es inhibida por dos grupos de fármacos: los inhibidores de la TI análogos de los nucleós(t)idos (ITIAN) y los inhibidores de la TI no análogos de los núcleós(t)idos (ITINAN). Los ITIAN son compuestos derivados de las bases purínicas (adenosina, guanosina e inosina) y pirimidínicas (citosina y timidina). Estos compuestos, una vez fosforilados y convertidos en nucleótidos, se incorporan en la cadena de ácido nucleico en formación y actúan como terminadores de cadena. Los ITINAN son moléculas con grupos químicos con acción inhibitoria específica de la TI del VIH. Los IP son fármacos muy potentes, con metabolismo dependiente del sistema citocromo-oxigenasa P450. Todos los IP pueden ser potenciados con dosis bajas de Ritonavir (RTV).

El TARGA en niños está basado en la actualidad en la combinación de tres o más fármacos ARV. Generalmente se utilizan la combinación de dos ITIAN asociados a un ITINAN o a un IP. También existen o se están desarrollando nuevas familias de fármacos cuya diana son otras enzimas o fases del ciclo biológico del VIH-1. Entre ellos, los inhibidores de la INT y los inhibidores de la entrada. El Raltegravir (RAL) es el único fármaco comercializado capaz de

inhibir la INT viral. El Maraviroc (MRV) impide la entrada del virus con tropismo R5, inhibiendo el correceptor CCR5. Actualmente se está estudiando su uso en pediatría. La Enfurvirtida (T-20) actúa evitando la entrada del VIH-1 en la célula, a través de su interacción con la glicoproteína viral gp41 encargada de la fusión de la membrana celular y viral. Su uso sólo esta aprobado en niños mayores de 6 años[105,106]. En la **Tabla 2.4** se resumen los fármacos actualmente disponibles en España para tratar la infección por VIH-1 en pediatría.

Tabla 2.4. Antirretrovirales aprobados en España para el tratamiento del VIH pediátrico.

<table>
<tr><th>Nombre genérico</th><th>Abreviatura</th><th>Nombre comercial</th><th>Fabricante</th><th>Tipo de Agente</th></tr>
<tr><td>Abacavir</td><td>ABC</td><td>Ziagen</td><td>GSK</td><td rowspan="7">ITIAN</td></tr>
<tr><td>Didanosina</td><td>ddI</td><td>Videx</td><td>BMS</td></tr>
<tr><td>Emtricitabina</td><td>FTC</td><td>Emtriva</td><td>Gilead</td></tr>
<tr><td>Lamivudina</td><td>3TC</td><td>Epivir/Zeffix</td><td>GSK</td></tr>
<tr><td>Estavudina</td><td>d4T</td><td>Zerit</td><td>BMS</td></tr>
<tr><td>Zidovudina</td><td>AZT, ZDV</td><td>Retrovir</td><td>GSK</td></tr>
<tr><td>Tenofovir[1]</td><td>TDF, TNF</td><td>Viread</td><td>Gilead</td></tr>
<tr><td>Efavirenz[1,2]</td><td>EFV</td><td>Sustiva</td><td>BMS</td><td rowspan="2">ITINAN</td></tr>
<tr><td>Nevirapina</td><td>NVP</td><td>Viramune</td><td>Boehringer-Ingelheim</td></tr>
<tr><td>Atazanavir[1]</td><td>ATV</td><td>Reyataz</td><td>BMS</td><td rowspan="6">IP</td></tr>
<tr><td>Darunavir[1]</td><td>DRV</td><td>Prezista</td><td>Tibotec-Janssen-Cilag</td></tr>
<tr><td>Fosamprenavir[1]</td><td>FPV</td><td>Telzir</td><td>GSK</td></tr>
<tr><td>Lopinavir</td><td>LPV</td><td>Kaletra</td><td>Abbott</td></tr>
<tr><td>Tipranavir[1]</td><td>TPV</td><td>Aptivus</td><td>Boehringer</td></tr>
<tr><td>Ritonavir</td><td>RTV</td><td>Norvir</td><td>Abbott</td></tr>
<tr><td>Enfuvirtide[1]</td><td>T-20</td><td>Fuzeon</td><td>Roche</td><td rowspan="2">Inhibidor entrada</td></tr>
<tr><td>Maraviroc[3]</td><td>MRV</td><td>Celsentri</td><td>Pfizer</td></tr>
<tr><td>Raltegravir[3]</td><td>RAL</td><td>Isentrees</td><td>MSD</td><td>Inhibidor INT</td></tr>
</table>

1. Indicado sólo para pacientes de determinada edad.
2. Solución sólo disponible por uso compasivo o ensayo clínico.
3. En estudio en pediatría.

TRATAMIENTO ANTIRRETROVIRAL DE INICIO EN PEDIATRÍA

Actualmente existen diferentes guías para el tratamiento del VIH pediátrico disponibles. Entre ellas están la Guía del Departamento de Sanidad y Servicios Sociales de EE.UU.[107] (DHHS), la de la Red Europea para el Tratamiento del SIDA Pediátrico[108] (PENTA), la de la Organización Mundial de la Salud[109] (OMS) y la de la Organización Panamericana de la Salud[110] (OPS). Existen también guías nacionales, como la del grupo Colaborativo Español para la Infección VIH

Pediátrica[28] (CEVIHP) y la del grupo de expertos de la Secretaría del Plan Nacional sobre el SIDA[29].

Todas estas guías comparten más similitudes que diferencias. No obstante, presentan características que las hacen útiles para distintos escenarios y necesidades. Las guías de la OMS y OPS son diseñadas para guiar el TAR en países de recursos limitados, por lo que se diferencian del resto de guías en objetivos y aproximación. En países de altos recursos, donde el seguimiento regular y la monitorización de linfocitos T CD4 y CV es la norma, las guías DHHS y PENTA son más detalladas y continuamente se actualizan con apartados específicos que tratan coinfecciones, toxicidad asociada al TAR, interacciones entre fármacos, manejo de coinfecciones y comorbilidades.

La **Tabla 2.5** compara las recomendaciones de inicio de TAR según la edad del niño infectado y la guía clínica. Según todas las guías de tratamiento elaboradas por distintos organismos y apoyándose en los resultados científicos publicados[97,100,111,112] se confirma que todos los lactantes menores de un año deben comenzar con el TAR en el momento de ser diagnosticados por VIH-1, independientemente de la cifra o porcentaje de CD4 o de la CV. Eso se justifica porque el riesgo de progresión y de mortalidad es menor cuanto antes se comience con el TAR[97,100,111,112].

En mayores de un año, la recomendación de inicio del TAR se basa en la edad y en criterios clínicos, inmunológicos o virológicos. Así, en mayores de 12 meses, el estadio clínico C es predictivo de mayor riesgo de mortalidad, por lo que siempre está indicado iniciar TAR[113]. En pacientes en estadio clínico B la consideración de inicio de TAR se hará por parámetros inmunológicos o virológicos.

En niños menores de cinco años el riesgo de progresión es más elevado que en los mayores de esta edad. Los parámetros inmunológicos son mejores predictores del riesgo de progresión que la CV. En menores de 5 años es preferible el porcentaje al valor absoluto porque está sujeto a una menor variabilidad fisiológica con la edad[114,115]. Todas las guías recomiendan el inicio de la terapia con un umbral inferior al 25%. Aún siendo un parámetro menos robusto, también debe tenerse en cuenta la cifra absoluta de linfocitos T CD4, que podría estimarse en menos de 1.000 CD4 en niños entre 1 y 3 años y por debajo de 750 CD4 en niños entre 3-5 años.

Tabla 2.5. Comparación de inicio de TAR en pediatría según edad y guías clínicas.

Edad	Estadio	CEVIHP 2012[28] y PENTA 2009[108]	DHHS 2011[107]	OPS 2010[110]	OMS 2010[109]
0-11 meses	Clínico Inmunológico Virológico	Todos	Todos	Todos	Todos (hasta 24 meses)
12-35 meses	Clínico Inmunológico Virológico	Estadio B o C CD4<25% o <1.000 céls/mm^3 Considerar CV>100.000 c/ml	Estadio B o C CD4<25% CV>100.000 c/ml	Todos	Estadio III/IV CD4<25% o 750 céls/mm^3
36-59 meses	Clínico Inmunológico Virológico	Estadio B o C CD4<25% o <750 céls/mm^3 Considerar CV>100.000 c/ml	Estadio B o C CD4<25% CV>100.000 c/ml	CD4≤25% o ≤750 céls/mm^3	Estadio III/IV CD4<25% o 350 céls/mm^3
> 5 años	Clínico Inmunológico* Virológico	Estadio B o C CD4<350-500 céls/mm^3 Considerar CV>100.000 c/ml	Estadio B o C CD4<500 céls/mm^3 CV>100.000 c/ml Considerar: N, A y CD4>500 céls/mm^3 y CV<100.000 c/ml	CD4≤350 céls/mm^3	Estadio III/IV CD4<350 céls/mm^3

*Las guías PENTA establecen el inicio del TAR cuando los CD4 están por debajo de 350 céls/mm^3 en niños mayores de 5 años.

En los mayores de 5 años, la cifra absoluta de linfocitos T CD4 es el mejor marcador de progresión al igual que en los adultos[114,115]. Los datos obtenidos de adultos indican que el riesgo de progresión y/o muerte es mayor cuando se inicia el TAR por debajo de 350 CD4, que cuando se inicia con niveles superiores[116,117]. No obstante, existe también una correlación entre la CV y el riesgo de progresión. Por ello algunas guías recomiendan que se deba considerar el inicio del TAR cuando la CV sea superior a 100.000 copias de ARN-VIH-1/ml en pacientes de cualquier edad[108,118]. Los datos que sustentan este debate provienen de cohortes de pacientes en las que se ha evaluado la mortalidad, la progresión a SIDA, la incidencia de enfermedades no definitorias de SIDA, la recuperación inmunológica y la toxicidad del tratamiento en función de la cifra de linfocitos T CD4 previa al inicio del TAR.

Las distintas guías pediátricas proponen iniciar la terapia empleando la triple combinación de fármacos ARV, incluyendo dos ITIAN y un ITINAN o como alternativa un IP. Sin embargo, siempre habrá que tener en cuenta las características de cada paciente y el ensayo de resistencias previo para elegir uno u otro ARV.

Elección de ITIANs: la combinación de ABC+3TC es la más potente y eficaz de las posibles combinaciones de ITIAN[119]. Otra opción, en caso de presentar hipersensibilidad a ABC (HLA-B*5701), sería emplear la combinación ZDV+3TC. En mayores de 12 años, La combinación ABC+3TC puede encontrarse como un único comprimido (Kivexa®). La combinación ZDV+3TC también esta disponible en una única pastilla (Combivir®) disponible para adolescentes.

Elección de ITINAN o IP como tercer fármaco: la eficacia entre emplear un IP (LPV/r) o un ITINAN (NVP) como tercer fármaco fue evaluada en el estudio PENPACT-1[120] sin encontrar diferencias. No obstante los ITINAN seleccionan resistencias con mayor frecuencia que los IP a sí mismos y a los ITIAN. Sin embargo, los IP tienen más toxicidad metabólica, y requieren más tomas de comprimidos. Aunque la NVP presenta mayor tasa de fracaso virológico en terapia de inicio en niños pequeños[121,122], es más fácil de administrar y la adherencia puede ser mejor, aumentando su eficacia terapéutica[123]. Los ITINAN disponibles para población pediátrica infectada actualmente son NVP y EFV (en mayores de 3 años), mientras que como IP se puede emplear LPV/r, ATV/r (en mayores de 6 años) o DRV/r (en mayores de 12 años). A pesar de que la elección entre LPV/r y NVP debe ser individualizada, los estudios realizados en países en vías de desarrollo, donde se utiliza NVP para prevenir la transmisión vertical del VIH-1, apoyan el uso de LPV/r[121,122].

Tabla 2.6. TAR de inicio en función de la edad del paciente**.**

Edad del paciente	Pautas de elección
Desde los 14 días a los 3 años	2 ITIAN+LPV/r
≥ 3 años	2 ITIAN+LPV/r 2 ITIAN+EFV
≥ 6 años	2 ITIAN +LPV/r 2 ITIAN+EFV 2 ITIAN+ATV/r
≥ 12 años	2 ITIAN+LPV/r 2 ITIAN+EFV 2 ITIAN+ATV/r 2 ITIAN+DRV/r

*Pautas de 2 ITIAN: 3TC+ABC o FTC+ABC (si HLA-B*5701 negativo) o ZDV+3TC (si HLA-B*5701 positivo).

Recientemente se ha puesto de manifiesto la posibilidad de comenzar TAR con cuádruple terapia. Los regímenes basados en cuatro fármacos de la familia ITNAN han demostrado una mayor respuesta virológica e inmunológica comparado con regímenes basados en triple terapia[124]. Según los resultados obtenidos, existe evidencia que demuestra que un régimen cuádruple provee una mayor respuesta virológica sostenida y presenta una mayor barrera genética para la selección de mutaciones de resistencia. Sin embargo, un mayor número de comprimidos diarios puede impactar en la adherencia al TAR. Se requieren estudios complementarios en cohortes pediátricas que confirmen estos resultados.

EPIDEMIOLOGÍA DE LA INFECCIÓN POR VIH EN EL MUNDO

El último informe conjunto de la Naciones Unidas y de la OMS, publicado a finales de 2012, calcula en 34 millones el numero de infectados por VIH globalmente[13]. De ellos 2,5 millones son niños menores de 15 años. En el último año, 350.000 jóvenes menores de 15 años fueron infectados y 250.000 fallecieron a causa de la infección por VIH. Especialmente dramática resulta la situación en la región de África subsahariana donde viven el 90% de los pacientes diagnosticados de infección por VIH-1 en el mundo[13].

En España, desde el inicio de la epidemia hasta Junio de 2012 se han notificado un total de 82.009 casos de SIDA, de los cuales 966 han sido declarados en pacientes pediátricos. En 2011, se notificaron 844 casos de SIDA, y se estima por retraso en la notificación que habrían sido 1.038.

En 2011 se diagnosticaron en España un total de 2.763 casos de infección por VIH. La ruta mayoritaria de infección fue la de hombres que mantienen relaciones sexuales con hombres (HSH; 54%), seguida de la heterosexual (31%) y de la de usuarios de drogas inyectadas (UDI; 5%). Sólo se diagnosticaron 8 casos de infección por VIH en pacientes pediátricos por transmisión materno-fetal. En la Comunidad de Madrid se diagnosticaron en 2011 un total de 778 casos de infección por VIH, 28.1% de los casos totales en España, siendo la ruta mayoritaria HSH (69,5%) seguida de la heterosexual (20,2%). La Comunidad de Madrid notificó en el año 2011 un total de 2 casos de infección por VIH por transmisión materno-infantil[125].

Hasta 1997 la proporción de nuevos diagnósticos en personas nacidas fuera de España estuvo por debajo del 3%, pero desde entonces ha subido progresivamente hasta alcanzar el 30% en 2011. Durante 2011, el 46% de estas personas extranjeras procedía de Latinoamérica y el 26% de África[125].

TRANSMISIÓN VERTICAL DEL VIH EN ESPAÑA

Desde que en 1997 se instauró la administración de TARGA en las madres durante el embarazo y la profilaxis en el parto y en los niños al nacer, ha disminuido la transmisión vertical a menos de un 2% en nuestro país, por lo que el número de nuevas infecciones pediátricas ha disminuido[126]. La profilaxis en el parto y en el niño, el diagnóstico temprano, el cuidado prenatal, la alimentación artificial, la cesárea electiva y la monitorización del embarazo también han reducido las tasas de transmisión vertical del VIH en países

desarrollados hasta el 1-2%[127-129]. Aún así, siguen ocurriendo casos de transmisión vertical del VIH, sobre todo en el colectivo de inmigrantes infectados originarios de países con altas prevalencia de la infección y en grupos sociales que rechazan la monitorización del embarazo[126]. El aumento de la inmigración también ha traído consigo nuevos diagnósticos de infección VIH en niños en España, a pesar de las medidas de prevención encaminadas a evitar nuevos casos de VIH pediátrico[126]. Así, durante el año 2011, ocho niños fueron diagnosticados de infección por VIH en todo el territorio nacional, cuatro originarios de África subsahariana y cuatro españoles[125]. En España, las tasas de nuevos diagnósticos han seguido la tendencia de otros países de Europa occidental. No obstante, debido al alto impacto que tuvo durante la década de los 80 y 90 el abuso de drogas por vía parenteral que impactó especialmente en la población femenina, se alcanzaron los valores más altos de incidencia de VIH de toda Europa occidental, causando una alta incidencia de transmisión vertical del VIH en nuestro país.

COHORTE NACIONAL DE PACIENTES PEDIÁTRICOS CON INFECCIÓN POR VIH

La Cohorte de la Red de Investigación en SIDA pediátrico (CoRISpe) es una cohorte nacional de carácter prospectiva-retrospectiva, nacional y multicéntrica nacional de niños y adolescentes infectados por VIH (http://www.corispe.org)[130]. Su objetivo es conocer la situación epidemiológica del VIH pediátrico en España, diseñar estudios estratégicos y terapéuticos para mejorar la situación clínica, inmunológica y virológica de los pacientes y aumentar su calidad de vida. Actualmente la mayoría de estos pacientes son adolescentes que han sobrevivido a la era previa al TARGA, presentando unas características clínicas, inmunológicas, virológicas y tratamiento diferentes a las del paciente VIH de la misma edad e infectados por otra vía.

En CoRISpe, participan 838 niños y adolescentes repartidos en 74 hospitales de todo el territorio nacional. Alrededor de dos tercios (64,8%) son menores de edad y se encuentran en seguimiento en unidades pediátricas. El 35,2% de la cohorte ha alcanzado la mayoría de edad y ha sido transferida a unidades adultos.

De los 536 pacientes actualmente en seguimiento, el 97% fueron infectados verticalmente, más de la mitad (52%) no muestran actualmente síntomas clínicos de progresión a SIDA (estadio clínico CDC N o A), el 89% mantienen un nivel de linfocitos T CD4>25% y el 66% presentan supresión viral con CV indetectable (CV<50 copias de ARN-VIH-1/ml). La mayoría (68%) son de origen español, pero también están representados pacientes originarios de África

subsahariana (20%) y de otras nacionalidades (12%). La mayoría recibe un tratamiento combinado basado en ITIAN+IP (46,6%) o en ITINAN+ITIAN (29,2%). Muchos de los 282 pacientes que han alcanzado la mayoría de edad y han sido transferidos a unidades de adultos han sufrido terapias subóptimas. De hecho, sólo el 17,5% de ellos comenzaron TAR con TARGA[130].

COHORTE DE NIÑOS Y ADOLESCENTES INFECTADOS POR VIH-1 DE LA COMUNIDAD DE MADRID

La infección por el VIH en niños comenzó a diagnosticarse en la Comunidad de Madrid a principios de los años 80. Se estima que 536 (51,6%) niños de los 1.039 infectados por VIH-1 en España desde el comienzo de la epidemia han estado bajo seguimiento clínico en la cohorte de niños y adolescentes infectados por VIH-1 de la Comunidad de Madrid desde 1995 hasta diciembre de 2012.

La cohorte de niños y adolescentes infectados por VIH de Madrid, iniciada en 2003, es una cohorte retrospectiva, prospectiva y multicéntrica en la que participan 9 hospitales públicos de la Comunidad de Madrid. En esta cohorte se incluyen los nuevos diagnósticos y se recogen los datos clínicos, epidemiológicos, analíticos, el TAR y la evolución clínica, inmunológica y virológica de los niños, entre otros datos.

En la actualidad están bajo seguimiento un total de 287 (53,5%) pacientes, con una edad media de 13,5 años y un rango entre 3 meses y 19 años. A diciembre de 2012, ciento treinta y ocho pacientes (25,8%) han alcanzado la mayoría de edad y han sido transferidos a Unidades de adultos donde se realiza su seguimento. Ciento cuarenta y nueve (27,8%) están bajo seguimento en unidades pediátricas, 179 (33,4%) han fallecido y de 70 (13%) se desconoce su situación actual. La mayor parte de los nuevos infectados son niños de procedencia extranjera[126]. Alrededor del 90% de los niños reciben TARGA en la actualidad, en su mayoría tienen buena situación clínica e inmunológica y las dos terceras partes de ellos han alcanzado carga viral plasmática indetectable en la última determinación disponible.

La cohorte de Madrid está integrada en la red de seguimiento de niños con infección VIH de España (CoRISpe), formando parte del Nodo I. Está financiada por becas de investigación de FIPSE y Fondo de Investigación Sanitaria y su coordinación se lleva desde la jefatura del Servicio de Pediatría del Hospital Universitario de Getafe. Asimismo, esta cohorte está en íntima relación con otras cohortes europeas de niños (Red PENTA, cohorte CHIPS, Encuesta

Perinatal Francesa, Registro Italiano, European Infant Collaboration Group), así como con cohortes europeas de adultos (COHERE, EUROSIDA) que pretenden dar respuesta a numerosos interrogantes en cuanto a la historia natural de la enfermedad y evolución en la edad adulta.

La cohorte de Madrid dispone de más 3.000 muestras biológicas secuenciales de los niños infectados por VIH-1 en la Comunidad de Madrid almacenadas en el BioBanco-VIH pediátrico integrado en la Red de investigación en SIDA (RIS), ubicado en el Hospital General Universitario Gregorio Marañón. Esta colección permite desarrollar proyectos de investigación. En nuestro caso nos ha permitido conseguir muestras clínicas pediátricas para realizar análisis virológicos con los que estudiar la epidemiología molecular del VIH-1, para identificar los subtipos y formas recombinantes del VIH-1, para identificar las mutaciones de resistencia a fármacos ARV transmitidas o adquiridas durante la terapia en los niños y adolescentes infectados por VIH-1 que forman parte de la cohorte de Madrid.

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OBJETIVOS

El objetivo general de esta Tesis consiste en:

Estudiar a nivel molecular el VIH-1 que infecta a la cohorte de niños y adolescentes infectados por VIH-1 de la Comunidad de Madrid desde 1993 a 2011, describiendo las variantes genéticas del VIH-1 y analizando la naturaleza y prevalencia de mutaciones de resistencia a fármacos ARV en presencia y ausencia de TAR.

Los objetivos específicos de esta Tesis son:

1. Describir las características clínicas, epidemiológicas y demográficas en la cohorte de niños y adolescentes infectados por VIH-1 de la Comunidad de Madrid (España) desde 1993 a 2011.

2. Analizar la presencia, naturaleza y año de entrada de las variantes no-B del VIH-1 en la cohorte de estudio identificadas mediante análisis filogenéticos de las secuencias virales generadas a partir de muestras clínicas cedidas por el BioBanco-VIH pediátrico y/o recuperadas de los hospitales de seguimiento de los pacientes.

3. Determinar la naturaleza y prevalencia de mutaciones de resistencia a fármacos antirretrovirales en el gen *pol* del VIH-1 de los virus que infectan a la cohorte pediátrica de Madrid. Evaluar la tasa y tipo de mutaciones de resistencia transmitidas (MRT) y adquiridas (MRA) en pacientes *naïve* y tratados.

4. Evaluar y comparar el grado de susceptibilidad genotípica a fármacos antirretrovirales tras introducir las secuencias del gen *pol* del VIH-1 generadas y/o recuperadas en herramientas bioinformáticas de interpretación genotípica.

5. Determinar la dinámica temporal de aparición de mutaciones específicas en el gen *pol* del VIH-1 que confieren resistencia o modifican la susceptibilidad a fármacos ARV en la cohorte de niños y adolescentes infectados por VIH-1 de la Comunidad de Madrid.

6. Evaluar la situación clínica y virológica de los adolescentes de la cohorte de Madrid al ser transferidos desde unidades pediátricas hospitalarias a unidades de adultos.

7. Estudiar los aspectos definidos en los objetivos 1 a 4 en una cohorte de niños infectados por VIH-1 de la ciudad de Nueva York (EE.UU.) y los objetivos 1 a 3 en dos cohortes de niños infectados por VIH-1 de El Salvador y Honduras.

8. Comparar los resultados obtenidos en las cohortes estudiadas de pacientes pediátricos infectados por VIH-1.

PUBLICACIONES

CAPÍTULO 1

Miguel de Mulder, Gonzalo Yebra, Leticia Martín, Luis Prieto, María José Mellado, Pablo Rojo, María Ángeles Muñoz Fernández, Santiago Jiménez de Ory, José Tomás Ramos y África Holguín. **Drug Resistance and HIV-1 Variant Characterization in Naïve and Pretreated HIV-1 Infected Pediatric Population in Madrid.** *The Journal of Antimicrobial Chemotherapy.* 2011; 66:2362-71. FI: 5,1.

CAPÍTULO 2

Miguel de Mulder, Gonzalo Yebra, Adriana Navas, Leticia Martín, María Isabel de José, María Luisa Navarro, Santiago Jiménez de Ory, Ignacio González-Granado, María José Mellado, José Tomás Ramos y África Holguín. **Trends in Drug Resistance Prevalence in HIV-1 Infected Children in Madrid: 1993-2010 Analysis.** *The Pediatric Infectious Disease Journal.* 2012; 31:e213-21. FI: 3,6.

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Miguel de Mulder, Gonzalo Yebra, Adriana Navas, María Isabel de José, María Dolores Gurbindo, María Isabel González-Tomé, María José Mellado, Jesús Saavedra Lozano, María Ángeles Muñoz-Fernández, Santiago Jiménez de Ory, José Tomás Ramos y África Holguín. **High Drug Resistance Prevalence among Vertically HIV-Infected Patients Transferred from Paediatric Care to Adult Units in Spain.** *PLoS One*. 2012; 7:e52155. FI: 4,1

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CAPÍTULO 5

África Holguín, Karen Erazo, Gustavo Escobar, **Miguel de Mulder**, Gonzalo Yebra, Leticia Martín, Jovel LE, Castaneda L, Elsy Pérez. **Drug Resistance Prevalence and HIV-1 Variant Characterization in HIV-1 Infected Pediatric Populations in Honduras and El Salvador During 1989-2009 Period.** *The Pediatric Infectious Disease Journal*. 2011; 30:e82-7. FI: 3,6.

CAPÍTULO 1

Prevalencia de resistencia a fármacos antirretrovirales y caracterización genética de variantes del VIH-1 en población pediátrica *naïve* y pretratada de la cohorte de niños y adolescentes de la Comunidad de Madrid

JUSTIFICACIÓN Y OBJETIVOS

El uso del TAR ha disminuido el riesgo de progresión a SIDA y ha reducido la morbilidad y mortalidad asociada a la infección por VIH-1 en pacientes pediátricos. Las mutaciones de resistencia a fármacos ARV afectan la efectividad del TAR. Para mantener las opciones terapéuticas en pacientes pediátricos que requieren un tratamiento de por vida se debe minimizar la selección de mutaciones de resistencia en el genoma del VIH-1. La identificación de subtipos y formas recombinantes del VIH-1 es importante, no sólo para ayudar a definir la epidemiología molecular del virus en una población, sino porque pueden influir en el diagnóstico de la infección, en el uso del TAR, en la determinación de parámetros clínicos y en la progresión de la enfermedad. Hasta el desarrollo de ésta Tesis, sólo se conocían las variantes del VIH-1 que circulaban en la Comunidad de Madrid en población adulta, pero ningún trabajo había analizado la población pediátrica de Madrid. Un único estudio, publicado en 2009, analizó la presencia de variantes no-B en 12 pacientes menores de 18 años de procedencia extranjera infectados entre 1988 y 2006. Ningún estudio previo había determinado la prevalencia y tipo de mutaciones de resistencia a fármacos ARV en pacientes de la cohorte de niños y adolescentes de la Comunidad de Madrid desde su inicio.

Los objetivos de este capítulo son:

- Describir las características epidemiológicas, clínicas y virológicas de los pacientes pediátricos de la cohorte de niños y adolescentes infectados por VIH-1 de la Comunidad de Madrid con muestra o secuencia disponible desde 1993 a diciembre de 2009.
- Caracterizar por primera vez las variantes virales que infectan a cada paciente pediátrico de la cohorte incluido en este estudio mediante análisis filogenético de secuencias del gen *pol* del VIH-1.
- Determinar la prevalencia global y la naturaleza de las mutaciones de resistencia a cada una de las tres familias de fármacos ARV (IP, ITIAN e ITINAN) en pacientes *naïve* (sin exposición previa a TAR) y pretratados durante el periodo 1993-2009.
- Identificar el número de pacientes de la cohorte infectados por variantes no-B del VIH-1.

CONCLUSIONES

- Hasta diciembre de 2009 se identificaron un total de 198 pacientes con secuencia *pol* del VIH-1 disponible. La mayoría (90%) eran europeos, un 65% presentaban síntomas moderados de SIDA y el 85% estaban bajo TAR en el momento de recogida de muestra. La mayoría (67%) fueron infectados en la década de los 90. La situación clínica inmunológica era buena, el 58,6% presentaban CV indetectable y el 65,7% alcanzaban valores de linfocitos T CD4 por encima de las 500 céls/mm^3.
- Uno de cada 10 pacientes de la cohorte de niños y adolescentes infectados por VIH-1 de la Comunidad de Madrid entre 1993 y 2009 estaba infectado por subtipos no-B y formas recombinantes del VIH-1. La introducción de este tipo de variantes comenzó en la primera mitad de los años 90 a través de madres infectadas originarias de países endémicos para variantes no-B del VIH-1.
- De los 19 pacientes infectados por variantes no-B, más de la mitad (58%) fueron formas recombinantes circulantes (CRF) diferentes: 8CRF02_AG, 1CRF08_BC, 1CRF12_BF y 1CRF13_cpx. El resto fueron subtipos no-B "puros": 1A2, 2C, 2D, 1F1, 1G y 1H.
- Un 13% de pacientes pediátricos *naïve* presentaron mutaciones de resistencia a fármacos que comprometían al menos a una familia de fármacos ART.
- Dos tercios (66%) de los pacientes pediátricos que habían recibido TAR estaban infectados por variantes virales resistentes al menos a una familia de fármacos y un 30% presentaban variantes virales resistentes a dos familias de fármacos.
- La prevalencia de mutaciones de resistencia para IP, ITIAN e ITINAN en pacientes pretratados fueron nueve (37% *vs.* 4%), siete (54% *vs.* 7%) y tres (35% *vs.* 10%) veces superior en pacientes pretratados que en pacientes *naïve*.
- La prevalencia global de mutaciones de resistencia en población *naïve* (13%) fue superior a la hallada en la cohorte pediátricas francesa y británica (10% y 6,8% respectivamente), pero similar a la determinada en población adultos infectada por VIH-1 de la Comunidad de Madrid (13,6%).
- La prevalencia global de mutaciones de resistencia en pacientes infectados por subtipo B era tres veces más frecuente que en pacientes infectados por variantes no-B (62% *vs.* 21,1%, $p<0,05$), siendo cinco veces mayor para los que presentaban resistencia a ITIAN (50,3% *vs.* 11,1%, $p<0,05$).

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Drug resistance prevalence and HIV-1 variant characterization in the naive and pretreated HIV-1-infected paediatric population in Madrid, Spain

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Background: Drug resistance mutations affect antiretroviral therapy (ART) effectiveness in HIV-1-infected children, compromising long-term therapy. HIV-1 variants and drug resistance mutations were identified in HIV-infected children from Madrid, Spain.

Methods: Patients from the Madrid cohort of HIV-infected children (1993–2009) with available *pol* sequences or infected samples stored at the Spanish HIV-1 BioBank were selected. Specimens were used to perform new *pol* sequences when not available. HIV-1 variants were characterized by phylogenetic analysis. Resistance mutations were identified according to the International AIDS Society–USA list (2009).

Results: In 198 patients, *pol* sequences were recovered from routine resistance testing ($n=98$) or newly performed using stored plasma, lymphocytes or DNA ($n=100$). Patients were mostly Europeans (90%), with moderate to severe AIDS symptoms (65%), on ART (85%) when the specimen was sequenced and infected by subtype B (90%). Among the 19 HIV-1 non-B variants found, 58% were recombinants (8CRF02_AG, 1CRF08_BC, 1CRF12_BF and 1CRF13_cpx) and the rest were 'pure' non-B subtypes (1A2, 2C, 2D, 1F1, 1G and 1H). Transmitted drug resistance (TDR) mutations were detected in 13% of naive children; 4%, 7% and 10% for protease inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), respectively. Global resistance prevalence was higher (66%) among ART-exposed children; 37% for PIs, 54% for NRTIs and 35% for NNRTIs.

Conclusions: HIV-1 non-B variants infected 10% of the cohort during 1993–2009. Resistant viruses were present in 26.5% and 66% of naive and pretreated children, respectively. Our data suggest that TDR prevalence in children could be higher than that reported in adults in Spain. The provided data will help to improve clinical management of HIV-infected children in Spain.

Keywords: HIV/AIDS, antiretroviral therapy, drug resistance, paediatrics

Introduction

The virological, immunological and clinical outcomes in HIV-infected children have improved greatly with the introduction of highly active antiretroviral therapy (HAART).[1] HAART significantly reduces the progression of HIV-1 disease and decreases AIDS-associated morbidity and mortality in the paediatric population.[2] Antiretroviral therapy (ART) leads to HIV RNA viral load (VL) suppression, which prevents the evolution of viral drug resistance and allows normal immune function, leading to normal growth and development in most infected children.[3] However, HIV-infected children must use long-term ART,[4] increasing the development of drug resistance mutations and toxic effects, which limit the long-term effectiveness of HAART, leading to the selection of drug-resistant HIV-1 variants and virological failure events.[5] Drug-resistant variants can also be selected

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during pregnancy in women treated with a sub-optimal regimen, which has serious implications for future ART success in infants.[6]

Spain has among the highest AIDS incidence rate and prevalence in women in Western Europe,[7,8] which has a direct impact on the spread of the infection in infants. The Autonomous Community of Madrid (CAM) is the area most affected by HIV infection in Spain and this mainly occurred during the 'epidemic of heroin use' in the 1990s. The rates of mother-to-child transmission (MTCT) in the CAM have followed the same trend as in other neighbouring countries due to the implementation of health policies, pregnancy follow-up and ART administration in infected mothers. During 2009, 1037 new cases of AIDS were declared in Spain, and only 4 (0.4%) of these were caused by MTCT. However, between 1981 and June 2009 a total of 946 cases of AIDS caused by MTCT were reported in Spain.[8] Of these, 237 cases were reported in the CAM.

Spain has become one of the main entrance points of Africans into the European Union, mainly from sub-Saharan African countries with high HIV infection rates and a high prevalence of HIV-1 non-B variants.[9,10] Owing to these population movements, all HIV-1 subtypes and multiple recombinants are circulating in Spain.[11–13] Infection with certain non-B variants can have clinical implications, accelerating disease progression.[14] Furthermore, non-B variants present clade-specific substitutions in positions related to drug resistance,[15] which can accelerate the emergence of drug-resistant viruses, change or induce alternative pathways of resistance[16] and influence the interpretation of genotypic resistance algorithms,[17] rapid subtyping tool reliability[13] and VL quantification.[18] Thus the proper detection and description of HIV-1 variants in representative cohorts is essential for further studies. The aims of this study were to identify the HIV-1 variants infecting patients from the Madrid cohort of HIV-infected children, summarize their clinical and epidemiological features and determine the prevalence of drug resistance mutations in viruses present in naive and ART patients.

Methods

Study population

The Madrid cohort of HIV-infected children has registered a total of 523 patients since the beginning of the epidemic in Spain. Today, 198 (38%) of these patients remain under clinical follow-up in paediatric units, 79 have transferred to adult units, 63 have been lost to follow-up and 183 have died. A total of 227 patients infected during their childhood were included in this study, selected according to sample [immortalized DNA, plasma or peripheral blood mononuclear cells (PBMCs)] or *pol* sequence availability. Available sequences from previous clinical routine drug resistance analysis performed since 1999 in hospitals where patients were under follow-up were recovered during 2010. HIV-1-infected specimens since 1993 from the remaining children without available *pol* sequences at December 2009 were recovered from the HIV-1 BioBank located in Hospital Gregorio Marañon, where they were stored and new sequences were performed. This study was approved by the Ethics Committees of all the institutions and hospitals involved.

HIV-1 diagnosis

Diagnosis of HIV-1 infection was previously confirmed in the study population by a positive RNA or DNA PCR for HIV in children aged less than 18 months and by two serial positive serological assays in children older than 18 months.

RNA and DNA extraction

For RNA extraction from HIV-1-infected plasma samples, an automated platform for the isolation of RNA based upon magnetic-silica technology was used employing NucliSENS® easyMAG® instrumentation (bioMérieux, Durham, NC, USA), according to the manufacturer's instructions. For viral DNA extraction from infected PBMCs, a commercial DNA isolation kit based upon column extraction was used (Qiagen Inc., Valencia, CA, USA).

HIV-1 *pol* sequencing and subtyping

HIV-1 RNA or proviral DNA amplification using an in-house reverse transcriptase (RT) nested PCR method was performed for the *pol* coding region (1121 bp), including the complete protease (PR) (297 bp) and partial RT gene as previously reported.[19] cDNA synthesis was carried out using the Access Quick™ RT–PCR system (Promega, Madison, WI, USA) and amplifications from generated cDNA or extracted proviral DNA using iProof™ High-Fidelity Master Mix (Bio-Rad, Hercules, CA, USA) or PCR Master Mix (Promega), following the manufacturers' instructions. All *pol* sequences were subtyped by phylogenetic analysis as previously described.[19] At least two representative sequences of each subtype and circulating recombinant form (CRF) within HIV-1 group M available at the moment of the analysis were taken as references. DNA sequences were aligned using the ClustalW program. The tree topology was obtained using the neighbour-joining method. The pairwise distance matrix was estimated using the Kimura two-parameter model within the DNADIST program, as implemented in the PHYLIP software package. Bootstrap re-sampling (1000 datasets) of the multiple alignments was performed to test the statistical robustness of the tree. The bootstrap cut-off was set at 700.

Genotypic drug resistance identification

Transmitted drug resistance (TDR) mutations among naive patients were classified using the WHO drug resistance mutation list[20] and drug resistance mutations among pretreated patients, defined by the International AIDS Society (IAS)–USA,[21] were manually located in each PR and RT sequence. Resistance mutations in the PR gene in pretreated patients were classified as primary (major) or secondary (minor) following the IAS–USA nomenclature.

Genotypic drug resistance interpretation was performed using the genotypic resistance algorithms provided by Stanford (v.4.3.7) available from http://hivdb6.stanford.edu/asi/deployed/hiv_central.pl?program=hivdb&action=showSequenceFormalgorithms.

Statistical analysis

Confidence interval (CI) tests were performed with Epidat 3.1 (Pan American Health Organization). Significance was set at $P<0.05$.

Results

Study population selection

A total of 227 patients infected during childhood with HIV-1 in the period 1993–2009 were enrolled in this study. HIV-1 *pol* sequences were obtained from genotypic resistance analysis in 98 patients from routine testing in the corresponding hospitals. In the remaining 129 children with available infected samples, 100 additional sequences were newly performed from successful

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pol gene amplifications using extracted RNA or DNA from recovered specimens. Resistance analyses were performed from plasma-associated RNA in 156 children and from proviral DNA in 42 (10 naive and 32 pretreated) due to unavailable plasma samples from these children. PCR amplifications were successfully achieved in 5/7 (71.4%) previously immortalized DNA samples, in 37/40 (92.5%) PBMC samples and in 58/82 (70.7%) plasma samples. In the remaining specimens insufficient sample volume, undetectable VL (<50 copies/mL) and poor quality due to long-term storage of samples could be the causes for no amplification. Twenty-six subtype B specimens with reported undetectable viraemia values (<50 HIV-1 RNA copies/mL) provided positive amplifications, suggesting underestimation or failure in viraemia values or high efficacy of nested amplification in specific cases.

A total of 198 HIV-1 subjects from eight hospitals in the CAM, infected during childhood mainly by vertical transmission during 1993–2009 and with available *pol* sequences, were included in the study. Baseline characteristics of the study population were recorded (Table 1). Patients were mainly female (59%), perinatally infected (96%), born in Spain (88.5%), and on ART (85%) by sample collection time. Most infections (67%) occurred during 1990–1999, although sequenced samples for the study were recovered in 76% of children after 1999.

Table 1. Baseline features of the 198 patients[a] at collection time of sequenced specimens

Features	Patients, *n* (%)
Demographics	
male gender	81 (41)
adopted	44 (22)
HIV-1 transmission	
perinatal	190 (96)
blood transfusion	5 (2.5)
sexual	1 (0.5)
unknown	2 (1)
Origin[b]	
Spain	175 (88.5)
other European countries	3 (1.5)
Africa	12 (6)
America	7 (3.5)
Asia	1 (0.5)
HIV-1 infection year	
1980–89	31 (16)
1990–99	133 (67)
2000–09	34 (17)
Year of specimens	
1993–99	36 (18)
2000–04	83 (42)
2005–09	67 (34)
unknown	12 (6)
ART status of specimens collected	
drug naive	30 (15)
under ART	168 (85)
PI experienced	166 (84)
NNRTI experienced	126 (64)
NRTI experienced	92 (46)
Infecting HIV-1 variants	
B subtype	179 (90)
non-B variants	19 (10)

[a]Patient distribution among the CAM hospitals; Hospital La Paz (*n*=51), Hospital Gregorio Marañón (*n*=47), Hospital 12 de Octubre (*n*=34), Hospital Carlos III (*n*=30), Hospital de Getafe (*n*=24), Hospital Niño Jesús (*n*=7), Hospital Príncipe de Asturias (*n*=4) and Hospital de Móstoles (*n*=1).
[b]Countries of origin; Spain (175), Portugal (1), Romania (1), Poland (1), Equatorial Guinea (6), Cameroon (2); Morocco (2), Mozambique (1), Nigeria (1), Ecuador (2), Guatemala (2), Honduras (1), Peru (1), Venezuela (1) and India (1). Six of the 175 children from Spain had a foreign mother or father coming from Equatorial Guinea (2), Morocco (1), Democratic Republic of Congo (1), Senegal (1) or Cameroon (1).

Clinical features of the study population

All clinical and epidemiological features from the 198 children included in the study by 31 December 2009 were recorded (Table 2). The mean age of the study cohort (*n*=198) was 15.2 years, which makes this one of the oldest paediatric cohorts in Europe. The majority were alive and under follow-up in paediatric units. Nearly two-thirds presented moderate to severe symptoms of AIDS. Of note, 60% of the patients had achieved undetectable VLs (<50 copies/mL), 79% presented relative CD4+ T cell counts ≥25% and 40% had >750 CD4+ cells/mm^3. The rate of children on ART by December 2009 increased with respect to the specimen collection time (89.9% versus 85%) (Tables 1 and 2). By December 2009, only 7 (3.5%) of the 198 children remained drug naive, and 11 (5.6%) were not receiving any ART, although they were drug-experienced. Among the 198 children, 61.6% had received ≥4 different ART regimens (range 4–21) during their follow-up.

Phylogenetic characterization of HIV-1 variants

HIV-1 subtype B was the most prevalent (90%) variant found by phylogenetic analysis of *pol* sequences. It was present in 179 of the 198 children under study infected during the 1993–2009 period (Table 1). The 19 non-B variants identified in the study cohort were confirmed with high bootstrap values (>700) in phylogenetic trees. Among the 19 (10%) HIV-1 non-B variants found by phylogenetic characterization, 42% corresponded to 'pure' non-B subtypes or sub-subtypes (1A2, 2C, 2D, 1F1, 1G and 1H) and 58% were inter-subtype recombinant strains ascribed to CRFs (8CRF02_AG, 1CRF08_BC, 1CRF12_BF and 1CRF13_cpx). No unique recombinant forms (URF) were found in the study population. Figure 1 shows the HIV-1 non-B subtypes and recombinants defined using PR and partial RT sequences (1302 bp) available for 17 non-B variants. The 19 non-B subtypes and recombinants found during the study period corresponded to 4 (21%) native children born to native Spanish parents and 15 (79%) children born to a foreign infected biological mother or father coming from India (1 case) or sub-Saharan Africa

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Table 2. Characteristics of the 198 patients with available *pol* sequence by December 2009

Features	Total (*n*=198)	Infected by resistant viruses (*n*=119)	Infected by wild-type virus (*n*=79)
Alive [*n* (%)]	194 (98.0)	116 (97.5)	78 (98.7)
Mean age, years (SD)	15.2 (5)	15.9 (4.8)	14.5 (5.1)
Carrying HIV-1 non-B variants [*n* (%)]	19 (9.6)	5 (4.2)	14 (17.7)
Clinical follow-up [*n* (%)]			
paediatric unit	151 (76.3)	89 (74.8)	62 (78.5)
adults unit	27 (13.6)	18 (15.1)	9 (11.4)
lost to follow-up	16 (8.1)	9 (7.6)	7 (8.9)
deceased	4 (2.0)	3 (2.5)	1 (1.2)
Clinical status (CDC[a]) [*n* (%)]			
asymptomatic, N1	1 (0.5)	0 (0)	1 (1.3)
low symptoms, A	65 (32.8)	38 (32.0)	27 (34.2)
moderate symptoms, B	47 (23.7)	26 (21.8)	21 (26.5)
severe symptoms, C	82 (41.4)	53 (44.5)	29 (36.7)
unknown	3 (1.6)	2 (1.7)	1 (1.3)
Viral load (HIV-1 RNA copies/mL) [*n* (%)]			
≤50	116 (58.6)	70 (58.8)	46 (58.2)
51–500	22 (11.1)	17 (14.3)	5 (6.3)
>500	53 (26.8)	29 (24.4)	24 (30.4)
unknown	7 (3.5)	3 (2.5)	4 (5.1)
Range of CD4+ T cell count (cells/mm^3) [*n* (%)]			
>1500	13 (6.6)	6 (5.0)	7 (8.9)
751–1500	66 (33.3)	41 (34.5)	25 (31.6)
500–750	51 (25.8)	37 (31.1)	14 (17.7)
200–499	27 (13.6)	16 (13.5)	11 (13.9)
<200	3 (1.5)	3 (2.5)	0 (0)
unknown	38 (19.2)	16 (13.4)	22 (27.9)
Range of CD4+ T cell count (%) [*n* (%)]			
<25	38 (19.2)	31 (26.1)	7 (8.9)
25–50	155 (78.3)	85 (71.4)	70 (88.5)
>50	2 (1.0)	1 (0.8)	1 (1.3)
unknown	3 (1.5)	2 (1.7)	1 (1.3)
ART by December 2009 [*n* (%)]			
drug naive	7 (3.5)	1 (0.8)	6 (7.6)
stopped treatment[b]	11 (5.6)	5 (4.2)	6 (7.6)
under ART	178 (89.9)	112 (94.2)	66 (83.5)
unknown	2 (1.0)	1 (0.8)	1 (1.3)
Sequenced specimen [*n* (%)]			
DNA-derived sequence[c]	42 (21.2)	19 (16.0)	23 (29.1)
RNA-derived sequence[d]	156 (78.8)	100 (84.0)	56 (70.9)
Numbers of ART regimens by December 2009 [*n* (%)]			
0	7 (3.5)	1 (0.8)	6 (7.6)
1	12 (6.1)	7 (5.9)	5 (6.4)
2	24 (12.1)	12 (10.1)	12 (15.2)
3	30 (15.2)	16 (13.5)	14 (17.7)
4–6	72 (36.4)	46 (38.7)	26 (32.9)
7–12	49 (24.7)	35 (29.4)	14 (17.7)
>12	1 (0.5)	1 (0.8)	0 (0)
unknown	3 (1.5)	1 (0.8)	2 (2.5)

[a]Classification according to the CDC. 1994 revised classification system for human immunodeficiency virus infection in children less than 13 years of age; Official authorized addenda: human immunodeficiency virus infection codes and official guidelines for coding and reporting ICD-9-CM. *MMWR* 1994; **43** (No. RR-12): 1–10.
[b]Patients with previous but not current exposure to ART.
[c]DNA-derived sequences were obtained from immortalized DNA (*n*=5) and PBMCs (*n*=37) available from HIV-1 BioBank.
[d]RNA-derived sequences were obtained from plasma samples newly performed in 58 patients and recovered from routine drug resistance analysis in 98 cases.

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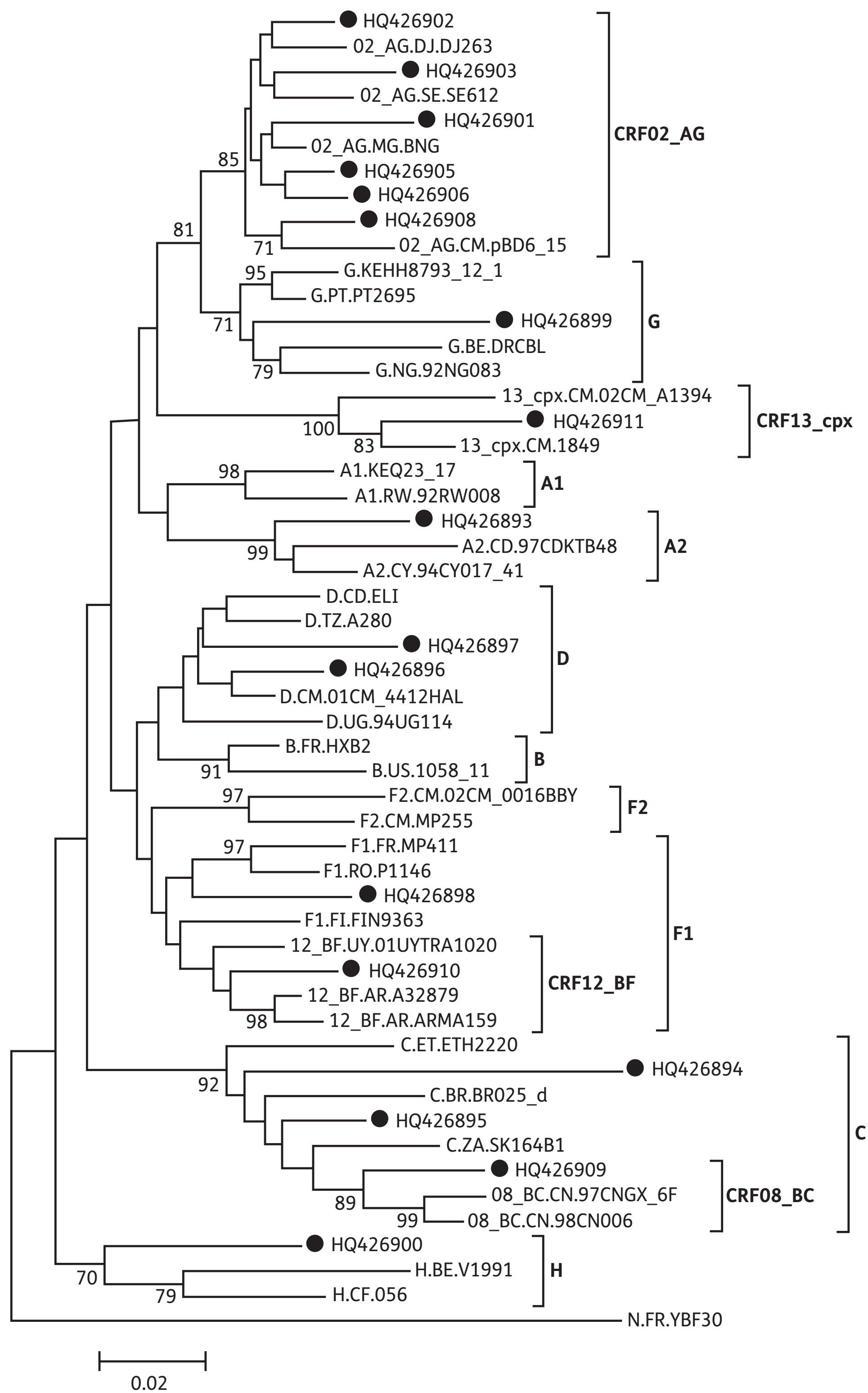

Figure 1. Phylogenetic analysis of *pol* sequences ascribed to HIV-1 non-B variants. The bootstrap cut-off was set at 700. Bootstrap values are shown as percentages. The tree was performed with the 17 non-B sequences including both PR and partial RT coding regions (1302 bp). The 19 non-B variants identified in the study cohort were confirmed with high bootstrap values (>700) in additional phylogenetic trees, including the two specimens with only PR or RT sequences available (data not shown).

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(14 cases); Equatorial Guinea (8), Cameroon (3), Nigeria (1), Democratic Republic of Congo (1) and Mozambique (1). Finally, among the four native Spaniards infected by non-B strains, two were infected by recombinant variants (1CRF02_AG and 1CRF12_BF) and two by non-B 'pure' variants (1A2 and 1D). HIV-infected patients harbouring non-B variants (A2, D, G, H and CRF02_AG) appeared in the study cohort in the first half of the 1990s. HIV-1 non-B variants were found in 17.7% of children infected by wild-type and 4.2% of those carrying resistant viruses (Table 2).

High rate of HIV-1 perinatally transmitted resistance

Among the 198 HIV-1-infected children under study, *pol* sequences were analysed in 30 (15%) before using any ART. Seven of these (23%) were infected by non-B subtypes (1A2, 2D, 1G and 3CRF02_AG) and the rest by subtype B strains. Three naive patients infected with non-B variants were born in Spain, while the rest came from Equatorial Guinea (3) and Nigeria (1). All of them acquired HIV-1 infection through MTCT, except two patients with unknown infection routes (Table 1). Sequences from 30 naive subjects were amplified from 16 plasma specimens (14 PR/RT and 2RT), 8 PBMC specimens (6 PR/RT, 1 RT and 1 PR), 2 DNA specimens (1 PR/RT and 1 RT) and the remaining 4 (all PR/RT) were recovered sequences. Four of the 30 naive children (13%) were infected with HIV-1 resistant variants carrying at least one resistance mutation to one or more drug family. For protease inhibitors (PIs), only major mutations were considered. One of the four children carrying resistant viruses was infected by a non-B variant (CRF02_AG), and they were infected in 1994 (1), 1997 (1), 2004 (1) and 2005 (1). Thus the global TDR mutations to any analysed drug family appeared in 13% of naive children; 4%, 7% and 10% for PIs, nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), respectively (Table 3).

Regarding TDR mutations for PIs, NRTIs and NNRTIs found among the 30 naive patients, M46L was the only major PI resistance mutation found, and was present in one virus from 1 of the 26 patients with available PR sequences. The frequencies of NRTI resistance mutations in naive children were 6.9% (for M41L) and 3.4% (for both L210W and T215S). The frequency of NNRTI resistance mutations was 3.4% (for both K103N and P225H). All TDR mutations from naive children were recovered from RNA-derived sequences. More than a quarter of 119 children carrying resistant virus presented <25% CD4+ T cells (Table 2).

High level of drug resistance among children on ART

One hundred and sixty-eight of the 198 children (85%) with analysed sequences were on ART by the time specimens were collected. Among these, 156 (93%) were infected by subtype B viruses and 12 patients harboured non-B variants (2C, 1F1, 1H, 5CRF02_AG, 1CRF08_BC, 1CRF12_BF and 1CRF13_cpx). Five of these non-B-infected children were native Spanish and the rest came from Equatorial Guinea (n=3), Mozambique (n=1), Cameroon (n=1), Congo (n=1) and India (n=1). PR/RT sequences were analysed in 125 pretreated children, only PR in 32 and only RT in 11. Sequences from pretreated children were amplified from 42 plasma specimens (33 PR/RT, 5 PR and 4 RT), 29 from PBMCs (22 PR/RT, 1 PR and 6 RT), 3 from DNA (2 PR and 1 RT) and 94 recovered sequences (70 PR/RT and 24 PR).

Table 3. Prevalence of HIV drug resistance mutations according to drug class family

Feature	Number of HIV-infected patients naive (n=30)	pretreated (n=168)	total (n=198)
Patients with available PR	26	157	183
Patients with available RT	29	136	165
Prevalence of drug resistance mutations (%) (95% CI)[a]			
global (to any class)	13 (4–31)	66 (59–73)	58 (51–65)
to PIs (major)	4 (0.1–20)	37 (29–45)	32 (25–39)
to NRTIs	7 (0.9–23)	54 (46–63)	46 (38–54)
to NNRTIs	10 (0.1–18)	35 (26–43)	29 (22–36)
to ≥2 classes	0	30 (23–38)	25 (19–32)

[a]Prevalence for naive patients was determined following the WHO TDR list[20] while for pretreated patients the IAS–USA 2009 list[21] was used.

Two-thirds of the 168 children on ART at specimen collection time were infected with mutants resistant to any drug family (Table 3). Three of the 111 children carrying resistant viruses were infected by non-B variants (1C, 1H and 1CRF02_AG). Thus global resistance prevalence was higher among ART-exposed children than in naive children (66% versus 13%). Considering drug families, 32% of children were infected by viruses resistant to PIs, 46% of children were infected by viruses resistant to NRTIs and 29% of children were infected by viruses resistant to NNRTIs (Table 3). As expected, more children were infected by viruses resistant to two or more drug classes among pretreated versus naive individuals; 30% (50/168 patients) versus 0% (0/30 patients) (Table 3). Among pretreated patients in the cohort, a similar high rate of global resistance provided by RNA (136 patients) and DNA (32 patients) analysis was found (68% versus 56%; P=0.273), although significantly higher for PIs (41% versus 18%; P=0.036) and for NNRTIs (42% versus 10%; P=0.002). According to the infecting HIV-1 variant, we observed a significantly higher global prevalence of drug-resistant mutations among children harbouring subtype B compared with non-B variants (Table 4), mainly due to a significantly higher NRTI resistance.

The drug resistance mutations for PIs, NRTIs and NNRTIs observed in pretreated children are shown in Figure 2. The major resistance mutations found in over 8% of children receiving ART were: for PIs, L90M (14.6%), D30N (12.6%), M46I (8.8%) and V82A (8.2%); for NRTIs, D67N (27.9%), M41L (25.7%), T215Y (22.8%), K70R (16.9%), L210W (16.9%), M184V (15.4%) and K219Q (11%); and for NNRTIs, K103N (16.2%).

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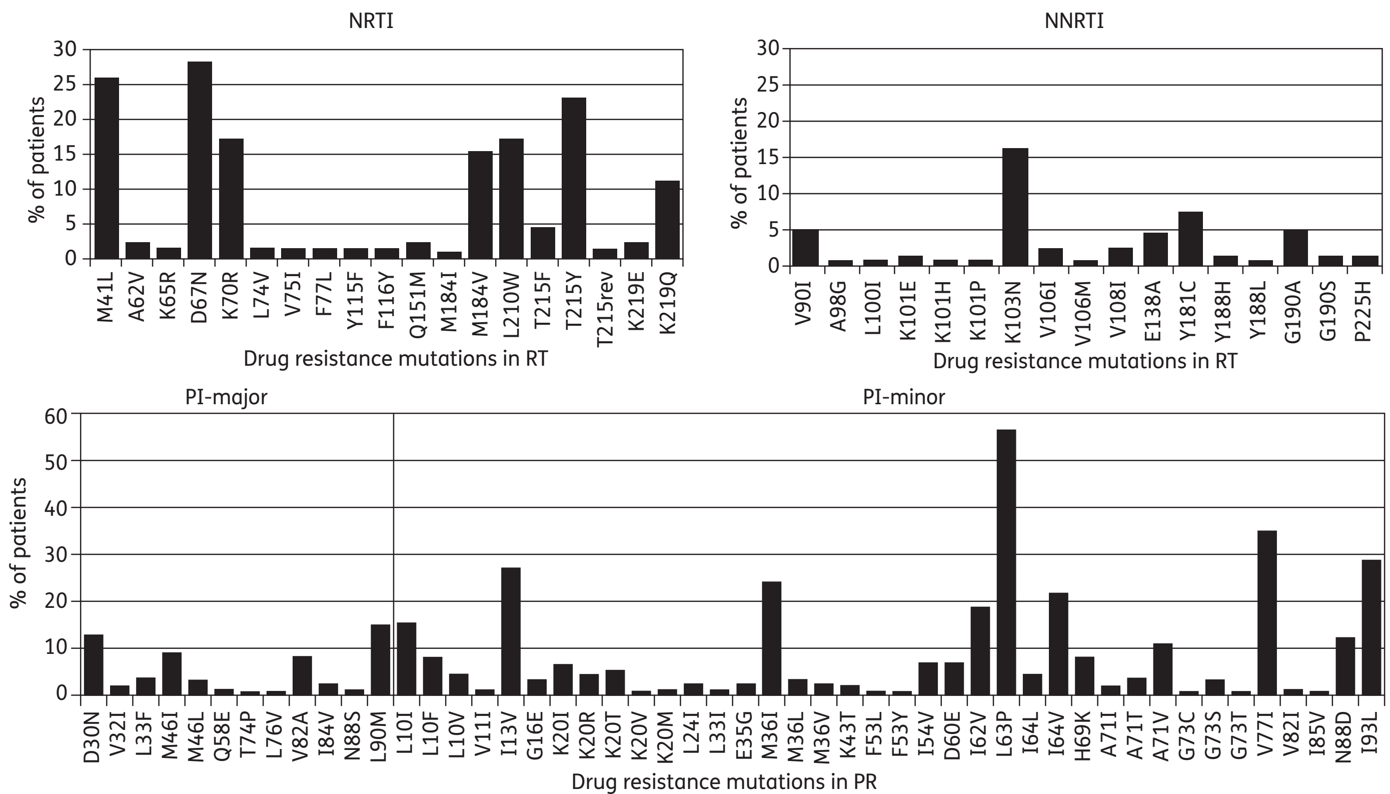

Figure 2. Substitutions in PR and RT associated with drug resistance in the pretreated paediatric HIV-1-infected population. Neither major PI drug resistance mutations nor RT drug resistance mutations found are natural polymorphisms in non-B variants according to previous reports.[17] Drug resistance mutations are listed according to IAS–USA.[21] Mutations not found and not shown in figure: for PIs, I47A/V, G48V, I50L/V, I54L/M and V82F/L/ST (major) and L33V, E34Q, I54A/S/T, I64M, N83D, L89V and I93M (minor); for NRTIs, T69insertion and K70E; and for NNRTIs, V106A, V179D/F/T, Y181I/V, Y188C and M230L. T215rev in RT means T215 revertants, including changes T215A/C/D/E/G/H/I/L/N/S/V. Available *pol* sequences among 169 pretreated patients; 157 PR and 136 RT. Naive patients are not included in this figure. Primary drug resistance mutations found among four naive patients according to the TDR list[20] were Patient 1 (M41L), Patient 2 (M46L), Patient 3 (M41L, L210W and T215S) and Patient 4 (K103N and P225H).

Table 4. Prevalence of drug resistance mutations according to drug class in children harbouring HIV-1 subtype B versus non-B variants

	Prevalence of drug resistance mutations, % (95% CI)			
	total (n=198)	B variants (n=179)	non-B variants (n=19)	P
Global (to any class)	58 (51–65)	62 (55–69)	21.1 (6–46)	<0.02
To PIs (major)	32 (25–39)	34 (26–41)	16.7 (4–41)	NS
To NRTIs	46 (38–54)	50.3 (42–59)	11.1 (1.4–35)	<0.02
To NNRTIs	29 (22–36)	31.3 (23–39)	11.1 (1.4–35)	NS
To ≥2 classes	25 (19–32)	27.4 (21–34)	5.3 (0.1–26)	NS

Ns, not significant.

Sequence data

All 198 PR and/or RT sequences from the HIV-1 *pol* gene from all infected subjects reported in this study have been submitted to GenBank. Accession numbers for subtype B sequences are HQ426714–HQ426892 and for non-B variants are HQ426893–HQ426911.

Discussion

This study describes for the first time the HIV-1 variants infecting patients enrolled in the Madrid cohort of children infected with HIV during the period 1993–2009, their clinical and epidemiological features and the prevalence of drug resistance mutations for different drug families in viruses present in naive and pretreated patients at sample collection.

HIV-1 non-B variants are present in the Madrid paediatric cohort

The major finding is that HIV-1 non-B variants infect almost 1 in 10 patients in this large Spanish paediatric cohort, most of whom are under current follow-up. The data reveal that non-B variants were mainly introduced through perinatal infection in the first half of the 1990s through foreign infected mothers, mainly coming from countries with a high prevalence of non-B variants.[22] Only 21% of the children infected by non-B variants

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had native Spanish parents. The observed prevalence of HIV-1 infections with non-B variants (10%) agrees with previous reports in infected adults from Madrid and Spain,[11,13] where an increasing prevalence has been reported in recent years.[11]

HIV-1 subtype diversity represents a challenge for clinical management of HIV-1-infected individuals.[23] The HIV-1 clade can impact on disease progression and response to ART.[24] Although no evidence of subtype-determined virological response to ART has been found in previous Paediatric European Networks,[25] differences in disease progression have been confirmed for adult and children infected with clade D variants,[14,26] as well as subtype-associated differences in the rate of CD4+ T cell decline.[23]

Since infection in newborns under suboptimal antiretroviral prophylaxis of their mothers increases the risk of drug resistance acquisition, early resistance genotyping of HIV-1-infected newborns is essential,[27] mainly in resource-limited settings where ART is being scaled up.[28] However, limited data on antiretroviral drug resistance rates are available in well-established paediatric cohorts. Our study reports for the first time the rate of HIV-1 drug resistance in naive and pretreated children in a large paediatric cohort, the Madrid cohort of HIV-infected children.

High rate of TDR among naive patients in the cohort of HIV-infected children in Madrid

The estimated TDR rate (13%) found among the naive paediatric cohort studied was similar to the reported TDR data for adults in Spain (13.6%), although significantly higher in non-B versus B subtypes (18.7% versus 10.6%).[17] The TDR rate in naive children in Spain was also similar to that reported in other adult cohorts across Europe and the USA, ranging from 5% to 15%.[29–31] TDR rates observed in our cohort were higher than in other surrounding countries, such as France, where 10% of perinatally infected children born during 1997–2004 carried resistant HIV-1 variants.[32] Lower TDR rates (6.8%) were also found in the multicentre Collaborative HIV Paediatric Study (CHIPS) cohort in the UK.[33] Regarding drug families, TDR for NRTIs and NNRTIs in the 30 naive subjects studied from the Spanish cohort was higher (7% and 10%, respectively) than that reported for 44 naive patients from the CHIPS cohort (6.8%) from 1998 to 2004.[33] The TDR rate observed in our cohort could be explained by the use of suboptimal antiretroviral prophylaxis in their mothers, mainly infected when only monotherapy was available. It could also be due to suboptimal ART regimens in foreign mothers coming from low-income countries, where prophylaxis is not always well implemented. Unfortunately, the maternal treatment history of children with TDR was not available.

High resistance in children on ART

Among the 198 children studied, 85% were on ART and 61.6% had been treated with 4–21 different ART regimens by December 2009, presenting good immunological and clinical status. The number of children with ≥200 CD4+ T cells was high, similar to other European paediatric cohorts with long-term outcomes,[32] and higher than in low-income areas such as Africa.[34]

This situation is different in low-income countries, such as those in sub-Saharan Africa, where 2.3 million children younger than 15 years have HIV infection[9] and <30% have received treatment.[35] In paediatric HIV treatment cohorts in sub-Saharan Africa, children started ART with a poorer immunological status and presented severe immunodeficiency in nearly 70%, compared with 41% in our Spanish cohort. Moreover, the lower number of ART regimens, shorter treatment experience, lack of guarantees of ART per life and lack of extended resistance and viraemia testing predict that infections with resistance variants could be even higher among children infected in low-income countries.

Resistance mutations to PIs, NRTIs, and NNRTIs were nine (37% versus 4%), seven (54% versus 7%) and three (35% versus 10%) times higher, respectively, when comparing pretreated and naive children in the study cohort. As a limitation, this study may not be extrapolated to other settings due to the special characteristics of our study cohort and the small sample size of naive patients. In our cohort, resistant viruses were found in two-thirds (66%) of pretreated children, as in multicentre adult cohorts receiving ART.[36] Almost one in three (30%) was infected with viruses resistant to two or more drug classes, reinforcing the importance of the use of new therapies not always approved for paediatric use.[37] Another limitation is that although the majority of resistance analyses were performed in plasma-associated RNA, in 42 (10 naive and 32 pretreated) of the 198 children with no available plasma specimens, resistance was analysed in proviral DNA, resulting in lower resistance to PIs and NNRTIs. Resistance mutations found in proviral DNA can reveal hidden archived drug resistance from previous regimens, providing a more expansive historic record of drug resistance relative to plasma. Trends of drug resistance mutations over time were not analysed due to the difficulty in establishing time periods with an equal number of specimens.

In children failing first-line therapy, selection of specific mutants is differentially affected by distinct HIV-1 subtypes.[38] HIV-1 non-B variants present natural polymorphisms in drug-associated positions in the absence of ART selection,[15,39] which can affect the susceptibility to certain drugs and can accelerate the selection and persistence of strains carrying certain primary drug resistance mutations.[40–42] However, the impact of each natural polymorphism on the efficacy of ART remains unknown.

The literature also reports the longer presence of variants carrying resistance mutations in patients infected by certain HIV-1 non-B variants, including the K103N mutation, in children on NNRTIs.[34] In our study K103N was the most prevalent NNRTI resistance mutation observed among pretreated children, in agreement with a previous study performed in different cities in Spain.[43] Due to the increasing frequency of patients infected with non-B variants in our country,[11] and their unknown long-term ART evolution, all infected children should be tested for HIV-1 subtypes, mainly those with mothers from endemic countries for non-B variants.

There is an urgent need for resistance testing as well as the development of new therapies based on different retroviral targets in drug-experienced children with therapeutic failure events. It is essential to use optimized ART for each individual, based on resistance information, to avoid suboptimal regimens and early resistance acquisition and to reduce long-term consequences of incomplete virological control. The reported data will help improve clinical management of the HIV-infected paediatric population in Spain.

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Members of the Madrid cohort of HIV-infected children

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Transparency declarations

None to declare.

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CAPÍTULO 2

Dinámica de prevalencia de resistencia a fármacos antirretrovirales en la cohorte de niños y adolescentes infectados por VIH-1 de la Comunidad de Madrid: análisis de 1993 a 2010

JUSTIFICACIÓN Y OBJETIVOS

El estudio de resistencias a fármacos ARV constituye uno de los parámetros más importantes al manejar y monitorizar la infección por VIH-1. A pesar de que el significado clínico de la resistencia a fármacos antirretrovirales ha sido bien documentado en población adulta infectada, todavía existen importantes limitaciones en los estudios que analizan la prevalencia y los patrones mutacionales de resistencia a fármacos ARV desarrollados específicamente para población pediátrica. La determinación de la susceptibilidad genotípica a fármacos ARV y la presencia de mutaciones de resistencia permiten evaluar las posibilidades terapéuticas presentes y futuras en pacientes multitratados que requieren tratamiento de por vida. Por esto, en este capítulo la determinación de la prevalencia de resistencias a ARV global y por familias de fármacos ARV, el tipo de mutaciones de resistencia y el grado de susceptibilidad predicho por herramientas bioinformáticas para cada ARV se estudiaron en cuatro periodos temporales distintos: P1 (1993-1999), P2 (2000-2004), P3 (2005-2008) y P4 (2009-2010). De esta forma pretendemos observar como han evolucionado las tasas de resistencia a ARV con el tiempo en esta cohorte y las mutaciones de resistencia más frecuentes, así como su potencial impacto en la eficacia de los fármacos ARV.

Los objetivos de este capítulo son:

- Describir las características clínicas y epidemiológicas de los niños y adolescentes infectados por VIH-1 de la Cohorte de Madrid incluidos en este estudio con secuencia o muestra clínica disponible hasta finales de 2010.
- Determinar la presencia y naturaleza de las mutaciones de resistencia a cada una de las tres familias de fármacos antirretrovirales más utilizadas (IP, ITIAN e ITINAN) para el tratamiento de la infección por VIH-1 en los cuatro periodos de estudio establecidos desde 1993 hasta finales de 2010.
- Determinar la dinámica temporal de prevalencia de resistencia a fármacos en población pediátrica infectada por VIH-1 pretratada en Madrid en los cuatro periodos establecidos.
- Determinar el grado de susceptibilidad a 19 fármacos antirretrovirales en población pediátrica infectada por virus resistentes en cada uno de los cuatro periodos analizados.
- Estimar la tasa de infecciones por variantes no-B del VIH-1 en niños nacidos antes y después del año 2000, caracterizadas por análisis filogenéticos del gen *pol* del VIH-1.

CONCLUSIONES

- Hasta finales de 2010 se seleccionaron un total de 232 pacientes con secuencia del gen *pol* del VIH-1 disponible. Su edad media era de 14,9 años, la mayoría (63,3%) estaban bajo seguimiento en unidades pediátricas y casi un cuarta parte de ellos (23,3%) habían sido transferidos a unidades de adulto.
- La situación clínica e inmunológica de los pacientes era buena. El 59% presentaban CV indetectable y el 79,7% un recuento de linfocitos T CD4+ >25%. La mayoría (91,3%) de los niños estaban bajo TAR, eran europeos (84%) y estaban infectados principalmente por subtipo B del VIH-1 (89%).
- La prevalencia de mutaciones de resistencia transmitidas en los 44 pacientes *naïve* a toma de muestra fue del 4,8% para IP, 9,3% para ITIAN y 11,6% para ITINAN.
- La prevalencia global de mutaciones de resistencia adquiridas en 188 pacientes pretratados fue de 39,9% para IP, 66,5% para ITIAN y 35,3% para ITINAN. Sorprendentemente, se encontró una alta prevalencia de virus con resistencia a las tres familias de fármacos entre pacientes pretratados, alcanzando el 21% durante el último periodo de estudio (2009-2010) y un 15,9% entre 1993 y 2010.
- En el periodo P1 (1993-1999) la prevalencia de resistencia se debió principalmente a fármacos ITIAN. Durante P2 (2000-2004) aumentó la prevalencia de resistencia a fármacos en todas las familias ARV alcanzando sus niveles máximos y en P3 (2005-2008) descendió. Entre 2009 y 2010 (P4) se mantuvieron los niveles de resistencia del periodo anterior, con un ligero aumento del nivel de resistencias a ITINAN e IP.
- Se encontraron diferencias estadísticas en la prevalencia de virus resistentes entre el periodo P1 (1993-1999) y P4 (2009-2010) para IP (13% *vs.* 39% $p<0,05$) y para los ITINAN (6% *vs.* 42%, $p<0,05$), siendo la diferencia no significativa para los ITIAN (44% *vs.* 63%, p=NS).
- La interpretación de las dinámicas temporales de susceptibilidad genotípica a fármacos ARV también reveló un incremento temporal en el grado de resistencia a las distintas familias de los mismos. Se encontró que los fármacos cuyo uso estaría más comprometido serían NFV (IP), 3TC y FTC (ITIAN) y DLV, EFV y NVP (ITINAN) todos por encima del 20% de resistencia entre los pacientes de la cohorte.
- Hasta diciembre de 2010, entre los 232 pacientes de la cohorte analizados, se encontraron 24 pacientes infectados por variantes no-B. Esto refleja que, aunque el subtipo B era el mayoritario (89%), una de cada diez infecciones en la cohorte de niños

y adolescentes de la Comunidad de Madrid estaría causada por subtipos no-B o formas recombinantes del VIH-1.

- La tasa de infecciones por variantes no-B aumentó significativamente en niños nacidos después del año 2000 con respecto a los nacidos antes de ese año (39% *vs.* 4,2%, $p<0,05$).
- La tasa de pacientes infectados por formas recombinantes del VIH-1 en la cohorte casi duplicó la tasa de infectados por subtipos no-B puros en niños nacidos entre 2000 y 2010 (24,4% *vs.* 14,6%). La prevalencia de infecciones por formas recombinantes del VIH-1 en la población de estudio aumentó del 1,6% en niños nacidos antes del 2000 al 24,4% en niños nacidos después del año 2000.

Trends in Drug Resistance Prevalence in HIV-1–infected Children in Madrid: 1993 to 2010 Analysis

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Background: Drug resistance mutations compromise antiretroviral treatment (ART) effectiveness in HIV-1–infected children. Trends in drug resistance prevalence have not been previously evaluated in HIV-infected children in Spain.

Methods: HIV-1 variants, drug resistance prevalence dynamics and drug susceptibility were analyzed from 1993 to 2010 in HIV-infected children with available *pol* sequence, sample or drug resistance profile. HIV-1 variants were characterized by phylogenetic analysis. Resistance mutations in pretreated and naive patients were identified according to International AIDS Society-2010 and the World Health Organization list, respectively.

Results: In 232 patients, genotypic resistance profiles (n = 11) or *pol* sequences (n = 128) were recovered or newly generated from infected samples (n = 93). Patients were mainly in care at pediatric units (63%), were mostly Europeans (84%), with moderate AIDS symptoms (65%), on ART (91%) and infected by HIV-1 subtype B (89%). Transmitted major drug resistance mutations were selected in 6 (13.6%) of the 44 ART-naive children: 4.8%, 9.3% and 11.6%, for protease inhibitors, nucleoside reverse transcriptase inhibitors and nonnucleoside reverse transcriptase inhibitors, respectively. Overall resistance prevalence was higher (71.8%) among ART-exposed children: 39.9%, 66.5% and 35.3% for protease inhibitors, nucleoside reverse transcriptase inhibitors and nonnucleoside reverse transcriptase inhibitors, respectively. Resistance prevalence among ART-exposed children was higher in 2009 to 2010 relative to 1993 to1999 for nonnucleoside reverse transcriptase inhibitors (42% versus 6%; $P = 0.006$), protease inhibitors (39% versus 13%; $P = 0.004$) and nucleoside reverse transcriptase inhibitors (63% versus 44%; P = NS). Susceptibility to each drug in resistant viruses was predicted. The rate of non-B infections increased in the last years, mainly caused by recombinant viruses.

Conclusions: The increasing resistance prevalence among the HIV-infected pediatric population in Spain highlights the importance of specific drug resistance and drug susceptibility surveillance in long-term pretreated children to optimize treatment regimens.

Key Words: HIV-1, children, drug resistance, susceptibility, Spain

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The World Health Organization estimates that 3.4 million children below 15 years of age were living with HIV by the end of 2010.[1] Increasing access to services for preventing HIV mother-to-child transmission (MTCT) and the increased availability of antiretroviral therapy (ART), particularly in middle-income and low-income countries, have reduced the number of AIDS-related deaths by 20% and new HIV infections by 50% among children, from 2005 to 2010,[1,2] according to the latest update on the report on the global AIDS epidemic.

In developed countries, perinatal transmission rates have decreased to 1–2%, mainly due to the expanded access to highly active antiretroviral treatment (HAART) and prevention efforts in HIV testing, prenatal care, formula feeding, elective cesarean delivery and pregnancy monitoring.[3–7] However, MTCT still occurs in high-income countries mainly due to infected immigrants from countries with a high HIV prevalence, and within socially at-risk groups who have lower rates of pregnancy monitoring and HIV testing. In Spain, rates of MTCT have followed the same trend as in other neighboring countries due to the implementation of health policies, pregnancy follow-up and HAART administration in infected mothers.[8,9] Despite this, the heroin abuse during the 1980s and 1990s in Spain impacted women especially, reaching the highest AIDS incidence and prevalence in Western Europe, leading to a high incidence of MTCT in children born between 1980 and 1990.[10,11] Furthermore, Spain has become one of the main entrance points of Africans into the European Union, mainly from sub-Saharan African countries with high HIV infection prevalence.[12] Both phenomena have caused a high number of perinatally HIV-infected children with AIDS in Spain, reaching 958 cases between 1981 and 2010, of which 246 were reported in Madrid.[10]

Access to treatment has dramatically changed the course of infection, reducing morbidity, mortality, viremia and increasing the life expectancy of HIV-infected children.[13–15] Because of the use of HAART, an emergence of antiretroviral (ARV) resistance among treated patients has been observed,[16] mainly due to poor adherence or suboptimal drug dosing or regimens, limiting the therapeutic efficacy. Resistance genotyping has become a standard-of-care in HIV infection management.[7] Although the clinical significance of HIV drug resistance has been well documented in the adult populations, studies on the prevalence and patterns of drug resistance mutations in pediatric HIV-infected populations remain limited. Thus, the aims of this study were to describe temporal trends of prevalence for drug resistance mutations in both heavily pretreated and treatment-naive patients

from the Madrid cohort of HIV-infected children during a period of almost 2 decades (1993–2010).

MATERIALS AND METHODS

Study Population

Since the beginning of the epidemic in Spain, 530 patients have been registered in the Madrid cohort of HIV-infected children. By December 2010, 183 of them still remained in clinical care in pediatric units, 100 had been transferred to adult units, 62 had been lost to follow-up and 185 had died. A total of 232 patients infected during their childhood were enrolled in this study, selected according to availability of sample (immortalized DNA, plasma or peripheral blood mononuclear cells [PBMCs]), *pol* sequence or genotypic resistance profile. Genetic sequences and genotypic resistance profiles were recovered during 2010 from clinical routine drug resistance tests performed in hospitals where patients were or had been in care. Samples from patients were provided by the HIV BioBank integrated in the Spanish AIDS Research Network.[17] Samples were processed following current procedures and frozen immediately after their receipt. All patients participating in the study gave their informed consent, and protocols were approved by institutional ethical committees.

HIV-1 Diagnosis

All the children included in the study population were diagnosed with HIV-1 infection by a positive RNA or DNA polymerase chain reaction for HIV in children under 18 months of age, and by 2 serial-positive serologic assays in children over 18 months of age or by a positive quantitative-polymerase chain reaction (RNA) testing (viral load).

HIV-1 *pol* Genotyping

For RNA extraction from HIV-1–infected plasma samples, an automated platform for the isolation of RNA based on the magnetic-silica technology was used with NucliSENS easyMAG instrumentation (BioMerieux, Durham, NC), according to the manufacturer's instructions. For viral DNA extraction from infected PBMCs, a commercial DNA isolation kit based on column extraction was used (Qiagen Inc., Valencia, CA). HIV-1 *pol* sequencing (1,121 base pair), including the complete protease (PR; 297 base pair) and partial reverse transcriptase (RT) genes and HIV-1 subtyping by phylogenetic analysis were performed as previously reported.[18]

Drug Resistance Prevalence

The prevalence of transmitted drug resistance (TDR) was defined according to the list of mutations for TDR surveillance, as recommended by the World Health Organization[19] using the Calibrated Population Resistance tool.[20] Drug resistance mutations in pretreated patients, defined by the International AIDS Society-USA,[21] were manually located in each PR and RT sequence following the International AIDS Society-USA nomenclature. Genotypic interpretation of these resistance mutations was evaluated using the Stanford HIVdb Algorithm,[22] version 6.0.11 (Stanford University, Palo Alto, CA). Resistance was normalized in 3 levels: susceptible (S), intermediate (I) and resistant (R). Susceptibility was estimated for protease inhibitors (PI): atazanavir/r, darunavir/r, fosamprenavir/r, indinavir/r, lopinavir/r, nelfinavir (NFV), saquinavir/r and tipranavir/r where "/r" indicates coadministration with low-dose ritonavir for pharmacologic "boosting"; for nucleoside reverse transcriptase inhibitors (NRTI): lamivudine (3TC), abacavir, zidovudine (ZDV), stavudine (d4T), didanosine, emtricitabine (FTC), tenofovir (TDF); for nonnucleoside reverse transcriptase inhibitors (NNRTI): delavirdine (DLV), efavirenz (EFV), etravirine, nevirapine (NVP).

Statistical Analysis

Prevalence was expressed in percentage with 95% confidence interval. Confidence interval tests were performed with Epidat 3.1 (Pan American Health Organization, Washington DC). Categoric variables were compared using the χ^2 test. Significance was set at $P < 0.05$.

RESULTS

Study Population Selection

The study comprised a total of 232 subjects from 8 different public hospitals in Madrid infected with HIV-1 during childhood mainly by vertical transmission in 1980 to 2010. Criteria for inclusion required at least 1 available genotypic resistance profile, an infected sample or an HIV-1 gene *pol* sequence to analyze their genotypic resistance patterns. Characteristics of the study population at baseline (entry into the cohort study) are summarized in Table 1. Patients were mainly female (58%), perinatally infected (96.6%), born in Spain (82.3%) and on ART (81%) by sample collection time. Most infections (61.6%) occurred during the 1991 to 2000 period, although sequences were mainly obtained from specimens dated between 2000 and 2010.

Among the 232 selected pediatric patients, 60% had an HIV-1 *pol* sequence (n = 128) or an available genotypic resistance profile (n = 11), both obtained from clinical routine drug resistance analysis performed in hospitals where patients were or had been in care. We generated new *pol* sequences in the remaining 40% (n = 93) of patients with plasma (n = 24), RNA (n = 66) or DNA (n = 3) infected samples available at the HIV-1 BioBank.

Thus, this study was performed with 221 *pol* gene sequences (191 PR + RT, 21 PR and 9 RT) and 11 resistance profiles (6 PR+RT, 1 PR and 4 RT) derived from sequences obtained from plasma samples of HIV-infected children but with no fasta format sequences available at the study time.

Nearly two thirds (n = 145; 65.6%) of *pol* sequences had been previously reported and submitted to GenBank.[18] The 76 newly generated *pol* sequences in this study were performed from HIV-1 RNA or proviral DNA amplifications from new specimens (73 plasmas, 3 PBMC) recovered from the HIV-1 BioBank. Most (82.3%) of the 232 analyzed sequences were obtained from plasma samples and the remaining 41 DNA-derived sequences from PBMCs or immortalized DNA specimens (Table 1).

Clinical Features of the Study Population

Clinical and epidemiologic features of the 232 children included in the study by December 2010 are shown (Table 2). By then, the mean age of the study cohort was 14.9 years, 98.3% were still alive and 86.3% were in care in pediatric units (63%) or had been transferred to adult units (23.3%). Nearly all (97%) had a history of HIV-related symptoms according to the Centers for Disease Control and Prevention classification,[23] which were mild in 32.3% of cases. Of note, 59% patients had achieved undetectable viral load (less than 50 plasma RNA HIV-1 copies/mL), 79.7% had total CD4+ T cell counts over 25% and 46.6% had CD4+ counts greater than 750 cells/mm^3. The proportion of children receiving ART by December 2010 increased with respect to the specimen collection time (81% versus 91.3%; Tables 1 and 2). By December 2010, only 8 (3.4%) of the 232 children remained drug naive, and 11 (4.7%) were off treatment but were drug-experienced. Among the 212 children on ART, nearly all (90.1%) were receiving HAART,

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TABLE 1. Baseline Features of the 232 Selected Patients

Features	Patients (n [%])
Demographics	
Female gender	135 (58)
Adopted	50 (21.6)
HIV-1 transmission	
Perinatal	224 (96.6)
Blood transfusion	5 (2.2)
Sexual	1 (0.4)
Unknown	2 (0.8)
Origin[*]	
Spain	191 (82.3)
Other European countries	4 (1.7)
Africa	23 (10)
America	13 (5.6)
Asia	1 (0.4)
HIV-1 infection year	
1981–1990	54 (23.3)
1991–2000	143 (61.6)
2001–2010	35 (15.1)
Specimens collection year	
1993–1999	39 (16.8)
2000–2004	76 (32.8)
2005–2008	62 (26.7)
2009–2010	55 (23.7)
ART status of specimens collected	
Drug-naive	44 (19)
Under ART	188 (81)
Sequenced specimen	
DNA-derived sequence	191 (82.3)
RNA-derived sequence	41 (17.7)
Ethnicity	
White	181 (78)
Black	24 (10.3)
Hispanic	8 (3.5)
Romani	9 (3.9)
Others	10 (4.3)
Infecting HIV-1 variants	
B subtype	197
non-B variants	24
Unknown[†]	11

Patient distribution among hospitals in Madrid. Hospital Universitario La Paz (n = 57), Hospital General Universitario Gregorio Marañon (n = 52), Hospital Universitario Doce de Octubre (n = 49), Hospital .Universitario Carlos III (n = 30), Hospital Universitario de Getafe (n = 28), Hospital Principe de Asturias (n = 7), Hospital Universitario del Niño Jesus (n = 7), Hospital Universitario de Móstoles (n = 2).

[*]Countries of origin: Spain (n = 191), Equatorial Guinea (n = 11), Ecuador (n = 4), Morrocco (n = 3), Guatemala (n = 3), Honduras (n = 3), Nigeria (n = 3), Cameroon (n = 3), USA (n = 1), Portugal (n = 1), Romania (n = 1), Poland (n = 1), Ukraine (n = 1), Mozambique (n = 1), Democratic Republic of Congo (n = 1), Senegal (n = 1), India (n = 1), Peru (n = 1), Venezuela (n = 1).

[†]No infected samples or *pol* sequences from these patients were available to define HIV variant.

TABLE 2. Characteristics of the 232 Selected Patients by December 2010

Features	Patients (n [%])
Mean age (SD)	14.9 (4.9)
Alive (n [%])	228 (98.3)
Clinical follow-up (n [%])	
Pediatric unit	146 (63)
Adult unit	54 (23.3)
Lost to follow-up	28 (12)
Deceased	4 (1.7)
Clinical status (CDC[23]; n [%])	
Asymptomatic, N1	2 (0.8)
Low symptoms, A	75 (32.3)
Moderate symptoms, B	60 (25.9)
Severe symptoms, C	90 (38.8)
Unknown	5 (2.2)
Viral load (HIV-1 RNA copies/mL; n [%])	
≤50	137 (59)
51–500	27 (11.6)
>500	63 (27.2)
Unknown	5 (2.2)
Range of CD4+ T cell counts (cells/mm^3; n [%])	
>1500	13 (5.6)
751–1500	95 (41)
500–750	65 (28)
200–499	30 (12.9)
<200	4 (1.7)
Unknown	25 (10.8)
Range of CD4+ T cell counts (n [%])	
<25	42 (18.1)
25–50	182 (78.5)
>50	3 (1.2)
Unknown	5 (2.2)
ART experience	
Drug-naïve	8 (3.4)
PI experienced	199 (85.8)
NNRTI experienced	179 (77.2)
NRTI experienced	224 (96.6)
ART by December 2010 (n [%])	
HAART	209 (90.1)
Combined	1 (0.4)
Monotherapy	2 (0.8)
Stopped treatment or untreated[*]	19 (8.3)
Unknown	1 (0.4)
Number of ART regimens by December 2010 (n [%])	
0	8 (3.4)
1	18 (7.8)
2	23 (9.9)
3	34 (14.6)
4–6	84 (36.2)
7–12	63 (27.2)
>12	2 (0.9)

[*]Patients with previous but not current exposure to ART. SD indicates standard deviation.

and 64.3% had received more than 4 different ART regimens (range: 4 –12) during their clinical follow-up.

High Prevalence of HIV-1 Transmitted Drug Resistance

Among the 232 HIV-1–infected patients under study, 44 (19%) were treatment-naive when samples were obtained. None of these 44 had received neonatal ARV, according to clinical records. Forty-two of them had available *pol* sequences (40 PR + RT, 2RT), and 2 had resistance profiles (1PR and 1RT). Six children harbored TDR mutations. Thus, global TDR mutations prevalence (ie, to any ARV drug class) among the 44 naive patients was 13.6% (Table 3). The prevalence of TDR for each of the available drugs was: 4.8% for PI, 9.3% for NRTI and 11.6% for NNRTI. D30N, N88D and M46L (all 2.3%) were the only major PI mutations found within the naive population. TDR mutations found for reverse transcriptase inhibitors within the naive population were for NRTI: M41L, K70R and T215S/Y (all 4.6%) and M184V and L210W (all 2.3%) and for NNRTI: K103N, E138G, P225H and Y181C (all 2.3%). The TDR pattern and sampling year among the 6 naive patients were: patient 1 (M41L, year 1996), patient 2 (M41L, L210W, T215S, year 1997), patient 3 (K70R, M184V, T215Y, year 1999), patient 4 (D30N, N88D, K70R, Y181C, year 2004), patient 5 (K103N, E138G, P225H, year 2004) and patient 6 (M46L, year 2007).

By December 2010, 3 of the 6 children who harbored TDR were lost to follow-up in pediatric units. Only 3 of the 6 had achieved undetectable viral load (mean: 7472 HIV RNA copies/mL), with a mean CD4+ cell count of 894 CD4+ T cell and a mean percentage of 30% of CD4+ T cells.

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Trends in Prevalence of HIV-1 Drug Resistance

At the time the samples were collected, 188 of the selected patients were drug-experienced. One hundred seventy-nine *pol* sequences (151 PR + RT, 21 PR and 7 RT) and 9 (6 PR + RT and 3 RT) resistance genotypic profiles were available to determine the prevalence of resistance mutations in pretreated patients. The prevalence of overall drug resistance mutations (DRMs) according to International AIDS Society-USA 2010 list[21] among the 188 ART-experienced patients with available *pol* sequence or resistance profile between 1993 and 2010 was 71.8% (Table 3). DRM prevalence among pretreated patients was 39.9% for PI, 66.5% for NRTI and 35.3% for NNRTI.

All (n = 188) available sequences or resistance genotypic data from pretreated patients were grouped in 4 time periods according to the date of sampling: P1 1993 to 1999 (n = 18), P2 2000 to 2004 (n = 68), P3 2005 to 2008 (n = 55) and P4 2009 to 2010 (n = 47). Global DRM prevalence in the pretreated population for each time period was 61%, 84%, 67% and 66%, respectively. Major PI drug resistance mutations[21] detected in over 5% of patients were: D30N (10.1%), M46I (12.8%), V82A (9%), I84V (5.3%) and L90M (18.1%). NRTI mutations occurring in over 5% were: M41L (26.6%), D67N (26.1%), T69D (8.5%), K70R (16%), L74V (6.9%), M184V (22.3%), L210W (17.6%), T215F (6.4%), T215Y (21.8%) and K219Q (10.6%). NNRTI mutations occurring in over 5% were: K103N (15.4%), Y181C (6.9%) and G190A (6.9%).

The prevalence of these DRMs was compared in the 4 time periods (Fig. 1). In P1, resistance prevalence was caused mainly by the presence of mutations for NRTIs, with higher prevalence of D67N, T69D and K70R. They were selected by the use of ZDV, the first approved drug to treat HIV-infected children in Spain in 1988. D67N and K70R mutations are considered thymidine-analog–associated mutations, which can also affect other NRTIs, such as d4T and TDF approved in Spain in 1996 and 2002, respectively. No mutations for NNRTIs were found in this period, and only a low-level PR mutation (M46I) was detected.

In the P2 period, there was a substantial increase in prevalence of mutations to all 3 classes of drugs, and a high prevalence of viruses harboring D30N mutation was observed, probably caused by the substantial use of NFV in that period. K103N was the most prevalent NNRTI mutation compromising the use of NVP and EFV, approved in Spain in 1998 and 1999, respectively.

In P3, a change of pattern was observed in the prevalence of certain drug resistance mutations to all drug classes, decreasing for D30N, L90M and I84V (for PIs), K70R and MI84V (for NRTI) and K103N (for NNRTI). By contrast, in P3, the prevalence increased for V82A in PR affecting all PIs: for thymidine-analog–associated mutations M41L, D67N, L210W and T215Y and K219Q, compromising the efficacy of some NRTIs and for G190A, affecting NNRTIs.

In P4, the prevalence of viruses carrying other resistance mutations at PR (M46I, V82A, L90M) and in RT (M41L, D67N, T69D, K70R, L210W, T215F/Y, K210Q, affecting NNRTI) decreased compared with the previous period. By contrast, the prevalence of NRTI-associated mutations L74V and M184V, of the 3 NNRTI-associated drug mutations K103N, Y181C and G190A and for the PI-associated I84V decreased.

Temporal trends in DRM prevalence were also assessed. Figure 2 shows the tendency of drug resistance prevalence for the 3 available drug ART classes during the studied period from 1993 to 2010. Prevalence of DRM for NNRTI, PI and NRTI was lower in P1 (6%, 13% and 44%, respectively) compared with the most recent period P4 (42%, 39% and 63%, respectively) reaching the highest prevalence during the years 2000 to 2004 (P2). When comparing

TABLE 3. Prevalence of HIV Drug Resistance Mutations According to Drug Class Family

	Number of HIV-infected Patients		
	Naive (n = 44)	Pretreated (n = 188)	Total (n = 232)
Patients with available PR	41	178	219
Patients with available RT	43	167	210
Prevalence of drug resistance mutations (%; [95% CI])*			
Global (to any class)	13.6 (2.4–24.9)	71.8 (65.1–78.5)	62.1 (55.6–68.5)
To PIs	4.8 (0.6–6.5)	39.9 (32.4–47.4)	33.3 (26.9–39.8)
To NRTIs	9.3 (2.6–22.1)	66.5 (59–73.9)	54.8 (47.8–61.7)
To NNRTIs	11.6 (3.9–25.1)	35.3 (27.8–42.9)	30.5 (24–36.9)
To 2 classes	2.3 (0.1–12)	40.4 (33.2–47.7)	33.2 (26.9–39.5)
To 3 classes	2.3 (0.1–12)	15.9 (10.5–21.5)	13.4 (8.8–17.9)

Only 6 of the 44 naive children harbored TDR mutations. *Prevalence for naive patients was determined following the WHO TDR list,[19] whereas for pretreated patients the International AIDS Society-USA 2010 list[21] was used. CI indicates confidence interval.

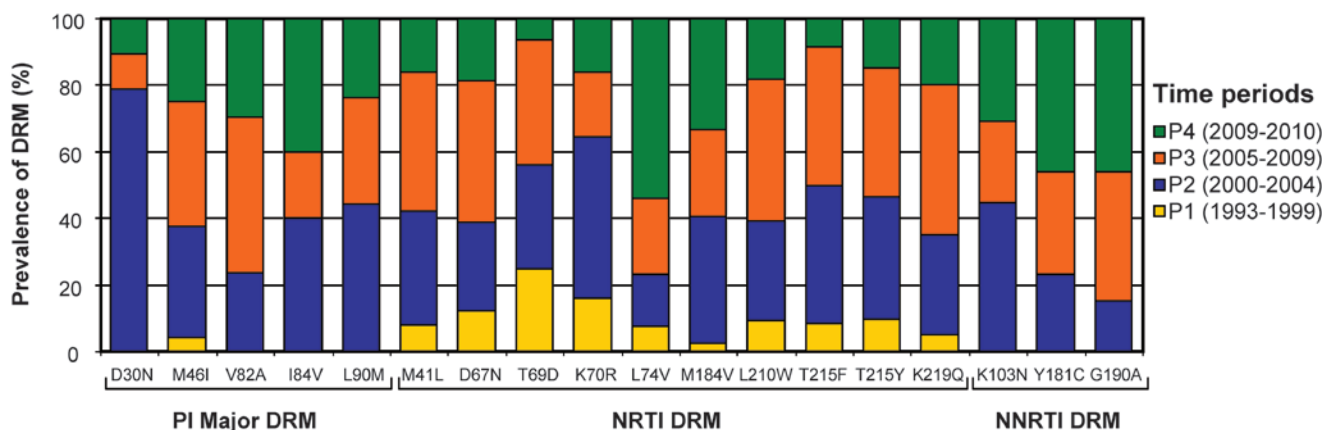

FIGURE 1. Temporal trends of DRM prevalence according to drug class. PI indicates protease inhibitors; RT, reverse transcriptase; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, nonnucleoside reverse transcriptase inhibitors. Asterisk indicates statistical differences between P1 (1993–1999) and P4 (2008–2010).

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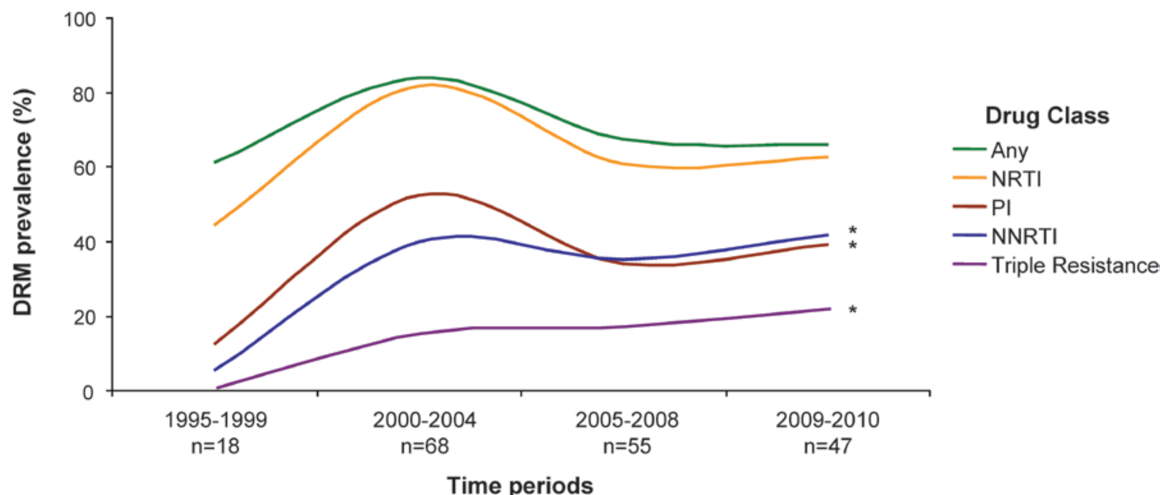

FIGURE 2. Prevalence of DRM positions for each drug class represented over 5% in the selected population.

the first (P1) and the latest period (P4), a statistically significant increase in prevalence of resistance to PIs (13% versus 39%; P = 0.004), and to NNRTIs (6% versus 42%; P = 0.006) was observed.

Resistance mutations to 2 drug classes were detected in 40.4% of pretreated children, a significant increase when comparing the 1993 to 1999 and 2009 to 2010 periods (5.5% versus 46.8%; P = 0.002). Specimens from 15.9% of the pretreated children had viruses with mutations conferring resistance to NRTI, NNRTI and PI (Table 3). Of note, prevalence of triple class resistance among pretreated children significantly increased from 0% in 1993–1999 to 21% in the 2009–2010 period (P < 0.03; Fig. 2).

Genotypic Drug Interpretation

Susceptibility for individual ARV drugs in each time period was analyzed for all of the pretreated samples available for this study (n = 188; Fig. 3). During P1 (1993–1999), a high susceptibility to all drugs was observed, mainly due to the small number of ART in use at that time for the management of pediatric HIV infection. The highest resistance was found in the NRTI family, highlighting the narrow therapeutic choices available in Spain before year 2000. By the end of the 1990s, 30% and 20% of pediatric patients were resistant to ZDV/d4T and to didanosine, respectively.

During P2 (2000–2004), an increase of resistance to all 3 drug families was observed, especially to abacavir, 3TC, FTC, NVP, EFV, DLV and NFV. Abacavir, approved in Spain in 2000, showed an 18-fold increase in resistance level from P1 (0%) to P2 (18%). There was also a significant increase in resistance to 3TC and to FTC (approved in Spain in 2000) from 0% in P1 to more than 35% in P2. Prevalence of resistance against EFV and NVP increased markedly from 0% in P1 versus 33% in P2; these NNRTI drugs were approved for pediatric use in Spain in 1998 and 1999, respectively. Finally, there was also a remarkable increase in resistance for certain PIs, especially NFV (5% in P1 versus 45% in P2).

During P3 (2005–2008), the prevalence of resistance to 3TC, FTC, TDF, EFV, DLV, NVP and NFV decreased despite an increasing resistance to the remaining drugs. Especially for lopinavir/r, approved in Spain in 2000, the increase of resistance from 0% to more than 15% of patients was noteworthy. There was also a notable increase in resistance to NRTI drugs ZDV and d4T from 27% to 33%, reaching almost 40%.

In P4 (2009–2010), there was a stabilization of both drug resistance prevalence, with a tendency for a decrease in resistance prevalence for some drugs such as ZDV, d4T, didanosine but an increase for others, including 3TC, TDF, FTC, EFV and DLV.

Phylogenetic Characterization of HIV-1 Variants

Phylogenetic analysis of 221 *pol* sequences revealed that HIV-1 subtype B was the most prevalent (89.1%) variant in the cohort. Among the 24 (10.9%) HIV-1 non-B variants, 45.8% corresponded to "pure" non-B subtypes or sub-subtypes (1 A1, 1 A2, 3 C, 2 D, 1 F1, 2 G, 1 H) and 54.2% were intersubtype recombinant strains ascribed to circulating recombinant forms (CRFs; 10CRF02_AG, 1CRF08_BC, 1CRF12_BF and 1CRF13_cpx). No unique recombinant forms were found in the study population.

The 24 non-B subtypes and recombinants found during the study period corresponded to 3 (12.5%) children born to native Spanish parents and 21 (87.5%) children with 1 foreign infected parent coming from Ukraine (1 case), India (1 case), Morocco (2 cases) or sub-Saharan Africa (17 cases). Among the 191 children infected before 2000, 8 were HIV-1 non-B variants coming from Spain (1 sub-subtype A2, 1 CRF02_AG, 1 CRF12_BF), Morocco (1 subtype D), Nigeria (2 subtypes G), Cameroon (1 subtype H) and Equatorial Guinea (1 CRF02_AG). Among the 41 patients infected after 2000, the 16 HIV-1 non-B variants came from Ukraine (1 A1), Cameroon (1 subtype C, 1 CRF02_AG), Morocco (1 subtype C), Mozambique (1 subtype C), Equatorial Guinea (1 subtype D, 7 CRF02_AG, 1 CRF08_BC), Democratic Republic of Congo (1 sub-subtype F1) and India (1 CRF13_cpx). All patients infected by non-B variants were perinatally infected except for the subtype D–infected patient from Morocco infected in 1995 by unknown route of infection.

An analysis of the infecting variant according to the year of infection highlights an interesting trend. The rate of non-B infections significantly increased among the 41 children born after year 2000 compared with the 191 children infected before 2000 (39% versus 4.2%; P < 0.05). Considering the 24 children carrying HIV-1 non-B variants, 8 (33.3%) and 16 (66.7%) were infected before or after 2000, respectively. Meanwhile, the rate of viruses carrying "pure" non-B subtypes versus recombinants was similar in the 191 children infected before 2000 (2.6% versus 1.6%); in children born from 2000 to 2010, the rate of recombinants almost duplicated the rate of pure non-B subtypes (24.4% versus 14.6%). Furthermore, the prevalence of recombinants in the study population increased from 1.6% before 2000 to 24.4% after 2000.

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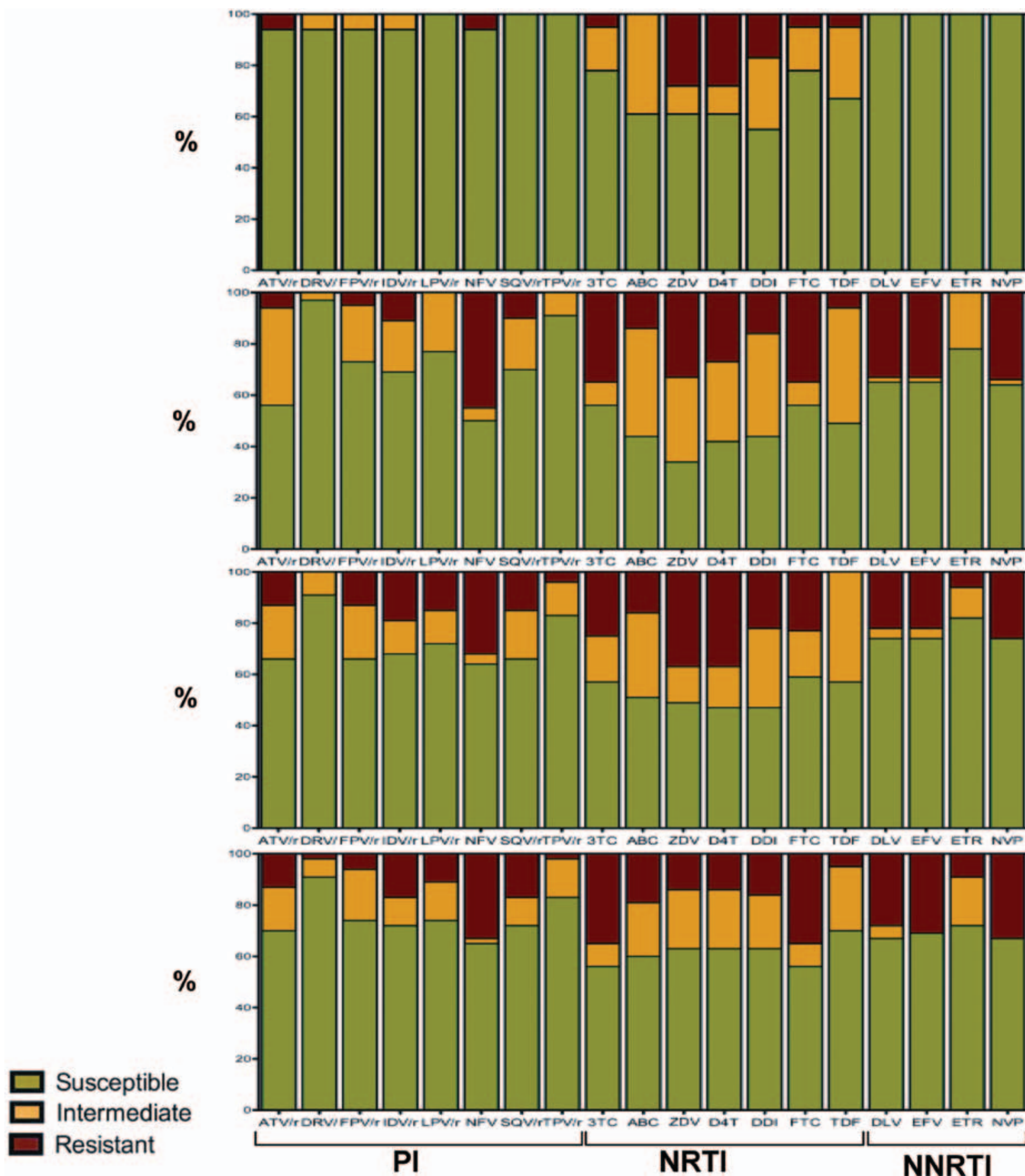

FIGURE 3. Predicted susceptibility to each of the drugs used as ART in pretreated patients. Top represents patients from 1993 to 1999 period (n = 18), second panel from the top shows patients from period 2000 to 2004 (n = 68), third panel from the top, patients from 2005 to 2008 (n = 55) and bottom panel represents patients from the 2009 to 2010 period (n = 47). Susceptibility was estimated according to the HIVdb Interpretation Algorithm (Stanford University, Palo Alto, CA). PI indicates protease inhibitors: atazanavir/r (ATV/r), darunavir/r (DRV/r), fosamprenavir/r (FPV/r), indinavir/r (IDV/r), lopinavir/r (LPV/r), nelfinavir (NFV), saquinavir/r (SQV/r), tipranavir/r (TPV/r) and where "/r" indicates coadministration with low-dose ritonavir (RTV) for pharmacologic "boosting." NRTI, nucleoside reverse transcriptase inhibitors: lamivudine (3TC), abacavir (ABC), zidovudine (ZDV), stavudine (d4T), didanosine (ddI), emtricitabine (FTC), tenofovir (TDF). NNRTI, nonnucleoside reverse transcriptase inhibitors: delavirdine (DLV), efavirenz (EFV), etravirine (ETR), nevirapine (NVP); R, high level resistance; I, intermediate resistant; S, susceptible. The approval year for each drug in Spain were: 1988 (ZDV), 1993 (ddI), 1996 (d4T, 3TC, IDV, SQV), 1998 (NVP, NFV), 1999 (ABC, EFV), 2001 (LPV/r), 2002 (TDF), 2003 (FTC), 2004 (ATV/r, FPV/r), 2005 (TPV/r), 2007 (DRV/r) and 2008 (ETR; data available at www.aemps.gob.es).

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Sequence Data

All PR and/or RT sequences from the HIV-1 *pol* gene from the 221 infected subjects with available *pol* sequence reported in this study have been submitted to GenBank. Among them, 145 *pol* sequences had previously assigned accession numbers[18]: (HQ426714–HQ426719, HQ426721–HQ426722, HQ426724–HQ426728, HQ426730–HQ426731, HQ426733–HQ426734, HQ426736–HQ426737, HQ426741–HQ426743, HQ426745, HQ426749–HQ426752, HQ426754–HQ426755, HQ426758, HQ426760, HQ426762, HQ426765–HQ426769, HQ426771, HQ426774, HQ426776, HQ426777, HQ426779–HQ426780, HQ426782, HQ426784–HQ426788, HQ426790–HQ426793, HQ426798–HQ426827, HQ426829–HQ426833, HQ426839–HQ426840, HQ426842–HQ426863, HQ426866–HQ426869, HQ426874–HQ426888, HQ426890, HQ426893, HQ426895–HQ426900, HQ426903, HQ426905-HQ426906, HQ426908, HQ426910–HQ426911). The 76 newly generated *pol* sequences were submitted to GenBank with the following assigned accession numbers: JQ351951–JQ352026.

DISCUSSION

In this study, we describe for the first time the temporal trends in drug resistance prevalence in pediatric patients enrolled in the Madrid cohort of HIV-infected children during nearly 2 decades (1993–2010). Resistance prevalence increased substantially among pretreated children during 2009 to 2010 compared with the 1993 to 1999 period. The rate of HIV-1 non-B variants infections has also risen since 2000.

Because of the advent of combination ART and effective viral suppression with immunologic reconstitution, 23.3% of our perinatally infected children who have survived into their second decade of life have been transferred to adult units, as reported in other European pediatric cohorts.[24] This recent process described in Europe will eventually occur in developing countries, where access to treatment will enable more children to survive and be transferred to adult units. Due to the low number of new cases of HIV infection caused by MTCT in developed countries,[25] the number of perinatally infected children will decline.

HIV-1 Non-B Variants Infect One of Every Ten Children

The observed prevalence of HIV-1 non-B variants (10.9%) in our pediatric cohort is similar to others previously published in developed countries[18,26] and in the adult population in Spain.[27,28] It is interesting to note that 54.2% of non-B variants infecting children in our cohort in Madrid were intersubtype recombinants, reflecting the increasing heterogeneity of the HIV epidemic in our country and in the pandemic.[29] The high increase of recombinant forms among children born after 2000 reflects the increasing genetic complexity among HIV-1 variants, which was also observed by our group in recently HIV-1–infected adults at local[27] and national levels in Spain.[28] The intersubtype recombinant variants are being introduced into the adult population in Spain through both immigrants and natives, and represent majority (72%) of non-B infections.[28]

In agreement with our study, the increasing prevalence of non-B subtypes infecting newly diagnosed patients in Spain and other European countries has been reported.[27,30] Published articles highlight that HIV-1 diversity may confer distinct pathogenesis and impact in the disease progression.[31,32] Subtype-associated differences can induce differential levels of CD4+ apoptosis, causing faster rates of CD4 decline and subtype-induced activation, which could lead to higher rates of virologic failure, causing differential disease outcome or potentially affect the efficacy of HIV therapy.[32,33]

The increasing prevalence of non-B variants in developed countries due to migration flows, international travel and international child adoption results in greater numbers of HIV-1 non-B–infected patients. Clinicians should be aware of how HIV-1 non-B infection may differentially impact on HIV infection outcome, and monitor the spread of different subtypes and recombinant forms in their countries. As rapid subtyping tools have proven poor performers in characterizing some HIV-1 non-B variants and mainly recombinant forms,[34] we encourage the use of phylogenetic analysis to correctly identify HIV-1 variants.

Higher TDR Prevalence in Children Versus Adult Population in Madrid

In industrialized areas, such as Europe, with about 15 years of experience on ART, 4.8–20% of newly HIV-infected adults are infected with strains with some degree of ARV drug resistance.[30,35–39] However, few studies have assessed TDR prevalence in perinatally infected children, which ranges from 12% and 20%.[18,26,40–43] The infected cohort from Madrid, including 232 patients infected between 1993 and 2010, presented a similar global TDR prevalence (13.6%), slightly higher than that found (9.7%) among 732 recently infected adults from the same city during 1996 to 2010.[37] Although the reported global TDR rate in the adult infected population in Spain decreased from 11.3% in 2004 to 2006 to 8.4% in 2007 to 2010, TDR increased among sub-Saharan Africans and female patients.[37] Unfortunately, due to the low number of naive children, we could not estimate the temporal TDR rate in the present pediatric cohort. These low numbers are a result of lack of approval of genotypic resistance testing in naive patients in Spain during the period when most plasma specimens from the study cohort were collected and the practice of initiating ART in children was performed, as they had confirmed HIV diagnosis.

Because the presence of TDR may compromise the success of ART and therapy roll-out programs, surveillance for prevalence of TDR is necessary. Thus, further prospective studies including more naive and pretreated children from Spain are required for a solid statistical support of TDR and DRM trends.

High Resistance Levels Among Pretreated Patients

The emergence of drug resistance due to incomplete viral suppression is a major obstacle to effective treatment.[44] As expected, this study reveals that most (71.8%) pretreated patients of this heavily pretreated cohort were exposed to all available drug classes carrying resistance mutations, in agreement with other reports in Europe.[45]

The reported prevalence of viruses carrying resistance mutations to 2 drug classes in the pretreated patients from the Madrid Cohort (global 40.4%, 46.8% in 2009–2010) is similar to other European studies that found 52% of dual-resistance levels in perinatally infected children over 10 years of age.[24] The reported triple-class resistance prevalence (15.9% globally, 21% in 2009–2010) is in agreement with the 12–32% three-class resistance levels reported for other European Pediatric cohorts.[24,43,45] Clinicians will face problems of managing heavily pretreated perinatally infected patients with many resistance mutations, not completely adherent to the treatments or with previous suboptimal regimens. The increasing resistance prevalence among the HIV-infected pediatric population in Spain highlights the importance of specific drug resistance and drug susceptibility surveillance in long-term pretreated children to optimize treatment regimens.

Drug Susceptibility

Treatment failure in children during ART is frequent, develops fast and with more extensive drug resistance than in adults,[46]

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leading to virologic treatment failure and immunologic damage. To date, 17 compounds have been licensed for HIV infection in the pediatric population,[47] including the recently approved although still being evaluated fosamprenavir, tipranavir, and darunavir.[48–50] Our data reveal that these 3 drugs, together with TDF, showed the highest likelihood of susceptibility during 2009 to 2010, suggesting that they could be a good option as a component of future ART regimens in perinatally infected individuals. This fact could reflect the change of treatment choices by clinicians. Note that the increase in drug resistance levels between P3 and P4 in TDF, FTC and EFV could be probably explained by the use of Truvada (Gilead Sciences, Inc., Foster City, CA), approved in Spain in 2006.

Close surveillance of adherence has to be a priority in these heavily pretreated perinatally infected children. Genetic resistance to newer ART drug classes such as integrase and fusion inhibitors was not expected due to limited previous use in the treatment of HIV pediatric infection, suggesting its possible usefulness to treat HIV infection in children.

Active surveillance efforts will be required to monitor the trends of drug resistance among highly pretreated HIV perinatally infected populations. It still remains important to assess the evolution of resistance patterns, to prevent the transmission of resistant strains, to monitor non-B infections and to maintain a close surveillance of adherence to reduce virologic failures in these children and adolescents requiring lifelong treatment.

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ERRATUM

Trends in Drug Resistance Prevalence in HIV-1–infected Children in Madrid: 1993 to 2010 Analysis: ERRATUM

In the article that appeared on page e213 of volume 31, issue 11, the figure legends were incorrect for Figures 1 and 2. The figures and legends are reprinted below correctly.

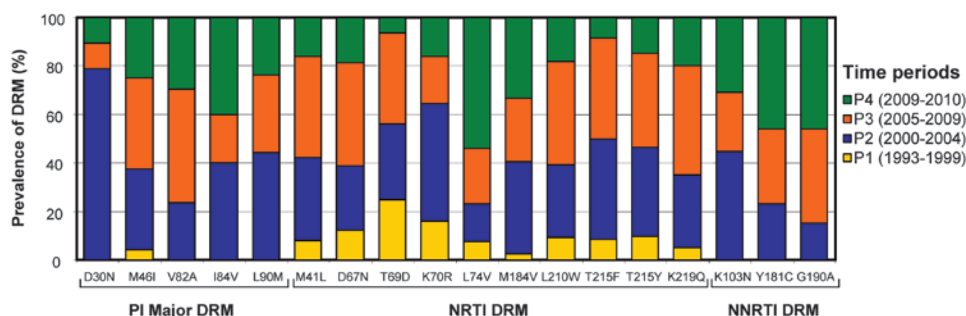

FIGURE 1. Prevalence of DRM positions for each drug class represented over 5% in the selected population.

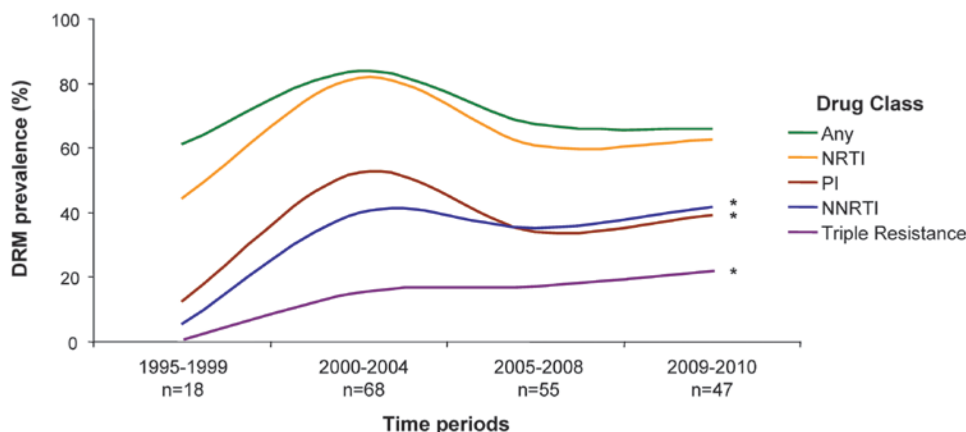

FIGURE 2. Temporal trends of DRM prevalence according to drug class. PI indicates protease inhibitors; RT, reverse transcriptase; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, nonnucleoside reverse transcriptase inhibitors. Asterisk indicates statistical differences between P1 (1993–1999) and P4 (2008–2010).

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CAPÍTULO 3

Prevalencia de resistencia a fármacos antirretrovirales en pacientes verticalmente infectados por VIH-1 transferidos desde unidades pediátricas a unidades de adultos en la Comunidad de Madrid

JUSTIFICACIÓN Y OBJETIVOS

En aquellos países desarrollados donde el acceso al TARGA es universal, la infección por VIH-1 se ha convertido en una enfermedad crónica. En el contexto de la infección pediátrica, se ha observado un incremento de pacientes que están alcanzando la mayoría de edad y por ello deben ser transferidos a unidades de adultos para continuar con su seguimiento clínico. Los adolescentes infectados verticalmente por VIH-1 que hoy acceden a las unidades de adultos representan un reto único e irrepetible. Estos pacientes han sufrido un largo historial de TAR, con cambios de regímenes al desarrollarse y aprobarse nuevos fármacos y familias de ARV para su uso en pediatría. La transición de estos adolescentes a unidades de adultos refleja los avances clínicos y farmacológicos desarrollados en los últimos años para tratar la infección por VIH-1. En los capítulos anteriores observamos como existía un aumento gradual del número de pacientes de la cohorte de niños y adolescentes infectados por VIH-1 de la Comunidad de Madrid que habían sido transferidos a unidades de adultos para continuar con su seguimiento clínico. Por ello en este capítulo nos propusimos:

- Describir las características clínicas y demográficas de los pacientes adolescentes infectados por VIH-1 en la cohorte de niños y adolescentes de la Comunidad de Madrid que habían sido transferidos a unidades de adultos entre 1997 y 2011.
- Comparar la situación clínica, inmunológica y virológica de éstos adolescentes transferidos con aquellos pacientes de la cohorte que todavía estaban bajo seguimiento clínico en unidades pediátricas a diciembre de 2011.
- Describir el historial de TAR, la resistencia a fármacos antirretrovirales y el grado de susceptibilidad genotípico en pacientes transferidos.
- Evaluar las opciones terapéuticas disponibles para tratar la infección por VIH-1 según los datos obtenidos en la predicción genotípica de susceptibilidad a fármacos ARV en pacientes pretratados de la población de estudio.
- Determinar la prevalencia de variantes no-B del VIH-1 en aquellos pacientes de la cohorte transferidos a unidades de adultos con respecto a los que aún continuaban en unidades pediátricas a diciembre de 2011.

CONCLUSIONES

- Desde el inicio de la pandemia hasta finales de 2011, un total de 534 pacientes pediátricos estuvieron bajo seguimiento clínico en la cohorte de niños y adolescentes infectados por VIH-1 de la Comunidad de Madrid. De ellos, 112 (21%) habían alcanzado la mayoría de edad y habían sido transferidos a unidades de adultos.
- Éstos pacientes fueron en su mayoría de origen español (91,9%), con una edad media de 18,9 años e infectados verticalmente (93,7%) entre 1985 y 1994 (81,3%).
- Los pacientes transferidos habían sufrido estadios avanzados de inmunosupresión. Dos tercios habían alcanzado <15% de linfocitos T CD4 y el 57,1% llegaron a tener menos de 200 céls/mm^3. Al menos el 98,2% de los pacientes presentaron alguna manifestación clínica de SIDA durante su seguimiento clínico, siendo moderada en el 75% de ellos.
- En el momento de la transición los pacientes transferidos tenían una adecuada situación inmune con recuento medio de linfocitos T CD4 de 627,5 céls/mm^3 y el 64,2% presentaba más de 350 céls/mm^3. Cerca de la mitad (47,3%) tenían la carga viral controlada con menos de 200 copias ARN-VIH-1/ml.
- La mayoría de los transferidos (84,8%) recibían TARGA en el momento de la transferencia. Sin embargo, el 59,8% comenzó su primer TAR con monoterapia y sólo el 14,3% con TARGA.
- La prevalencia de global mutaciones de resistencia en pacientes transferidos pretratados fue alta (81%), siendo 50,9% frente a IP, 76,9% frente a ITIAN y 36,5% frente a ITINAN. El porcentaje de pacientes infectados con virus resistentes a las tres familias de fármacos ARV de la población transferida fue similar a la población no transferida (17,3% vs. 17,6%). La prevalencia de mutaciones de resistencia a la combinación IP+ITIAN en población transferida fue significativamente mayor a la encontrada en no transferidos (19% *vs.* 8,4%, $p<0,05$).
- Las predicciones genotípicas de susceptibilidad a fármacos ARV revelaron que cerca de la mitad de los pacientes transferidos eran resistentes a NFV, AZT y d4T y entre el 20-30% a SQV, IDV, ATV, FPV, 3TC, FTC, ddI, EFV y NVP.
- Se encontró diferencia estadísticamente significativa en el nivel de resistencia a fármacos ARV de las familias IP (NFV, SQV, IDV, ATV, FPV y LPV) y a ITIAN (AZT y d4T) entre pacientes transferidos y no transferidos.

- Se observó una baja prevalencia de infección por variantes no-B en pacientes transferidos comparado con aquellos pacientes de la cohorte no transferidos e infectados más recientemente (1,9% *vs.* 11,4%).

PLOS | ONE

High Drug Resistance Prevalence among Vertically HIV-Infected Patients Transferred from Pediatric Care to Adult Units in Spain

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Abstract

Background: Antiretroviral treatment (ART) has contributed to increased life expectancy of HIV-1 infected children. In developed countries, an increasing number of children reaching adulthood are transferred to adult units. The objectives were to describe the demographic and clinical features, ART history, antiviral drug resistance and drug susceptibility in HIV-1 perinatally infected adolescents transferred to adult care units in Spain from the Madrid Cohort of HIV-1 infected children.

Methods: Clinical, virological and immunological features of HIV-1 vertically infected patients in the Madrid Cohort of HIV-infected children were analyzed at the time of transfer. *Pol* sequences from each patient were recovered before transfer. Resistance mutations according to the International AIDS Society 2011 list were identified and interpreted using the Stanford algorithm. Results were compared to the non-transferred HIV-1 infected pediatric cohort from Madrid.

Results: One hundred twelve infected patients were transferred to adult units between 1997 and 2011. They were mainly perinatally infected (93.7%), with a mean nadir CD4+-T-cells count of 10% and presented moderate or severe clinical symptoms (75%). By the time of transfer, the mean age was 18.9 years, the mean CD4+T-cells count was 627.5 cells/ml, 64.2% presented more than 350 CD4+T-cells/ml and 47.3% had ≤200 RNA-copies/ml. Most (97.3%) were ART experienced receiving Highly Active ART (HAART) (84.8%). Resistance prevalence among pretreated was 50.9%, 76.9% and 36.5% for Protease Inhibitors (PI), Nucleoside Reverse Transcriptase Inhibitors (NRTI) and Non-NRTI (NNRTI), respectively. Resistance mutations were significantly higher among transferred patients compared to non-transferred for the PI+NRTI combination (19% *vs.* 8.4%). Triple resistance was similar to non-transferred pediatric patients (17.3% *vs.* 17.6%).

Conclusion: Despite a good immunological and virological control before transfer, we found high levels of resistance to PI, NRTI and triple drug resistance in HIV-1 infected adolescents transferred to adult units.

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Introduction

By the end of 2010, of the 34 million people living with human immunodeficiency virus (HIV), there were 3.4 million children below the age of 15 years [1]. During 2010, 390.000 children were infected with HIV and 250.000 died from AIDS related causes [1]. In Western Europe and North America the HIV epidemic has remained stable since 2004. In 2010, one million infected individuals lived in Western and Central Europe, including 6,000 infected children [1]. In the WHO European Region of the 646 children who acquired the HIV infection through mother-to-child transmission (MTCT) [2], 19% of them originated from countries with generalized epidemics (in sub-Saharan Africa, the Caribbean and Asia).

Due to the expanded access to highly active antiretroviral treatment (HAART) and prevention efforts in HIV testing, prenatal care, formula feeding, elective Caesarean and pregnancy monitoring [3–5], few children were newly infected with HIV (<500) or died from AIDS-related illnesses (<500) in Western Europe in 2010. This reflects the extensive provision of services that can prevent MTCT of HIV [6,7]. Despite the success of preventive measures, MTCT still occurs in high-income countries [8–10] mainly due to infected immigrants from countries with a high HIV prevalence and within social compartments that refuse pregnancy monitoring and HIV testing. A total of 80.827 cases of AIDS had been declared in Spain [11] by the end of 2010; these 958 were children infected through MTCT. During 2010, a total of 2907 new HIV infections cases were notified in Spain, twelve of them caused by MTCT (0.4%) mainly (8/12 cases) among foreign patients. In the region of Madrid, a total of 805 HIV infection cases were reported in 2010, 2 of them caused by MTCT [11].

In developed settings with access to HAART, perinatally acquired HIV-1 infection has become a chronic disease of childhood with increasing numbers of adolescents surviving to adulthood and transitioning from pediatric to adult services. Perinatally infected adolescents have been heavily pretreated, have a long history of treatment with many switches and variable levels of adherence to the treatment have been reported. Sub-optimal treatments and non-complete compliance can increase the prevalence of drug resistance mutations in HIV thus, compromising the success of present and future treatment options.

Successful transition to adult services has become a necessity in these heavily pretreated patients. Teenagers growing up with HIV/AIDS have common problems related to social difficulties and to side-effects of HIV and HAART which play an impact on their growth and development. The objectives of this study were to describe the demographic and clinical features, antiretroviral therapy (ART) history, antiviral drug resistance and susceptibility to drugs in HIV-1 perinatally infected adolescents transferred to adult units in Spain from the Madrid Cohort of HIV-1 infected children.

Patients and Methods

Study Population

Since the beginning of the HIV epidemic in Spain, a total of 534 patients have been registered in the Madrid cohort of HIV-infected children established in 2003. By the end of December 2011, 175 of them still remained under clinical follow-up in pediatric units, 112 had been transferred to adult units, 62 had been lost to follow-up and 185 had died. In this study we selected the 112 patients from the cohort that had reached adolescence and been transferred to adult units in 8 public hospitals from 1997 to December 2011. Clinical and epidemiological features of all transferred patients were recorded from the database of the cohort.

An additional cohort of HIV-1 infected patients was used to compare results on drug resistance and drug sensitivity. The selected cohort consisted of the HIV Madrid cohort of non-transferred perinatally infected patients previously described [12,13].

This study was part of a project approved by the review board of the Hospital Universitario Ramón y Cajal Clinical Research Ethical Committee. It was designed to protect the right of all subjects involved under the appropriate local regulations. To maintain subject confidentiality, a unique number was assigned to each specimen, and written consent obtained for each patient by clinicians.

Drug Resistance Analysis

For the drug resistance study, we selected those transferred patients according to *pol* sequence, genotypic resistance profile or sample availability by December 2011. Most genetic sequences and genotypic resistance profiles were previously reported [12,13] or recovered from clinical routine drug-resistance tests performed in hospitals where patients were or had been under follow-up. When more than one sample was available per patient, we selected the closest and previous to the time of transference to adult units among transferred subjects and the most recent for non-transferred patients.

Previously reported genotypes were performed from infected samples (immortalized DNA, plasma or peripheral blood mononuclear cells, PBMCs) kindly provided by the HIV BioBank integrated in the Spanish AIDS Research Network (RIS) [14,15]. Samples from patients were processed following current procedures and frozen immediately after their reception. Sample collection, processing and storing were performed under international guidelines for biological storing and under supervision of a Scientific and Ethical Committee. HIV-1 subtyping of new sequences was performed by phylogenetic analysis (phy) as previously described [13].

The prevalence of transmitted drug resistance among naïve patients was defined according to the list of mutations for Transmitted Drug Resistances (TDR) surveillance, as recommended by the WHO [16] using the Calibrated Population Resistance tool [17]. Drug-resistance mutations (DRM) in pretreated patients were defined by the International AIDS Society-USA list (IAS) [18]. Drug susceptibility was estimated for each available antiretroviral according to the HIVdb Interpretation Algorithm version 6.0.11 (Stanford University, Palo Alto, CA, USA) [19].

Statistical Analysis

Prevalence was expressed in percentage with a 95% confidence interval (CI). CI tests were performed with Epidat 3.1 (Pan American Health Organization). Statistical significance was set at $p<0.05$.

Results

Baseline Features of the Transferred Population

A total of 112 patients of the Madrid cohort of HIV-infected children transferred from pediatric services to adult units in different hospitals in Madrid between 1997 and December 2011 were selected for this study. Baseline characteristics of the non-transferred (n = 131), total transferred (n = 112), and transferred patients with available genotypic profile (n = 63) are summarized in **Table 1**. All transferred patients were HIV-1 diagnosed at childhood (mean 2 years of age), the majority were born in Spain (91.9%) and mainly infected through MTCT (93.7%). Only a few (12.5%) were adopted. Most (81.3%) were diagnosed along the 1985–1994 period. The median duration of follow-up was 13.2 (Standard Deviation, SD 5.2), 15.6 (SD 4.5), and 16.7 (SD 3.6) years for non-transferred, transferred (n = 112) and transferred with available resistance genotypic profile, respectively.

Advanced stages of immunosuppression were observed as a result of the long term infection and scarce effective antiretroviral availability before 1996. Over two thirds of transferred patients reached less than 15% CD4+ cell counts and half (57.1%) reached <200 cells/mm^3. The mean nadir CD4+ T-cells count was 10%. Monotherapy was the first ARV treatment in 59.8%, mainly with AZT (79.1%), 23.2% started with combined therapy (including AZT backbone in 88.4% of them) and only 14.3% with HAART.

Table 1. Baseline characteristics of the non-transferred, transferred and transferred with available genotypic profile patients.

Features	Non-transferred n = 131	Transferred n = 112	Transferred with genotype* n = 63
	[n (%)]	[n (%)]	[n (%)]
Adopted	31 (23.7)	14 (12.5)	8 (12.7)
Female gender	76 (58)	60 (53.6)	34 (54)
Median age until diagnosis (years)	0.5	2	1.4
Non-B variants prevalence (%)	11.6	–	1.9
Demographics			
Caucasian	100 (76.3)	98 (87.5)	59 (93.6)
Hispanic	6 (4.6)	4 (3.5)	2 (3.2)
Romani	4 (3.1)	3 (2.7)	1 (1.6)
African**	18 (13.7)	2 (1.8)	–
Other	2 (1.5)	2 (1.8)	1 (1.6)
Unknown	1 (0.8)	3 (2.7)	–
Origin[a]			
Europe	112 (85.5)	105 (93.7)	61 (96.8)
North America	1 (0.8)	–	–
South and Central America	8 (6.1)	5 (4.5)	2 (3.2)
North Africa	2 (1.5)	–	–
Sub-Saharan Africa	7 (5.3)	2 (1.8)	–
Asia	1 (0.8)	–	–
Year of HIV diagnosis			
1985–1989**	1 (0.8)	31 (27.7)	16 (25.4)
1990–1994**	37 (28.2)	60 (53.6)	39 (61.9)
1995–1999**	54 (41.2)	18 (16)	7 (11.1)
2000–2004**	28 (21.4)	3 (2.7)	1 (1.6)
2005–2009	10 (7.6)	–	–
Unknown	1 (0.8)	–	–
Route of infection			
Perinatally	127 (96.9)	105 (93.7)	61 (96.8)
Transfusion	3 (2.3)	5 (4.5)	2 (3.2)
Unknown	1 (0.8)	2 (1.8)	–
Year of transfer			
1997–1999	–	3 (2.7)	–
2000–2002	–	9 (8)	1 (1.6)
2003–2005	–	27 (24.1)	13 (20.7)
2006–2008	–	33 (29.5)	21 (33.3)
2009–2011	–	40 (35.7)	28 (44.4)
Nadir CD4 count achieved		Mean 10%	Mean 11%
<15%	67 (51.1)	75 (67)	43 (68.3)
15–24%	42 (32.1)	24 (21.4)	13 (20.6)
≥25%	19 (14.5)	13 (11.6)	7 (11.1)
Unknown	3 (2.3)	–	–
Nadir CD4 count achieved (cells/mm^3)			
<200**	35 (26.7)	64 (57.1)	31 (52.4)
200–499	63 (48.1)	37 (33)	26 (38.1)
≥500**	30 (22.9)	11 (9.9)	6 (9.5)
Unknown	3 (2.3)	–	–

[a]Origin of patients by country: Spain (n = 103), Portugal (n = 1), Romania (n = 1), Honduras (n = 2), Argentina (n = 1), Mexico (n = 1), Peru (n = 1), Cape Verde (n = 1), Equatorial Guinea (n = 1).
*Transferred to adult units with available resistance genotyping profile.
**Statistical differences ($p<0.05$) have been found between transferred and non-transferred patients for these features. HIV-1 non-B variants include HIV-1 non-B subtypes and recombinants.
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In the transferred cohort compared to the non-transferred, a statistical significant lower number of African patients were found (1.8% *vs.* 13.7%, $p<0.05$), a significant higher number of children reached nadir CD4-Tcell values below 200 (57.1 *vs.* 26.7, $p<0.05$) and a lower number of patients achieved CD4 T-cells over 500 (9.9% *vs.* 22.9%, $p<0.05$). No statistical differences were found in the baseline studied characteristics between transferred patients with and without available genotype (**Table 1**).

Features of the Population at the Time of Transfer

Characteristics of the study population at the time of transfer to adult units are shown in **Table 2**. By the time of transfer, the mean age was 18.9 years and the mean CD4+T-cells count 627.5 cells/ml. 5.4% presented less than 15% CD4+T-cells, 66% more than 25% and 55.3% more than 500 CD4+ cells/ml counts. Nearly all (98.2%) had presented signs and symptoms of AIDS according to CDC classification [20], which were severe in 34.8% of cases. Immunological status at the time of the transfer revealed an immunologically severe suppression in 66.9% (CDC stage 3). Among the 102 patients with available viral load data, 56.2% had ≤500 RNA-copies/ml and 38.4% undetectable viraemia (≤50 RNA-copies/ml).

Comparison among transferred and non-transferred patients by December 2011 revealed that transferred patients had a worst immunological-virological profile comparing to the non-transferred group. A lower number of transferred patients were categorized in clinical status A (23.2% *vs.* 37.4%, $p<0.05$) and achieved undetectable levels of viraemia (38.4% *vs.* 64.9%, $p<0.05$). T-cell CD4 counts (either ≥25% or >500 CD4+ T-cells) were also lower among transferred and a lower number of transferred were on HAART (84.8% *vs.* 94.6%, $p<0.05$). No statistical differences were found in studied clinical features by December 2011 between transferred patients with and without available genotype (**Table 2**).

ART Experience among Transferred Patients

The transferred cohort started any type of ART with a median age of 5.6 years (SD 3.5 years) and the median duration of ART was 11.5 years (SD 4.8 years). Only 3 (2.7%) of the 112 transferred individuals remained drug naïve at transfer and the rest (97.3%) were ART experienced. Most (84.8%) were receiving HAART, 9.8% had stopped treatment and 2.7% were receiving combined therapy at the transfer time according to clinical reports. The most commonly used HAART combinations in our cohort were 2NRTI+1NNRTI (32.6%) and 2NRTI+1PI (28.4%). The pretreated patients presented a long treatment history and had experienced several different ART combinations; the mean number of regimens was five, with at least 3 HAART regimens in 48% of them. The main ART families were NRTI, PI and NNRTI, 94.6%, 84.8%, and 72.3% respectively (**Table 2**). Triple class experience was found in almost two-thirds of patients. Use of other drug families was scarce and only three transferred patients had experience with the fusion inhibitor, enfuvirtide and one of them also had received raltegravir, an integrase inhibitor. Adherence history was assessed according to clinical charts data but was not available in all cases.

High Prevalence of HIV-1 Resistant Variants in Pretreated Transferred Patients

Among the 112 patients transferred before the end of December 2011, only in 63 (56.2%) subjects drug resistance genotypes could be analyzed. Among them, in 48 (76.2%) cases the *pol* sequence had been previously reported by our group [12,13]. In the remaining 15 patients, six patients had an available plasma specimen stored in the HIV-1 Spanish Biobank and new HIV-1 *pol* sequences were newly generated as previously reported [13]. Other 9 patients presented a genotypic resistance profile derived from *pol* sequences obtained from plasma specimens recovered from clinical routine drug-resistance tests performed in hospitals where the patients were or had been under follow-up. However, no fasta format sequences of any profile were available. Clinical specimens in all 63 genotyped samples were obtained before the transfer time to adult units: during 1993–1999 (5 genotypes), 2000–2004 (27 genotypes) and 2005–2010 (31 genotypes). The median age of patients when genotypic data was generated was 14.7 years old (SD 4.4). The 63 patients with available genotypic data included five genotypes (accession numbers HQ426734, HQ426806, HQ426860, HQ426867, HQ426893) performed before treatment was initiated. Out of these five, only 2 patients remained drug naïve until they were transferred to adult units while the other 3 received any ART during their follow-up. None of these sequences harboured TDR mutations.

Among the 63 transferred patients of the Madrid cohort of HIV-infected children with available *pol* sequences or resistance profiles, the prevalence of HIV drug resistance mutations was analyzed according to the drug class family. Fifty-eight were pretreated and 5 remained drug-naïve at sampling time. No TDR mutations were found among the *pol* sequences from the 5 transferred naïve subjects. The most prevalent DRM found in the 58 transferred ART-experienced patients were: for PI, L90M (27.5%), V82A (15.7%), M46I (13.7%) and D30N (9.8%); for NRTI, M41L (48.1%), D67N (40.4%), T215Y (40.4%), L210W (34.6%), M184V (25%), T69D (23.1%), and K219Q (23.1%); and for NNRTI, K103N (19.2%), Y181C (7.7%) and G190A (7.7%).

Global resistance prevalence among the 58 transferred ARV-exposed pretreated patients with available *pol* sequences or resistance profiles was 50.9% for PI, 76.9% for NRTI and 36.5% for NNRTI (**Table 3**). No primary drug resistance mutations were found among naïve subjects. According to our data, no statistical differences were found between the resistance rate and the duration of treatment (data not shown).

Higher Prevalence of Drug Resistance Mutations Among Transferred Patients than in Non-transferred

Besides assessing the global prevalence of DRM in the 58 pretreated patients of the transferred cohort, prevalence of DRM was also compared to the non-transferred cohort. The first included the 58 transferred and pretreated patients with available genotypic data compared to the 131 non-transferred pretreated pediatric patients from the Madrid cohort of HIV-1 infected children (**Table 3**). All 131 non-transferred pediatric patients had available resistance genotype, previously reported [12,13] and with available GenBank accession numbers.

Transferred patients tended to present higher DRM prevalence when compared to non-transferred children (**Table 3**). Drug resistance mutations were found in a higher number of transferred patients than in the non-transferred pretreated population under follow up in Madrid for PI (50.9% *vs.* 36.8%, p = NS) and for NRTI, 76.9% *vs.* 62.1%, p = NS) and lower for NNRTI (36.5% vs. 40.5%, p = NS). However, resistance prevalence was significantly higher among transferred patients for the PI+NRTI combination (19% *vs.* 8.4%, $p<0.05$). Triple resistance was similar to non-transferred pediatric patients (17.3% *vs.* 17.6%, p = NS).

Table 2. Characteristics of the non-transferred, transferred and transferred with available genotypic profile patients by December 2011.

Features	Non-transferred n = 131	Transferred n = 112	Transferred with genotype* n = 63
Mean age (years)	14.7	18.9	18.5
LTNP [n (%)]	1 (0.8)	5 (4.5)	2 (3.2)
Median ART duration [years (SD)]	12 (4.5)	11.5 (4.8)	13.5 (4.1)
Median follow-up [years SD)]	13.2 (5.2)	15.6 (4.5)	16.7 (3.6)
Immunological status [n (%)]			
1	8 (6.1)	4 (3.6)	2 (3.2)
2	41 (31.3)	31 (27.7)	18 (28.5)
3	79 (60.3)	75 (66.9)	43 (68.3)
Unknown	3 (2.3)	2 (1.8)	-
Clinical status [n (%)]			
N	-	2 (1.8)	1 (1.6)
A**	49 (37.4)	26 (23.2)	14 (22.2)
B**	35 (26.7)	45 (40.2)	23 (36.5)
C	43 (32.8)	39 (34.8)	25 (39.7)
Unknown	4 (3.1)	-	-
CD4 count (%) [n (%)]			
<15%	3 (2.3)	6 (5.4)	3 (4.8)
15–24%	18 (13.7)	26 (23.2)	15 (23.8)
≥25%**	105 (80.2)	74 (66)	43 (68.2)
Unknown	5 (3.8)	6 (5.4)	2 (3.2)
CD4 count (cells/ml) [n (%)]			
	Mean 770.5 cells/ml	Mean 627.5 cells/ml	Mean 654 cells/ml
≤200	3 (2.3)	4 (3.6)	1 (1.6)
201–350	6 (4.6)	7 (6.3)	5 (7.9)
351–500	13 (9.9)	10 (8.9)	4 (6.4)
>500**	99 (75.6)	62 (55.3)	36 (57.1)
Unknown	10 (7.6)	29 (25.9)	17 (27)
Viral load (HIV-1 RNA-copies/ml) [n (%)]			
≤20**	41 (31.3)	12 (10.7)	9 (14.2)
21–50	44 (33.6)	31 (27.7)	23 (36.5)
51–200	10 (7.6)	10 (8.9)	4 (6.4)
201–500	7 (5.3)	10 (8.9)	2 (3.2)
501–1,000	-	4 (3.6)	3 (4.8)
1,001–10,000	12 (9.2)	19 (17)	9 (14.3)
>10,000	13 (9.9)	16 (14.3)	11 (17.4)
Unknown	4 (3.1)	10 (8.9)	2 (3.2)
ART experience [n (%)]			
Drug naive	1 (0.8)	3 (2.7)	2 (3.2)
PI-experienced	116 (88.6)	95 (84.8)	54 (85.7)
NRTI-experienced**	130 (99.2)	106 (94.6)	59 (93.6)
NNRTI-experienced	99 (75.6)	81 (72.3)	47 (74.6)
FI-experienced	-	3 (2.7)	1 (1.6)
InI-experienced	-	1 (0.9)	1 (1.6)
PI+NRTI+NNRTI-experienced	88 (67.2)	72 (64.3)	42 (66.7)
Treatment status [n (%)]			
HAART**	124 (94.6)	95 (84.8)	54 (85.7)
Stopped-treatment	3 (2.3)	11 (9.8)	6 (9.5)
Naive	1 (0.8)	3 (2.7)	2 (3.2)

Table 2. Cont.

Features	Non-transferred n = 131	Transferred n = 112	Transferred with genotype* n = 63
Monotherapy	1 (0.8)		
Combined	2 (1.5)	3 (2.7)	1 (1.6)

SD, standard deviation; ART, antiretroviral therapy; PI, protease inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-NRTI; FI, fusion inhibitors, InI; integrase inhibitors.
*Transferred to adult units with available resistance genotyping profile.
**Statistical differences ($p<0.05$) have been found between transferred and non-transferred patients for these features.
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Higher Predicted Level of Resistance to PI and NRTI among Transferred Patients

Figure 1 shows the comparison of the predicted level of resistance to each drug in all pretreated patients from the two study cohorts. Analysis of the genotypic resistance interpretation revealed that transferred adolescents presented a significantly higher predicted level of resistance to all drugs from the PI and NRTI families probably explained by the long-term therapy history of these patients. Half of the transferred patients carried infecting HIV-1 variants resistant to NFV, followed by AZT and d4T (nearly 40% of them). Similar predicted resistance was observed for 3TC and FTC in the non-transferred and transferred study groups (around 20%). Between nearly 20% and 30% of the adolescents in Madrid were infected with variants resistant to the remaining PI drugs, except for TPV/r and DRV/r, the new PI drug generation. EFV and NVP were the NNRTI with the most compromised susceptibility in both cohorts (around 20–30%), being higher in non-transferred patients. Data revealed a low (2–5%) predicted resistance level to etravirine and rilpivirine, the new NNRTI drugs; resistance levels to these drugs remained low in a gap between 2% and 5% for both studied cohorts.

Low Prevalence of HIV-1 Non-B Variants in Transferred Patients

Most (98.1%) of the transferred patients were infected by subtype B, the most prevalent HIV-1 variant in North America and Western Europe, including Spain [13,21]. Only one transferred perinatally infected female carried a "pure" sub-subtype A2. She was a white-Caucasian, vertically infected and born of Spanish parents in 1992. Prevalence of HIV-1 non-B variants among the 54 transferred patients with available *pol* sequence was low (1.9%, 1/54), compared to the 11.5% (15 patients) found among the 131 non-transferred children of the same pediatric cohort (**Table 1**) or the 12.2% reported n the Spanish cohort of antiretroviral treatment-naïve HIV-infected patients [21]. Of interest, recombinant viruses were absent in transferred patients although found in 60% (9/15) of infections caused by non-B variants in the non-transferred pediatric cohort, respectively.

Sequence Data

Pol (PR and/or RT) sequences from 48 (88.8%) of the 54 transferred patients included in this study had been previously submitted to GenBank [13]: HQ426715, HQ426719, HQ426725, HQ426728, HQ426734, HQ426766, HQ426768, HQ426779, HQ426780, HQ426788, HQ426799, HQ426806, HQ426807, HQ426818, HQ426826, HQ426840, HQ426842, HQ426847,

Table 3. Comparison of HIV drug resistance mutations prevalence according to drug class family in the non-transferred and the transferred pediatric cohorts.

	Transferred patients[a] (n = 58)	Non-transferred children[b] (n = 131)
Patients with available PR	51	125
Patients with available RT	52	116
Prevalence of drug resistance mutations (%) [95% CI][c]		
Global (to any class)	81.0 [70.1–92]	69.5 [61.2–77.7]
To PIs	50.9 [36.3–65.7]	36.8 [27.9–45.7]
To NRTIs	76.9 [64.5–89.3]	62.1 [52.8–71.3]
To NNRTIs	36.5 [22.5–50.6]	40.5 [31.2–49.9]
To NRTI+NNRTI	12.1 [2.8–21.3]	12.2 [6.2–18.2]
To PI+NRTI**	19 [8–29.9]	8.4 [3.3–13.5]
To PI+NNRTI	–	0.8 [0.02–4.2]
To PI+NRTI+NNRTI	17.3 [6.7–27.8]	17.6 [10.7–24.5]

[a]Selected patients from the Madrid cohort of HIV-1 infected children that have been transferred to adult units by December 2011.
[b]Pretreated patients selected from the Madrid cohort of HIV-1 infected children excluding those transferred to adult units.
[c]Prevalence of drug resistance mutations was determined following the IAS-USA 2011 list [18]. PR, protease; RT, reverse transcriptase; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-NRTI; PI, protease inhibitors.
**Statistical difference ($p<0.05$) has been found between transferred and non-transferred patients for this feature.
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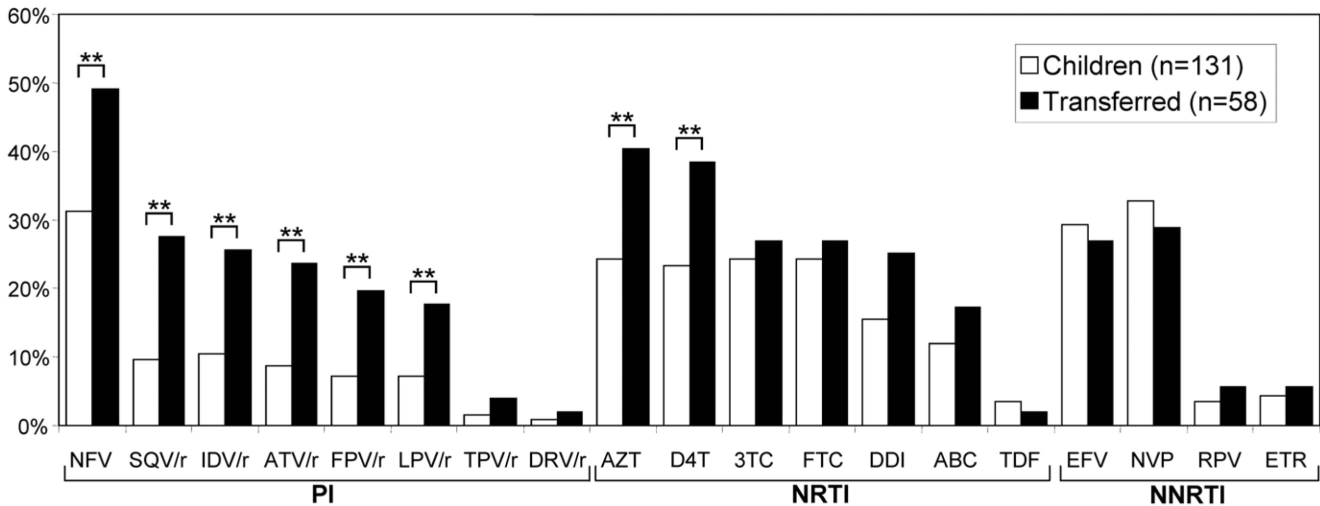

Figure 1. Predicted resistance level to antiretroviral drugs in pretreated patients from the two studied cohorts. Resistance level was estimated according to the HIVdb Interpretation Algorithm (Stanford University, Palo Alto, CA, USA) [19]. PI, protease inhibitors: nelfinavir (NFV), saquinavir/r (SQV/r), indinavir/r (IDV/r), atazanavir/r (ATV/r), fosamprenavir/r (FPV/r), lopinavir/r (LPV/r), tipranavir/r (TPV/r) and darunavir/r (DRV/r), where "/r" indicates co-administration with low-dose ritonavir (RTV) for pharmacological "boosting". NRTI, nucleoside reverse transcriptase inhibitors: zidovudine (AZT), stavudine (d4T), lamivudine (3TC), emtricitabine (FTC), didanosine (DDI), abacavir (ABC), tenofovir (TDF). NNRTI, non-nucleoside reverse transcriptase inhibitors: efavirenz (EFV), nevirapine (NVP), rilpivirine (RPV), etravirine (ETR). **Statistical differences ($p<0.05$) in resistance levels have been found between transferred and non-transferred patients for these drugs.
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HQ426850, HQ426857, HQ426860, HQ426861, HQ426866, HQ426867, HQ426868, HQ426869, HQ426874, HQ426879, HQ426880, HQ426883, HQ426889, HQ426890, and HQ426893. In reference [12]: JQ351951, JQ351960, JQ351984, JQ351986, JQ351988, JQ351989, JQ351995, JQ351997, JQ352005, JQ352006, JQ352010-JQ352012, JQ352014, and JQ352021. The 6 newly generated *pol* sequences for this study were submitted to GenBank with the following accession numbers: JQ828989-JQ828994. GenBank accession numbers for the 131 sequences from non-transferred children were previously reported [12,13].

Discussion

Due to the low number of new cases of HIV infection caused by MTCT in developed countries [2], perinatally infected cohorts will tend to reduce. However, several studies have assessed the current state of adolescent survivors of perinatally or early acquired HIV infection [22–33]. As ART becomes more widely available in the developing world, increasing the effective viral suppression with immunologic reconstitution, we can expect a steady increase in the number of children being transferred into adult units, as reported in European [24–26] and North American pediatric [22,29,32] cohorts. This recent process described in high income regions could eventually occur in developing countries, if the access to optimal treatment is maintained.

High Rate of Perinatally HIV-1 Infected Patients Transferred to Adults Units in Madrid

Features of the transferred patients from the Madrid cohort of HIV-1 infected children have been scarcely studied [34]. Among the 534 patients from the Madrid cohort of HIV-1 infected children by the end of December 2011, a total of 112 (21%) had reached adolescence and were transferred to adult units from 1997 through 2011. The first patient was transferred in 1997. The high percentage of transferred patients, even higher than in the British pediatric HIV CHIPS cohort [23] (n = 103; 16% *vs.* n = 112; 21%), is directly related to the high number of HIV-1 infections occurring in Spain in the early nineties due to the so called "heroin epidemic". The abuse of heroin during the 1980s and 1990s in Spain had a special impact in women, reaching the highest AIDS incidence and prevalence in Western Europe, which led to a high incidence of MTCT in children born between 1980 and 1990 [11,35]. Future transitioning programs will include a smaller number of patients due to the low number of new diagnoses [2,9] of HIV-1 among children in high income countries.

Higher Prevalence of Drug Resistant Strains among Transferred and Non-transferred Patients

The emergence of drug resistance due to incomplete viral suppression and incomplete adherence are the major obstacle for an effective ART [36]. Adolescents with perinatally acquired HIV are heavily pretreated, have a long history of treatment with many regimen switches, and present variable levels of adherence to the treatment, mainly during adolescence. However, there are few data about disease progression, response to ART and drug resistance prevalence in vertically HIV-infected adolescents in the era of effective therapy, even though their number is increasing in developed countries where ARV therapy is guaranteed. The results presented showed that two thirds of transferred patients from the Madrid cohort of HIV-1 infected children to adult units were triple class-experienced, higher than in other cohorts as in the UK (64.3% *vs.* 47%, respectively) [23]. Results may be explained by longer exposure to older, less efficacious treatments. In fact, HIV infected patients during childhood in our cohort were mainly infected during the early 1990's, and had to face the monotherapy and dual therapy regimens available at the time, thus increasing the risk of virological failures due to resistance development. The first patient reached adulthood in 1997 and thus had received previous, less efficacious treatments during his childhood.

Interestingly, DRM prevalence to all 3 drug classes among transferred patients was higher than for non-transferred infected children. This fact could be caused by regimen switches due to

therapeutic failure or because of the availability of new drugs during the infection period. As a consequence of their long treatment history and the treatment switches they have experimented, 81% of the patients transferred to adult units harboured resistant virus to at least one of the drug classes, higher than in non-transferred children (69.5%) mainly for NRTI, the first available drug class for clinical use, and for PI. This high rate of resistance could have compromised the susceptibility found in our data from both the PI and NRTI families.

In fact, the pediatric population (transferred and non-transferred) infected by viruses carrying triple resistance mutations was significantly higher than in pretreated adults from Madrid (17% *vs.* 8.6%), and moreover, was higher than in adolescents from the UK and Ireland (12%) [23] and the COHERE pediatric cohort of perinatally infected children in Western Europe aged less than 16 years (10%) [37]. On the other hand, DRM to NNRTI was slightly higher in the Madrid cohort of HIV-1 infected children than in the transferred group, so reflecting their preferential use in the pediatric population during the study period. These results on prevalence highlight the potential problem that clinicians have and will face in the near future with HIV-1 adolescents who are highly resistant to all drug classes.

Possible Candidates to Rescue Transferred Highly Pretreated Patients

Treatment failure in children during ART is frequent, develops fast and with more extensive drug resistance than in adults, leading to detectable viral loads and immunological damage [37–42]. Thus, keeping a close surveillance of adherence has to be a priority in these heavily pretreated adolescents requiring treatment for life [42,43]. Moreover resistance studies are also required to optimize ARV regimens. According to the predicted drug susceptibility, our data revealed than TDF (NRTI) and the new PI (TPV/r and DRV/r) and NNRTI (ETR and RPV) drugs could be good alternatives for inclusion in future ARV regimens to control the viraemia in highly pretreated transferred adolescents in Madrid. Adolescents could also benefit from the newly licensed drugs to treat HIV-1 infection for adults. Other drug families (cell-entry and integrase inhibitors), could be good candidates to control viraemia among pretreated transferred patients in Madrid due to their previous scarce exposure (<1%). However, the presence of X4-tropic variants in over 80% of the cohort of antiretroviral-experienced children and adolescents with vertical HIV-1 infection in Madrid has recently been reported [44]. The authors indicate a very limited role for CCR5 antagonists as part of salvage regimens for highly treatment-experienced vertically infected patients with extensive antiretroviral drug resistance [44]. Thus, integrase inhibitors could be the best rescue alternative in the cases of therapeutic failure with multiresistance, although additional genetic resistance studies should be performed to guarantee their usefulness.

Low Prevalence of HIV-1 Non-B Variants Infection among Transferred Patients

Epidemiological differences related to the nature of HIV-1 infecting variants were found between the non-transferred cohort of HIV infected children (11.4%) and with those transferred to adult units (1.9%). Pediatric patients that reached adulthood and were transferred to adult units were mainly infected by subtype B (98.2%). This fact is explained by the long term infection of our patients (mean age of 18.9 years), reflecting the local epidemiological situation in Spain at the time in which circulating variants other than B had not yet been detected in Spain.

Previous studies in the Madrid cohort of HIV-1 infected children and adolescents reported a non-B prevalence of 10% [12,13]. In fact, in Madrid an increase of HIV-1 non-B infections after year 2000 was reported among infected children [12] and among newly HIV infected adults [14]. Interestingly, the increasing complexity of the epidemic reported in HIV-infected adults [21,45] and in the pediatric population in Madrid [13] was not observed among the transferred population, in whom no recombinant strains were found. As a limitation, only half (n = 54) of the transferred cohort had an available *pol* sequence to perform HIV-1 variant characterization. Prevalence of HIV-1 non-B variants infecting children and adults from Madrid has been estimated as about 10% [12,13,21]. Similar results have been published for other pediatric cohorts, where non-B infections ranged from 5 to 15% [46,47].

Conclusions

Understanding the progress of HIV-1 infected children through pediatric care until they reach adolescence in developed countries could help to improve and plan adequate clinical and psychological transitioning services for the HIV-1 infected children that will reach adolescence [23,24,26,33] in the future. The increasing resistance prevalence among the HIV-infected-pediatric population in Spain highlights the importance of specific drug-resistance and drug-susceptibility surveillance in long-term pretreated children to optimize treatment regimens. Clinicians in Spain should consider that young adults infected during childhood do not present same clinical features as those young adults infected by other routes and that they require a specific clinical follow up.

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Author Contributions

Conceived and designed the experiments: MDM GY AN JTR AH. Performed the experiments: MDM. Analyzed the data: MDM GY AN AH JTR. Wrote the paper: MDM AH GY AN. Provided clinical records of the study population: MIDJ MDG MIGT MJM JSL. Provided clinical samples from HIV-1 biobank: MAMF. Provided statistical analysis and cohort data: SJDO.

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CAPÍTULO 4

Determinación de la variante viral infectante, prevalencia de resistencia y susceptibilidad a fármacos antirretrovirales en pacientes pediátricos infectados por VIH-1 de la cohorte Jacobi de Nueva York, Estados Unidos

JUSTIFICACIÓN Y OBJETIVOS

Según los cálculos de la OMS, en el mundo había un total de 34 millones de personas infectadas por el VIH-1 a finales de 2012 de los cuales 3,4 millones eran niños menores de 15 años. La transmisión vertical del VIH-1 es responsable del 10% de las nuevas infecciones anuales y causa un total de 390.000 nuevas infecciones en niños menores de 15 años, principalmente en países de recursos limitados. A pesar de los esfuerzos encaminados para reducir la transmisión vertical del virus, aún siguen ocurriendo casos de transmisión del VIH-1 en países desarrollados. A finales de 2009, vivían en el estado de Nueva York un total de 127.384 personas infectadas por VIH-1, entre los cuáles había 2.915 infectados por transmisión vertical. Durante el año 2009 se registraron un total de 4.000 nuevos diagnósticos del VIH-1, de las que 10 fueron niños infectados verticalmente.

El distrito del Bronx, en el área metropolitana de Nueva York, es una de las zonas urbanas con mayor prevalencia de infecciones por VIH-1 de EE.UU. A finales de 2009, 22.782 personas infectadas por VIH-1 vivían en este distrito. De ellos, 821 eran niños y adolescentes infectados verticalmente por el virus. El centro médico Jacobi ubicado en el Bronx se encarga, entre otras funciones, del seguimiento clínico de pacientes pediátricos infectados por VIH-1 (cohorte Jacobi). Hasta este estudio, no se habían caracterizado las variantes del VIH-1 ni la resistencia a fármacos ARV en población pediátrica del Bronx. Durante una estancia de investigación en San Francisco, EE.UU., se estableció una colaboración con el personal clínico de esta cohorte, que cedió muestras clínicas de los pacientes para que pudiéramos cumplir los siguientes objetivos:

- Determinar las características clínicas, virológicas, inmunológicas y demográficas de aquellos niños y adolescentes infectados por VIH-1 bajo seguimiento clínico en el centro médico Jacobi localizado en el distrito del Bronx de la ciudad de Nueva York, EE.UU.
- Estimar por primera vez en esta cohorte la prevalencia de mutaciones de resistencia a fármacos ARV y determinar el grado de susceptibilidad a 19 fármacos ARV en los virus de pacientes pretratados mediante herramientas de predicción genotípica.
- Comparar los perfiles de resistencia a fármacos ARV obtenidos a partir de muestras de plasma con aquellos obtenidos a partir de sangre seca tomadas en paralelo en los pacientes seleccionados.
- Realizar el primer estudio filogenético de las variantes del VIH-1 que infectaban a los población pediátrica de estudio.

CONCLUSIONES

- Entre agosto y noviembre de 2011 se seleccionaron un total de 47 pacientes infectados verticalmente por VIH-1 y bajo seguimiento clínico en el *Jacobi Medical Center* localizado en el distrito del Bronx de la ciudad de Nueva York, EE.UU.
- La mayoría de pacientes (61,7%) incluidos en el estudio se infectaron entre 1985 y 1994. La cohorte Jacobi, al igual que otras cohortes pediátricas de países desarrollados, está envejecida presentando una edad media de 17,7 años. Por lo tanto, es previsible la necesidad de desarrollar un plan de transición de pacientes pediátricos de esa cohorte a unidades de adultos.
- La situación clínica e inmunológica de los pacientes estudiados fue buena, presentando una media de linfocitos T CD4 de 21,1% o 486 céls/mm^3. El 59,6% presentaban una CV <1.000copias/ml, siendo indetectable en el 40,9% de los pacientes.
- La mayoría de los pacientes (97,8%) estaban infectados por variantes B del VIH-1 por lo que las infecciones por subtipos no-B y formas recombinantes en la cohorte Jacobi fue muy baja (2,7%).
- Se encontró una prevalencia de resistencia del 27,6% para PI, 54,1% para ITIAN y 27% para ITINAN. El 64.9% de los pacientes estaban infectados por virus con mutaciones de resistencia al menos a una familias de fármacos, el 27% a dos familias y el 5,4% a las tres familias de fármacos ARV.
- La interpretación genotípica de resistencias a ARV mostró un alto grado de sensibilidad a las tres familias de fármacos evaluadas. Se observó un 30% de resistencia a FTC y 3TC (ITIAN). Entre los ITINAN, la NVP y el EFV estaban comprometidos en el 19% y 16% respectivamente de los pacientes. El NFV resultó ser el fármaco IP con mayores valores de resistencia (20%).
- El uso de muestras biológicas de sangre seca para la amplificación y detección de mutaciones de resistencia en el gen *pol* demostró su validez frente a secuencias del gen *pol* obtenidas a partir de plasma sanguíneo. La similitud nucleotídica media entre secuencias obtenidas de plasma y de sangre seca (DBS) fue del 97,9%.

HIV-1 Drug Resistance Prevalence, Drug Susceptibility and Variant Characterization in the Jacobi Medical Center Paediatric Cohort, Bronx, NY, USA

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Background: With the advent of combined ART (cART), perinatally HIV-infected children are surviving into adolescence and beyond. However, drug resistance mutations (DRM) compromise the viral control, affecting long term ART effectiveness.

Methods: Paired plasma and dried blood specimens (DBS) were obtained from HIV-1 perinatally infected patients attending the Jacobi Medical Center, New York. Clinical, virological and immunological features were analyzed. HIV-1 p*ol* sequences were generated from samples to identify DRM according to IAS-2011 list.

Results: Forty-seven perinatally-infected patients were selected, with a median age of 17.7 years and 97.4% carrying subtype B. They presented 3143 HIV-1-RNA copies/ml mean viral load and 486 mean CD4 cells/µl at sampling time. Nineteen (40.4%) patients achieved undetectable viremia (<50 copies/mL) and 40.5% more than 500 CD4 cells/µL. Most (97.9%) had received cART, including protease inhibitors (PI)-based regimens in 59.6% cases.

DRM prevalence was 54.1%, 27.6% and 27% for nucleoside reverse transcriptase inhibitors (NRTI), PI and non-NRTI (NNRTI), respectively. Almost two thirds (64.9%) of the patients harboured DRM to at least one drug class and 5.4% were triple resistant. Mean nucleotide similarity between plasma and DBS sequences was 97.9%. Identical DRM profiles were present in 60% of plasma-DBS paired sequences. A total of 30 DRM were detected in plasma and 26 in DBS, being 23 present in both.

Conclusion: Although more perinatally HIV-1 infected children are reaching adulthood due to advances on cART, our study cohort presented a high prevalence of resistant viruses, especially to NRTI. DBS specimens can be used for DRM detection.

INTRODUCTION

In 2010, an estimated 34 million people were living worldwide with HIV-1. Mother to child transmission (MTCT) of HIV-1 is responsible for more than 10% of new HIV-1 infections globally, causing 390,000 new HIV infections in infants and children [1]. Policies towards increasing coverage of treatment to children and to pregnant women to prevent MTCT of HIV are being created. However, only 48% of HIV-1 infected women receive HAART (highly active antiretroviral therapy) during pregnancy [2]. Thus, more efforts towards scaling-up access to treatment are still needed to prevent MTCT [2].

Over the past decade, a dramatic reduction in MTCT of HIV has occurred in the developed world [3-6] due to testing and treating pregnant women for HIV-1. However, MTCT still occurs at a low frequency in high-income countries each year [7-9], reporting MTCT rates below 2% in the United States and Europe [3-7].

According to the Centers for Disease Control and Prevention (CDC), about 1.2 million people are living with HIV-1 in the United States (US) [9]. The HIV incidence has been relatively stable in the past few years. An estimated 48000 people were diagnosed with HIV infection in 2010, being African Americans and Latinos the most affected groups (67%) [9]. Since the epidemic started, a total of 9809 children have been diagnosed of AIDS, 2437 of them in the state of New York [9]. By the end of 2009, 4986 people with perinatally HIV-1-infected patients were living in the US with an AIDS diagnosis, with approximately half residing in New York (1296, 53.2%) [9].

Treatment of HIV-1-infected individuals with combined antiretroviral therapy (cART) has dramatically changed the course of the infection, reducing mortality and morbidity events associated with this disease [10-11]. The use of ART in high-income countries has been associated with the development of drug resistance [12,13]. Resistance genotyping has become a standard of care in HIV infection management [14-16]. Drug resistance in HIV-1 infected children and infants may result either from a drug resistant strain transmitted from the mother or from a drug-resistant strain developed after paediatric or maternal ART administration used for preventing MTCT [17,18]. Development of drug resistance in children on ART is usually related to poor adherence, use of suboptimal regimens, or subtherapeutic drug levels [19,20]. Although the clinical significance of HIV drug resistance has been well documented in the adult populations, studies on the prevalence and patterns of drug-resistance mutations in the paediatric HIV-infected populations remain limited.

Thus, the aims of this study were to estimate the prevalence of antiretroviral drug resistance mutations, to determine drug susceptibility and to characterize the infecting variants in a cohort of HIV-infected children under medical care at Jacobi Medical Center located in the Bronx, New York, USA.

MATERIALS AND METHODS

Study Population

A total of 47 HIV-1 perinatally infected individuals from the Jacobi Medical Center located in the Bronx, New York City, USA were enrolled in this study. Patients attending clinic from August through November 2011 were selected. Blood samples from these patients were shipped to the Division of Experimental Medicine in San Francisco, CA. Prior to processing, blood samples were blotted onto 903 filter papers (GE Healthcare Barcelona, Spain), dried and sent to a reference laboratory in Madrid, Spain, for further analysis. Blood samples were centrifuged, providing isolated plasma that was saved at -80ºC until use.

This study was approved by the Ethical Committees of all institutions involved in the US. It was designed to protect the rights of all subjects included under the appropriate local regulations. To maintain subject confidentiality, a unique ID number was assigned to each specimen, and written consent for each patient over 18 years old or legal guardian (<18 years) were obtained by clinicians.

Dried Blood Spots

Dried Blood Spots (DBS) specimens were prepared adding 50-70ul of blood to fill each circle of 903 filter paper cards (GE Healthcare Barcelona, Spain). Cards were dried overnight at room temperature and stored at -80ºC until they were shipped at room temperature to Madrid, Spain for testing. After their arrival, they were stored at -80ºC until nucleic acid isolation.

Blood from 3 DBS circles per patient was eluted in 2ml lysis buffer (BioMerieux, Durham NC, USA) for 2 hours with gentle rotation and processed using an automated platform for the isolation of RNA based upon the magnetic-silica technology was used employing NucliSENS® easyMAG® instrumentation (BioMerieux, Durham, NC, USA) according to the manufacturer´s instructions.

Plasma Specimens

HIV-1 viral ARN was recovered from plasma samples using QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA, USA) following manufacturer´s instructions.

HIV-1 *pol* amplification and sequencing from infected samples

HIV-1 RNA either extracted from DBS or plasma was amplified by nested-polymerase chain reaction (PCR). cDNA synthesis was carried out using the Qiagen One Step RT-PCR Kit (Qiagen, Valencia, CA, USA), and further amplifications using Phusion® High-Fidelity PCR Master Mix (Thermo Fischer scientific, Madrid, Spain), following manufacturer's instructions.

A 835-base pair fragment of the complete protease (PR) and a 775-base pair fragment of partial reverse transcriptase (RT) were amplified using specific primers designed by the Public Health Agency of Canada (PHAC) and proposed by World Health Organization (WHO) in the manual for HIV drug resistance testing using dried blood spot specimens [21]. Conditions for RT-PCR and nested PCR have been previously published [22]. HIV-1 *pol* sequences obtained from DBS samples were sequenced by Macrogen Inc. (Gasan-dong, Geumchun-gu, Seoul, Korea). Quintara Biosciences (Albany, CA, USA) provided sequences obtained from plasma samples.

HIV-1 subtyping

For subtype or CRF characterization, phylogenetic analyses (phy) using *pol* sequences were carried out using reference HIV-1 sequences belonging to HIV-1 group M variants available at the GenBank when the analysis was performed. The tree topology was obtained using the Neighbour-Joining method. Alignment of DNA sequences was performed using the ClustalW program. The pairwise distance matrix was estimated using the Kimura two-parameter model within the MEGA v5 software package. Bootstrap re-sampling (1000 data sets) of the multiple alignments was performed to test the statistical robustness of the tree.

Genotypic Drug Resistance identification

Drug resistance mutations (DRM) in pretreated patients, defined by the 2011 International AIDS Society (IAS)-USA [23], were manually located in each PR and RT sequence following the IAS-USA nomenclature. Genotypic interpretation of these resistance mutations was evaluated using the Stanford HIVdb Algorithm [24], version 6.0.11 (Stanford University, Palo Alto, CA). Resistance was normalized in three levels: susceptible (S), intermediate (I), and resistant (R).

Statistical analysis

Prevalence was expressed in percentage with 95% confidence interval (CI). CI tests were performed with Epidat 3.1 (Pan American Health Organization). Significance was set at $p<0.05$.

Accession Numbers

PR and/or RT sequences from the study population were submitted to GenBank with the following accession numbers: KC470320 to KC470366.

RESULTS

Clinical features of the study population

Forty-seven HIV-1 perinatally infected patients during the period 1989-2009 and under clinical follow-up during 2011 in the Jacobi Medical Center in the Bronx, New York, were selected. Paired plasma and DBS specimens were taken from August to November 2011. All available clinical and epidemiological features from the study population at sampling time were recorded (**Table 1**). All patients were perinatally infected mostly (61.7%) during the 1985-1995 period and all were born in the US. The mean age of the study cohort was 17.7 years and all were alive by December 2011.

Over half of the patients (53.2%) reached relative CD4+ cell counts higher than 25% and 40.5% of them more than 500 CD4+ cells/mm^3. The mean absolute and relative CD4+ cell counts were 486 cells/ml and 21.1%, respectively.

Table 1. Clinical and epidemiological features of the study population at sampling time.

Features	Patients [n (%)]
Age (years) Mean 17.7 years SD (4.4)	
0-5	1 (2.1)
6-15	13 (27.7)
16-18	6 (12.8)
>18	27 (57.4)
Gender	
Female	25 (53.2)
Year of infection	
1985-1989	9 (19.2)
1990-1994	20 (42.5)
1995-1999	16 (34)
2000-2009	2 (4.3)
Infecting HIV-1 variant[a]	
B subtype	36 (76.6)
CRF02_AG recombinant	1 (2.1)
unknown [b]	10 (21.3)
Viral load (copies/ml) Mean 3143 copies/ml	
< 50	19 (40.4)
50-100	3 (6.4)
101-1000	6 (12.8)
1001-10000	10 (21.3)
10001-100000	5 (10.6)
>100000	3 (6.4)
unknown	1 (2.1)
CD4 count (%) Mean 21.1%	
<15	7 (14.9)
15-24	14 (29.8)
25-49	25 (53.2)
unknown	1 (2.1)
CD4 count (cells/ml) Mean 486 cells/ml	
<200	8 (17)
200-499	19 (40.4)
500-750	9 (19.2)
>750	10 (21.3)
unknown	1 (2.1)
Tropism[c]	
Dual/Mixture	11 (23.5)
R5-tropic	16 (34)
X4-tropic	1 (2.1)
unknown	19 (40.4)
Current ART	
1 drug family	**6 (12.8)**
1NRTI	3 (6.4)
2NRTI	3 (6.4)
2 drug families	**28 (59.6)**
2NRTI+PI	22 (46.8)
2NRTI+NNRTI	4 (8.5)
3NRTI+PI	2 (4.3)
3 drug ffamilies	**12 (25.5)**
2NRTI+NNRTI+PI	6 (12.8)
2NRTI+PI+InI	3 (6.4)
3NRTI+PI+InI	1 (2.1)
2NRTI+NNRTI+InI	1 (2.1)
NRTI+NNRTI+PI+InI	1 (2.1)
unknown	1 (2.1)

SD, Standard Deviation; DBS, Dried Blood Spot; [a]Infecting variants characterized by phylogenetical analysis. [b]No *pol* sequences from these patients were available to characterize HIV variant. [c]Tropism determined by Trofile® assay (Monogram Biosciences Inc. SF, CA, USA).

Viral load (VL) levels were undetectable (less than 50 HIV-1 ARN copies/ml) in 19 (40.4%) patients and VL over 1000 copies/ml in 38.3%. HIV-1 viral tropism determined by the Trofile® assay, gold standard in tropism testing, was determined in 28 patients revealing that 16 (34%) subjects were infected by HIV-1 CCR5-tropic variants, 11 by dual-tropic variants and 1 harboured a CXCR4-tropic variant.

Most (97.9%) patients of the paediatric cohort were treatment experienced at sampling time, majority (59.6%) under a 2 drug family scheme. The most (46.8%) used combination was 2NRTI+1PI. Twelve patients (25.5%) were prescribed a 3 family-based regimen including NRTI as a backbone and adding NNRTI, PI or an Integrase Inhibitor (InI). The second most used regimen in the cohort was the three drug family combination of 2NRTI+NNRTI+PI prescribed in 6 patients (12.8%) and 2NRTI+NNRTI+InI in other 6. In 4 cases the prescribed InI was Raltegravir and in 2 was Dolutegravir. Monotherapy with either one or two NRTI drugs was used in six patients (12.8%). No records of ART history or previous ART schemes among the cohort were available.

Among the 47 selected paediatric patients, HIV-1 *pol* sequences were recovered from plasma and/or DBS samples from 37 subjects (10 of them with undetectable VL). Among the 10 non-successfully amplified specimens, nine had less than 50 HIV-1 RNA-copies/ml. PR and RT viral coding regions were amplified in 29 patients and in 8 only RT. In 27 of 37 patients, viral sequences were recovered from plasma (20 PR/RT and 7 RT) and 20 from DBS specimens (19 PR/RT and 1 RT). HIV-1 sequences were recovered from both DBS (5PR/RT, 4PR and 1RT) and plasma (9PR/RT and 1RT) specimens in 10 of the 37 subjects.

The 37 sequences (29 PR/RT and 8 RT sequences) derived from plasma specimens were used to analyze drug resistance prevalence, HIV-1 infecting variant and drug susceptibility.

Phylogenetic characterization of HIV-1 variants

All but one of the 37 patients included in the the Jacobi cohort with available HIV-1 sequence carried subtype B virus by phylogenetic analysis, all infected during the 1985-2009 period. The only non-B variant was a CRF02_AG recombinant found in a patient born in 1995. Prevalence of non-B variants among these patients was 2.7%. The patient infected by CRF02_AG variant harboured no resistance mutations.

Prevalence of HIV-1 Drug Resistance

All 47 patients from the Jacobi cohort were drug-experienced. The prevalence of DRM to at least one drug class among the 37 ART-experienced patients with available *pol* sequence was 64.9% (**Table 2**). The prevalence of DRM by drug classes was, 54.1% (20 patients) for NRTI, 27.6% (8 patients) for PI and 27% (10 patients) for NNRTI. Thus, over six of each ten patients (64.9%) were infected with mutants resistant to at least one drug family (**Table 2**).

Table 2. Prevalence of HIV drug resistance mutations according to drug class family.

	Number of patients from the Jacobi cohort (n=47)
Patients with amplified PR	29
Patients with amplified RT	37
Prevalence of drug resistance mutations (%) [95% CI][a]	
global (to any class)	64.9 [48.1-81.6]
to PIs	27.6 [9.6-45.6]
to NRTIs	54.1 [36.6-71.5]
to NNRTIs	27 [11.4-42.7]
to 2 classes	27 [11.4-42.7]
to 3 classes	5.4 [0.7-18.2]

[a]Prevalence for pretreated patients was determined following the IAS-USA 2011 list[23]. PR, protease; RT, reverse transcriptase; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; PI, protease inhibitors; CI, confidence intervals. Prevalence for DRM was determined using sequences obtained from plasma samples.

Drug resistance prevalence to two drug families (to PI+NRTI or to PI+NNRTI or to NRTI+NNRTI) was detected in 10 (27%) patients and triple class resistance (to PI+NRTI+NNRTI) in 2 subjects (5.4%).

Major PI drug resistance mutations found according to IAS-2011 list [23] were: L90M (20.7%), D30N, M46I and I54V (all 6.9%) and M46L, I50V, V82A, V82L and I84V (all 3.5%). NRTI mutations were: M184I/V (29.7%), M41L and T215F/Y (both 18.9%), D67N, T69D/S and K219E/Q (all 13.5%), K70R and L210W (both 5.4%) and L74V, F116Y and Q151M (all 2.7%). Resistance mutations for NNRTI were: K103N (18.9%) and A98G, L100I, K101E, V108I, Y181C and M230L (all 2.7%).

Drug susceptibility interpretation

Using the HIV-1 *pol* sequences obtained from 37 patients we analyzed the genotypic resistance interpretation to each antiretroviral drug **(Figure 1)**.

Globally, we found a low level of resistance to each drug within each class. We observed the highest resistance rate for NRTI family, mainly to FTC and 3TC, with nearly 30% of resistant patients.

Ninety two percent of the 37 patients with available *pol* sequence in the cohort (n=34) currently used FTC (n=27) or 3TC (n=7) as backbone of their ART. For NNRTI, NVP and EFV accounted for the highest levels of resistance (19% and 16%, respectively). Four patients were receiving EFV as part of their current NNRTI-based treatment and none NVP.

For the PI class, the highest predicted level of resistance was for NFV (20%) followed by ATV/r, FPV/r and SQV/r (all 10%). Twelve patients (32.4%) were receiving ATV as part of their PI-based treatment.

For TPV/r and DRV/r, the new PI drug generation, the genotypic resistance interpretation provided no level of resistance. Data also revealed an absent level of predicted resistance to ETR and RPV, the new NNRTI drugs; total susceptibility levels to these drugs remained above 90% in all patients. Low levels of resistance (below 5%) were also detected for LPV/r, ABC and TDF. No drug resistance interpretation for Integrase inhibitors were performed because *integrase* gene was not amplified in the study cohort.

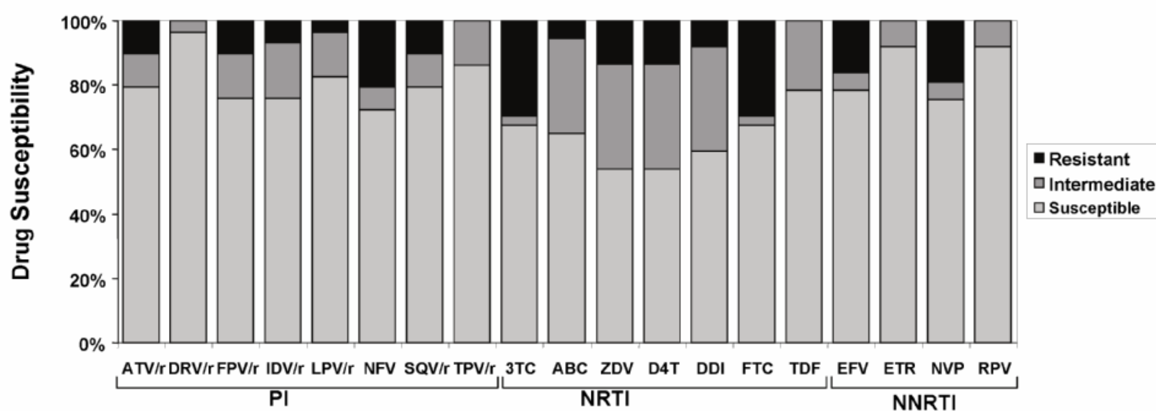

Figure 1. Predicted susceptibility to each of the drugs used as ART in pretreated patients. Susceptibility was estimated according to the HIVdb Interpretation Algorithm (Stanford University, Palo Alto, CA, USA).

PI, protease inhibitors: atazanavir/r (ATV/r), darunavir/r (DRV/r), fosamprenavir/r (FPV/r), indinavir/r (IDV/r), lopinavir/r (LPV/r), nelfinavir (NFV), saquinavir/r (SQV/r), tipranavir/r (TPV/r) and where "/r" indicates co-administration with low-dose ritonavir (RTV) for pharmacological "boosting". NRTI, nucleoside reverse transcriptase inhibitors: lamivudine (3TC), abacavir (ABC), zidovudine (ZDV), stavudine (d4T), didanosine (ddI), emtricitabine (FTC), tenofovir (TDF). NNRTI, non-nucleoside reverse transcriptase inhibitors: delavirdine (DLV), efavirenz (EFV), etravirine (ETR), nevirapine (NVP); R, high level resistance; I, intermediate resistant; S, susceptible. Prevalence for DRM was determined using sequences obtained from plasma samples.

Table 3. Comparison of drug resistance profiles obtained from plasma and dried blood spots specimens.

Patients	logVL (HIV-1 RNA copies/ml)	Infection Year	DRM detected in plasma			DRM detected in DBS		
			PI Major	NRTI	NNRTI	PI Major	NRTI	NNRTI
CC092591	4.8	1991	M46L, I54V, V82A, L90M	M41L, L74V, M184V, L210W, T215Y	L100I, K103N	M46L, I54V, V82A, L90M	M41L, L74V, M184V, L210W, T215Y	L100I, K103N
TN090293	5.1	1993	-	M184V, T215L	M230L	-	M184V, T215L	M230L
AC070893	5.7	1993	n.a.	T215IL, K219Q	-	M46I**, I50V**	T215IL, K219Q	-
CM120388	4.7	1988	-	K219Q	K103N	-	K219Q	K103N
EJ120388	3.8	1988	-	M41L, T69D	K103N	-	M41L, T69D	K103N
JD101997	3.6	1997	-	M41L*, M184V, T215Y	-	-	M184V,L210W*,T215Y	-
JW112393	3.3	1993	L90M*	-	-	-	-	-
AC060892	3.8	1992	-	M41L, D67N, T69D, M184V, T215Y	-		n.a.	
EV032591	3.1	1991	-	-	-	-	-	-
JV110892	4.7	1991	-	-	-	-	-	-

VL, viral load; DRM, drug resistance mutations; PI, protease inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-NRTI; DBS, dried blood spots; dash, no DRM found; n.a., region not amplified; *, This DRM was not found either in plasma or DBS.

Comparison between genotypic profiles obtained from plasma *vs.* DBS

Among the 37 patients with available PR and/or RT sequence from plasma samples, in 10 subjects sequences from both plasma and DBS specimens were obtained. In more detail, HIV-1 sequences were recovered from both DBS (5PR/RT, 4PR and 1RT) and plasma (9PR/RT and 1RT) specimens in 10 of the 37 subjects. Of the ten plasma-DBS sequence pairs compared the mean nucleotide similarity between them was 97.9%. Identical DRM profiles were present in 60% (6 out of 10) of plasma-DBS paired sequences (**Table 3**). A total of 30 DRM were detected in plasma and 26 in DBS, being 23 present in both. Of the seven DRM present in plasma samples but undetected in DBS, in 5 DRM no *pol* gene region was available from DBS samples. Two DRM (L90M in patient 7 and M41L in patient 6) were found in plasma but were not present in DBS. Of the 3 mutations detected in DBS absent in plasma, two of them were not found because we were unable to amplify by PCR this region. The remaining mutation, L210W in patient 6 was present in DBS but not found in plasma. Summarizing, DRM profiles obtained form DBS provided a good concordance with those obtained from plasma samples. Discrepancies rise in two patients, two mutations were detected in plasma but not in DBS and one mutation was detected in DBS but not in plasma.

DISCUSSION

This study analyzes the clinical and immunological features of a cohort of HIV-1 perinatally infected children (Jacobi Medical Center cohort). It also describes the HIV-1 infecting variants, drug resistance prevalence, drug susceptibility and viral tropism in the study population.

Because of the advent of combined ART and effective viral suppression with immunologic reconstitution, many of the perinatally infected children from the Jacobi Medical Center cohort have survived into their second decade of life. Thus, long-term observation to monitor the effects of prolonged exposure to both HIV and HAART and transition pathways from paediatric units to adult services have been studied [25-28], although further research is still required.

HIV-1 non-B variants are present in the Jacobi Medical Center cohort

Our results show a low prevalence of non-B HIV-1 infecting variants. The presented data revealed that only one patient, born in 1995, was infected by a non-B variant, representing a prevalence of non-B infections of 2.7%. It was lower than the previously reported among HIV-1 perinatally infected children born in New York between 1998-1999 and 2001-2002 (4.4% and 16.7% non-B prevalence, respectively) [29-30]. Also it was lower (9.5%) than in other cohort of infants of less than 6 months infected between 2002 and 2005 in the United States [31].

Differences in HIV non-B variants prevalence could be explained by the long-term infection of our patients (mean age of 17.7 years), reflecting the local epidemiological situation in New York City prior to the detection and spreading of HIV-1 non-B variants. Similar low HIV-1 non-B variants prevalence values (1.9%) have been detected for perinatally infected children transferred to adult units in Spain at a mean age of 18.9 years [27] compared to non-transferred patients of the same cohort more recently infected with a non-B variant prevalence of 11.5% [27]. Prevalence of HIV-1 non-B variants in perinatally infected children has been reported in European countries, ranging 10-36.4% [32-34]. Clinicians should be aware of how HIV-1 non-B infection may differentially impact on HIV infection outcome, in disease progression, and in the rate of CD4+ T cell decline [35-37].

Drug Resistance Prevalence of HIV-1 Drug Resistance

As expected, this study reveals high levels of drug resistance in pretreated patients. Most patients (64.9%) harboured resistance mutations to at least one drug family. Prevalence of drug resistance mutations is in agreement with other reports in Europe where resistance

to at least one drug family ranged from 66% to 82% [27,33,38,39]. The reported prevalence of viruses carrying resistance mutations to 2 drug classes in the Jacobi Cohort (27%) is half than in the British CHIPS cohort 52% [28] but closer to paediatric cohorts from Spain showing 30-40% levels of dual resistance [32,33]. Triple-class levels of resistance was lower (5.4%) compared to publish data from other European Paediatric cohorts where it ranged (12–27%) [27,28,33,39].

Limited data on HIV-1 drug resistance on third generation drugs (cell-entry and integrase inhibitors) are available from paediatric cohorts [40-42]. HAART-failing patients from the Jacobi Medical cohort could still benefit from CCR5 antagonists-based strategies, even in the case of triple-class virologic failure. Over half of our patients (57.1%) with an HIV-1 tropism test performed were R5-tropic and could benefit from the use of fusion inhibitors. However, further studies are needed to evaluate the efficacy and the tolerability of CCR5 antagonists in this paediatric population. Also, integrase inhibitors could be the best rescue alternative in the cases of therapeutic failure with multiresistance, although additional genetic resistance studies should be performed to guarantee their usefulness.

Drug susceptibility interpretation

Treatment failure in children during ART is frequent, develops fast and with more extensive drug resistance than in adults, leading to virologic failure and immunologic damage [43-45]. Thus, a correct usage and formulations of licensed compounds to treat HIV in children are required, a close surveillance of adherence and resistance studies are required in these heavily pretreated perinatally infected children.

According to the predicted drug susceptibility, our data revealed that recently approved PI compounds could be good candidates to control viraemia in our cohort. Low levels of resistance to TPV/r and DRV/r prove there clinical utility as a first-line or second-line PI, as they have proved for HIV-1 infected children from the UK and the USA [46,47]. Similar high levels of susceptibility for the new NNRTI (ETR) were found. ETR-based regimens could also be good alternatives for inclusion in future ART as it has proven a sustained antiviral response and improved immunological parameters in paediatric and adolescent patients [41].

Comparison between genotypic profiles obtained from plasma *vs.* DBS

Many previously published studies have aimed to compare the nucleotide sequences generated from paired DBS and plasma specimens [48]. Reported concordance between nucleotide sequences generated from two specimens ranged 98.1% to 99% [48]. Previous studies highlighted that genotyping was reliable in samples with viral loads above 4 log [49] but in our study samples with 3.1 log provided positive amplifications. This amplification success may be explained by the in-house RT-PCR assays designed by the WHO to amplify small genome fragments avoiding larger nucleic acid fragments that are more sensitive to degradation. Despite limitations, DBS genotyping strategy has been implemented and proven their reliability in resource limited settings [21,22].

DBS have proven to provide reliable data for monitorizing HIV in constrained settings but they can be a practical, cost-effective way for HIV diagnoses, determination of drug resistance prevalence, viral load quantification and molecular epidemiological surveillance studies that track the global distribution and spread of HIV-1 variants. DBS technology can be used to improve large scale HIV national plans [50].

CONCLUSION

This study describes the clinical, epidemiological and virological features in HIV-1 perinatally infected children enrolled in the Jacobi Cohort of New York, showing the prevalence of drug resistance mutations, the drug susceptibility interpretation and the HIV-1

infecting variants among pretreated patients at sampling collection time in 2011.

The subject investigated in this manuscript is of public health importance especially under the current scenario of high access to mother to child preventive measures therapy and few cases of vertical transmission of HIV in developed countries. There is an urgent need for resistance testing as well as the development of new therapies based on different retroviral targets in drug-experienced children with therapeutic failure events. It is essential to use optimized ART for each individual, based on resistance information, to avoid suboptimal regimens and early resistance acquisition and to reduce long-term consequences of incomplete virological control. The reported data will help improve clinical management of the HIV-infected paediatric population in New York.

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DISCLOSURE STATEMENT

Authors declare that no competing interests exist.

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CAPÍTULO 5

Prevalencia de resistencia a fármacos antirretrovirales en pacientes pediátricos infectados por VIH-1 en Honduras y El Salvador en el periodo 1989-2009

JUSTIFICACIÓN Y OBJETIVOS

La región caribeña y los países de Centroamérica son dos de las zonas mundiales más castigadas por la infección por VIH-1. En ciertas regiones la prevalencia de infección por VIH-1 llegó a alcanzar el 2% de la población. La tasa de infecciones es variable en los países de Centroamérica y entre colectivos. Las características sociales, geográficas, políticas y el acceso al TAR en cada país centroamericano determinan la epidemiología del VIH-1 y la selección de resistencias a ARV. El aumento del acceso al TAR para tratar la infección por VIH-1 en países de recursos limitados ha provocado la reducción de la morbi-mortalidad asociada a la infección y el aumento de la esperanza de vida en población infectada. Sin embargo, también ha favorecido la aparición de variantes virales resistentes a los ARV administrados. Éstas ponen en riesgo el futuro del TAR e incrementa el riesgo de padecer eventos de fracaso terapéutico, especialmente en pacientes pediátricos que requieren tratamiento de por vida. Las mutaciones de resistencia, junto con la limitada disponibilidad y variedad de fármacos ARV para población pediátrica, comprometen el propio tratamiento y generan un gran problema de salud pública.

La puesta en marcha de programas nacionales de acceso al TAR en Honduras desde 2002 y en El Salvador desde 2004 ha mejorado la situación clínica de los pacientes. Sin embargo, su impacto en la selección de variantes virales del VIH-1 resistentes a los fármacos ARV en uso no había sido estudiado. De hecho, en El Salvador no está instaurada la detección de mutaciones de resistencia en la rutina clínica. Por todo lo anterior, y aprovechando la visita de dos pediatras de Honduras y de El Salvador financiados por el programa ESTHER (Red de Solidaridad Hospitalaria contra el SIDA, por sus siglas en francés), permitió sentar las bases de una colaboración científica con el laboratorio. Así, en conjunto nos propusimos:

- Recoger muestras de sangre seca (DBS) de una parte de la cohorte de niños infectados por VIH-1 en seguimiento clínico por dichos pediatras en sus respectivos países.
- Describir las características clínicas y epidemiológicas de esos niños y adolescentes infectados por VIH-1 desde 1989 hasta 2009 bajo seguimiento clínico en Honduras (Clínica de Atención Integral del Hospital Dr. Mario Catarino Rivas en San Pedro Sula) y en El Salvador (Centro de Excelencia para Niños con Inmunodeficiencia, Hospital Nacional de Niños Benjamín Bloom en San Salvador).
- Determinar la prevalencia de mutaciones de resistencia a fármacos antirretrovirales en pacientes de El Salvador y Honduras infectados.

- Caracterizar las variantes virales del VIH-1 que infectan a la población de estudio.
- Determinar las diferencias en regímenes usados para el tratamiento de la infección por VIH-1 en las dos cohortes estudiadas.

CONCLUSIONES

- Desde julio a diciembre de 2009 se recogieron muestras de sangre seca (DBS) de 80 pacientes, 54 de Honduras y 26 de El Salvador.
- La edad media de la población de estudio de Honduras fue de 9,6 años y la de El Salvador de 8 años. La mayoría eran niños (65%), infectados por vía vertical (96%) principalmente (82,5%) entre 1989 y 2004. Casi un tercio (32,5%) eran huérfanos.
- El 70% de los 80 pacientes seleccionados presentaban síntomas moderados o severos de progresión a SIDA. El retraso diagnóstico fue elevado: 72,7% en Honduras y 57,7% en El Salvador. El 45% de los pacientes de ambas cohortes no habían sido diagnosticados de la infección hasta la edad de 4 años.
- El 45% de pacientes de Honduras y el 60% de El Salvador presentaban un recuento de linfocitos T CD4+ por debajo de las 500 céls/mm^3 al comenzar el TAR. El 87,3% de los niños habían recibido TAR durante 2 a 9 años. Sin embargo, la mayoría (65,1%) mantenían su primer régimen de TAR en el momento de la toma de muestra.
- La prevalencia global de resistencia a fármacos ARV en pacientes *naïve* fue del 16,6% en Honduras y del 10% en El Salvador. En pacientes pretratados la prevalencia global fue del 74,4% en Honduras y del 66,7% en El Salvador.
- Uno de cada 10 pacientes del estudio en ambos países estaba infectado por variantes virales resistentes a las tres familias de fármacos ARV.
- Todos los pacientes analizados estaban infectados por subtipo B del VIH-1.
- Este ha sido el primer estudio que analiza la prevalencia de mutaciones de resistencia y caracteriza las variantes virales del VIH-de personas infectadas por VIH-1 en El Salvador.

Drug Resistance Prevalence in Human Immunodeficiency Virus Type 1 Infected Pediatric Populations in Honduras and El Salvador During 1989–2009

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Background: Emergence of viral resistance is a major obstacle for antiretroviral treatment (ART) effectiveness. Human immunodeficiency virus type-1 (HIV-1) variants and drug-resistance mutations were identified in naive and antiretroviral drug-experienced children with virologic failure, in Honduras and El Salvador.

Methods: Dried blood spots (DBS) from 80 individuals (54 from Honduras, 26 from El Salvador) infected during their childhood between 1989 and 2009 were collected in 2009. The HIV *pol* region was amplified and sequenced to identify antiretroviral-resistant mutations according to the 2009 International AIDS Society. The genotypic drug resistance interpretation was performed using the Stanford algorithm. HIV-1 variants were characterized by phylogenetic analysis and subtyping tools.

Results: HIV-1 protease and reverse transcription sequences were obtained from DBS specimens in 71 and 66 patients, respectively, of the 80 patients. All children were native Central Americans carrying subtype B, with a mean age of 9 years, most were male (65%), perinatally infected (96%), with moderate/severe AIDS symptoms (70%), and receiving first line ART at the time of sequencing (65%). Diagnostic delay was frequently observed. Infected children from Honduras presented longer ART experience and clinical outcomes, and more frequent severe symptoms. Resistant variants infected 1 of 11 naive children from El Salvador but none of the perinatally infected naive children from Honduras. Resistance was higher among ART-exposed individuals in both countries and similar for protease inhibitors (16%), nucleoside reverse transcription inhibitors (44%–52%), and nonnucleoside reverse-transcription inhibitors (66.7%). One in 10 pretreated children in each country was infected with resistant viruses to the 3 drug families.

Conclusions: Our data support the need for continued surveillance of resistance patterns using DBS at national levels among naive and pretreated children to optimize the ART regimens.

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The expanding use of antiretroviral drugs for treatment of human immunodeficiency virus type-1 (HIV-1) infections favors the emergence of primary resistance mutations in viruses from patients under antiretroviral therapy (ART) and in treatment-naive patients who have been infected by pretreated subjects. ART resistance can lead to ART failure.[1] Treatment failure in children during ART is frequent and is of fast development.[2] It reflects an incomplete viral suppression, the inadequate dosing, adherence difficulties, and the reduced number of approved antiretrovirals with pediatric formulations.[3,4] It represents a serious public health problem, especially in perinatally infected pediatric populations, because of their need of therapy from birth, and the restrictions in available antiretrovirals in therapeutic use in adults. There have been few studies monitoring resistance in HIV-1-infected children, mainly in regions with limited resources such as Central America. Dried spots made of whole blood (dried blood spots [DBS]) on filter paper is an affordable and practical alternative specimen source to liquid plasma, for drug-resistance HIV-1 genotyping and HIV-1 subtyping, mainly in the context of low- and middle-income countries.[5,6]

National ART programs began to scale-up access to HIV therapy in 2002 in Honduras and in 2004 in El Salvador, favoring the appearance of viruses with drug-resistance mutations in both countries. Honduras has one of the highest HIV-1 prevalences in Latin America[7] (1.9%), but it is lower among pregnant women[8] (0.3%–1.7%) and higher in specific minority ethnic groups.[9] The HIV prevalence in El Salvador is lower (0.4%), with a vertical transmission rate of 0.5%, although during 2008–2009, nearly 11% of newborns from HIV-1-infected mothers acquired HIV-infection.[10] We report the first study to examine the prevalence of resistance mutations in HIV-1 *pol* sequences and the HIV-1 variants in DBS specimens collected in 2009 from 80 children HIV-1-infected between 1989 and 2009 from El Salvador and Honduras, naive or under antiretroviral-therapy.

METHODS

Study Population

A total of 80 individuals (54 from Honduras, 26 from El Salvador) infected during their childhood in the 1989–2009 period were enrolled in the study. Patients were selected from 2 HIV/AIDS clinics: Clínica de Atención Integral del Hospital Dr. Mario Catarino Rivas, San Pedro Sula, in Honduras, and Centro de Excelencia para Niños con Inmunodeficiencia, Hospital Nacional de Niños Benjamín Bloom, San Salvador, in El Salvador. DBS

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were collected from July to December 2009 after parents or guardians of the enrolled children less than 18 years of age had given their consent. Four individuals infected during childhood but aged more than 18 years when DBS were collected, signed their own informed consent. This study was part of a project approved by a review board and Ethical Committee of the collaborating institutions in Madrid, Spain, Honduras, and El Salvador. Most of the pretreated children had virologic failure, defined as the HIV-1-infected patient who did not reach <1000 HIV-1 RNA copies/mL after 24 weeks under ART. Viral load was quantified in the corresponding HIV/AIDS clinics where children were under follow-up.

DBS Specimens

DBS were prepared by adding 1 to 2 drops of blood to fill each circle of 903 filter paper cards (Schleicher & Schuell, Bio-Science GmbH, Barcelona, Spain). Cards were dried overnight at room temperature and stored at −20°C before they were shipped to Madrid at room temperature for testing. After their arrival, they were stored at −20°C until nucleic acid isolation. Then, blood from 1 to 2 DBS circles per patient was eluted in 2 mL of NucliSENS lysis buffer (BioMerieux, Madrid, Spain) for 2 hours with gentle rotation and processed to extract RNA according to the manufacturer's instructions using NucliSENS easyMAG instrumentation (BioMerieux).

HIV-1 *pol* Sequencing

Direct sequencing of nested polymerase chain reaction (PCR) purified products from viral RNA was home-performed in the HIV-1 *pol* coding region. *Pol* sequences included the complete protease (codons 1–99) and part of the reverse transcriptase (codons 1–232). HIV-1 RNA was amplified using an in-house reverse transcription (RT)-nested PCR method. cDNA synthesis was carried out using the Access Quick RT-PCR system (Promega, Madison, WI), and amplifications using PCR Master Mix (Promega), following manufacturer's instructions. A 1121 base pair of the complete protease (PR) (297 bp) and partial RT genes were amplified using the outer primers Prot1[11] and NEI35m.[12] Inner primers were Prot3[11] and RT4mnew (5′-AGG ATG GAG YTC ATA YCC CAT CCA AAG-3′, positions 3231–3257 HXB2). In some specimens, alternative inner primers sets were used for separate amplifications of PR and RT. A 514 base pair amplification including the complete PR was performed using Prot3[11] and Prot4.[11] For inner partial RT amplifications (653 bp), primers were RT1m (3′-CCA AAR GTT AAA CAA TGG CCA TTG ACA-3′, positions 2604–2630 HXB2) and RT4mnew. In some specimens, positive amplification was only achieved using different primers sets for PR[11] and RT separately in the first PCR. For outer RT amplification, primers A35[12]/RT4m or 54RU (5′-AGT TTG CCA GGA AGA TGG AAA CCA-3′, positions 2361–2384 HXB2)/NEI35m; and for inner amplification, RT1m/RT4m were used. In all cases, PCR conditions were incubation at 94°C for 5 minutes, 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. Annealing temperature was 57.5°C for nested PCR using Prot3/Prot4.

HIV-1 Subtyping

Sequencing was performed by Macrogen (Gasan-dong, Geumchun-gu, Seoul, Korea). *Pol* sequencing was performed using the same primers as the corresponding inner amplifications. They were subtyped by phylogenetic analysis, as previously reported,[13] including reference sequences from Latin American countries available in GenBank 2010.

To identify recombinants at *pol*, the following 5 recombination programs were used: SCUEAL (http://www.hyphy.org/pubs/SCUEAL), Recombination Identification Program (RIP, http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html), jpHMM (http://jphmm.gobics.de/jphmm.html), Recombination Detection Program (RDP, http://darwin.uvigo.es/rdp/rdp.html), and SimPlot (http://sray.med.som.jhmi.edu/SCRoftware/simplot/). In Simplot, windows of 300 nucleotides (nt) were used moving in 10 nucleotides increments, as recommended.[14] To perform the bootscanning, sequences obtained from GenBank, with the same geographical origin as the analyzed sequences, were used when possible.

Genotypic Drug Resistance Identification

Drug-resistance mutations defined by the 2009 International AIDS Society-United States of America (IAS-USA)[15] were manually located in each PR and RT sequence. Resistance mutations at PR gene were classified as primary (major) or secondary (minor), following the IAS-USA nomenclature. The genotypic drug-resistance interpretation was performed using the genotypic resistance algorithms provided by Stanford (v.4.3.7), available at: http://hivdb6.stanford.edu/asi/deployed/hiv_central.pl?program=hivdb&action=showSequenceFormalgorithms.

RESULTS

Epidemiology and Clinical Features of the Study Population

A total of 80 HIV-1 subjects infected in childhood were enrolled in the study. Most of the children were diagnosed as HIV-1 positive by serologic testing in 2 HIV/AIDS clinics located in 2 Central America countries. The 54 children from Honduras were from 3 departments, mainly (93%) Cortés. Twenty four of the 26 patients from El Salvador with known procedence came from 9 departments: San Salvador (9), La Libertad (4), La Paz (3), Usulután (2), San Miguel (2), Sonsonate (1), Auachapán (1), Santa Ana (1), and Cuscatlán (1).

Table 1 records all clinical and epidemiologic features from the 80 children enrolled in the study. All patients were native Central Americans, with a mean age of 9 years, and mainly presenting severe or moderate symptoms (70%), according to the Centers for Disease Control and Prevention classification system.[16] Nearly a third of them were orphans. Sexual HIV-1 transmission occurred in the following 3 cases from Honduras: a 15-year-old girl infected by her sexual partner, and two 12- and 13-year-old boys by suspected sexual abuse. Patients acquired HIV-1 infection during a 21 year period (1989–2009), mainly before 2005 (94.4% in Honduras, 57.8% in El Salvador). In the HIV-infected pediatric population from Honduras, severe symptoms were more frequent and clinical follow-up was longer, maybe due to a larger number of children who acquired the HIV-1 infection during the period 1989–1999 (Table 1).

ART Differences in Infected Children From Honduras and El Salvador

Among the 80 individuals, 63 (78.7%) were receiving ART at the time of DBS collection. All ART experienced children presented at least 1 virologic failure event during their clinical follow-up, mostly explained by a poor adherence to ART or by toxicity to drugs. Nearly two-thirds of them had received non-nucleoside reverse-transcription inhibitors (NNRTI)-based regimens (Table 1). The number of ART regimens, years on ART, line ART, the mean baseline $CD4^+$ cell count before the first ART, and the specific drug-experience in both cohorts at DBS collection are recorded in Table 2. A higher rate of children receiving ART was found in Honduras than in El Salvador. In Honduras, most of the 48 pretreated children were on first or second line ART. In El Salvador, most of the 15 pretreated children were on first or third

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TABLE 1. Baseline Characteristics of the Study Population at DBS Collection Time

	Honduras	El Salvador	Total
No. children (n)	54	26	80
Demographics			
Male gender	64.8%	65.4%	65%
Mean age (mo)	115.3	96.2	109.1
Orphan	27.8%	42.3%	32.5%
Risk groups			
Perinatal transmission	94.4%	100%	96.2%
Others	5.6%	0%	3.8%
HIV-1 infection year			
1989–1999	51.8%	23.1%	42.5%
2000–2004	42.6%	34.6%	40%
2005–2009	5.6%	42.3%	17.5%
HIV-1 diagnostic year			
1989–1999	7.4%	0%	5%
2000–2004	50%	23%	41.2%
2005–2009	42.6%	77%	53.8%
Diagnostic tool			
HIV-1 serology	87%	100%	91.3%
HIV-1 PCR	13%	0%	8.7%
Serologic diagnostic delay (≥24 mo after birth) only in perinatal transmission	72.7%	57.7%	67.1%
Clinical status			
Asymptomatic, N	9.3%	11.5%	10%
Low symptoms, A	20.4%	19.2%	20%
Moderate symptoms, B	22.2%	42.3%	28.7%
Severe symptoms, C	48.1%	27%	41.3%
Clinical follow-up			
≥3 HIV-1 viremia determinations	70.4%	57.5%	66.25%
≥3 $CD4^+$ cell count determinations	50%	57.5%	52.5%
Treatment at DBS collection			
Drug-naive	11%	42.3%	21.3%
Under ARV therapy	48 (89%)	15 (57.7%)	63 (78.7%)
PI-based regimen	29.2%	26.7%	28.6%
NNRTI-based regimen	68.7%	73.3%	69.8%
On treatment suspension	2.1%	0%	1.6%

*Virologic failure is considered when HIV-1 infected patient does not reach undetectable plasma viremia or <1000 HIV-1 RNA copies/mL after 24 weeks under antiretroviral treatment or does not present 2 log reduction in viral load values after 3 months of therapy. Clinical status categories according to Centers of Diseases Control, 1994.[16]

HIV indicates human immunodeficiency virus; PCR, polymerase chain reaction; DBS, dried blood spots; ARV, antiretroviral; PI, protease inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors.

TABLE 2. ART History Among the 63 ARV-experienced Children at DBS Collection Time

	Honduras	El Salvador	Total
No. pretreated children	48	15	63
Under virologic failure	98%	100%	98.4%
Years on ARV			
≤1	2.1%	33.3%	9.5%
2–4	35.4%	46.7%	38.1%
5–9	58.3%	20%	49.2%
10–12	4.2%	0	3.2%
ARV therapy			
On first-line	62.4%	73.3%	65.1%
On second line	23%	6.7%	19%
On third line	4.2%	13.3%	6.3%
On fourth line	4.2%	6.7%	4.7%
On fifth line	6.2%	0	4.7%
Mean baseline $CD4^+$ cell count before first ARV treatment			
>1500	15.1%	20%	16.7%
751–1500	24.2%	13.3%	20.8%
500–750	15.1%	6.7%	12.5%
200–499	30.3%	46.7%	35.4%
<200	15.1%	13.3%	14.6%
Specific drug-experience			
NRTI			
AZT-experienced	100%	86.7%	96.8%
3TC-experienced	100%	100%	100%
DDI-experienced	12.5%	6.6%	11.1%
D4T-experienced	14.6%	26.7%	17.5%
ABC-experienced	25%	6.6%	20.6%
TNF-experienced	18.7%	0	14.3%
NNRTI			
EFV-experienced	100%	40%	85.7%
NVP-experienced	0%	66.7%	15.9%
PI			
LPV/r-experienced	48%	26.6%	46%
NFV-experienced	8.3%	0%	6.3%

$CD4^+$ cell counts values available when DBS was collected.

ART indicates antiretroviral therapy; ARV, antiretroviral; DBS, dried blood spots; NRTI, nucleoside reverse transcriptase inhibitors; AZT, zidovudine; 3TC, lamivudine; DDI, didanosine; D4T, stavudine; ABC, abacavir; TNF, tenofovir; NNRTI, non-nucleoside reverse transcriptase inhibitors; EFV, efavirenz; NVP, nevirapine; PI, protease inhibitors; LPV, lopinavir; NFV, nelfinavir.

line treatment. The mean HIV-1 viremia in pretreated children from Honduras and El Salvador was 5.3 log and 4 log HIV-1 RNA copies/mmL, respectively.

Before the first ART therapy in both countries, 13% to 15% of pretreated children had less than 200 $CD4^+$ T-cell counts and around 15% to 20% had up to 1500 $CD4^+$ T-cells. Pretreated children in Honduras were receiving ART for longer periods of time. A 62.5% of pretreated children in Honduras had had ART experience for 5 to 12 years, while 80% children in El Salvador had less than 5 years (Table 2).

The HIV/AIDS program began to scale-up access to HIV therapy in Central America in 2002. Following international guidelines for adults[17] and children,[18] an NNRTI-based regimen with an AZT + 3TC backbone was the first option in Honduras and El Salvador. All 48 pretreated children in Honduras had zidovudine (AZT), lamivudine (3TC), and efavirenz (EFV) in at least 1 of their ART regimens, and none had experience with nevirapine (NVP). In contrast, although the 15 pretreated children in El Salvador also had 3TC-experience and most (86.7%) of them AZT-experience, 66.7% had NVP, and 40% had EFV experience. Didanosine, abacavir (ABC), and tenofovir (TNF) were the most used nucleoside reverse transcription inhibitors (NRTI) in Honduras, and stavudine (d4T) in El Salvador. Among NNRTI-based regimens, EFV was most frequently used in Honduras and NVP in El Salvador. Lopinavir (LPV/r) was the most frequent drug in protease inhibitors (PI)-based regimens in both countries. Only TNF and nelfinavir (NFV) had been included in ART in children from Honduras (Table 2).

Differences in first- and second-line ART regimens were also observed in both countries, the most frequent being NNRTI-based first-line regimens. AZT + 3TC + EFV regimen was used in 94% of pretreated children in Honduras versus 20% from El Salvador, whereas AZT + 3TC + NVP or d4T + 3TC + NVP were the first regimens in 67% of pretreated children in El Salvador. Second-line regimens were based on d4T in El Salvador and were more diverse in Honduras. LPV/r was present in second-line regimens in 10/18 and 3/4 children from Honduras and El Salvador, respectively.

HIV-1 Diagnostic Delay in the Study Population

Most children were diagnosed by serologic assays, and diagnostic delay was observed in both cohorts (Table 1). In nearly 45% of perinatally infected children, the HIV diagnosis was delayed up to 4 years (range, 48–56 months). In nearly a third of

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children diagnosed by anti-HIV antibodies detection in Honduras, the first serologic test was performed before 18 months of age. Most of these children acquired HIV-1 infection from their mothers before 2004. Genetic HIV diagnosis was performed within 18 months after birth in 7 cases from Honduras and in 9 cases from El Salvador, using the Amplicor COBAS Taqman, a real time RNA-PCR (Roche Diagnostics, Panama) (Table 1).

Low Rate of Transmitted Resistance in HIV-1-infected Naive Children

Among the 17 naive children under study (6 from Honduras, 11 from El Salvador), PR or/and RT sequences were analyzed in 16 (94%) of them (Table 3). A naive 8-year-old girl from El Salvador was perinatally infected with an NNRTI-resistant variant including V108I change. The only naive child from Honduras carrying resistant virus was a 12-year-old boy who probably acquired the infection by sexual abuse and was infected with a PI-resistant variant harboring PR M46I. Thus, considering only naive children who acquired the HIV infection from their mothers, the global transmitted primary drug-resistance (TDR) mutations to any analyzed drug family appeared in 1 child in El Salvador and in none from Honduras (Table 3).

High Level of Drug Resistance Among Children Under ART in Central America

In the study cohort, 48 and 15 patients from Honduras and El Salvador, respectively, were receiving ART at specimen collection time. All had undergone at least 1 virologic failure event. Of the 63 children receiving ART at DBS collection time, two-thirds were infected with resistant mutants to any drug family according to the IAS 2009 list[15] (Table 3). Global primary drug-resistance prevalence (ie, to any antiretroviral drug class) in viruses collected in 2009 from patients infected during their childhood in the 1989–2009 period was similar in both countries, that is, 74.4% in Honduras and 66.7% in El Salvador. Infection with resistant viruses among ART-exposed children was higher than that in naive—74.4% versus 16.6% in Honduras and 66.7% versus 10% in El Salvador (Table 3). Similar prevalence of drug-resistance mutations was observed in both countries. Around 16% of pretreated children with virologic failure were infected by resistant viruses for PI, 44% to 52% for NRTI, and 66.7% for NNRTI. However, single resistance was nearly 2-fold more frequent to NRTI or NNRTI in pretreated children from El Salvador, and dual NNRTI-NNRTI resistance in children from Honduras (Table 3), probably explained by longer ART outcomes. Of note, nearly 1 in 10 pretreated children in each country was infected with viruses carrying resistance mutations to the 3 drug families: PI + NRTI + NNRTI.

TABLE 3. Frequency of HIV Drug Resistance Mutations According to Drug Class Family, Among Naive and Pretreated Pediatric Populations From Honduras and El Salvador

	No. HIV-infected Patients			
	Honduras		El Salvador	
	Naive* (n = 6)	Pretreated (n = 48)	Naive* (n = 11)	Pretreated (n = 15)
Patients with available PR (n)	6	43	10	12
Patients with available RT (n)	5	42	10	9
Prevalence of DRM (%)				
Global (to any class)	16.6%†	74.4%	10%	66.7%
To PI (major)	16.6%†	16.3%	0	16.7%
To NRTI	0	52.4%	0	44.4%
To NNRTI	0	66.7%	10%	66.7%
Single resistance				
PI	16.6%†	7%	0	8.3%
NRTI	0	4.8%	0	11%
NNRTI	0	14.3%	10%	22%
Dual resistance				
NRTI + NNRTI	0	35.7%	0	22%
PI + NRTI	0	0	0	0
PI + NNRTI	0	0	0	0
Triple resistance				
PI + NRTI + NNRTI	0	9.5%	0	11%

*Of the 6 naive children from Honduras, 3 were perinatally infected. All naive children from El Salvador acquired HIV-1 infection by perinatal route.

†The only child carrying resistant HIV-1 variant was infected by sexual route.

HIV indicates human immunodeficiency virus; n, number; PR, protease; RT, reverse transcriptase; DRM, drug-resistance mutations according to IAS 2009; PI, protease inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors.

Different Patterns of Drug Resistance in Honduras and El Salvador

The nature and frequency of drug-resistance mutations for PI, NRTI, and NNRTI observed in 63 pretreated children in Honduras and El Salvador are shown in Figure 1. The major resistance mutations for PI found in children receiving ART was M46I in Honduras and M46I and V82A in El Salvador. Multiple minor PI-resistance mutations were observed in a high frequency in the study population. The most frequent resistance mutations in RT for NRTI or NNRTI resistance found were: K103N and D67N, K70R, V106I, K219Q, and P225H in Honduras and K103N and M184V in El Salvador. Of note, K103N (associated with virologic failure to NVP and EFV) and M184V (associated with virologic failure to NRTI) were the main NNRTI and NRTI-resistance mutations detected in children from both countries (Fig. 1). Despite all pretreated children in Honduras and 40% from El Salvador having EFV experience, the P225H EFV-resistance mutation was found in only 12.8% of children from Honduras, and was absent in children from El Salvador.

Phylogenetic Characterization of HIV-1 Variants

Phylogenetic analysis of *pol* sequences revealed, as expected, that HIV-1 subtype B variant was present in all patients infected during 1989–2009, in Honduras and El Salvador, in all available PR (n = 71) or RT (n = 66) sequences under study. No recombination events at *pol* were found after using the following specific programs for recombinant variants detection: SCUEAL, RIP, RDP, jpHMM, and SimPlot.

Accession Numbers

The GenBank accession numbers of PR and RT sequences from Honduras were HQ010598 to HQ010649, and from El Salvador, HQ010650 to HQ010672.

DISCUSSION

As the emergence of viral-resistant strains is the major obstacle for effective treatment, the aim of this study was to describe the prevalence of antiretroviral drug resistance in naive and antiretroviral drug experienced children with virologic failure in 2 Central American countries. In the study cohort, nearly 45% and 60% of children from Honduras and El Salvador, respectively, had had less than 500 CD4 cells count when they started ART. This is in contrast to European PENTA Guidelines,[4] which recommend that ART should be started in all infants regardless of clinical or immunologic stage, as soon as the diagnosis of infection is confirmed.

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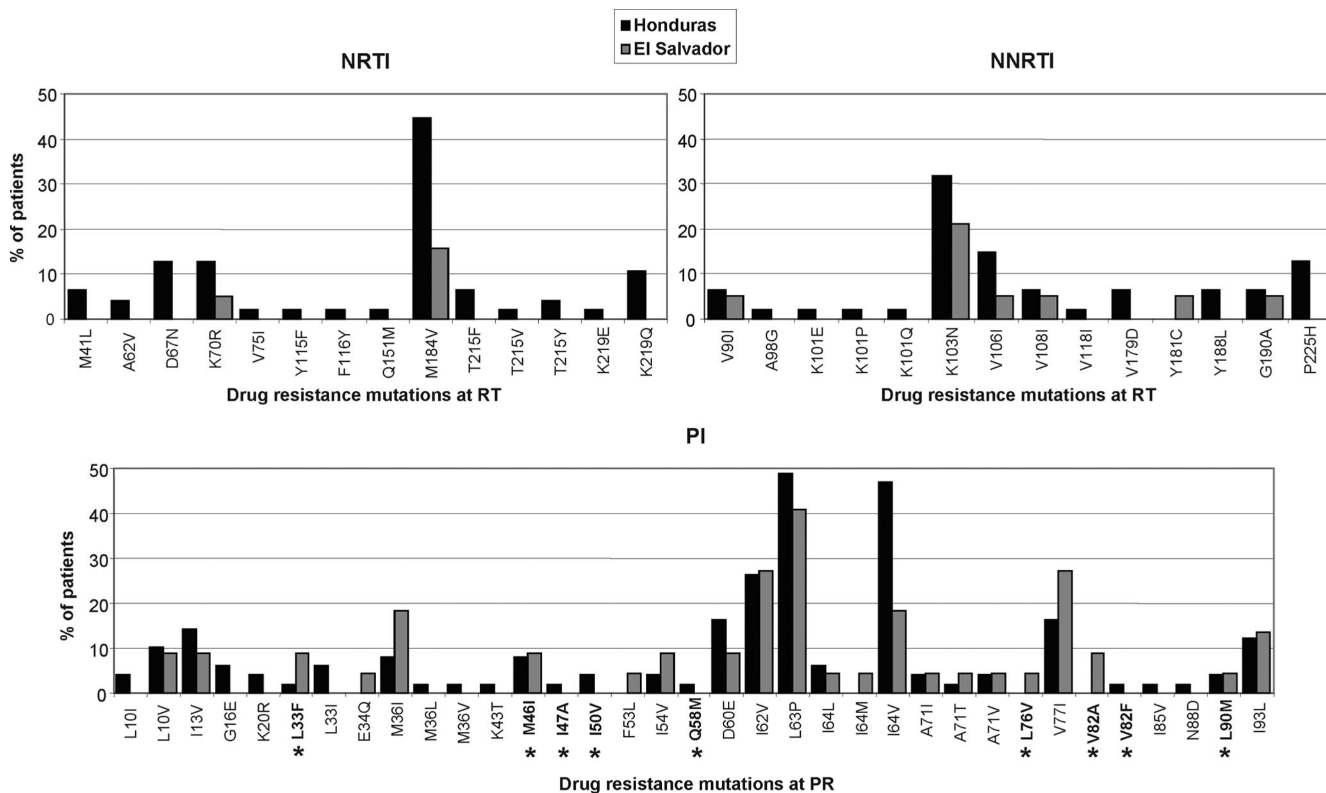

FIGURE 1. Substitutions in PR and RT associated with drug resistance in HIV-1 infected children receiving ART, from Honduras and El Salvador.

TDR rates from 5% to 15% have been reported in naive adult subjects in European countries and the United States,[19–23] but few TDR studies have been conducted in Latin America.[24–30] These studies were mainly in adults, and the TDR rates ranged from 0% in Panama[31] to 16% in certain areas of Mexico.[24] In Honduras, the reported TDR rate was 9.2% during 2002–2003[32] and 7% during 2004–2007.[33] Although ART during pregnancy has reduced the rate of HIV perinatal transmission, it increases the transmission of resistant strains to the babies.[1] Our study reveals just 1 case of TDR in each country. We found lower TDR rates in Central America than in other pediatric cohorts from Spain (26.5%, 2003–2009)[34] or France (20%, 1997–2004),[35] but higher than in the United Kingdom (6.8%, 1998–2004).[36]

Most pretreated children in our study population had virologic failure events and presented a high frequency of antiretroviral resistance, in agreement with other multicenter pediatric cohorts,[34,36,38] limiting the therapy success.[39] The lower global resistance rate found in children from El Salvador could be explained by a shorter HIV-1 infection time and fewer ART switches in that country. K103N/M184V changes were present in 32% and 21% of pretreated children from Honduras and in 45% and 16% children from El Salvador, respectively. One in 10 children receiving ART was infected with viruses resistant to 3 drug classes, reinforcing the importance of the use of new therapies based on different retroviral targets. In our study, the low viral load in some specimens or RNA degradation during the collection, transport, or storage for DBS could explain the no PR or RT amplification in some specimens. Moreover, we cannot exclude the possibility that some subtype B viruses had mutations in the primer binding sites, explaining the negative amplifications.

This work confirms the homogenicity of the HIV epidemics in both countries, dominated by subtype B. It has provided the first results on HIV-1 subtyping in El Salvador, and reinforces previous reports in Honduras.[32,33,40,41] Due to the high frequency of HIV-1 genetic recombination,[42] we cannot rule out that these viruses carrying HIV-1 subtype B sequences at *pol* could be intersubtype recombinant carrying other subtypes in nonanalyzed viral genetic regions.

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DISCUSIÓN

COMPARATIVA CLÍNICA DE LOS PACIENTES DE LAS COHORTES PEDIÁTRICAS ESTUDIADAS

Las características clínicas, virológicas e inmunológicas de los pacientes pediátricos infectados por VIH-1 en cuatro cohortes de cuatro países distintos se comparan en la **Tabla 3.1**. Las **Figuras 3.1** y **3.2** muestran la localización y distribución de los pacientes incluidos en las cohortes de El Salvador, Honduras y Nueva York. Los datos más llamativos muestran la alta edad media de las cohortes de Madrid y Nueva York (14,9 y 17,7 años, respectivamente). El aumento de la esperanza de vida y la reducción de la morbi-mortalidad asociada a la infección por VIH-1 en pacientes verticalmente infectados en países desarrollados se debe principalmente al uso expandido de TAR[1-3]. La mayoría de los pacientes de las cohortes de Nueva York y de Madrid (84,9% y 97,9% respectivamente) se infectaron en el periodo 1981-2000. La situación en Honduras y El Salvador es completamente distinta, ya que el 51,9% de las infecciones en Honduras y el 73,1% en El Salvador ocurrieron entre 2001 y 2010, y por ello la edad media de las dos cohortes no supera los 10 años.

Figura 3.1. Situación geográfica y distribución de pacientes pediátricos de las cohortes de Honduras y El Salvador.

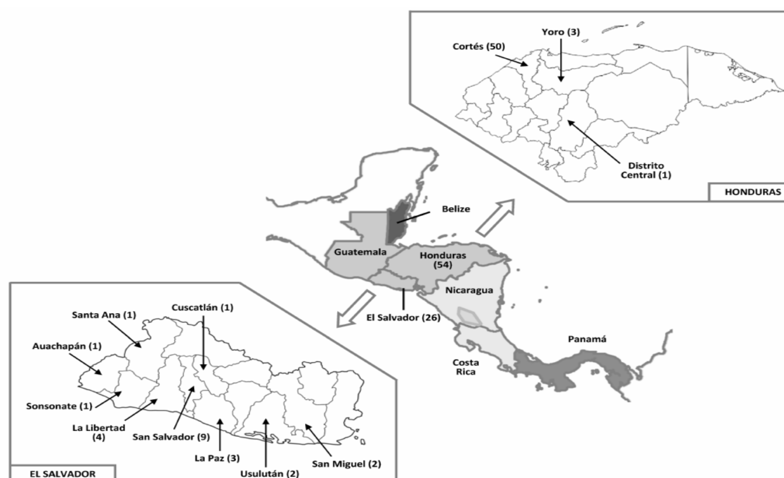


Figura 3.2. Situación geográfica y localización del Jacobi Medical Center en el distrito del Bronx en el área metropolitana de Nueva York.

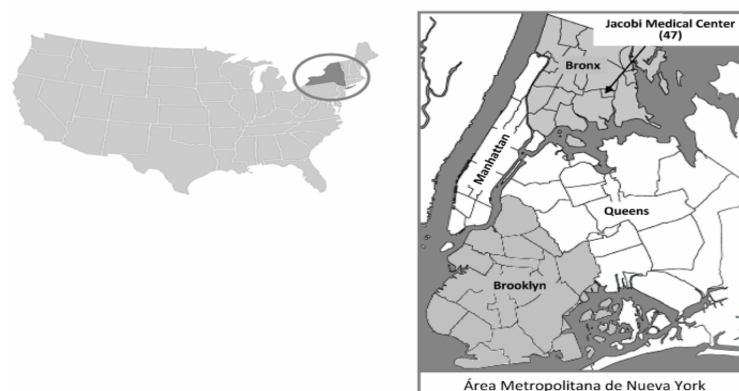


Tabla 3.1. Situación clínica, virológica e inmunológica de los pacientes pediátricos de las cuatro cohortes de pacientes pediátricos infectados por VIH-1 analizadas en esta Tesis.

	España (Madrid)	**Honduras (San Pedro Sula)**	**El Salvador (San Salvador)**	**Estados Unidos (Nueva York)**
Número de pacientes	232	54	26	47
Año(s) de toma de muestra	1993-2011	2009	2009	2011
Demográficas				
Sexo masculino	42%	64,8%	65,4%	46,8%
Edad media [DT]	14,9 [4,9]	9,6 [4,4]	8,0 [7,8]	17,7 [4,4]
Adoptado	21,6%	-	-	-
Vía de transmisión				
Perinatal	96,6%	94,4%	100%	100%
Transfusión	2,2%	-	-	-
Sexual	0,4%	-	-	-
Otros	0,8%	5,6%	-	-
Origen				
España	82,3%	-	-	-
África	10%	-	-	-
Centroamérica	5,6%	100%	100%	-
Norteamérica	-	-	-	100%
Otros países europeos	1,7%	-	-	-
Asia	0,4%	-	-	-
Periodo de infección				
1981-1990	23,3%	3,7%	-	25,6%
1991-2000	61,6%	44,4%	26.9%	72,3%
2001-2010	15,1%	51,9%	73,1%	2,1%
Variante VIH infectante				
Subtipo B	89,1%	100%	100%	97,3%
Variantes no-B	10,9%	-	-	2,7%
Estadio inmunológico				
1	5,6%	14,8%	19,2%	-
2	31,5%	22,2%	34,6%	-
3	61,6%	51,9%	42,3%	-
Desconocido	1,3%	11,1%	3,9%	-
Estadio clínico				
Asintomático, N	0,8%	9,3%	11,5%	-
Síntomas leves, A	32,3%	20,4%	19,2%	-
Síntomas moderados, B	25,9%	22,2%	42,3%	-
Síntomas severos, C	38,8%	48,1%	27%	-
Desconocido	2,2%	-	-	-
Tratamiento a toma de muestra				
Naïve	19%	11%	42,3%	-
En TAR	81%	89%	57,7%	100%
Recuento Linfocitos T CD4 (céls/mm^3)				
≤200	1,7%	7,4%	7,7%	17%
201-500	12,9%	14,8%	23,1%	40,4%
501-750	28%	18,5%	26,9%	19,2%
751-1.500	41%	14,8%	23,1%	19,2%
>1.500	5,6%	5,6%	-	2,1%
Desconocido	10,8%	38,9%	19,2%	2,1%
Carga viral (copias VIH-1/ml)				
≤50	59,1%	-	-	40,4%
51-1.000	14,1%	-	3,8%	19,2%
1.000-10.000	9,5%	40,7%	46,2%	21,3%
>10.000	15,1%	40,7%	50%	17%
Desconocida	2,2%	18,6%	-	2,1%

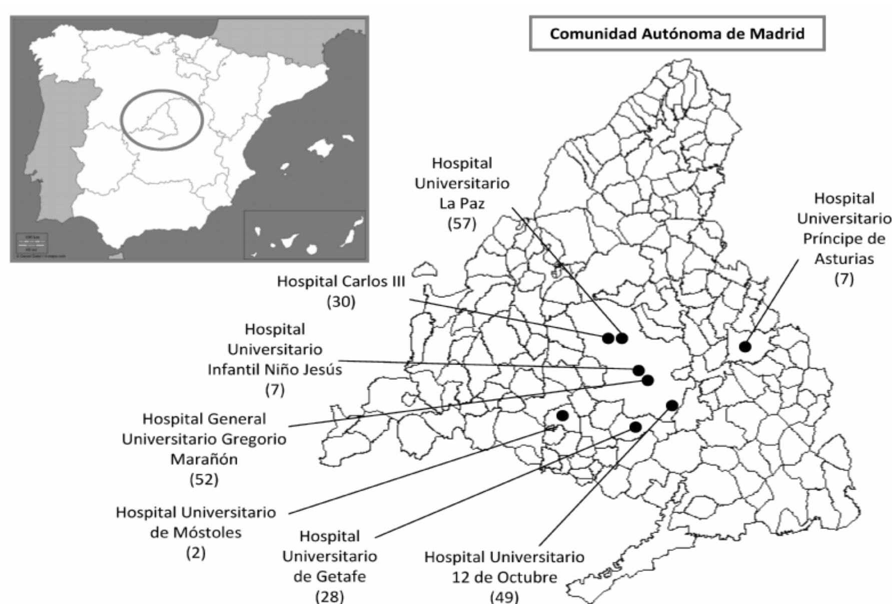
Debido al desarrollo de medidas para prevenir la transmisión del VIH-1 a sus hijos en mujeres embarazadas, la tasa de nuevos diagnósticos de VIH-1 en pacientes pediátricos en países desarrollados ha sufrido una drástica disminución. Esto ha provocado un envejecimiento de las cohortes pediátricas del VIH-1 en países desarrollados, lo que ha permitido la posibilidad de presenciar como pacientes verticalmente infectados han llegado a alcanzar la edad adulta. Estos casos han permitido analizar estos pacientes únicos e irrepetibles, proporcionando datos útiles para el correcto manejo clínico de pacientes pediátricos que alcancen la mayoría de edad. Todo este conocimiento adquirido podrá ser aplicado a otras cohortes pediátricas, especialmente a aquellas de niños infectados por VIH-1 en países de recursos limitados.

A pesar de que las guías para el manejo de la infección en pediatría en América Central recomiendan el inicio de TAR en todos los lactantes, es llamativo que en la cohorte de El Salvador un 42,3% de los niños infectados no habían recibido TAR a fecha de toma de muestra. Además su situación clínico virológica no era la más adecuada, ya que un 42,3% de los pacientes presentaban síntomas clínicos moderados y estadio inmunológico 3 (**Tabla 3.1**). En Honduras, pese a que el acceso a TAR era mayor (89%), la situación clínica era más grave, ya que el 48,1% de los niños mostraban síntomas severos de progresión de la enfermedad. La situación virológica tampoco era la más deseable en ninguno de los dos países, puesto que el 81,7% de los pacientes de Honduras y el 96,2% de El Salvador presentaban valores de CV superiores a las 1.000 copias de ARN-VIH-1/ml. Además de mostrar claros síntomas de progresión de la enfermedad y de falta de control de la viremia, la situación se complicaba al observar como alrededor del 7% de los pacientes de ambas cohortes presentaban unos niveles de linfocitos T CD4+ por debajo de las 200 céls/mm^3, dando una idea de lo alarmante de su situación. En la cohorte de Madrid únicamente un 1,7% de los pacientes se encontraban por debajo del umbral de 200 céls/mm^3, mientras que en la cohorte de Nueva York el número de pacientes con recuentos de linfocitos T CD4 era mayor (17%) que en Honduras y El Salvador. Únicamente el 24,6% y el 38,3% de la población pediátrica infectada de Madrid y Nueva York presentaban una CV por encima de las 1.000 copias/ml. Por tanto, a pesar de estar infectados durante periodos de tiempo más largos, los pacientes de Madrid y de Nueva York presentaban un mejor control de la infección, probablemente debido a un acceso temprano al TAR. En la cohorte de Madrid, cerca del doble de pacientes (46,6%) que en el resto de las cohortes superaba los 750 células/mm^3. Además, fue la única cohorte con el menor número de pacientes (1,7%) con niveles de CD4 inferiores a 200 céls/mm^3 y con el mayor número de sujetos (59,1%) con CV por debajo de las 50 copias/ml (**Tabla 3.1**).

INFECCIONES POR VARIANTES NO-B DEL VIH-1 EN PACIENTES PEDIÁTRICOS DE LA COMUNIDAD DE MADRID

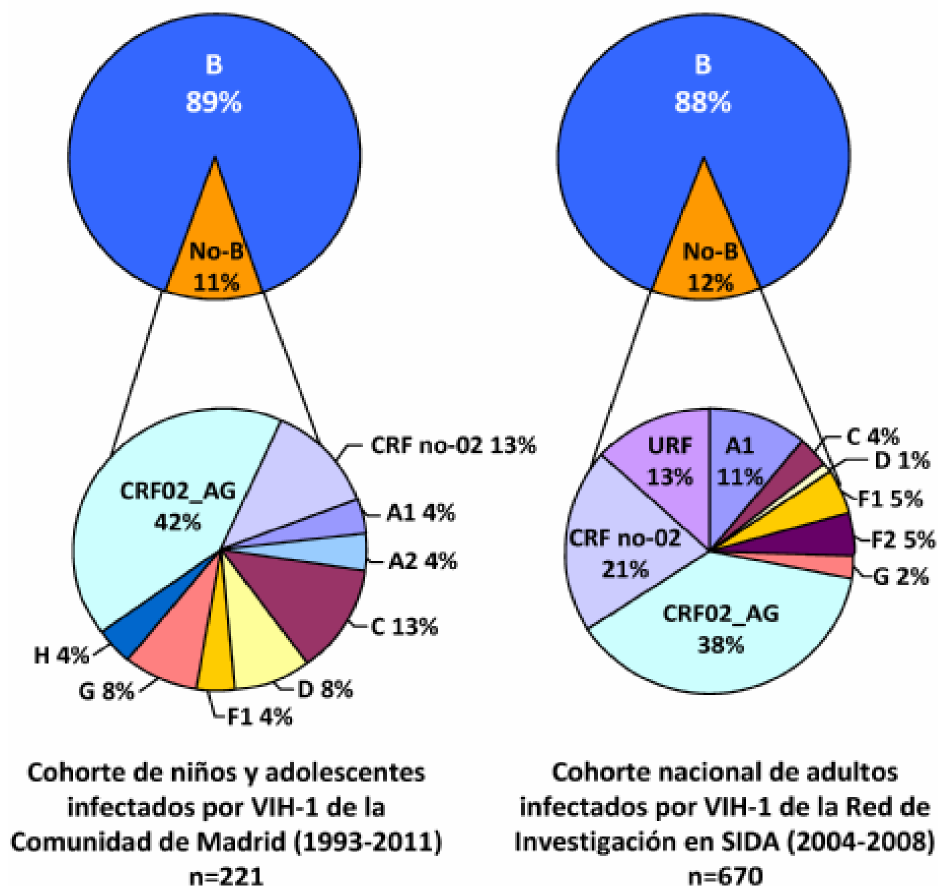
A finales de diciembre de 2011, la cohorte de Madrid de niños infectados por VIH-1 contaba con 534 pacientes, de los cuales 175 estaban en seguimiento clínico en unidades pediátricas de 8 hospitales de la Comunidad Autónoma de Madrid (**Figura 3.3**). Afortunadamente, 112 pacientes del total de niños que forman parte de la cohorte habían alcanzado la adolescencia y, por lo tanto, habían sido transferidos a unidades de adultos donde actualmente se continúa con su seguimiento clínico. Lamentablemente, 185 de los niños habían fallecido y 62 se encontraban en situación de pérdida de seguimiento.

Figura 3.3. Distribución de hospitales públicos en la Comunidad de Madrid donde los pacientes pediátricos están bajo seguimiento clínico.



La prevalencia de infecciones causadas variantes no-B del VIH-1 en la cohorte de niños y adolescentes infectados por VIH-1 en la Comunidad de Madrid durante el periodo 1993-2011 fue del 10,9%. Los análisis filogenéticos (AF) realizados empleando 221 secuencias del gen *pol* del VIH-1 disponibles a finales de 2011 indicaron que 9 de cada 10 infecciones en la cohorte estaban causadas por subtipo B del VIH-1, mientras que las restantes se debían a subtipos no-B y formas recombinantes del VIH-1. Entre las 24 variantes no-B detectadas mediante AF se encontraron representantes de subtipos no-B "puros": A1, A2, C, D, F1, G y H y recombinantes: CRF02_AG, CRF08_BC, CRF12_BF y CRF13_cpx. En la **Figura 3.4** se comparan la distribución y naturaleza de las 24 variantes no-B halladas en esta Tesis con las 82 variantes no-B detectadas en 670 pacientes adultos infectados por VIH-1 entre 2004 y 2008 de la cohorte nacional CoRIS identificadas también por nuestro grupo empleando AF[4].

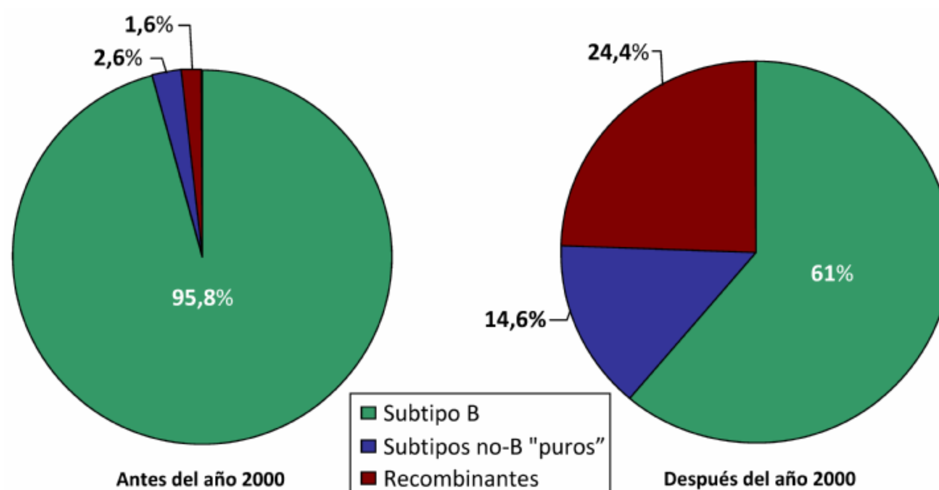
Figura 3.4. Distribución y naturaleza de las variantes VIH-1 encontradas en la cohorte nacional de adultos (CoRIS) y en la cohorte de niños y adolescentes infectados por VIH-1 de la Comunidad de Madrid.



La presencia de variantes no-B entre los pacientes infectados por VIH-1 de la Comunidad de Madrid, se podría explicar por el fenómeno de la inmigración desde países endémicos para esas variantes. La mayoría (87,5%) de pacientes infectados verticalmente durante la década de los años 90 por variantes no-B del VIH-1 eran de procedencia extranjera o al menos uno de sus progenitores lo era. Sin embargo, se identificaron este tipo de variantes en tres pacientes de origen español con ambos progenitores nativos.

El aumento en la tasa de formas recombinantes que infectan a pacientes pediátricos nacidos a partir del año 2000 refleja el aumento en la complejidad genética de las variantes del VIH-1, proceso observado por nuestro grupo en pacientes adultos recientemente diagnosticados de infección por VIH-1 tanto a nivel local[5] como a nivel nacional[4]. En la **Figura 3.5** se muestra la prevalencia de variantes no-B del VIH-1 en pacientes pediátricos nacidos antes y después del año 2000.

Figura 3.5. Distribución de variantes del VIH-1 entre los pacientes pediátricos diagnosticados antes (n=191) y después (n=41) del año 2000 en la Comunidad de Madrid.



Los datos presentados vienen a demostrar las tendencias ya publicadas por nuestro grupo al describir el aumento de cepas no-B entre los nuevos diagnósticos de VIH-1 entre población nativa española desde 2000 hasta 2007[5]. Además, el aumento de las infecciones por variantes no-B entre los nuevos diagnósticos de VIH-1 entre población nativa ya estaba descrito en países europeos que reciben inmigrantes de países con alta incidencia de VIH-1 y donde las cepas no-B son mayoritarias, entre ellos Francia, Reino Unido y EE.UU.[6-10]. Otro punto interesante que observamos fue un aumento de la complejidad de las variantes no-B que infectaban a niños de la cohorte de Madrid, ya que el 54,2% de las 24 variantes no-B del VIH-1 encontradas correspondían a formas recombinantes circulantes (CRF). Incluso algunas de las formas recombinantes detectados en pacientes pediátricos (CRF08_BC y CRF13_cpx) aún no habían sido descritas en población adulta infectada de la cohorte CoRIS, a pesar de encontrarse 12 CRF diferentes[4]. Ello da idea de la heterogeneidad de variantes que se encuentra instalada dentro de nuestras fronteras. Esta parece que será la tendencia que siga la pandemia en los próximos años, pues el número de variantes recombinantes identificadas sigue en aumento en España y en el resto de países desarrollados que reciben inmigración de zonas endémicas para subtipos no-B y recombinantes del VIH-1.

La infección por variantes no-B del VIH-1 tiene implicaciones clínicas, como se explicó en las páginas 21 y 22 de la Introducción. Así, la diversidad genética del VIH-1 puede afectar a la evolución natural e impactar en la patogénesis de la enfermedad[11]. También se ha demostrado el impacto de la diversidad viral en niveles diferenciales de apoptosis de linfocitos T CD4

causando tasas más rápidas de progresión de la enfermedad y afectando potencialmente la eficacia del TAR[12-15].

Los médicos especialistas en enfermedades infecciosas y enfermedades tropicales deberían conocer cómo la infección por algunas de estas variantes no-B puede incidir en la historia natural de la infección como, por ejemplo, el subtipo D[11]. Se debería monitorizar su diseminación en los países y su tendencia en el tiempo. Hoy en día, la única técnica validada estadísticamente para caracterizar las variantes virales del VIH-1 son los AF. Ya se ha demostrado que las páginas electrónicas de subtipaje que permiten identificar variantes virales en poco tiempo carecen de la robustez estadística que presenta el AF y cometen errores en la identificación de ciertas variantes no-B que infectan a una población de estudio si se comparan con los obtenidos por AF[16]. En un estudio recientemente publicado por nuestro grupo[16], al comparar la distribución de cada subtipo y recombinante proporcionada por cada una de las ocho páginas de subtipaje rápido disponibles con la generada mediante AF empleando las mismas secuencias *pol* de 670 pacientes de la cohorte de adultos infectados CoRIS, se observó que ninguna de las páginas electrónicas disponibles proporcionó una distribución correcta de las variantes del VIH-1. Según la página de subtipaje rápido empleada, se sobrestimaban determinadas variantes o se subestimaban otras. Por ello, a pesar de ser complicados, largos y requerir de personal cualificado, los AF son la única técnica que permite identificar una variante viral de forma fiable siempre que se realice empleando una secuencia genética de tamaño adecuado y unas secuencias de referencia actualizadas. Por todo esto, los AF están considerados como la técnica de referencia en los estudio de epidemiología molecular y son los que se han empleado en esta Tesis Doctoral.

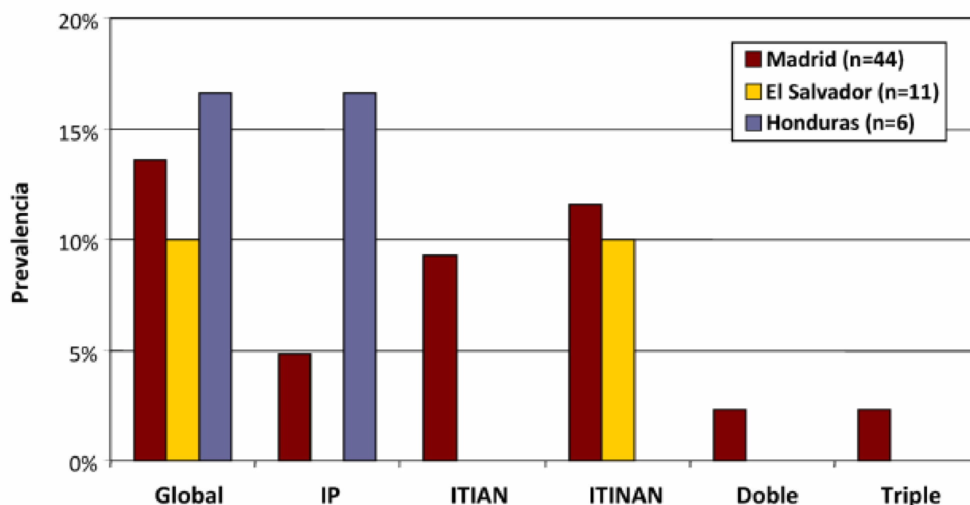
Los AF de secuencias genéticas del gen *pol* del VIH-1 a partir de muestras clínicas mostraron una alta (11%) prevalencia de variantes no-B en la cohorte de Madrid, que fue muy baja (2,7%) en la cohorte de Nueva York y no se encontraron en las cohortes de El Salvador y Honduras. En un análisis más detallado de la cohorte de niños y adolescentes de Madrid, se observó que en aquellos adolescentes transferidos a unidades de adultos la prevalencia de variantes no-B del VIH-1 era baja (1,9%) mientras que para aquellos pacientes pediátricos no transferidos, más jóvenes e infectados más recientemente, la tasa de infección por variantes no-B fue mayor (11,5%). Ello viene a reforzar de nuevo la teoría del aumento de infecciones por variantes no-B entre los nuevos diagnósticos de VIH-1 en España, como ya se publicó previamente para población adulta infectada[4,5].

COMPARATIVA DE PREVALENCIA DE MUTACIONES DE RESISTENCIA TRANSMITIDAS A FÁRMACOS ANTIRRETROVIRALES EN LAS CUATRO COHORTES ANALIZADAS

En zonas industrializadas como Europa, con una experiencia acumulada de 15 años con el TAR, entre el 4,8 y 20% de los nuevos diagnósticos en adultos están infectados por variantes del VIH-1 con algún grado de resistencia a los fármacos ARV[17-22]. Los estudios que analizan este fenómeno en adultos son abundantes en países desarrollados. Sin embargo para población pediátrica infectada por VIH-1 los estudios aún son escasos. Los estudios disponibles que analizan la tasa de mutaciones de resistencia transmitidas (MRT) en pacientes pediátricos señalan una tasa variable entre el 6,8% detectada entre 1998 y 2004 en el Reino Unido[23] hasta el 20% detectado en recién nacidos en Francia entre 1997 y 2004[24].

En la cohorte de niños y adolescentes infectados por VIH-1 de la Comunidad de Madrid se analizaron 232 pacientes infectados por VIH-1 entre 1993 y finales de 2010. De ellos, 44 pacientes no habían recibido TAR a fecha de toma de muestra. La prevalencia global de mutaciones de resistencia transmitidas en estos 44 pacientes fue del 13,6% (**Figura 3.6**). Por familias, 4,8% a IP, 9,3% a ITIAN y 11,6% a ITINAN. Las MRT más prevalentes encontradas en los pacientes *naïve* de la Comunidad de Madrid por familias fueron frente a IP: D30N, N88D y M46L; frente a ITIAN: M41L, K70R y M184V, L210W y T215S/Y; frente a ITINAN: K103N, E138G, Y181C y P225H. Los valores de prevalencia global de MRT obtenidos para la cohorte de Madrid hasta diciembre de 2010 (13,6%) fueron ligeramente superiores a los hallados en 732 pacientes adultos infectados en Madrid entre 1996 y 2010 (9,6%)[20]. En la cohorte Jacobi todos los pacientes habían recibido TAR, por lo que no se pudo determinar la prevalencia de MRT.

Figura 3.6. Prevalencia de mutaciones de resistencia transmitidas en los pacientes *naïve* de las cuatro cohortes de estudio.



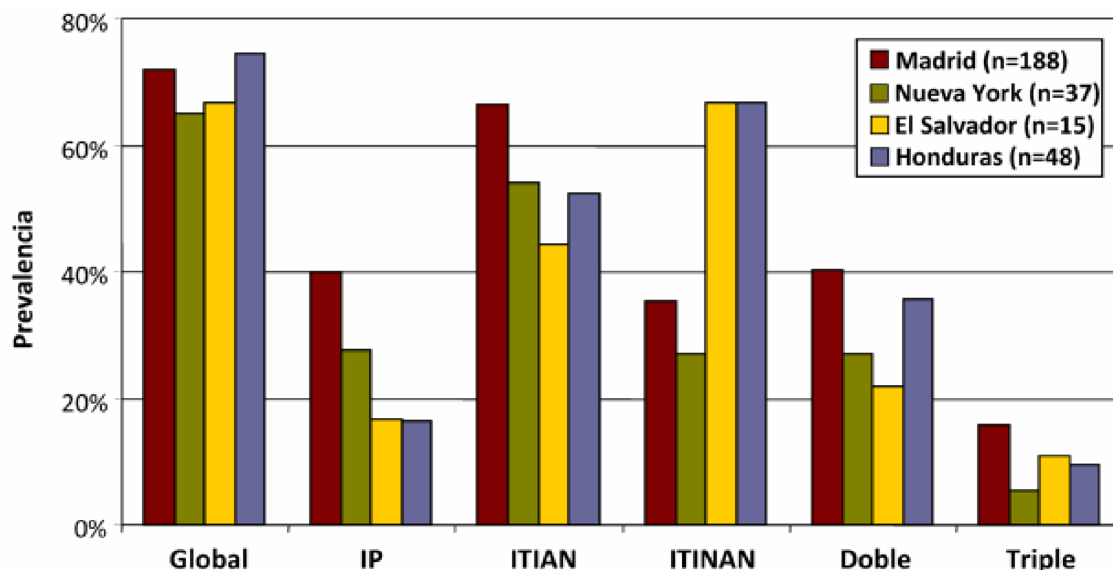
Son pocos los estudios disponibles que analizan la tasa de MRT en pacientes pediátricos de Centroamérica. Los datos obtenidos de estudios previos en adultos muestran un rango de MRT que va desde su ausencia en Panamá[25] hasta el 16% en determinadas regiones de México[26]. Los estudios previos en población adulta recién diagnosticada en Honduras revelaron una tasa de MRT del 9,2% entre 2002 y 2003[27] y del 7% entre 2004 y 2007[28]. En nuestro estudio, 17 de los 80 pacientes estudiados eran *naïve* al TAR en el momento de recogida de muestra (6 de Honduras y 11 de El Salvador). De ellos, sólo 2 estaban infectados por virus con algún tipo de resistencia transmitida a fármacos ARV. Estos dos pacientes, uno de El salvador y otra de Honduras, estaban infectados por variantes virales que conferían resistencia a IP (mutación M46I en el paciente hondureño) e ITINAN (mutación V108I en la paciente salvadoreña). La prevalencia global de MRT para El Salvador fue del 10%, mientras que en Honduras fue del 16,6% (**Figura 3.6**). Aún así, la monitorización de la prevalencia de MRT en poblaciones pediátricas y adultas debe ser prioritaria, ya que pueden comprometer la eficacia presente y futura del TAR.

COMPARATIVA DE PREVALENCIA DE MUTACIONES DE RESISTENCIA ADQUIRIDAS A FÁRMACOS ANTIRRETROVIRALES EN LAS CUATRO COHORTES ANALIZADAS

La selección de mutaciones de resistencia debida a una incompleta supresión viral constituye el mayor obstáculo para un TAR eficaz[29]. La prevalencia global de mutaciones de resistencia adquiridas (MRA) en pacientes diagnosticados entre 1989 y 2009 en pacientes pretratados de Honduras (n=48) y El Salvador (n=15) fue similar en ambos países: 74,4% en Honduras y 66,7% en El Salvador (**Figura 3.7**). En la cohorte de Madrid la prevalencia global de MRA en 188 pacientes pretratados infectados entre 1981 y 2010 entre fue del 71,8% y la de los 37 pacientes pretratados con secuencia *pol* del VIH-1 disponible de la cohorte de niños y adolescentes infectados por VIH-1 del centro médico Jacobi (Nueva York, EE.UU.) fue del 64,9% (**Figura 3.7**). Estos datos de prevalencia global de MRA en pacientes pediátricos son similares a los determinados en otras cohortes pediátricas de niños infectados por VIH-1 en Gran Bretaña[23] y Francia[30].

La prevalencia de MRA por familias de fármacos fue de 16,7%, 16,3%, 39,9% 27,6% frente a IP, 44,4%, 52,4%, 66,5% y 54,1% frente a ITIAN y de 66,7%, 66,7%, 35,3% 27% frente a ITINAN, respectivamente en las cohortes de El Salvador, Honduras, Madrid y Nueva York, respectivamente (**Figura 3.7**).

Figura 3.7. Prevalencia de mutaciones de resistencia adquiridas en pacientes pretratados de las cuatro cohortes de estudio.

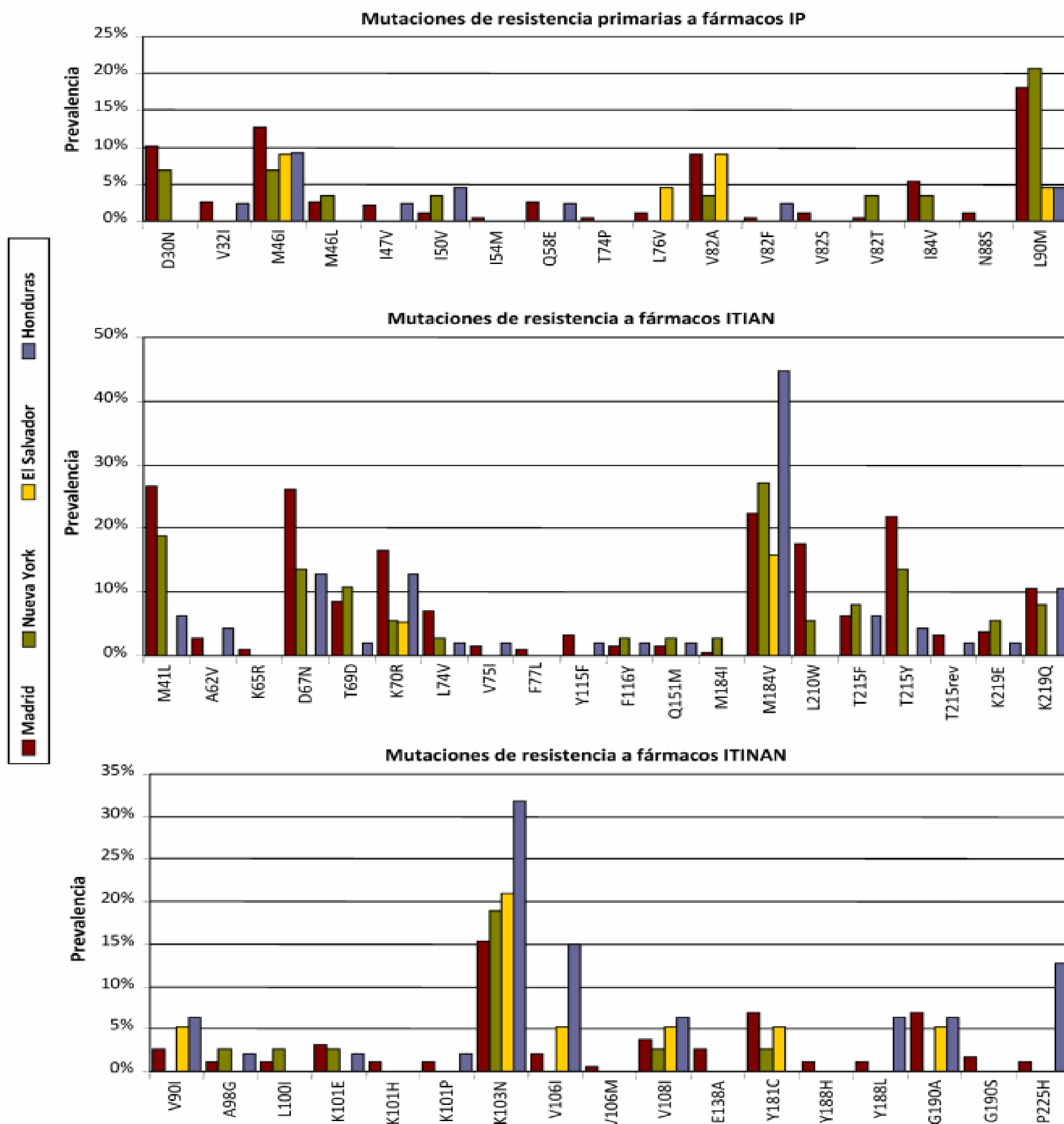


La prevalencia de pacientes infectados por virus que seleccionaron mutaciones de resistencia para las tres familias de fármacos a la fecha de toma de muestra fue de 9,5% para Honduras y 11% para El Salvador, siendo superior (15,9%) en la cohorte de niños y adolescentes infectados por VIH-1 de la Comunidad de Madrid. Esta diferencia puede deberse a los largos periodos de tratamiento y a la falta de adherencia al TAR. La triple resistencia en la cohorte Jacobi fue inferior (5,4%), sólo encontrada en 2 de los 37 pacientes estudiados. Las tasas de triple resistencia determinadas en los estudios presentados son variables pero, en cualquier caso, preocupantes pues limitan las opciones terapéuticas futuras de estos pacientes. Sin embargo, los datos presentados son similares a los publicados en otras cohortes de niños y adolescentes, donde los valores de triple resistencia oscilan entre el 12 y el 32%[23,30,31].

Además de analizar globalmente la prevalencia de resistencia a familias de fármacos en pacientes pretratados, se analizó y representó en las cuatro cohortes estudiadas la prevalencia de cada una de las mutaciones de resistencia incluidas en el listado de la Sociedad Internacional de SIDA de EE.UU. para cada familia de fármacos ARV (**Figura 3.8**). Las mutaciones de resistencia más prevalentes (por encima del 5%) a IP en las cuatro cohortes fueron M46I y L90M. En las cohortes de Madrid y Nueva York estaban, además, representadas las mutaciones D30N y V82A, esta última también presente entre pacientes de El Salvador. Las MRA más prevalentes frente a ITIAN presentes en las cuatro cohortes fueron K70R y M184V, alcanzando esta última valores por encima del 20% en tres de las cohortes. También las MRA M41L, D67N y T215Y fueron altamente prevalentes en las cohortes de Madrid y Nueva York. La MRA frente a

ITINAN más prevalente fue la K103N, presente entre el 15-30% de los pacientes de las cuatro cohortes.

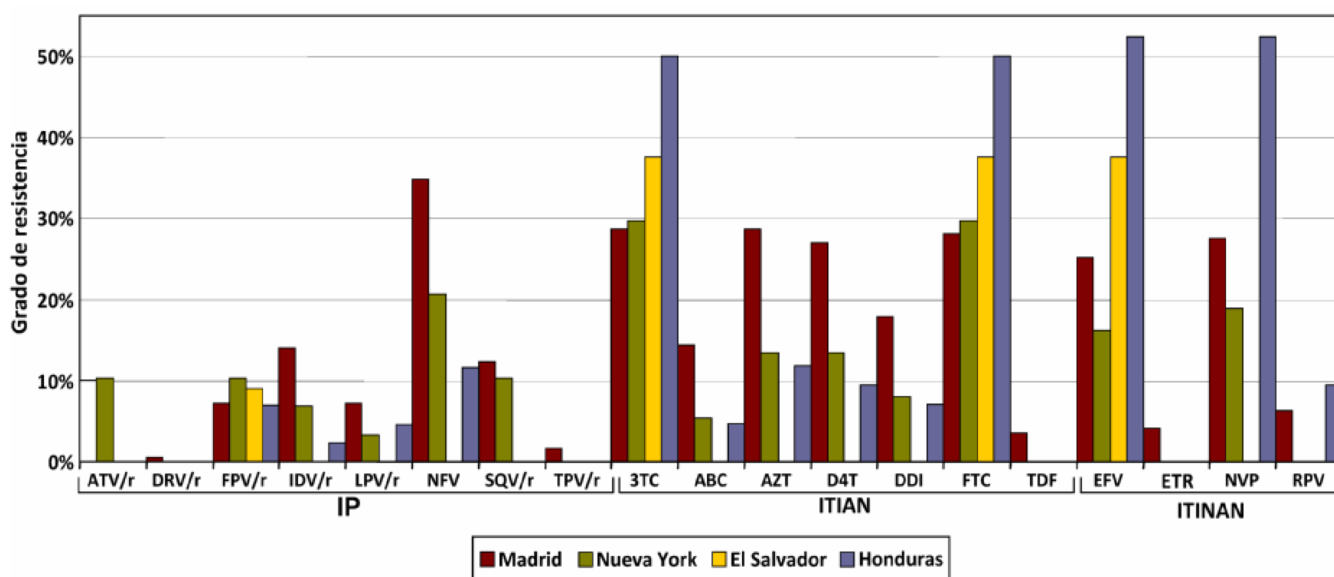
Figura 3.8. Prevalencia de mutaciones en el gen *pol* del VIH-1 asociadas a resistencia a los fármacos ARV en pacientes pretratados de las cuatro cohortes de estudio.



COMPARATIVA DE LA SUSCEPTIBILIDAD GENOTÍPICA A FÁRMACOS ANTIRRETROVIRALES EN LAS CUATRO COHORTES ANALIZADAS

El análisis de la interpretación genotípica de susceptibilidad a fármacos antirretrovirales en las cuatro cohortes analizadas reveló un alto grado de resistencia a fármacos de las familias ITIAN e ITINAN (**Figura 3.9**). Entre la familia de los IP, el NFV reveló un alto grado de resistencia en las cohortes de Madrid (25%) y de Nueva York (20%), bajo en Honduras (20%) y ausente en El Salvador. El fármaco IP cuya eficacia estaría más comprometida en las cuatro cohortes sería el FPV/r. Para la familia ITIAN, los fármacos 3TC y FTC presentaron un grado de resistencia predicho entre el 28 y el 50% en función de la cohorte analizada. Para los ITINAN, el fármaco EFV estaría comprometido en las cuatro cohortes en unos valores del 5 al 50% y el fármaco NVP también estaría comprometido en tres de ellas, alcanzando niveles de resistencia superiores al 50% en la cohorte de Honduras.

Figura 3.9. Predicción comparada del grado de resistencia a fármacos antirretrovirales en pacientes pretratados de las cuatro cohortes estudiadas.



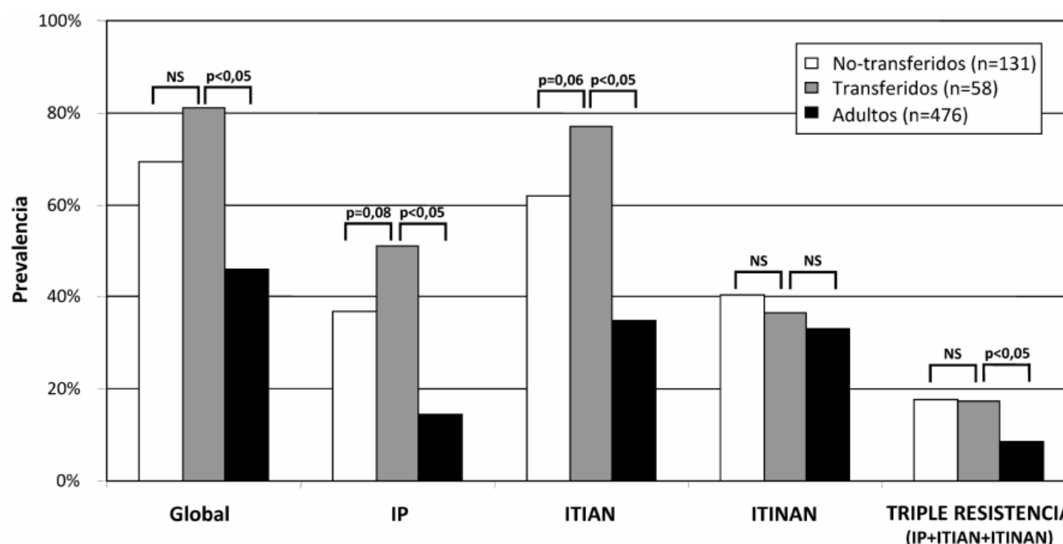
COMPARATIVA ENTRE ADOLESCENTES TRANSFERIDOS A UNIDADES DE ADULTOS, NIÑOS NO TRANSFERIDOS Y ADULTOS INFECTADOS POR VIH-1 EN MADRID

De los 534 pacientes incluidos en la cohorte de niños y adolescentes infectados por VIH-1 en la Comunidad de Madrid a finales de 2011, un total de 112 (21%) habían sido transferidos a unidades de adultos entre 1997 y 2011. Estos datos son ligeramente superiores a los de la cohorte británica de niños infectados por VIH-1 (Collaborative HIV Paediatric Study, CHIPS)

donde, desde 1996 hasta 2007, se habían transferido un total de 103 niños, que representan el 16% de la cohorte pediátrica infectada por VIH-1 del Reino Unido[31] e inferiores a los de la cohorte perinatal francesa donde se habían transferido un total de 210 niños (9,5%) nacidos antes de diciembre de 1993[32]. No existen, que sepamos, más estudios en cohortes de adolescentes infectados verticalmente que hayan sido transferidos a unidades de adultos.

La prevalencia de mutaciones de resistencia a fármacos ARV en los 112 pacientes transferidos a unidades de adultos en la cohorte de Madrid fue comparada con los 131 niños de la cohorte que continuaban su seguimiento en unidades pediátricas a diciembre de 2011. También se comparó con una cohorte de 476 adultos infectados con VIH-1 por vía no vertical y que estaban en seguimiento clínico en la región de Madrid (**Figura 3.10**). La comparativa entre estas tres cohortes nos permitió observar que los adolescentes transferidos presentaban más mutaciones de resistencia globales (81% *vs.* 46%, p<0,05) y a las familias de IP (50,9% *vs*. 14,5%, p<0,05) e ITIAN (76,9% *vs*. 34,9%, p<0,05). La prevalencia de mutaciones de resistencia a la familia ITINAN fue similar en las tres cohortes.

Figura 3.10. Prevalencia de mutaciones de resistencia a fármacos ARV en adolescentes transferidos, niños no transferidos y adultos infectados por VIH-1 de la Comunidad de Madrid.

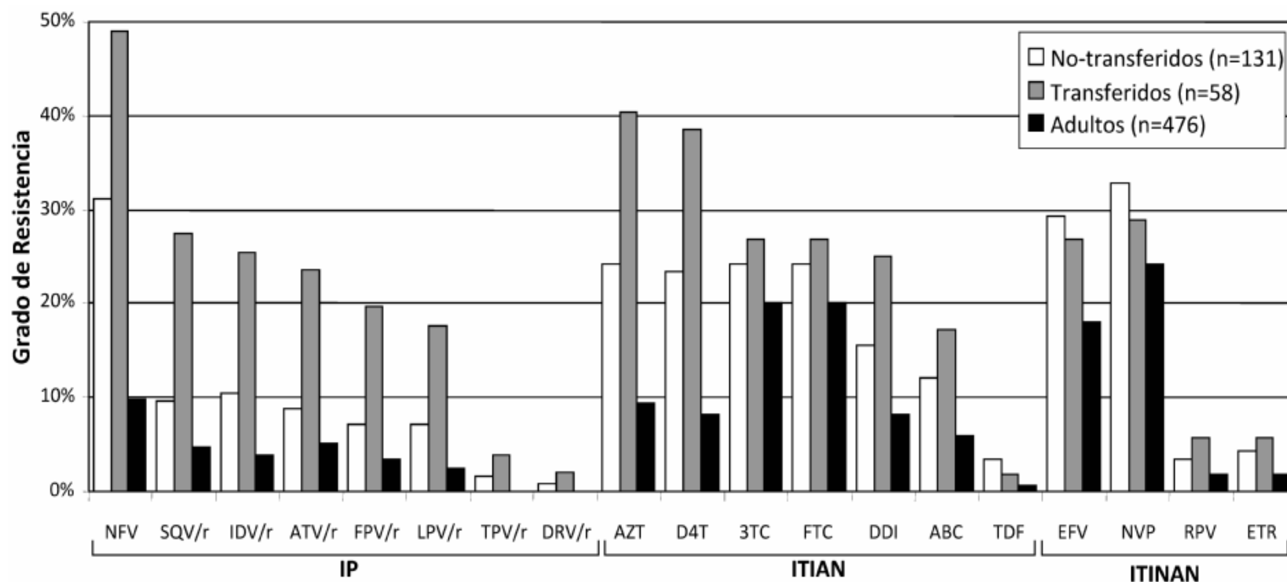


La prevalencia de virus resistentes a las tres familias de fármacos fue similar entre pacientes transferidos (17,3%) y no transferidos (17,6%). Sin embargo, fue significativamente mayor a la detectada en población adulta infectada (17,3% *vs.* 8,6%, p<0,05) y también mayor que la detectada en la cohorte británica CHIPS de adolescentes infectados por VIH-1 y transferidos a unidades de adultos (12%)[31]. Los pacientes adultos presentaron niveles más bajos de prevalencia de resistencia a todas las familias de fármacos analizados (**Figura 3.10**).

Las diferencias observadas en la cohorte de adolescentes transferidos con respecto a las cohortes de adultos y de no transferidos podrían explicarse por los diferentes tiempos de infección con respecto a las otras dos cohortes. También a que los adolescentes verticalmente infectados habían sido tratados intensamente durante muchos años con cambios frecuentes en los regímenes terapéuticos y con niveles variables de adherencia al TAR, especialmente durante la adolescencia. Los resultados obtenidos muestran que dos tercios (64%) de la cohorte de transferidos hasta diciembre de 2011 habían sido tratados con las tres familias de fármacos analizadas, datos ligeramente superiores a los hallados en la cohorte de transferidos británica (64,3% *vs.* 47%, respectivamente)[31]. Además, muchos de los pacientes transferidos se infectaron durante la década de los 90 y debieron recibir tratamientos considerados subóptimos actualmente (monoterapia o biterapia). Ello pudo haber aumentado el riesgo de fracaso virológico debido a la selección de mutantes del VIH-1 resistentes al TAR. Los resultados presentados resaltan el problema al que deberán enfrentarse los clínicos de adultos que reciban en sus consultas a estos pacientes adolescentes infectados por vía vertical o durante su infancia, ya que muchos se encuentran multitratados, infectados hace largos periodos de tiempo y con altos niveles de resistencia a fármacos ARV.

Además de evaluar la situación de los adolescentes transferidos nos propusimos analizar las opciones terapéuticas en éstos con respecto a la de niños no transferidos y a adultos. Los resultados obtenidos mediante la interpretación genotípica de susceptibilidad a fármacos a partir de secuencias del gen *pol* se compararon en los pacientes pretratados en las tres cohortes (**Figura 3.11**). Así se observó que los adolescentes transferidos presentaban un nivel de resistencia significativamente mayor a todos las fármacos de la familia IP e ITIAN que en niños no-transferidos y que en adultos en seguimiento en Madrid, probablemente explicado por su largo historial de tratamiento. La mitad de los pacientes transferidos estaban infectados por variantes virales resistentes a NFV y el 40% a AZT y d4T. Entre el 20-30% de los pacientes transferidos eran resistentes a todos los IP excepto a los de nueva generación (TPV y DRV). En las tres cohortes el EFV y la NVP fueron los fármacos de la familia ITINAN más comprometidos, siendo resistentes entre el 20% y el 30% de los pacientes y alcanzando niveles mayores en pacientes transferidos (**Figura 3.11**).

Figura 3.11. Grado de resistencia a ARV en pacientes transferidos a unidades de adultos y en niños no transferidos de la cohorte pediátrica de Madrid con adultos infectados por VIH-1 en seguimiento en Madrid.



Estos estudios resaltan la prioridad de mantener adecuados niveles de adherencia al TAR en población pediátrica y adulta para mantener opciones terapéuticas futuras, ya que requerirán tratamiento de por vida. Los datos de susceptibilidad genotípica revelan que los fármacos TDF (ITIAN), TPV/r y DRV/r (IP) y ETR y RPV (ITINAN) podrían ser buenas alternativas en futuros regímenes ARV debido a su alto nivel de susceptibilidad predicha en pacientes transferidos y no transferidos. Por último, los adolescentes transferidos también podrán aprovecharse de los nuevos fármacos aprobados para el tratamiento de la infección por VIH autorizados para su uso en adultos. Las nuevas familias de fármacos ARV (inhibidores de la entrada o inhibidores de la INT) también podrían ser buenos candidatos por su poco uso en pacientes transferidos de la Comunidad de Madrid.

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CONCLUSIONES

CONCLUSIONES

1. La situación clínica e inmunológica de los pacientes de la cohorte de niños y adolescentes infectados por VIH-1 de la Comunidad de Madrid analizados en esta Tesis era generalmente buena. Seis de cada diez presentaban CV indetectable y tres cuartas partes presentaban recuentos de linfocitos T CD4 superiores a 500 céls/mm^3.
2. La prevalencia de mutaciones de resistencia transmitidas en pacientes *naïve* de Madrid fue baja. Aún así, se recomienda realizar un *test* de resistencias a todo niño diagnosticado por VIH-1.
3. Un tercio de los pacientes pretratados de la cohorte de niños y adolescentes de la Comunidad de Madrid analizados entre 1993 y 2010 estaban infectados por virus resistentes a IP y a ITINAN. Dos tercios eran resistentes a ITIAN y una sexta parte de los pacientes a las tres familias de fármacos.
4. Uno de cada diez pacientes de la cohorte de niños y adolescentes de la Comunidad de Madrid están infectados por variantes no-B del VIH-1.
5. La tasa de infecciones por variantes no-B del VIH-1 en población pediátrica de Madrid se ha incrementado casi 10 veces entre niños nacidos antes y después del año 2000.
6. La complejidad de las variantes del VIH-1 detectadas en la cohorte de Madrid también ha aumentado en los últimos años. Así, se ha incrementado el número de pacientes infectados por recombinantes del VIH-1, que ha pasado del 1,6% en niños nacidos antes del 2000 al 24,4% en nacidos después del año 2000.
7. Hasta diciembre del 2011 un total de 115 adolescentes infectados por vía vertical en la Comunidad de Madrid habían alcanzado la mayoría de edad, por lo que fueron transferidos a unidades de adulto para su seguimiento clínico. A pesar de haber sufrido estadios avanzados de inmunosupresión durante su control en unidades pediátricas, los pacientes transferidos presentaban una adecuada situación inmune en el momento de la transición.
8. La situación virológica de los pacientes transferidos es preocupante, ya que 8 de cada 10 presentaron algún tipo de mutación de resistencia a fármacos ARV en el momento de la transferencia. Tres cuartas partes fueron resistentes a ITIAN, la mitad a IP y un tercio a ITINAN. Uno de cada seis pacientes transferidos presentaba resistencia a las tres familias de fármacos ARV. Esto refuerza la necesidad de realizar un esfuerzo en este colectivo para aumentar el cumplimiento farmacológico y justifica el uso de las nuevas familias de fármacos disponibles para tratar la infección.

9. La prevalencia de infección por variantes no-B del VIH-1 en pacientes transferidos en la Comunidad de Madrid fue baja (1,9%), comparada con los pacientes pediátricos no transferidos e infectados más recientemente (11,4%).
10. La cohorte pediátrica Jacobi del distrito del Bronx en Nueva York es semejante a la cohorte de Madrid y a la de otros países desarrollados, por incluir pacientes de una alta edad media, mayoritariamente infectados en la década de los 90, con gran experiencia a distintas combinaciones de ARV y con necesidad de planes de transferencia a unidades de adultos.
11. Dos tercios de los pacientes de la cohorte Jacobi eran resistentes a algún tipo de fármaco ARV. Tres de cada diez pacientes pretratados eran resistentes a IP e ITINAN y más de la mitad lo eran a ITIAN.
12. Las cohortes de El Salvador y Honduras, pese a incluir pacientes más jóvenes e infectados más recientemente que los de las cohortes de Madrid o Nueva York, presentaban estadios avanzados de la enfermedad. El 70% de los pacientes mostraban síntomas moderados o severos de progresión a SIDA. Además se observó un notable retraso diagnóstico que alcanzaba los cuatro años en el 67% de los pacientes, lo que contribuye a un mayor deterioro clínico de los niños infectados.
13. La prevalencia global de resistencia a fármacos ARV en pacientes *naïve* de Honduras y de El Salvador, fue similar a la determinada en Madrid (13,6%). Tres cuartas partes de los pacientes pretratados de Honduras y dos tercios de los de El Salvador eran resistentes a alguna familia de fármacos ARV y uno de cada 10 pacientes de ambas cohortes lo eran a las tres familias de fármacos. Todos los pacientes analizados estaban infectados por el subtipo B del VIH-1.
14. El uso de muestras biológicas de sangre seca o *dried blood spots,* recomendado por la Organización Mundial de la Salud para la monitorización de la infección por VIH-1 en pacientes de países de recursos limitados, demostró su validez en la detección de variantes del VIH-1 y en la monitorización de resistencias a ARV.

ANEXO I
OTRAS PUBLICACIONES

Miguel de Mulder y África Holguín. **Utilidad de los dried blood spots para monitorizar la infección por virus de la inmunodeficiencia humana en los programas de salud pública de países en desarrollo. (Dried blood spots for monitoring HIV infection in Public Health Programs in developing countries).** *Enfermedades Infecciosas y Microbiología Clínica*. 2013; 31:100-107. FI: 1,5.

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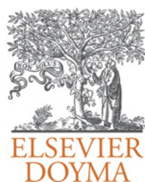

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Revisión

Utilidad de los *dried blood spots* para monitorizar la infección por virus de la inmunodeficiencia humana en los programas de salud pública de países en desarrollo

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RESUMEN

A medida que el acceso a tratamiento antirretroviral avanza globalmente, son necesarios esfuerzos encaminados a simplificar la toma y el envío de muestras a laboratorios de referencia para realizar técnicas de diagnóstico serológico y genético, análisis de carga viral y resistencia a fármacos antirretrovirales en países con recursos limitados. Por tanto, es necesario implementar métodos más baratos y prácticos de obtención, almacenaje y transporte de muestras biológicas y/o sanguíneas. La recogida de muestras en papeles de filtro es una alternativa económica y práctica al plasma para la monitorización del tratamiento antirretroviral, principalmente en laboratorios con equipamientos mínimos y sin acceso a cadenas de frío y/o refrigeración. En esta revisión abordaremos algunas de las aplicaciones clínicas del uso de sangre total seca recogida en papeles de filtro (DBS, *dried blood spots*). Nos centraremos en su aplicabilidad en la monitorización de la infección por VIH para su posible aplicación por los programas nacionales de salud de los países en vías de desarrollo. Haremos un repaso a los distintos estudios que han empleado DBS en la cuantificación de la carga viral, en la monitorización de mutaciones de resistencias a antirretrovirales, en el diagnóstico perinatal temprano, en el diagnóstico serológico en adultos, en la detección de antígeno p24 viral e incluso en la identificación de variantes virales para estudios de epidemiología molecular del VIH. Hablaremos de las variables que afectan a las determinaciones hechas con DBS en el campo del VIH y explicaremos cómo se pueden optimizar los procedimientos usando DBS para aumentar su sensibilidad. El objetivo final será dar a conocer las aplicaciones y ventajas del uso de los DBS en el campo del VIH, sobre todo en países de recursos limitados.

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Dried blood spots for monitoring HIV infection in Public Health Programs in developing countries

Keywords:
Human immunodeficiency virus (HIV)
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Monitoring
Viral load
Sub-types

ABSTRACT

As access to antiretroviral treatment increases in the developing countries, efforts towards making it easier and less costly to collect, store, and deliver the biological samples to reference laboratories, where the serological and genetic diagnosis techniques are performed, have become a high priority. Blood sampling on filter papers is an inexpensive and practical alternative to plasma for antiretroviral treatment monitoring in countries with limited resources and no access to cold chains or refrigeration. The main clinical applications and uses of blood-sampling onto filter papers (dried blood spots [DBS]) are reviewed, focusing on how these can be applied in monitoring HIV infection, particularly for use in National Health Programs in developing countries, or in resource-limited settings. A review is presented of studies that have used the DBS technique for quantifying viral load, analysis of antiretroviral drug-resistance mutations, early infant diagnosis, adult serological diagnosis, detection of viral p24 antigen, and molecular epidemiology of HIV-1, in different geographical locations. Those variables that could affect the use of DBS, particularly in the HIV field, as well as explaining how these procedures can be optimised to increase their sensitivity are also reviewed. The aim of this study was to review the advantages of implementing the DBS technique in the HIV field, especially in resource-constrained regions.

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Introducción a los *dried blood spots* (DBS)

De los más de 34 millones de infectados por el virus de la inmunodeficiencia humana tipo 1 (VIH-1) en el mundo, el 93% viven en países de bajos recursos[1]. A medida que el acceso al tratamiento antirretroviral (TAR) avanza globalmente, también son necesarios esfuerzos encaminados a monitorizar correctamente la terapia reduciendo costes y simplificando procedimientos. La recogida de muestras en papeles de filtro está considerada una alternativa barata y práctica al plasma para la monitorización del TAR, principalmente en laboratorios con equipamientos mínimos y sin acceso a cadenas de frío y/o refrigeración[2]. Los tipos de tarjetas existentes para la recogida de muestras biológicas se detallan en la tabla 1. Las muestras secas, ya sean de sangre total (DBS), plasma (DPS), suero (DSS) o incluso leche materna[3] de pacientes infectados y recogidas en papeles de filtro Whatman 903[4] son un método accesible de toma, almacenaje y envío de muestras a laboratorios de referencia. Pueden emplearse para la cuantificación de la viremia o carga viral (CV)[5–19], monitorización de mutaciones de resistencias a antirretrovirales[18–29], diagnóstico perinatal[7,11,30–32], diagnóstico serológico en adultos[33], detección de p24 viral[34] e identificación de variantes del VIH[18,22–25,29]. La experiencia de cribado en neonatos empleando DBS ha mostrado ser eficaz y sencilla[35]. Los DBS también han sido empleados para el diagnóstico de diversas enfermedades genéticas, metabólicas e infecciosas[35,36]. Actualmente no está aprobado el uso de los DBS para cuantificar linfocitos CD4+ por inmunoensayo, por la poca correlación con los valores obtenidos mediante citometría de flujo. Algunas aplicaciones de los DBS se resumen en la tabla 2.

El uso de los DBS en lugar de sangre tiene la ventaja de emplear un menor volumen de sangre total (50-100 μl) recogido mediante un pequeño pinchazo en el talón o en el dedo, evitando manejar jeringuillas y reduciendo el gasto en infraestructuras y material de laboratorio. Además, las muestras sanguíneas recogidas en papel de filtro no son infecciosas. Por ello el empleo de los DBS podría simplificar la monitorización de la infección por VIH en los programas de salud pública de países en desarrollo. El objetivo de esta revisión es detallar las posibles aplicaciones de los DBS en el diagnóstico y la monitorización de enfermedades, profundizando en su uso para el diagnóstico y la monitorización de la infección por el VIH.

Extracciones de ácidos nucleicos a partir de dried blood spots para la amplificación viral

Métodos de extracción de ácidos nucleicos (ARN, ADN) a partir de dried blood spots

La mayoría de las técnicas de cuantificación de CV, detección de resistencias, diagnóstico genético temprano perinatal y detección de variantes empleando DBS se basan en la amplificación de ácidos nucleicos, principalmente por la reacción en cadena de la polimerasa (PCR). El ARN viral (de virus circulantes) y/o el ADN proviral (de virus integrados en células sanguíneas infectadas) extraído del DBS también se puede usar para otros estudios que requieran secuenciación viral o clonaje molecular del VIH, incluso ensayos de resistencia fenotípica o detección molecular de otros virus del paciente. Sin embargo, todos ellos requieren un paso previo de extracción de material genético del DBS.

No todas las técnicas de extracción (comerciales y no comerciales) disponibles tienen la misma eficacia[37]. De hecho, los fallos en la extracción del ARN viral son la causa más frecuente de obtener valores de CV infraestimados, viremias negativas o fallos de

Tabla 1
Tipos de tarjetas para la recogida de muestras biológicas

Tarjetas	Fabricante	Indicaciones	Muestras empleadas	Información
Whatman 903	Whatman (GE Healthcare)	ARN ADN Anticuerpos Proteínas Otros	Sangre Otros fluidos corporales	www.whatman.com/903ProteinSaverCards.aspx
FTA	Whatman (GE Healthcare)	Solo ADN	Sangre Células Tejidos animales Tejidos vegetales Cultivos celulares Microorganismos	www.whatman.com/FTANucleicAcidCollectionStorageandPurification.aspx
IsoCode Stix	Schleicher & Schuell Bioscience	Solo ADN	Sangre Otros fluidos corporales	https://es.vwr.com/app/catalog/Catalog?parent_class_id=9&parent_class_cd=60949&frmls=x

Los tres dispositivos consisten en una pequeña «tarjeta» portadora que presenta un recorte de papel de algodón o nitrocelulosa adherido a un formulario para recoger la información identificativa del paciente. Las tarjetas 903 están especialmente diseñadas para la recogida y el almacenamiento de sangre para los programas de cribado neonatal. Las proteínas, ARN y otras biomoléculas se pueden archivar en papel 903 y almacenar entre 4 °C y −80 °C. Las tarjetas FTA están tratadas con formulaciones químicas que desnaturalizan proteínas, protegen de la degradación de ácidos nucleicos por las nucleasas y facilitan la unión de ADN. Los FTA se emplean para archivar ADN a temperatura ambiente de manera indefinida. Las tarjetas Isocode son tarjetas 903 modificadas que permiten la elución y amplificación del ADN por la reacción en cadena de la polimerasa (PCR), al tener impregnados inactivadores de muchos de los inhibidores de la PCR presentes en la sangre.

Tabla 2
Algunas aplicaciones prácticas del uso de los DBS

Aplicaciones de los DBS	Ejemplos
Diagnóstico de infecciones (serológico/genético)	VIH VHC CMV HVS-2 *Treponema pallidum* (sífilis) *Tripanosoma cruzi* (Chagas) *Plasmodium falciparum* (malaria) *Toxoplasma gondii* Esquistosomiasis etc.
Monitorización de la infección por HIV	Cuantificación de la carga viral Identificación de mutaciones de resistencia Identificación de subtipos y recombinantes del VIH-1 Estudios farmacocinéticos de antirretrovirales Cuantificación de antígeno p24 viral
Estudios epidemiológicos de enfermedades infecciosas	
Estudio de marcadores de alteraciones genéticas y metabólicas[a]	Anemia falciforme, fenilcetonuria, fibrosis quística, hipotiroidismo neonatal, hiperfenilalalinemia, talasemia, hidrocefalia congénita, leucemia, galactosemia, diabetes tipo 1, etc.
Estudios de haplotipo	
Estudios de marcadores de enfermedad	
Detección y cuantificación de compuestos y drogas por HPLC	

VIH: virus de la inmunodeficiencia humana; VHC: virus de la hepatitis C; CMV: citomegalovirus; HVS-2: virus herpes simplex tipo 2; HPLC: cromatografía líquida de alta eficacia.

[a] Principalmente en neonatos, por el pequeño volumen de sangre requerido usando DBS.

Tabla 3
Variables que afectan a las determinaciones hechas con DBS en VIH

Toma, manipulación y secado correcto del DBS
Volumen de sangre recogida en cada DBS
Temperatura, humedad y tiempo de almacenamiento hasta su uso
Método de extracción del material genético (ARN y/o ADN) del DBS
Número de círculos de sangre seca usados en la extracción
Preservación del material genético extraído hasta su uso
Uso del ADN proviral (más estable) vs. ARN viral (resistencias/diagnóstico/detección de variantes)
Técnica de cuantificación viral y de determinación de resistencias usada
Tamaño del fragmento viral amplificado del DBS
Variabilidad genética del VIH
Interferencia del ADN proviral en las determinaciones

ADN proviral: integrado en las células infectadas de la sangre del DBS; ARN viral: extraído del plasma de la sangre del DBS.

amplificación en la detección de resistencias en muestras de pacientes donde se esperaban CV altas. La mayoría de estudios de determinación de la CV, resistencias y epidemiología molecular y diagnóstico genético empleando DBS extraen ácidos nucleicos por técnicas basadas en el uso de sílice[38], principalmente mediante el empleo del NucliSens isolation Kit (BioMérieux).

Otros métodos de extracción (comerciales y no comerciales) de ácidos nucleicos en DBS alternativos al uso de la sílice también se han usado para cuantificar la viremia del VIH[6,7,10,15,16,37] y para detectar resistencias[22]. La extracción por NucliSens resultó ser más precisa y sensible que otras durante la cuantificación de la viremia[5] y la detección de resistencias[37].

Integridad del material genético en los dried blood spots

Aunque los DBS se pueden almacenar a temperatura ambiente, hay factores que hay que tener en cuenta para preservar la integridad del material genético a extraer del fluido seco en el DBS (tabla 3), y han sido extensamente estudiados[6,7,13,14,26,32,37,39–42]. Se ha comparado la integridad del material genético extraído del DBS considerando distintas temperaturas y grado de humedad durante la toma de la muestra, el transporte y el almacenamiento de los DBS. También se ha evaluado la eficacia de las distintas técnicas en DBS almacenados durante distintos tiempos hasta su uso, comparando las metodologías en DBS, DPS y plasma y empleando distintos números de círculos de sangre seca en la extracción. Aunque normalmente se usan dos círculos para CV y resistencias, la sensibilidad puede aumentar extrayendo ARN de un mayor número de círculos, al relacionarse con una mayor cantidad de muestra de sangre seca a partir de la cual se hará la extracción.

Para una mejor recuperación del ARN viral para la detección de resistencias o de CV es muy importante señalar que los DBS deben ser almacenados lo antes posible en un congelador a –20 °C, y preferiblemente a temperaturas menores (ultracongelador de –80 °C). Ello contribuirá a mantener la integridad del ARN de la muestra hasta su procesamiento. Sin embargo, cuando la criopreservación no es posible, los DBS pueden ser almacenados o transportados a temperatura ambiente hasta 14 días después de su toma. Después de ese tiempo deben ser procesados o almacenados a –80 °C (preferentemente) o a –20 °C hasta su uso. Cabe destacar que, en muestras con baja CV, a mayor tiempo de almacenaje a temperatura ambiente, mayor es el riesgo de degradación del ARN viral del DBS.

Se sabe que el ADN es más estable que el ARN, y ello se aplica también a los DBS[11]. El ARN viral se degrada a mayor tiempo de almacenaje del DBS hasta su uso y, por tanto, la eficacia de cuantificación viral o de detección de resistencias puede verse afectada[41]. El ARN en los DBS y DPS puede ser estable al menos durante un año a –70 °C[18,40] o incluso más. Debemos recalcar que la estabilidad del ARN viral recuperado y el rendimiento de la cuantificación de CV y monitorización de resistencias a partir del DBS será mayor cuanto menores sean la temperatura, la humedad y el tiempo de almacenamiento del DBS hasta su procesado. La sensibilidad del ensayo aumentará cuanto mayor sea el volumen de sangre en cada círculo y el número de círculos usados para la extracción del ARN. Por otro lado, cuanto menor sea el tamaño de la región del VIH a amplificar, mayor será la eficacia de amplificación por PCR necesaria para la monitorización de resistencias y diagnóstico genético en niños. Si es posible, los DBS deberían ser almacenados una vez secos en nevera o preferentemente en congelador en el punto de recogida de muestras, almacenados en bolsas de plástico cerradas e individuales, y deberían contener una bolsa de material desecante para reducir la humedad hasta su uso.

Carga viral y dried blood spots

Carga viral del VIH: uso clínico

La cuantificación de la CV del VIH en plasma sanguíneo es la herramienta de laboratorio recomendada para monitorizar la respuesta al TAR en pacientes infectados, detectar fracaso terapéutico y evaluar la necesidad de cambio de tratamiento. Sin embargo, en contextos con bajos recursos, esta técnica no se implementa y se siguen otros parámetros para este seguimiento. La cuantificación de la CV también ha sido empleada para el diagnóstico temprano en niños[7,30,31,34]. Valores de CV indetectables en plasma reflejan un adecuado control de la replicación viral en pacientes en TAR. Por el contrario, niveles detectables de viremia pueden indicar un

Tabla 4
Comparación de distintas técnicas de cuantificación de la carga viral del VIH-1 comerciales usando DBS vs. plasma

Autor y año	País	Pacientes (n)	Círculos usados	Describen variantes del VIH-1	Método de extracción de ARN	Ensayos de cuantificación de ARN VIH-1	Correlación CV (plasma/DBS)
Álvarez-Muñoz 2005	México	108	2	No	NucliSens manual	NucliSens HIV-1 QT assay (en DBS) Amplicor HIV-1 Monitor v1.5 (en plasma)	r = 0,95
Lira 2010	México	57	2	Subtipo B	NucliSens manual	COBAS Amplicor HIV-1 Monitor v1.5 (plasma) NucliSens HIV-1 QT assay (DBS)	r = 0,79
Garrido 2009	España	103	1	Subtipo B	NucliSens EasyMAG y Abbott m2000sp automatizados	NucliSens EasyQ HIV-1 v1.1 Abbott m2000rt real-time HIV	r = 0,87 r = 0,70
van Deursen 2010	Francia, Países Bajos	224	2	Subtipo B	NucliSens EasyMAG automatizado	NucliSens EasyQ HIV-1 v2.0	r = 0,95
Reigadas 2009	Francia	86	1	Varios	QIAamp viral RNA minikit manual	Generic HIV Charge Virale Amplicor COBAS® TaqMan® HIV-1	r = 0,86
Marconi 2009	Italia	169	2	No	Abbott m2000sp automatizado	Abbott m2000rt real-time HIV	r = 0,88
Andreotti 2010	Malaui	108	1	No	NucliSens miniMAG manual	COBAS Taqman real-time RT-PCR	r = 0,96
Mbida 2009	Camerún	41	2	No	Abbott m2000sp automatizado	Abbott m2000rt real-time HIV assay	r = 0,98
Lofgren 2009	Tanzania	137	2	No	Abbott m2000sp automatizado	Abbott m2000rt real-time HIV assay	r = 0,97
Johanssen 2009	Tanzania	98	2	No	NucliSens manual	NucliSens EasyQ HIV-1 v1.2	r = 0,75
Kane 2008	Senegal	41	2	No	NucliSens MiniMAG manual	NucliSens EasyQ HIV-1	r = 0,98
Waters 2007	Uganda	306	Nd	No	Primagen manual	COBAS Taqman real-time RT-PCR (en DBS) Amplicor HIV-1 Monitor v1.5 (en plasma)	r = 0,72
Lelawiwat 2009	Tailandia	167	1	CRF01_AE, subtipo B'	NucliSens manual	Amplicor HIV-1 Monitor v1.5	r≈0,94
Uttayamakul 2005	Tailandia	30	1	No	NucliSens manual	NASBA HIV-1 QT assay	r = 0,82

Nd: no determinado; VERSANT® HIV-1 RNA bDNA (Siemens Medical Solution); Amplicor HIV-1 Monitor y COBAS Taqman real-time RT-PCR (Roche Diagnostics); Abbott RealTime m2000rt (Abbott Molecular Diagnostics); NASBA HIV-1 QT assay (Organon Técnica); NucliSens EasyQ® HIV-1 (Biomerieux); NASBA o NucliSens manual: empleando su buffer de lisis; Generic HIV Charge Virale (Biocentric); técnica manual, no automatizada; Abbott m2000sp: sistema automatizado de extracción de ácidos nucleicos en muestras (Abbott sample preparation system m2000sp). Todas las correlaciones de medidas de CV entre plasma y DBS fueron significativas ($p < 0,001$).

mal control de la infección, fallos de adherencia o emergencia de virus con mutaciones de resistencia a fármacos en uso.

Ensayos de determinación de la carga viral

Existen 2 metodologías disponibles para medir ARN del VIH-1 en las que se basan las técnicas de cuantificación viral comerciales en plasma. La primera metodología requiere una extracción previa de ácidos nucleicos de la muestra infectada para su amplificación posterior. La amplificación puede realizarse por transcripción inversa o RT-PCR (Amplicor HIV-1 Monitor) que puede emplear amplificación a tiempo real (Amplicor COBAS® TaqMan® HIV-1; Abbott Real Time™ m2000rt) y mediante la técnica NASBA (NucliSENS EasyQ® HIV-1). La segunda metodología se basa en métodos de amplificación de señal (VERSANT® HIV-1 RNA bDNA) y no requiere extracción previa de ácidos nucleicos.

La determinación de CV en plasma presenta altos costes y limitaciones técnicas para países con bajos recursos, y se necesitarían ensayos baratos, rápidos y fáciles de usar en países con instalaciones limitadas. Por ello se han desarrollado métodos no comerciales de menor coste[22,32,43–47]. Algunos están basados en la primera metodología: Generic HIV-1 viral load (Biocentric), Primagen Retina-Rainbow, RT-PCR manuales (caseras o *in house*) o RT-PCR a tiempo real[32,44,47]. Otras técnicas de cuantificación alternativas miden la actividad de la transcriptasa inversa de VIH-1 (Cavidi ExavirLoad v2 y v3)[48]. No obstante, la detección de falsos positivos, las necesidades de equipamiento caro y de personal cualificado siguen siendo las principales barreras para su puesta en práctica en laboratorios poco equipados o con poca experiencia en estas técnicas[45]. Las ventajas e inconvenientes de varios métodos de cuantificación de CV comerciales y no comerciales ya han sido publicadas[43].

Variabilidad entre técnicas de carga viral en plasma y en dried blood spots

Las técnicas de cuantificación de CV del VIH-1 presentan diferencias en precisión y sensibilidad. Aunque la mayor parte de los artículos muestran buena correlación entre los valores de CV de plasmas y DBS[5–18], algunos han observado falsos positivos[10], atribuidos al efecto del ADN proviral en la cuantificación a partir de DBS en algunas muestras con CV plasmática baja[5,8,13]. El bajo volumen de sangre recogido en los DBS puede limitar la sensibilidad de la cuantificación de CV si se compara con la cuantificación convencional usando volúmenes de plasma mucho mayores. La sensibilidad de cualquier técnica puede disminuir si la muestra no ha sido bien conservada y principalmente en muestras con CV más bajas. No es fácil definir en qué grado las diferencias de sensibilidad empleando DBS vs. plasma observadas en estos

Tabla 5
Algunos estudios que monitorizan VIH-1 resistentes a fármacos antirretrovirales en DBS

Referencia	País	Número de muestras (plasmas/DBS)	Describen variantes del VIH-1	Método de extracción de ARN	Círculos Usados (n)	Método de amplificación ARN *pol*	Genotipo completo en DBS (PR + RT)[a]	Concordancia en secuencias (plasma, DBS)	Rango de CV (log copias ARN-VIH/ml)
Lira 2010	México	57	Subtipo B	NucliSens	4	ViroSeq	42,1%	96,4%	2,67-5,98
Bertagnolio 2007	México	103	Subtipo B	NucliSens	1	Nested RT-PCR	90,1% (PR o RT)	99,9%	3,7-5
Holguín 2011	Honduras El Salvador	80 Solo DBS	Subtipo B	NucliSens	2	Nested RT-PCR	94% (PR y/o RT)	Nd	1,7-6,4
Youngpairoj 2009	España	40	Subtipo B	Nuclisens	1	ViroSeq Nested RT-PCR	57,5% (ViroSeq) 95% (Nested RT-PCR)	94,5%	2,7-5,8
Masciotra 2007	España	60	Subtipo B	Nuclisens	1 o 2	ViroSeq	83%	98,5%	1,9-5,8
Buckton 2008	Reino Unido	33	Varios	Sílice	2	Nested RT-PCR	57,6% en PR 66.7% en RT	83%	1,7-5
Hallack 2008	EE. UU.	33	Subtipo B	NucliSens	1	TruGene	78,8%	≈99%	3-5,6
McNulty 2007	Camerún	40	No	NucliSens	1	Nested RT-PCR	92,5% en PR 60% en RT	98,5%	2,8-5,8
Steegen 2007	Kenia	60	Varios	QIAamp DNA blood mini kit	1	Nested RT-PCR	93%[b]	97,9%	1,8-> 5
Johannessen 2011	Tanzania	36	Varios	NucliSens	2	ViroSeq	47%	94%	3,1-6,5

Nd: no descrito; PR: proteasa; RT: transcriptasa inversa; NucliSens: EasyMag y MiniMag (Biomerieux); QIAamp DNA blood mini kit (Qiagen); TruGene; TruGene HIV-1 genotyping assay (Siemens Healthcare Diagnostics; 1,3 kb); ViroSeq HIV-1 genotyping system (Abbott Molecular; 1,8 kb); Nested RT-PCR: técnica de amplificación de la PR y RT virales no comercial en uno o 2 amplicones.

[a] Genotipo completo en DBS: amplificación por PCR positiva en la proteasa y transcriptasa inversa virales. Variable según método de amplificación, carga viral de la muestra y condiciones de almacenamiento del DBS hasta su uso.

[b] Menor en muestras con viremias indetectables.

estudios pueden ser atribuidas al método de extracción, al ensayo de CV específico, a la conservación de la muestra o incluso a la experiencia del laboratorio, al observarse también variabilidad de resultados entre laboratorios empleando las mismas muestras[40]. La tabla 4 resume algunos trabajos representativos que comparan la CV a partir de plasma y DBS empleando varias técnicas, principalmente comerciales. A pesar de todo, el rango de sensibilidad y la correlación de los valores de plasma deben ser determinados para cada ensayo comercial y versión. Independientemente del uso de plasma o sangre seca, es importante que no se cambie de técnica de cuantificación de viremia a la hora de monitorizar a un paciente durante su seguimiento clínico.

Límite de detección de carga viral en plasma vs. dried blood spots

La cuantificación de CV en DBS no puede alcanzar los mismos niveles de sensibilidad que empleando plasma. Mientras que en el DBS se emplean normalmente uno o 2 círculos de sangre seca, cada uno saturado por 50-100 μl de sangre total, la nueva generación de ensayos de CV emplea 1 ml de plasma equivalente a 2,5 ml de sangre total. Por tanto, empleando plasma se pueden alcanzar límites de detección de 40-50 copias ARN-VIH/ml (1,3-1,7 log) y en DBS se establece próximo a las 1.000 copias ARN-VIH/ml (3 log)[6,7,14], aunque algunos estudios detectaron hasta 200 copias ARN-VIH/ml (2,3 log)[15,32].

Resistencias y dried blood spots

Eficacia de amplificación y ensayos de resistencia genotípica

La Organización Mundial de la Salud (OMS) recomienda el empleo de DBS en instalaciones con recursos limitados para la vigilancia de resistencias transmitidas a fármacos del VIH-1 en pacientes *naïve* y para monitorizar resistencias en pacientes en TAR[19]. La tabla 5 resume algunos de los estudios que evalúan resistencias genotípicas a antirretrovirales empleando DBS[18,20–29].

La tasa de éxito de amplificación de material genético del VIH empleando DBS difiere según la CV de las muestras, y depende de las condiciones de almacenamiento de los DBS antes de su procesamiento, del método de extracción de ARN empleado y de la longitud de la región de *pol* del VIH amplificada. Como ocurre para la CV, la eficacia de amplificación para resistencias en DBS es mayor con CV superiores a 3 log de copias ARN-VIH/ml, reduciéndose drásticamente en muestras con CV indetectables (< 1,7 log), como ocurre usando plasma.

Los ensayos de resistencia genotípica comerciales en *pol* (ViroSeq HIV-1 genotyping system; Abbott Molecular y TruGene HIV-1 genotyping assay; Siemens Healthcare Diagnostics) se han empleado para determinar resistencias en DBS[18,25,26,28]. Estos amplifican la proteasa y transcriptasa inversa virales en uno o 2 amplicones de 1,8 kb (ViroSeq) y 1,3 kb (TruGene), respectivamente. Sin embargo, son más numerosos los estudios que detectan resistencias en DBS empleando métodos no comerciales o caseros de amplificación basados en nested RT-PCR, de menor coste y que parecen ser tanto o más eficientes que ViroSeq o TruGene al tener mayores tasas de éxito de amplificación (tabla 5). Ello se debe a que en muchas ocasiones los PCR caseros amplifican la proteasa y transcriptasa inversa por separado, y la amplificación de regiones más cortas es más eficaz que las largas empleando DBS.

El éxito de amplificación del fragmento de 1,8 kb por ViroSeq usando DBS fue variable en diversos estudios, dependiendo de las condiciones de almacenamiento del DBS a su uso y de su CV. En un estudio, ViroSeq consiguió amplificar el 79% de muestras con CV mayores de 5,1 log copias RNA-VIH/ml, del 42,8% entre 4,2-5 log, y ninguna amplificación con CV inferiores a 4,1 log[18]. En otro estudio, ViroSeq amplificó las 46 muestras con viremias entre 2,7 y 5,8 log en DBS almacenados a −20 °C durante 6 meses, pero la amplificación falló en 26 de ellas cuando los DBS de los mismos pacientes se almacenaron a 4 °C durante 12 meses hasta su uso[26]. En otro trabajo, ViroSeq amplificó el 75% de muestras con viremias mayores a 4 log, pero solo el 13% con viremias inferiores[28] en DBS almacenados a −20 °C cerca de 4 meses, transportados a temperatura

ambiente durante 20 días y guardados otro mes a –20 °C hasta su procesamiento. Otros lograron genotipar por ViroSeq las 38 muestras en DBS con viremias mayores de 3,3 log, pero solo el 54,5% con CV inferiores en DBS transportados y almacenados a –20 °C durante una media de 5 meses, aunque consiguieron genotipar el 95% de las 46 muestras empleando una técnica no comercial de RT-PCR[23]. TruGene pudo amplificar el 90,5% de muestras en DBS con viremias mayores a 3,7 log[25].

Por todo ello, a pesar de que los kits comerciales son eficientes en muestras con altos niveles de viremia, como en pacientes en fracaso terapéutico o en la mayoría de los pacientes sin tratamiento, los ensayos de nested RT-PCR caseros que amplifican la proteasa y transcriptasa reversa por separado parecen dar más amplificaciones positivas en un porcentaje mayor de muestras con CV bajas. Además, el coste de las técnicas no comerciales normalmente es bastante inferior a las comerciales. En cualquier caso, cualquier técnica de detección de resistencias, tanto casera como comercial, requiere una buena integridad del ARN y, por tanto, las mismas condiciones favorables de conservación de los DBS.

Comparación de genotipos de resistencia obtenidos a partir de dried blood spots y plasma

Además de analizar el éxito de amplificación a partir de muestras de DBS, la mayoría de los estudios tienen como objetivo comparar las secuencias generadas a partir de DBS y plasma de las mismas muestras. Existe una alta concordancia (83-99,9%) entre mutaciones de resistencia a fármacos encontradas en plasma y DBS en los diversos estudios[18–28] (tabla 5). Algunas mutaciones adicionales solo fueron detectadas en DBS principalmente como mezclas de aminoácidos incluyendo el residuo mutante y salvaje, pudiendo también deberse a la influencia del ADN proviral amplificado de las células infectadas junto al ARN viral en el DBS. Ello reflejaría mutaciones archivadas en el ADN proviral y que podrían volver a expresarse en las condiciones adecuadas de presión selectiva.

Interferencia del ADN proviral recuperado de los dried blood spots en la carga viral y determinación de resistencias

El ADN proviral extraído de sangre seca contribuye significativamente al producto de la amplificación de ácidos nucleicos en ausencia de transcriptasa inversa[21,23,41]. Su contribución sería más relevante a mayor CV de la muestra[21] y a mayor tiempo de almacenamiento de los DBS hasta la determinación de la resistencia[41], confirmando la degradación más rápida del ARN respecto del ADN en los DBS. Aunque el papel FTA puede emplearse en la monitorización de resistencias, el papel 903 es más adecuado para recuperar ARN[24].

Algunos autores han descrito interferencias del ADN proviral en la cuantificación basada en amplificación por RT-PCR y en los resultados genotípicos de resistencia, que puede conducir a problemas a la hora de valorar o interpretar un resultado positivo[10]. Ello ocurre principalmente cuando se emplean extracciones de ácidos nucleicos que recuperan tanto ADN como ARN al no incluir tratamiento con DNasas, como son los métodos basados en sílice, como el NucliSens. Ello se puede minimizar tratando los DBS previamente con DNasas para asegurar que solo se amplifica y cuantifica ARN viral[41]. Así se consigue que las CV usando una técnica de RT-PCR a tiempo real (Generic HIV Change Virale, Biocentric) y las eficiencias de amplificación por PCR de las mismas muestras tomadas en DBS y DPS almacenados 2 meses a 20 o 37 °C sean comparables[41]. La influencia del ADN proviral en la cuantificación de la CV no se confirma en otros estudios en los que se extrae el material genético con el extractor m2000sp (Abbott) cuantificando con Abbott real-time HIV-1[6] o se extrae el ARN usando el QIAmpviral RNA mini Kit (Quiagen) cuantificando con Amplicor COBAS® TaqMan® HIV-1[16]. En ambos estudios encontraron cuantificaciones similares en DBS y DPS sin amplificación de ADN proviral en los DBS y sin usar DNasa.

Por otro lado, cuantificando la CV por NucliSens se evita la amplificación del ADN proviral extraído del DBS durante la extracción previa por sílice, ya que esta técnica usa la ARN polimerasa de T7 para amplificar selectivamente el ARN de los ácidos nucleicos extraídos del DBS con una amplificación isoterma (4 °C). En ausencia de desnaturalización por calor (esencial para cualquier amplificación de material genético por PCR), la doble cadena de ADN que pudiera existir en la extracción no puede participar en el proceso de amplificación, no sobrestimando los valores de ARN viral de la muestra en DBS.

El efecto de la interferencia de ADN proviral en la CV también está condicionado por la eficacia del tratamiento antirretroviral en el paciente[22]. Así, en pacientes en fallo virológico se tiende a encontrar más mutaciones de resistencia a fármacos en plasma que en células infectadas, especialmente cuando las CV son bajas. Por el contrario, en pacientes que han interrumpido el tratamiento, el ADN proviral obtenido a partir de células mononucleares de sangre periférica (CMSP) puede actuar como un archivo de mutaciones de resistencia de tratamientos pasados, y los DBS darían más información que el plasma en este tipo de pacientes.

Diagnóstico infantil empleando dried blood spots

Según datos de la OMS, 390.000 niños menores de 15 años fueron infectados por VIH-1 en 2010[1]. El diagnóstico temprano de la infección perinatal por VIH-1, principalmente en hijos de madres seropositivas, es esencial para instaurar el TAR, prevenir infecciones oportunistas y reducir la mortalidad infantil. Recientemente, en países en desarrollo se ha empezado a extender el uso de DBS en lugar de sangre para el diagnóstico perinatal. El diagnóstico en niños menores de 18 meses requiere detectar el material genético de VIH-1 (de ARN viral o ADN proviral) tras amplificarlo por PCR cualitativa preferiblemente en 3 regiones[30,31] o por PCR cuantitativa[7,11,32], aunque su sensibilidad puede variar con la edad[31]. Antes de 18 meses de edad las técnicas serológicas convencionales no deben emplearse, ya que los anticuerpos maternos pasan desde la madre al neonato y pueden persistir hasta entonces en el niño. Los DBS también se han usado para el diagnóstico serológico del VIH-1 por ELISA ultrasensible de detección de antígeno viral p24 en niños[34] y para el diagnóstico serológico en adultos[33].

Uso de *dried blood spots* en estudios epidemiológicos

Las variantes no-B (subtipos no-B y recombinantes) del VIH-1 causan el 90% de los 34 millones de infecciones por VIH-1 y son prevalentes en gran parte de los países en desarrollo, en los que conviene implantar el uso de los DBS por sus ventajas a nivel metodológico. La alta variabilidad genética del VIH-1 puede afectar la eficacia de técnicas de amplificación por PCR del material genético viral extraído del DBS para cuantificar la CV, para detectar resistencias[24] o para el diagnóstico perinatal. Las variantes no-B pueden ser responsables de falsos negativos o de fallos de sensibilidad de las técnicas diagnósticas, al estar principalmente diseñadas y validadas para el subtipo B, prevalente en países industrializados. Por ello, aunque pueden emplearse para otros subtipos, la sensibilidad puede variar. Así, se han descrito CV subestimadas en cierta muestra de plasma de subtipos no-B y recombinantes del VIH-1 empleando diferentes métodos de cuantificación comerciales[49].

La toma de muestras en DBS constituye una técnica barata y práctica para diseñar grandes estudios de epidemiología molecular que analicen la distribución global y diseminación de variantes del VIH en el mundo. El análisis filogenético de las secuencias virales recuperadas de muestras infectadas es el método más adecuado para identificar variantes del VIH. Así, se puede subtipar a partir de la secuencia generada para identificar resistencias, y también de las secuencia de productos amplificados por PCR en cualquier región para el diagnóstico genético en niños. También existen páginas web gratuitas para el subtipaje rápido: se introduce la secuencia y se identifica la variante del VIH-1 en pocos segundos. Aunque estas son útiles para identificar subtipos B, presentan limitaciones importantes en la caracterización de ciertas variantes no-B del VIH-1, principalmente recombinantes complejos, cada vez más prevalentes en la pandemia[50].

Aún hay pocos artículos que incluyan la información sobre el subtipo o recombinante del VIH-1 de las muestras de estudio cuando comparan eficacia de distintas técnicas empleando sangre seca infectada por VIH tomada en DBS (tablas 4 y 5). Ya que la mayoría de los pacientes con VIH están infectados por variantes no-B del VIH-1, es importante validar todos los métodos de monitorización del VIH-1 con paneles que incluyan un alto número de muestras de cada subtipo y recombinante circulante. Así se podrá determinar la sensibilidad y la especificidad de cada técnica a emplear para cada uno de ellos y se podrán diseñar bien los *primers* o sondas para una amplificación eficaz del genoma viral recuperado del DBS para todas las variantes del VIH-1.

Conclusión

A lo largo de esta revisión hemos expuesto cómo los DBS son útiles en la monitorización del VIH, sobre todo en países con recursos limitados. Su toma es sencilla, barata, requiere entrenamiento mínimo del personal. Evita venopunciones y uso de jeringuillas, requiere volúmenes mínimos de sangre —lo que favorece su uso en neonatos—, ocupan poco espacio durante su almacenaje y son fáciles de transportar y de manejar. Su validez respecto al plasma ha sido demostrada por distintos estudios a nivel internacional. Por ello, la OMS (http://www.who.int/hiv/topics/drugresistance/dbs_protocol.pdf) recomienda su uso en países de recursos limitados para la toma de muestras y monitorización de la infección del VIH cuando la recogida de sangre por venopunción, extracción de plasma, almacenamiento y transporte a laboratorios de referencia conlleva dificultades a nivel práctico. Además, más de 6,7 millones de personas están recibiendo tratamiento antirretroviral en estos países[1]. Así, se requiere implantar o reforzar los sistemas de monitorización del tratamiento empleando la detección de resistencias y favoreciendo el diagnóstico precoz de la infección. En ese contexto, el uso de los DBS es una gran alternativa. Sin embargo, en la mayoría de los países de bajos recursos donde la prevalencia de la infección por VIH es más elevada, su uso no está implantado. Por ello, es necesario unificar y validar procedimientos de laboratorio empleando los DBS. Se requiere invertir tiempo y recursos para optimizar los métodos de extracción, amplificación y cuantificación de ARN viral empleando DBS al menor coste posible. Se debe reforzar la capacitación técnica de los laboratorios en estos países para que se puedan implementar correctamente el uso de los DBS y obtener su máximo rendimiento. Es la única manera de garantizar unas buenas prácticas de toma, almacenamiento y empleo de los DBS para mantener la integridad del material genético y aumentar el éxito de los procesos. Por todo ello, invertir en extender y optimizar el uso de los DBS con las técnicas de monitorización del VIH aprobadas en cada país podría ser más rentable en costes para los planes nacionales de salud que invertir en logística tradicional de muestras.

Todo ello, unido al gran número de aplicaciones empleando DBS en el diagnóstico serológico y genético de otras infecciones, refleja el enorme potencial a nivel epidemiológico y clínico que su uso puede tener en países de recursos limitados.

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High Prevalence of X4/DM-Tropic Variants in Children and Adolescents Infected With HIV-1 by Vertical Transmission

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Background: We studied HIV coreceptor tropism in vertically HIV-infected children and adolescents with the objective of predicting the proportion of children and adolescents that could be treated with CCR5 (R5) antagonists.
Methods: One hundred eighteen multidrug-resistant pediatric patients (36 children and 82 adolescents) were enrolled in a cross-sectional study. Viral tropism was assessed using the new phenotypic HIV-1 tropism coreceptor assay information and Trofile.
Results: Of 118 antiretroviral-experienced HIV-infected children and adolescents, 49 (57.0%) had dual-tropic and 20 (23.3%) had X4-tropic viruses by tropism coreceptor assay information testing. Only 17 (19.7%) showed R5-tropic variants. HIV-1 coreceptor usage was not detectable in 32 of 118 (27%) patients. Among 24 children and 62 adolescents with tropism coreceptor assay information results, 17 (70.8%) children and 51 (82.2%) adolescents showed viruses with dual-tropic or X4-tropic variants. Additionally, Trofile (ES) was performed in 42 of 118 patients with HIV-1 RNA > 1000 copies/mL. No patient showed X4-tropic variants; dual-tropic viruses were observed in 12 (28.6%) patients. In 6 (14.3%) patients, HIV tropism could not be determined. X4-tropic variants were more common in children ($P = 0.031$). $CD4^+$ T cell percentage was significantly lower in children ($P = 0.011$) and adolescents ($P = 0.027$) with R5-tropic viruses than in those with X4-tropic viruses.
Conclusions: The presence of X4-tropic variants in more than 80% of our cohort of antiretroviral-experienced children and adolescents with vertical HIV-1 infection indicates a very limited role for CCR5 antagonists as part of salvage regimens for highly treatment-experienced vertically HIV-1–infected patients with extensive antiretroviral drug resistance and limited treatment options.

Key Words: HIV tropism, maraviroc, children, adolescents

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To initiate cellular infection, HIV-1 needs to use the primary receptor CD4 and a secondary chemokine coreceptor, typically CCR5 (R5) or CXCR4 (X4). Based on coreceptor usage, HIV-1 variants are classified as R5-tropic, X4-tropic or dual-tropic (DM).[1] Early in primary infection, R5-tropic virus predominates, irrespective of the transmission route.[2,3] X4, DM and mixed populations appear in more advanced disease and have been associated with a faster decrease in $CD4^+$ T-lymphocyte (CD4) cell count and progression to AIDS.[2]

Currently, most vertically infected children in resource-rich settings are school-aged or older and have often received serial, nonsuppressive antiretroviral (ARV) regimens. Use of low-activity, early ARV regimens and incomplete adherence to ARV medications have resulted in large numbers of HIV-infected children and adolescents who have extensive resistance to most available ARV drugs. Development of a new ARV drug offers important novel options for creating suppressive ARV regimens for such children.

The epidemiology of R5-tropic and X4-tropic variants is of particular relevance because of the development of HIV-1 coreceptor inhibitors. Maraviroc is the first CCR5 antagonist to block the interaction with the CCR5 coreceptor. It is only active against R5-tropic variants and fails in the presence of X4-tropic or DM-tropic viral strains.[4] The promising results obtained in the MOTIVATE-1 and MOTIVATE-2 trials[5–7] led to the approval of maraviroc for treatment-experienced HIV-infected adults in 2007.[8] Maraviroc is not yet licensed for pediatric patients but could be considered as an option for highly active antiretroviral therapy (HAART) in HIV-1–infected children and adolescents without other viable treatment options.

HIV tropism has been extensively studied in HIV-infected adults.[9–11] However, the few studies evaluating HIV tropism in pediatric patients have been performed in newborns, mainly using older assays (non–syncytia-inducing or macrophage tropic).[3,12] No data about HIV tropism in vertically infected older children and adolescents have been reported. We aimed to predict the proportion of HIV-1–infected children and adolescents who could benefit most from treatment with maraviroc.

MATERIAL AND METHODS

Study Population and Design

We performed an epidemiologic, multicenter, cross-sectional, noninterventional study of 118 vertically HIV-1–infected

children (1–12 years of age) and adolescents (13–18 years of age) born between 1991 and 2008 who were receiving HAART and who were in care at 1 of 7 Spanish hospitals (Annexe) in the Spanish HIV Pediatric Cohort node 1 (CoRISpe-1) from September 1, 2009 to October 30, 2010. To be included, patients should not have modified their HAART regimen during the previous 6 months or have received treatment with CCR5-receptor antagonists. Patients with suppressed viral load (<50 HIV-1 RNA copies/mL) or with persistent detectable viral load (>50 HIV-1 RNA copies/mL) during the previous 6 months were included. Samples were provided by the Pediatric HIV BioBank of the Spanish AIDS Research Network. Peripheral blood mononuclear cells were isolated on a Ficoll-Hypaque density gradient (Rafer, Zaragoza, Spain) following the current procedures of Spanish HIV BioBank.[13,14] Informed consent was obtained from parents or legal representatives, and the local ethics committees approved the study.[13,14]

Quantification of HIV-1 RNA

HIV-1 RNA was measured in fresh plasma samples from patients and in cell-free supernatants by quantitative polymerase chain reaction using the commercial quantitative Amplicor Monitor assay (Roche Diagnostic Systems, Brandenburg, NJ) according to the manufacturers' instructions (lower detection limit of 50 HIV-1 RNA copies/mL). CD4 percentages and absolute counts were measures in total blood samples by flow cytometry using a Beckman Coulter FC-500 Cytometer (Beckman Coulter, Inc., Brea, CA).

HIV-1 Subtyping

HIV-1 subtypes and circulating recombinant forms (CRF) were identified by phylogenetic analysis of pol sequences (complete protease and/or partial reverse transcriptase). At least 2 representative sequences of each of the 9 subtypes and the 48 CRFs of HIV-1 group M available from GenBank at the time of the analysis were taken as references (http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html). The tree topology was obtained using the neighbor-joining method. DNA sequences were aligned using the ClustalX 2.0.11 program. The pairwise distance matrix was estimated using the Kimura 2-parameter model within the DNADIST program, as implemented in the PHYLIP software package. Bootstrap resampling (1000 data sets) of the multiple alignments was performed to test the statistical robustness of the tree. The bootstrap cutoff was set at 700.

Determination of HIV-1 Coreceptor Usage

Viral tropism was assayed with the phenotypic HIV-1 tropism coreceptor assay information (TROCAI), with some modifications.[15] Briefly, fresh peripheral blood mononuclear cells from patients were cocultured with phytohemagglutinin (Boehringer Ingelheim, Ingelheim, Germany)-stimulated HIV-1–seronegative donor peripheral blood mononuclear cells. HIV-1 RNA was determined in cell-free supernatants at days 7 and 14 to perform further infection assays. Afterward, viral tropism was determined using U87 cell lines (U87-CD4+CCR5+ and U87-CD4+CXCR4+). U87 cell lines were infected overnight with a range of 103–106 HIV-1 RNA copies of cell-free viral supernatant. The cells with the virus inoculum were then spinoculated at 2500 rpm for 1 hour. The medium was changed at day 3 and tested for HIV-1 RNA (copies/mL) at day 6 in the supernatant of the cultures of X4 and R5 cell lines. Results were presented as the proportion of viruses isolated using the X4 or R5 coreceptor.[15] A result was considered X4 when virus was detected in the U87-CD4+CXCR4+ T cell supernatant and was not detected in U87-CD4+CCR5+ T cell supernatants. A result was considered R5 when HIV was detected in U87-CD4+CCR5+ T cell supernatants and it was not detected in U87-CD4+CXCR4+ in T cell supernatants. A result was considered DM when the proportion of X4 virus in the U87 cell line supernatant was >0.5%, as previously described.[15] The results were categorized using the virologic response to short-term maraviroc monotherapy previously evaluated in HIV-1–infected adults (maraviroc clinical test [MCT][16]; Fig. 1). According to this categorization, the correlation between TROCAI results and virologic response was 100% when the percentage of X4 was fixed at a cutoff value of 0.5%.[15]

In patients with more than 1000 HIV-1 RNA copies/mL, the PhenoSense HIV Entry assay for coreceptor tropism (Monogram Biosciences Inc., South San Francisco, CA) was also applied according to the manufacturers' instructions.[17] This new version of Trofile TM (ESTA or Trofile [ES]) claims to have greater sensitivity, detecting X4 strains when present in levels as low as 0.3%.[18] However, discordant rates (15%) were found between the virologic response observed in MCT and Trofile (ES),[19] similar to discordant rates observed in a previous study between phenotypic assays.[20]

Statistical Analysis

Baseline characteristics of the study population were summarized. The association between qualitative variables was tested using the χ^2 test or Fisher exact test, as appropriate. Differences between groups were analyzed using the Mann–Whitney *U* test for continuous variables. Statistical significance was set at $P < 0.05$. All statistical analyses were performed using SPSS v18.0 (SPSS Inc., Chicago, IL).

RESULTS

Baseline Characteristics of the Study Population

Of 118 vertically HIV-infected participants who fulfilled the inclusion criteria, 36 (30.5%) were children and 82 (69.5%) were adolescents. Median age was 14.6 years (range: 10.9–17.0). Most were female (59.3%), white (83.9%) and from Spain (93.3%).

Seventy-six (64.4%) of 118 patients had plasma HIV-1 RNA levels (viral load) <50 copies/mL. Median plasma HIV-1 RNA in patients with a detectable viral load was 2596 ($3.4\log_{10}$) copies/mL, ranging from 1379 to 16,275 copies/mL. Median CD4 count (percentage) was 685 (32%) cells/μL, ranging from 514 to 1025 cells/μL and the nadir of the CD4 count (percentage) was 352 (15.1%), ranging from 179 to 518 cells/μL. All patients received HAART, and it was prescribed for a median (range) of 9.2 (5.2–11.1) years and included a median of 5 nucleoside analog reverse transcriptase inhibitors, 1 nonnucleoside analog reverse transcriptase inhibitor and 3 protease inhibitors. Fourteen percent

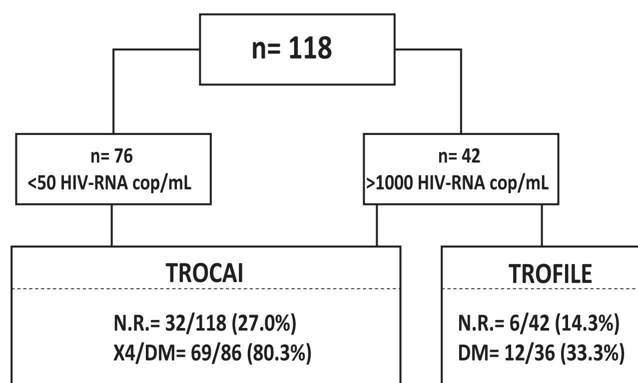

FIGURE 1. Flow diagram showing distribution of the patients and tropism results. DM indicates dual/mixed tropism; X4, CXCR4 tropism; NR, nonreportable.

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(17/118) of the treated patients were receiving a nonnucleoside everse transcriptase inhibitor–based regimen, 79% (93/118) were receiving a protease inhibitor–based regimen and 7% (8/118) a triple nucleoside regimen. The main characteristics of the study population are shown in Table 1.

HIV-1 Coreceptor Usage

The TROCAI test was performed in 118 patients. According to the TROCAI, HIV-1 coreceptor usage was not detectable in 32 of 118 (27%) patients, all of whom had plasma viral load <50 copies/mL. The overall prevalence of DM or X4 viruses was 80.3%, including 57.0% (n = 49/86) DM and 23.3% (n = 20/86) X4. Only 19.7% (n = 17/86) showed R5-tropic variants (Tables, Supplemental Digital Content 1 and 2, http://links.lww.com/INF/B295 and http://links.lww.com/INF/B296). When viral tropism was analyzed separately in children (n = 36) and adolescents (n = 82) with results by the TROCAI (n = 24 and n = 62, respectively), 70.8% (n = 17/24) of children and 82.2% (n = 51/62) of adolescents had viruses with DM or X4-tropic variants (Fig. 1).

Trofile (ES) was also performed in 42 of 118 patients who had plasma viral load >1000 copies/mL (Fig. 1). No patients showed X4-tropic variants, and 33.3% (n = 12/36) showed DM variants. HIV tropism was not detectable by Trofile in 14.3% (n = 6/42) of patients. Discrepancies between methods were observed in 22.2% (n = 8/36) of patients, in whom tropism was classified as R5 by Trofile (ES) but as DM or X4-tropic variants by TROCAI (Table, Supplemental Digital Content 1, http://links.lww.com/INF/B295).

TABLE 1. Baseline Characteristics of the Study Population

	n = 118
Age in years; median (IQR)	14.6 (10.9–17.0)
Girls; n (%)	70 (59.3)
Geographic origin; n (%)	
Western Europe (Spain)	110 (93.3)
Central America (Ecuador)	2 (1.7)
North Africa (Morocco)	1 (0.8)
Equatorial Africa (Nigeria, Guinea)	4 (3.4)
Southern Asia (India)	1 (0.8)
HIV subtype	
B	85 (72.2)
C	2 (1.7)
F	1 (0.8)
CRF02_AG	4 (3.4)
CRF12_BF	1 (0.8)
CRF13_cpx	1 (0.8)
CRF43_02G	1 (0.8)
URF_A2/D	1 (0.8)
URF_B	1 (0.8)
URF_BC	1 (0.8)
URF_BF	1 (0.8)
Unknown	19 (16.3)
HIV-1 RNA copies/mL (>50 copies/mL); median (IQR)	2596 (1379–16275)
HIV-1 RNA log10 copies/mL (>50 copies/mL); median (IQR)	3.4 (3.1–4.2)
$CD4^+$ T cell count, cells/μL; median (IQR)	685 (514–1025)
$CD4^+$ T cell, %; median (IQR)	32.0 (25.1–38.8)
$CD8^+$ T cell count, cells/μL; median (IQR)	945 (705–1250)
$CD8^+$ T cell, %; median (IQR)	40.9 (33.1–47.5)
Nadir $CD4^+$ T cell count, cells/μL; median (IQR)	352 (179–518)
Nadir $CD4^+$ T cell, %; median (IQR)	15.1 (10.0–22.5)
Immune category; n (%)	
≥500 cells/μL	72 (61.0)
200–499 cells/μL	34 (28.8)
<200 cells/μL	12 (10.2)
Clinical category; n (%)	
A	35 (29.7)
B	36 (30.5)
C	47 (39.8)
Years of HAART; median (IQR)	9.1 (5.2–11.1)
NRTI DRM, median (IQR)	2 (0–5)
NNRTI DRM, median (IQR)	1 (0–1)
PI DRM, median (IQR)	1 (0–5)
gp41 DRM, median (IQR)	0

IQR indicates interquartile range; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor; DRM, drug resistance mutation; URF, unique recombinant form.

Association Between HIV-1 Coreceptor Usage and Epidemiologic and Clinical Variables

No association was observed between HIV-1 coreceptor usage and gender in children and adolescents (Table 2). No association was observed between age and HIV tropic variants in adolescents. However, children who harbored X4/DM-tropic variants were younger, 7.3 years (median interquartile range: 5.1–10.3 compared with children who harbored R5-tropic variants; P = 0.031; Table 2). Between HIV-1 coreceptor usage and plasma viral load, no association was noticed either in children or in adolescents (P = 0.722 and P = 0.556, respectively; Table 2).

However, a significant correlation was found between coreceptor usage and CD4 percentage in both groups. It is interesting to note that patients with R5 variants showed significantly lower CD4 percentage (25.0% children and 26.6% adolescents) than patients infected with X4-tropic variants (36.4% children and 33.0% adolescents; P = 0.011 and P = 0.027, respectively). The nadir CD4 percentage was similar in children with R5-tropic and X4-tropic variants (18.5% and 21.9%, respectively; P = 0.533) and in adolescents (15.8% and 14.1%, respectively; P = 0.298; Table 2). However, a significant correlation was observed between nadir CD4 count and HIV tropism in children, with higher nadir median CD4 count (652 cells/mL) in children with X4-tropic variants than nadir median CD4 count (263 cells/mL) in children with R5-tropic variants (P = 0.001; Table 2).

DISCUSSION

The main objective of this study was to determine the proportion of vertically HIV-1–infected children and adolescents who had the R5 tropism that would be required for treatment with CCR5 antagonists. The preferential transmission of R5 against X4 viruses has been observed in the 3 main routes: sexual, blood products and mother-to-child transmission.[21] It was shown that all vertically HIV-infected children carried the R5 virus at the time of diagnosis of HIV infection independent of the mothers carrying X4 or R5 viruses.[12] However, if the mothers were carrying the X4 virus, although the child had R5 virus, the virus switched from R5 to X4 or to R5X4 sooner than if the mothers were carrying R5 virus at the moment of the delivery. Therefore, this points to a link between the presence of an X4 virus in the mother and the emergence of an X4 virus in her offspring.[12] We also know that immunologic deterioration in HIV-1–infected children precedes the viral phenotypic switch from R5 to X4 or R5X4-tropic variants.[12] We found that more than 80% of older children (7–8 years) and adolescents harbored DM or X4-tropic variants. These results are consistent with prior evidence that R5-tropic variants are responsible for the early establishment of HIV-1 infection as observed in newborns, whereas the emergence of X4 variants tends to occur later, as disease progresses.[22,23] Our results have important clinical implications because only 20% could benefit from maraviroc. This agent is well tolerated and safe in adults, which can facilitate better adherence. It has already been in use for adolescents and should soon be available for children. These advantages are particularly

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TABLE 2. Association Between HIV-1 Coreceptor Usage With Epidemiologic and Clinical Characteristics

	Antiretroviral-experienced Children n = 24			Antiretroviral-experienced Adolescents n = 62		
	R5	X4	*P*	R5	X4	*P*
Epidemiologic						
Female, n (%)	5 (71.4)	12 (60.0)	0.678	6 (54.5)	32 (58.2)	1.000
Age years, median (IQR)	11.0 (8.6–12.0)	7.3 (5.1–10.3)	0.031	15.0 (13.0–17.5)	16.2 (16.2–17.5)	0.413
Clinical						
Median detectable plasma HIV-RNA, log copies/mL (IQR)	3.8 (3.3–4.2)	3.3 (3.2–3.7)	0.722	3.6 (3.2–4.1)	3.5 (3.0–4.3)	0.556
Median CD4 count, cells/mL (IQR)	693 (536–739)	1168 (685–1675)	0.094	464 (337–563)	685 (557–985)	0.003
Median CD4 percentage, cells/mL (IQR)	25.0 (18.4–28.2)	36.4 (33.3–38.7)	0.011	26.6 (18.3–30.3)	33.0 (27.0–40.0)	0.027
Nadir CD4 count, cells/mL (IQR)	263 (63–372)	652 (416–1005)	0.001	284 (150–328)	311 (156–456)	0.588
Nadir CD4 percentage, cells/mL (IQR)	18.5 (15.1–22.2)	21.9 (17.0–7.0)	0.533	15.8 (8.2–26.8)	14.1 (7.6–18.0)	0.298

IQR indicates interquartile range.

relevant for very experienced patients with high levels of drug-resistant viruses and of toxicity to other drugs, for whom CCR5 antagonists could be one of the few treatment options.

The presence of X4 viruses has been linked to faster progression to AIDS and a rapid fall in $CD4^+$ T cell count in adults and young children in the natural history of the infection and during the first years of ARV treatments.[22] However, our results show that vertically HIV-infected older children and adolescents who harbor X4-tropic viruses have higher CD4 counts than patients with R5 variants. However, the observed CD4 counts in both groups were consistent with immunocompetent status. In fact, all the patients included in the study had CD4 levels equal or above 25% and, so, were not immunosuppressed. Furthermore, the nadir $CD4^+$ percentage was similar in patients with R5-tropic and X4-tropic variants. Taken together, these observations suggest that the positive impact of current HAART regimens is the dominant factor related to disease progression in contrast to the observed association of X4 tropism with more rapid disease progression in children in the pretreatment or early antiretroviral therapy era. One additional explanation for this finding could be that when all the studied patients were analyzed, patients with R5 virus had been diagnosed for a longer period of time, which was associated with lower $CD4^+$ T cell levels.

It was interesting to note that children and adolescents with undetectable viral load harbored higher proportion of X4 virus compared with patients with detectable viral load. A possible explanation for this finding could be that long-term HAART could induce the emergence of X4-tropic virus, although this premise is controversial. Several studies suggest that HAART could facilitate the necessary conditions that stimulate the gradual emergence of X4-tropic variants through infection. In a study performed in 28 patients infected with class-C viruses, the emergence of X4-tropic variants correlated with the use of ARV treatment.[24] In a longitudinal study performed in 32 patients with suppressed HIV-1 viremia receiving HAART for more than 5 years, the emergence of X4-tropic variants was observed in 11 of 23 patients, whereas X4-tropic variants were maintained in the 9 patients with X4-tropic variants at baseline.[25] The lack of longitudinal follow-up in this cohort does not allow us to conclude that HAART facilitates the gradual emergence of X4 variants through infection.

Complete concordance has been demonstrated between the TROCAI test[26] and MCT in HIV-infected adults.[16] However, a discordant rate of 15% was observed between the virologic response observed in MCT and Trofile,[19] similar to a study previously performed between 2 well-characterized recombinant phenotypic coreceptor assays.[20] Therefore, the discordant rate between Trofile and TROCAI would be expected to be around 15%.[10,15,27] The increase tendency between Trofile and TROCAI in the discordant rate observed in nonresponder HIV patients could therefore explain the discordant rate (22%) observed in our study.

Finally, as described earlier, recognition of X4-tropic variants at the beginning of HIV-1 infection is rare. However, in approximately half of all adult patients, progression of HIV-1 infection from asymptomatic stages to AIDS is associated with a switch in viral coreceptor usage from R5-tropic isolates to X4-tropic isolates associated with depletion of $CD4^+$ T cells.[28] The pathogenicity of X4-tropic strains may be explained by the ability of these isolates to infect thymocytes, the precursor cells of mature $CD4^+$ T lymphocytes.[22,29] Although HIV-1 infection affects the thymus in children and adults,[30] the consequences of thymic inhibition may be worse in children,[31] thus jeopardizing the production of new T lymphocytes and leading to poorer immune reconstitution. Moreover, in young children, the presence of X4-tropic variants could jeopardize their capacity to generate an adequate T cell repertoire. This, together with an immature immune system, could result in greater vulnerability to infection by HIV-1 or other pathogens.

In conclusion, X4-tropic variants are present in more than 80% of antiretroviral-experienced vertically HIV-infected older children and adolescents enrolled in the CoRISpe node 1 cohort. These variants may limit the usage of the CCR5 antagonist family. The consequences of this observation could be fatal for patients who present a large number of resistant mutations and, therefore, exhaust options with previously used drug families.

ANNEXE

Hospital General Universitario "Gregorio Marañón", Madrid; Hospital Universitario "Virgen del Rocío," Sevilla; Hospital Universitario "Ramón y Cajal," Madrid; Hospital Regional Universitario "Carlos Haya," Málaga; Hospital Universitario "La Paz," Madrid; Hospital Universitario de Getafe, Madrid; Hospital Universitario "Carlos III," Madrid; Hospital Universitario "Doce de Octubre," Madrid.

HIV Spanish Pediatric Cohort Node 1 (CoRISpe-1): Participating Hospitals and Personnel

Hospital General Universitario "Gregorio Marañón": VB, DG, A. García-Torre, S. J. de Ory, D. Gurbindo, MLN, J. Saavedra and MAM-F. Hospital Universitario "Virgen del Rocío": GM-L, ER-M, JALL and ML. Hospital Universitario "Ramón y Cajal": M de M, G. Yebra and A. Holguín. Hospital Regional Universitario "Carlos Haya": D. Moreno, E. Nuñez-Cuadros. Hospital Universitario "La Paz": MI de J. Hospital Universitario de Getafe: JTR. Hospital Universitario "Carlos III": MJM. Hospital Universitario "Doce de Octubre": M. Gómez-Tomé, Pablo Rojo.

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Characterization of the B-cell response against HERV-K (HML-2) TM protein during HIV-1 infection

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Conflict of Interest: HAM, SGD and DFN are listed as inventors on a patent application in relation to some of this work.

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Abstract

Human Endogenous Retroviruses (HERVs) comprise about 8% of the human genome. They have lost their ability to replicate or to produce infectious particles after having accumulated mutations over time. In HIV-1 infected subjectsthere is an increase of HERV-K (HML-2) mRNA coding for Env and Gag in the plasma of infected patients. In this study, we assessed the kinetics of expression of HERV-K (HML-2) EnvSU and TM subunit specific mRNA transcripts and proteins production in HIV-1 infection, and mapped the humoral response to these subunit proteins. We found that HIV-1 preferentially induces HERV-K (HML-2) TM mRNA expression, resulting in the expression of a fully N-glycosylated HERV-K (HML-2) envelope protein on the cell surface. Serological mapping of HERV-K (HML-2) envelope protein linear epitopes revealed two major immunogenic domains, one on SU and another on the ectodomain of TM. We showed that HIV-1 infection influences the humoral response to both these domains. The titers of anti-TM antibodies were increased in HIV-1 infected subjects, and correlated with HIV-1 plasma viral load in viremic non-controllers. Furthermore, elite controllers who naturally suppress HIV-1, had a high titer response against TM, compared to HAART suppressed and healthy donors. This suggests that anti-TM antibodies are involved in the pathogenesis of HIV-1 infection, and provide a new therapeutic opportunity.

INTRODUCTION

Current combination antiretroviral drug therapies have helped to reduce morbidity and mortality from AIDS but fail to eradicate the virus from latent reservoirs, necessitating lifelong treatment. The inability to eliminate virus is thought to be due to cellular viral persistence and limited drugbio-distribution,especially in lymphoid tissues or secondary mucosal associated lymphoid structures[1].While HIV-1 stimulates a vigorous cell mediated and humoral immune response which helps to contain viral replication, these responses are unable to clear virus from sequestered reservoirs. This has stimulated the search for alternate antigens which might mark an HIV-1 infected cell, and create an opportunity for vaccine induced responses to stimulate non-HIV-1 antigen immunity which can still target and eliminate HIV-1 infected cells as a prophylactic or immunotherapeutic vaccine[2,3,4,5].

Human Endogenous Retroviruses (HERVs) comprise about 8% of the human genome[6]. They have lost their ability to replicate or to produce infectious particles after having accumulated mutations over time. They are now considered as stably integrated, largely silent, and transmitted in a Mandelianfashion[7]. However, among the different HERV families, HERV-K (HML-2) is the most recently active HERV, with many elements unique to humans[8]. Integration of the different HERV-K families occurred from 12 million years ago for the most ancient to 0.12 million years for the most recent[9]. This diversity providesheterogeneity in their genomic structures, the presence of origin of replication (ORF) and their ability to express proteins.Their genomic organization is similar to exogenous viruses. The *gag*, *pol*, *pro*, and *env*genes are flanked by two Long Terminal Repeat (LTR), with ORFs for all genes for the most recent HERVs such as HERV-K (HML-2) 113,which is able to produce viral particles *in vitro*[10].

Despite the predominant transcriptional silencing, HERV activities are observed in several diseases and physiological states. HERV-K (HML-2) is actively studied in autoimmune diseases[11,12,13,14,15,16]and cancers[17,18,19,20,21,22],and mRNA and protein expression are frequently found in blood or tissue *in vivo*. HERV translated products caninduce an immune responsethat correlateswith disease progression or regression in some cancers[23,24,25,26,27,28].

In HIV-1 infected subjects, our lab and others have shown an increase of HERV-K (HML-2) mRNA coding for Env and Gag in the plasma of infected patients compared to uninfected patients[2,29,30]. However, to date, there areno reports of HERV-K (HML-2) Env expression on HIV-1-infected cells *in vivo* or *in vitro*.HERV-K Gag expression has been found in CD3+CD4+ cells from HIV-1 infected patients, but didnot correlate to viral load or CD4+ T cell count[31]. HERV-K (HML-2) Env is composed of three proteins, the signal peptide (SP), the surface unit (SU) and the transmembrane (TM) protein or gp36. Recently, the SU protein was found in plasma froman HIV-1 infected patient[29].

The exact mechanisms of HERV-K (HML-2) reactivation are not well understood, but may befrom activity of HIV-1 proteins Vif and Tat.Vif promotes the degradation of

APOBEC3G that participates in the silencing of HERV-K (HML-2)[32,33]. A recent study shows the HIV-1 Tat protein induces expression of mRNA coding for the HERV-K (HML-2) gag protein[34]. These studies strengthen the concept that HIV-1 specifically induces the transcription of HERV-K (HML-2) mRNA which results in the expression of HERV-K (HML-2) proteins in infected cells.

In this study, we assessed the kinetics of expression of HERV-K (HML-2) EnvSU and TM subunit specific mRNA transcripts and proteins production in HIV-1 infection, and mapped the humoral response to these HIV-1 induced neoantigens, or HIV-1 Associated Neo-Antigens (HANA). We found evidence that HIV-1 preferentially induces HERV-K (HML-2) TM mRNA expression, resulting in the expression of a fully N-glycosylated HERV-K (HML-2) envelope protein on the cell surface. Serological mapping of HERV-K (HML-2) envelope protein linear epitopes revealed two major immunogenic domains, one on SU and another on the ectodomain of TM. We showed that HIV-1 infection influences the humoral response to both these domains. The titers of anti-TM antibodies were dramatically increased in HIV-1 infected subjects, and correlated with HIV-1 plasma viral load in viremic non-controllers. Furthermore, elite controllers who naturally suppress HIV-1,had a high titer response against TM, compared to HAART suppressed and healthy donors. This suggests that anti-TM antibodies are involved in the pathogenesis of HIV-1 infection, and provide a new therapeutic opportunity.

RESULTS

Evidence of TM trans-activation and post-transcriptional modification

To investigate the mechanism by which HIV-1 infection modifies the transcription or the expression of HERV-K (HML-2) (HK) Env, we first measured the expression of HK TM or SU mRNA in plasma before and after HIV-1 infection. We designed two pairs of primers to perform nested PCR on plasma-isolated viral RNA from a subject on whom samples were available preceding and during acute HIV-1 infection. This subject had a rapid rise in HIV-1 plasma viremia which peaked at day 42. He elected to have antiretroviral drug therapy with HAART, and viral load declined from the initiation of treatment at d76 (Fig. 1A). There was a strong signal at d42 for TM mRNA (Fig. 1A), which corresponded to the peak of HIV-1 viremia. At d104, after treatment initiation and decrease in HIV-1 plasma viral load, mRNA was detected for both TM and SU (Fig. 1A). These results show a differential transactivation of HK TM and SU mRNA transcription during HIV-1 infection.

We next investigated whether transcription of HK Env products in an HIV-1 infected cell resulted in protein expression *in vitro*. Using two commercially available anti-HK TM and SU antibodies[29,35], we detected protein expression in HIV-1 infected Hela-T4 cells stably transfected with CD4 (Fig. 1B). The two sub-units were found at different molecular weights (MW) as expected, with one precursor (high MW) and one mature protein (low MW). Uninfected Hela-T4 cells showed SU, but not TM expression (column 1, Fig 1B), whereas HK transfected cells showed two bands for TM (precursor 70-100KDa) and mature TM (40KDa) (column 2, Fig. 1B). HIV-1 infected-HK Envuntransfected cells had the TM precursor protein (column 3, Fig. 1B). As the HKEnv TM protein has N-glycosylation sites, we investigated glycosylation of

the HK Envproteins by using Tunicamycin, a N-glycosylation inhibitor. The TM protein was modified (column 4, Fig. 1B). These results indicate that HIV-1 infection acts mainly on the HKEnvTM precursor form resulting in a glycosylated TM. A recent study which reconstituted a functional HERV-K (HML-2) TM gp36 from a consensus HERV-K (HML-2) sequence showed that the N-glycosylation state affects the membrane location and function of gp36[36]. We next assessed whether there was an increase in HK-Env TM on the cell surface by immunofluorescence. The data supports an HIV-1 induced neo-antigenic epitope on HK-Env TM through glycosylation of the TM protein on PBMCs *in vitro* (Fig. 1C).

Identification of two linear antibody epitopes in HERV-K (HML-2)Env

As HIV-1 infection revealed a new HKEnv TM epitope expressed on the surface of infected cells we hypothesized that the serological response to HK Env would differ between HIV-1 infected and uninfected subjects. We mapped humoral responses on the HKEnv protein using a set of 172 15mer HK Env peptides in a peptide-based ELISA assay. We initially used sera from 4 HIV-1 infected subjects and 2 from uninfected healthy donors (HD). Two strong immunogenic domains were identified, one with homology to a domain previously described on the HK Env SU protein, and a new epitope on gp36 (Fig 2A). We then tested the sera using SU or TM recombinant proteins. Selected sera from HIV-1 positive or negative subjects were tested against the corresponding recombinant protein and concordance of responsiveness to protein and peptide was noted (Fig. 2B). We identified two immunodominantHK Envhumoral domains.

A longitudinal study of anti-HK humoralresponse before and after HIV-1 infection

In order to better assess the effect of HIV-1 infection on HERV antibody responses, we measured anti-HK Env TM and SU antibodies in one high risk subject before and after infection with HIV-1. Before infection, he had a low titer of anti-SU IgG antibodies, and a modest anti-TM IgG titer. These levels remained static at day 10, the time of the first detection of HIV-1 plasma viremia (Fig. 3A). One month later, viremia had increased to 2 x 10^6 RNA copies/ml.The anti-HK TM response increased in parallel with HIV-1 plasma viremia, while the level of anti-HK SU antibodies decreased below the level of detection. After initiation of antiretroviral treatment the anti-TM response decreased at the same rate as decrease in plasma viremia, but the anti-SU response increased to pre-infection titer (Fig. 3A).

We then compared the titers of anti-SU or TM response in 4 additional subjects before and after HIV-1 infection. The anti-SU response decreased or remained undetectable after infection (Fig. 3B) whilst the anti-TM response increased or remained stable (Fig. 3C). The mean of the difference of antibody titer (after infection – before infection) showed a significant difference between TM and SU kinetics (Fig. 3D).

Detection of HK Env specific B-cells

As we had detected differences in serological responses to TM and SU, we next assessed the magnitude of the HK Env specific B-cell response using flow cytometry in one healthy donor and one HIV-1 infected subject(OP-115). For OP115 we used

three time points: an early time point (d116 post infection), a later time point (d352 post infection), and one time point when the patient was on anti-retroviral treatment (d383 post infection). The HIV-1 infected patient had a higher percentage of B cells for both anti-SU and anti-TM B-cell (4.24% and 3.46%, respectively) compared to the healthy donor (Fig. 4A). Interestingly, the percentage of anti-SU B-cells remained stable over time, while the percentage of anti-TM B-cells increased with sustained high viral load and decreased on HAART (Fig. 4A). Based on membrane IgD and CD27 expression, we observed that the composition of the anti-TM and SU B-cell sub-classeswere similar at d116. However, as the percentage of specific TMB cells increased over time, the population of unswitched and switched memory B-cells (respectively IgD+ CD27+ and IgD- CD27+) were also increased (Fig 4B). With HAART, the percentage and composition of anti-TM B-cells decreased to baseline levels. These results corroborate the serological studies, and demonstrate the induction of an anti-TM response after HIV-1 infection, which decreases in titer with successful HAART therapy.

Comparison of the humoral response against HERV-K Env SU and TM in healthy donors and in HIV-1 infected subjects

We next performed a serological screen of HK Env responses in 80 chronically untreated HIV-1 infected subjects, and 40 healthy HIV-1 uninfected donors (HDs) in a cross sectional study. The titration of the two responses showed that 15 percent of the HDs had anti-SU antibodies, whilst 65 percent had anti-TM antibodies at a low titer (1:200) (Table-1). Interestingly, the antibody responses to SU and TM were differentially modified in HIV-1-infected subjects. At an equivalent titer, the mean of the anti-SU titer was significantly decreased in HIV-1 infection ($p=0.0049$) (Fig. 5A), while the mean of anti-TM antibody was significantly increased compared to HDs ($p<0.0001$) (Fig 5B). The titration of the anti-TM response showed responses up to 1:3200 (Table 2B). A comparison of within subject anti-TM or -SU responses showed exclusivity of specificity, with those making anti-TM responses in general not making SU responses and vice versa (Fig 5C).

We next determined whether there was any relationship between anti-HK Envhumoral response and clinical status in 40 elite controllers (EC) and 40 viremic non-controllers (VNC). We found no difference between EC and VNC for the anti-SU response (data not shown). However, VNC had a significantly higher titer of anti-TM antibodies (up to 1/3200) compared to EC (up to 1/1600) (Table 2B), and both HIV-1 infected groups had a higher response compared to the HDs (Fig 5D). In subjects on HAART, there was an increase in anti-SU responses and a decrease in anti-TM response (Fig. 5E,F).

There was no correlation between the anti-SU response and plasma viremia or CD4+ T cell count. However, there was a very strong correlation between the anti-TM humoral response and the plasma viral load ($p=0.0083$), and an inverse correlation with the CD4+ T cell count ($p=0.003$) (Fig 6 A,B). This cross sectional study of 120 HIV-1 positive subjects confirms the results from the early infection study.

DISCUSSION

In this study, we investigated how infection with HIV-1 influences HERV-K (HML-2) envelope SU and TM subunit mRNA and protein expression,and assessed the humoral response to these two HERV-K (HML-2) Envproteins. We showed for the first time that the HERV-K (HML-2) TM protein expression was induced by HIV-1 infection, and this expressed protein stimulated an anti-TM antibody response. We mapped the major antigenic epitope in the TM protein, and describe this as anHIV-1 associated neo-antigen(HANA), as it is a self-antigen preferentially expressed in the context of an HIV-1 infected cell. In determining potential correlations between HERV-K (HML-2) Env antibody responses and HIV-1 viral load in HIV-1 infected subjects at different clinical stages, we found that HIV-1 infected elite controllers had high titers of anti-TM antibodies compared to HIV-1 infected patients on effective HAARTor healthy uninfected donors. These findings suggest that the anti-TM humoral response may play a role in HIV-1 pathogenesis, and provides a new target for immunotherapeutic strategies.

We initially determined how HIV-1 infection impacted HERV expression. An *in vitro* infection with the lab-adapted strain HIV_{LAI} inducedfully glycosylated TM protein expression in CD4+ cells, but did not affect SU expression. The state of glycosylation is crucial for the production of a functional glycoprotein TM gp36, anda previousstudy had shown that a fully glycosylated gp36 can reach the cellular membrane (HML-2)[36]. In the normal physiological state, TM is absent from the membrane, probably because ofweakgene expression and a leak of N-glycosylation. With HIV-1 infection, the induction of TM protein expression was enough to stimulate a specific TM humoral response, and break tolerance.

In this study we identified two major immunogenic antibody epitopeson HERV-K (HML-2) EnvSU and TM by using overlap peptides and recombinant proteins. Prior studies of anti-HERV-K (HML-2) Envhumoral responseshave been based mainlyon anti-SU detection. The presence of adistinct epitope on each sub-unit may explain the heterogeneity of results presented in the literature. Thepresence and quantity of the humoral response against HERV-K (HML-2) during HIV-1 infection is controversial[37]. Some studies have shown an increase of antibodies against HERV-K (HML-2)[38,39], while others saw no differences between HIV-1-infected and non-infected patients[23,40,41]. The first study describing an anti-HERV-K (HML-2) humoral response showed no difference between HIV-1 infected and uninfected patients[41]. They tested the antibodyresponse by western blot, detecting serumpositive for recombinant SU. They found fifteen percent of HIV-1 infected patients were seropositive for SU,concurring with our data.Another study mapped the anti-SU response in healthy subjects and patients with autoimmune disorders and, interestingly, they found a similar epitope on SU[12] (Fig. 7A).

The only prior study that demonstrated an anti-HK TM gp36 antibody response was in pregnancy, wheregp36 was found to be expressed in extravillouscytotrophoblast. In the TM epitope identifiedin this report, theamino acid sequence DWNTS (in which the N residue is known to be glycosylated[36])is nestledbetween the two cysteines flanking the ectodomain(Fig. 7B,and Supplemental figure 1). This sequence is well

conserved among the HERV-K families (Fig. 7C), and recognized by sera independent of disease stage. Interestingly, there is some sequence homologybetween the TM epitope and an HCV polyprotein (Fig. 7D). We found no significant difference between HIV-1 positive HCV positive orHIV-1 positive HCV negative subjectsintheir anti-TM response (Supplemental fig.2). The SU epitope also shares some limited homology with the HIV-1 TAT protein (Fig. 7D).

The longitudinal and cross-sectional studies showed that HIV-1 infection induced an increase in anti-TM response, in agreement with the induction of TM coding mRNA and protein induction. Following infection, the anti-TM response increased, whilst the SU response decreased. After antiretroviral treatment initiation, as the viral load in plasma decreased, the TM response decreased in parallel, andthe SU response increased. These results from plasma were corroborated by the study of specific SU and TM B-cells. Furthermore, a small fraction of anti-SU CD19+ IgD- CD27high plasmablast cells werefound after treatment was started, supporting the rebound of anti-SU antibody titer in sera.

“Neo” TM expression appears to be induced by HIV-1 infection, and the change in anti-TM response post infection reflective of increased TM protein production. We do not know if other infections or pathologies also induce TM expression. In assessing a potential role for anti-TM antibodiesin pathogenesis, we observed a correlation between the anti-TM response and loss of CD4+ T cells(and increasing plasma viremia) for viremicnon-controllers (VNC),suggesting that the anti-TM response is driven by active HIV-1 replication. During viremicreboundanti-TM antibodies could target TM-expressing HIV-1 infected CD4+ cells and accelerate CD4+ cell depletion through mechanisms such as ADCC or CDC. However, the presence of an intermediate high anti-TM level in elite controllers in the absence of detectable viremia suggests that theanti-TM response could also play a role in the control of virus. Interestingly, the ratio of anti-TM/totalIgGand anti-SU confirmed the absolute value observed in the cross-sectional study and showedthat the anti-HERV-K (HML-2) TM humoral response was not increased due to polyclonal expansion,showingthat elite controllershave anti-TM production even in absence of a detectable viremia (Supplemental fig. 3). Further investigations are needed to understand if this anti-TM response is due to a memory response or driven by low level viral replication.

The expression of the TM protein during HIV-1 infection could have a direct role in immunopathogensis. Some viral glycoproteins are known to be immunosuppressive and some studies have shown HERV-K (HML-2) gp36 contains an immunosuppressive domain[35,42,43,44]. The increase of expression in viral non-controllersmight be linked to a progression of the disease, while the antibodies present in elite controllersmight help to inhibit a gp36 immunosupressive effect or even help in reducing the latent reservoir burden.

The use of HERV-K (HML-2) proteins as tumor- or viral-associated antigens has already been investigated in different models. Recently, we have shown the immunogenicity and safety of an endogenous retro-transposable elements-based vaccine therapy in non-human primates. The vaccine, a combination of DNA and

adenovirus coding for Simian-ERV-K, induced specific T-cell and B-cell responses, notably an anti-TM antibody response[3]. This opens the perspective of new vaccine strategies in the context of HIV-1.

HERV-K (HML-2) Env has also been shown to elicit antibodies in patients with breast cancer[27,45]or melanoma[24], and HERV-K (HML-2) SU has been suggested as a prognostic marker or therapeutic target. A mouse monoclonal antibody directed against SU showed strong anti-tumoral activity *in vitro* and *in vivo* in a mouse model[28,46]. In melanoma, pancreatic or prostatecancer, thetumor-associated antigen HERV-K-MEL has been proposed as a specific target for tumor cells[19,26,47].

So far, genetic determinants such as HLA class I may explain around 20 percent of the variance of host control. For example, B57 and B27 alleles are known to be disease progression protective alleles, but some B57+ patients may lose the control and become progressors[48]. This shows part of the complexity of HIV-1 viremia control. Two important factors in plans towards a functional cureare the inability of standard drug regimens to eliminate virus in latent reservoirs, and viral sanctuary sitesthan cannot be reached by anti-retroviral drugs. This has led to a renewed focus on mobilizing immune responses to augment drug regimens. It is interesting to speculate that anti-HERV (HML-2) antibodies could target infected latent reservoircells.

We have found a novel HERV (HML-2) Envelope TM neo-antigen only expressed in the context of an HIV-1 infected cell, which stimulates a specific humoral response. As elite controllers have higher titers of this antibody compared to progressors, this suggests that this antibody response may be of importance in viral control. These findingscould lead to a new approach to HIV-1 vaccines or immunotherapeutics.

MATERIALS AND METHODS

Study populations

Samples of peripheral blood mononuclear cells (PBMCs) were selected from participants in two different San Francisco-based HIV-1-infected cohorts: Options (n=5) and SCOPE (n=120). Samples from HIV-1-negative controls were obtained from the Blood Center of the Pacific of San Francisco (n=80). The study was approved by the local institutional review boards (University of California San Francisco Committee on Human Research) and individuals gave written informed consent. Studies were performed on cryopreserved PBMCs and sera.

PBMC and sera samples were obtained from the following categories of chronically HIV-1-infected individuals: 40 elite controllers (EC: naive for treatment, undetectable viral load for two years, CD4>350); 40 highly active antiretroviral therapy (HAART: Viremic suppressed with undetectable viral load for at least two years, CD4>350), and 40 untreated virologic non-controllers (naive for treatment, viral load >2000 copies/mL).

Table 1 describes baseline subject characteristics.

HIV-1$_{LAI}$ stock virus

Stocks of HIV-1$_{LAI}$, a CXCR4-tropic laboratory strain, were obtained from the AIDS Research and Reference Reagent Program and amplified on stimulated PBMCs. HIV-1-infected cells were pelleted at 3,000 rpm for 20 min, and supernatant fluid was passed through a 0.2-µm filter and frozen in aliquots at −80°C. The titers of stocks were determined using TZM cells [49].

mRNA isolation and Nested RT-PCR

Viral mRNA were isolated from 140µL of plasma using QIAamp® Viral RNA Mini Kit from Qiagen. mRNA obtained were directly used in One-Step RT-PCR Kit (Qiagen) according to the manufacturer. Briefly, 5µl of viral RNA equivalent to 15µl of plasma was reverse transcribed at 50^0C for 30 min. The 1st round PCR was performed in 20 cycles consisting of 94^0C for 30 sec; an annealing step at 55^0C for both pairs of outer primers (SU Forward: GTATCAATGGTGGTAAGTCTCC; SU Reverse: CACTGCAATTAAAGTAAAAAT; TM Forward: GCCATTTTATACTRTCGTCCTAA; TM reverse: GACAAAACCRCCATCGTACTCAT) for 30 sec; and an extension step of 90 sec at 72^0C. PCR product were next diluted at 1:50 and used as template for the 2nd round PCR. The second round was performed using Phusion® High-Fidelity PCR Master Mix in 35 cycles consisting of 98^0C for 10 sec; an annealing step at 60^0C for both pairs of inner primers (SU Forward: TGGATAATCCTATAGAARTAT; SU Reverse: TATGTTTGTCTAAACTTTCTGT; TM Forward: GCTGTAGCAGGAGTTGCATTG; TM reverse: TAATTGTAGTACTTCCAATGGTC) for 30 sec; and an extension step of 60 sec at 72°C. PCR products were separated on 1% agarose gels.

Transfection and HIV-LAI infection/N-glycosylation inhibition.

Hela-T4 were plated in 12well plates at 0.8×10^6 cells/well and transfected with HERV-K (HML-2) Env coding plasmid using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer protocol. Briefly, plasmid and Lipofectamin™ were mixed at 1:2 ratio for 20min at RT and incubated with cells for 16h. Untransfected and transfected cells were washed with PBS and infected with HIV-$_{LAI}$ or mock infected with 10µg/ml of tunicamycin (Sigma) or grow medium for 16h.

Western Blot

Hela T4 cells are lysed in presence of anti-protease cocktail (Sigma) in n-Dodecyl ß-D maltoside (Sigma) diluted at 0.1mg/ml according the manufacturer protocol in dH2O/0.05M-TRIS HCl/0.15M-NaCl lysis buffer. Prior of loading, the samples were mixed with lamely buffer 2x and boiled at 95ºC for 5 minutes. Approximately, 25µg of total proteins assayed with BCA Proteins assay kit (Thermo Scientific) were loaded on 4-16% gradient precast gels (Pierce). PVDF (Biorad) membranes were blocked 1h at RT in PBS/0.05%-Tween 20/10%-non fat dry milk and incubated with mouse monoclonal anti-HERV-K TM and anti-HERV-K SU primary antibodies, respectively HERM-1811-5 and HERM-1821-5 (Austral Biologicals) in PBS/0.05%-Tween 20/5%-non fat dry milk at 1/1000 over-night at 4ºC. Membranes are then washed 3 times in PBS/0.1%-Tween 20 and incubated with an HRP-conjugated anti-mouse or anti-human IgG (Abcam) 2 hours at room temperature. After 6 washes, the membranes

were incubated with ECL-plus (GE Healthcare) and exposed at different time points on Kodak® Biomax™ MR film.

Immunofluorescence

PBMCs were stimulated with 2 µg/ml of phytohemagglutinin (PHA-P; Sigma, St. Louis, MO) for 48h in RPMI-10% FBS complemented with IL-2 70U/ml before the addition of HIV-1_{LAI} at a MOI of 0.005 for 7 days in RPMI-10% FBS.

PBMCs were fixed in PBS/PFA 2% (Electron Microscopy Sciences) and coated on slides using Cytospin (300rpm 2minutes), washed twice with PBS and blocked 15 minutes in PBS/0.5%-BSA. Cells were incubated with anti-HERV-K TM (HERM-1811-5, Austral Biologicals) at 1:50 dilution in a humidified dark room at 37ºC for 1 hour and then washed 5 times with PBS. Then, the cells were incubated with an Alexa-555 anti-mouse IgG (Invitrogen) in a humidified dark room at 37ºC for 1 hour and then washed 5 times with PBS. DNA was then stained with DAPI (0.5µg/ml) for 5 minute at RT and the cells are washed 5 times in PBS and 1 time with dH20. Coverslips are next mounted with PermaFluor (Thermo Electron Corporation) on microscope slides (Fischerbrand) and dried in the dark. Slides were analysed on LEICA DM6000B microscope and photo acquired on Image-pro 6.2 (Scientific Computing).

ELISA

A set of 172 overlapping 15-mer HERV-K (HML-2) Env peptides (JPT Peptide Technologies, Berlin, Germany) was used to comprehensively map the humoral responses. Positive signals were next confirmed by peptides produced by two other companies (New England Peptide and Gene Script). Two peptides corresponding to the immuno-dominant epitopes defined on SU (RPKGKPCPKEIPKES) and TM (HRFQLQCDWNTSDFC) were next used for the whole study (Gene Script). 96 microtiter wells plate (Nunc-Immuno Plate MaxiSorp Surface) were coated for 1 hour at 37ºC with peptides at 10µg/ml in PBS or over-night at 4ºC with recombinant protein (GeneArt) at 5µg/ml in PBS. Plates were then washed 3 times with 200µL of PBS/0.05%-Tween 20 and blocked with 100µL of blocking buffer (PBS/2.5%-BSA) at room temperature (RT). The samples were diluted in blocking buffer and incubated 2h at RT in duplicates. Plates were then washed 3 times with 200µL of PBS/0.05%-Tween 20. An anti-human IgG HRP-conjugated secondary antibody was diluted at 1:1000 in blocking buffer and incubated at RT for 1 hour. Plates were then washed 6 times with 200µL of PBS/0.05%-Tween 20 and incubated for 10 minutes with 100µL of TMB (Invitrogen). Addition of 50µL H2SO4 2M stopped the reaction. The plates were read at 450nm and 690 nm for the background on a plate reader. Background from 690nm uncoated wells and PBS-BSA as negative controls was subtracted from the mean absorbance of the coated wells.

Tetramer preparation and B-cell staining

Adapted from [50]

All tetramers were prepared freshly for each experiment. Biotinylated-SU or -TM peptides (Gene Script) were incubated with premium-grade phycoerythrin-labeled streptavidin (Molecular Probes) for at least 20 minutes on ice at a molar ratio of 4:1.

Before cell staining, tetramer preparations were centrifuged for 10 minutes at maximum speed to remove aggregates.
PBMCs were isolated by standard Ficoll-Hypaque density gradient centrifugation on fresh blood samples and immediately cryopreserved in fetal calf serum (HyClone, Logan, UT) containing 10% DMSO (Sigma Aldrich, St. Louis, MO) in liquid nitrogen. The cryo-preserved cells were stored in liquid nitrogen until they were used.

Cells were thawed, washed, counted, and resuspended in PBS/5% FCS. For memory B-cell labeling, cells were enriched with the use of Human B Cell Enrichment Kit (Miltenyi). After enrichment, cells were adjusted to a cell density of $5x10^6$ cells/mL and stained with SU-Tet or TM-Tet and incubated on ice for 30 minutes with intermittent gentle vortexing. Cells were costained with CD3-Alexa700 (BD Biosciences) CD21-PeCy5 (BD Biosciences) CD38-ECD (Invitrogen) IgM-FITC (BD Biosciences) IgD-APC (BD Biosciences) CD27-PacificBlue (BD Biosciences) CD19-APC-H7 (BD Biosciences) for an additional 20 minutes on ice. LIVE⁄DEAD® Fixable Dead Cell Stain Kit was used to discriminate live and dead cells.

For all flow cytometry experiments, data were acquired with an LSR-II system (Becton Dickinson). At least 100,000 events were collected and analyzed with FlowJo software, version 9.0 (Tree Star, Ashland, OR).

Statistical analyses
Humoral responses assayed by ELISA were compared between groups using the Kruskal-Wallis, Dunn’s multiple comparison, or two-tailed Mann-Whitney t tests. Linear regression and Spearman correlation analyses were used to measure associations between humoral response and HIV-1 viral load or CD4+ T cells count. All tests were conducted using GraphPad Prism, version 4.00 (GraphPad Software, San Diego, CA), with the statistical significance of the findings set at a p value of less than 0.05.

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Table1. Characteristics of study subjects.
The table summarizes the demographic characteristics of the subjects.

Table 2.Titration of the anti-HERV-K (HML-2) Env responses.
Antibody titer for both SU and TM epitopes were determined by 2 fold serial dilution of the sera in a peptide based-ELISA. The higher dilution presenting a positive signal was retained as titer. An OD higher than four times the background defines a positive signal. The tables represent the % of positive patient for SU (A) or TM (B) epitopes at the highest dilution of serum.BD: Below detection; Total: Sum of the seropositive patients; HD: Healthy Donors; HIV+: Patients positive for HIV-1 infection without treatment. EC: Elite Controllers; VNC: Viremic Non Controllers; HAART; Infected patients under highly active anti-retroviral therapy.

Fig. 1.Evidence of trans-activation and post-transcriptional modification of TM.
A) Viral RNA was extracted from 140µl of plasma. The figure shows the amplicons obtained after the second round of PCR (around 500pb). TM mRNA is over-expressed during the peak of viremia at d42. Antiviral treatment induces the transcription of both SU and TM mRNA at a similar level. HD: healthy donor; OP-1830: HIV-1 seroconverter patient (d0: before infection; d12, d42, d76: after infection without treatment, d104: after treatment); water: the non-template well. (B) Assumed precursor proteins at 75 to 90 kDa and TM subunits at 32 to 38 kDa are visible. Hela-T4 cells are infected by HIV-1_{LAI} in presence (4) or not (3) of 10µl/ml of tunicamycin. 24h post-infection, cells are lysed and HERV-K Env TM and SU detected by western blot using Austral antibodies HERM-1811-5 (anti-TM) and HERM-1821-5 (anti-SU). An anti-ß Actin is used as loading control. HERK-Env transfected cells (2) are used as positive control of HERV-K TM and SU expression. Uninfected-untransfected cells were used as control for endogenous HERV-K basal expression (1). (C) Representative images of HERV-K TM extracellular expression on PBMCs. Increase of TM protein expression is detected after in vitro HIV-1_{LAI} infection.

Fig. 2.Identification of two linear epitopes on HERV-K (HML-2) Env.
(A) 4 sera from chronically HIV-1 infected patients (black) and 2 sera from healthy uninfected patients (grey) were used for the epitope identification by ELISA. The 3 sub-units signal peptide (SP), surface-unit protein (SU) and trans-membrane protein (TM) are represented by 172 redundant 15mers. The lines represent the average of the signal (OD) for each individual. (B) Sera from patients presenting antibodies reacting only with the SU-peptide (HIV#1 and #2), only with the TM-peptide (HIV#3 and #4) or negative for both epitopes (HIV#5, #6 and HD#1) were used to confirm the signal obtained with the peptide-based ELISA. Plain columns represent the peptides and the hatched columns represent the recombinant protein; SU in white and TM in gray. The ELISA was performed in duplicate and the bar errors represent the SEM.

Fig. 3.Kinetics of anti-HERV-K Env antibodies after HIV-1 infection.
A) Total IgG anti-HERV-K TM (◆) and SU (●) were assayed by ELISA on the same plate for 5 time points (1:200 dilution) for the patient OP-1830. The thin black line represents the CD4+ T cell count (cells/mm3) and the hatched line the $\log_{10}$ of the

HIV-1 copy number/mm^3.BC) Comparison of the anti-SU (B) and anti-TM (C) response before and after HIV-1 infection in 4 seroconverted patients. The time between the two time points does not excess 1 month. 1:200 sera dilution was used for this essay. D) The evaluation of the kinetic was determined by the subtraction of the OD of the time point after infection by the OD of the time point before infection. A negative result means a decrease of the response while a positive result means an increase of the response. The comparison of these two factors shows two opposite kinetics for the anti-SU and anti-TM responses. Statistical analysis by Mann & Whitney t-test; * $p<0.05$. Results are typical of three independent experiments.

Fig. 4.Anti-HERV-K TM and SU specific B-cell responses.

The staining of the specific HERV-K TM and SU B-cells is detailed in the materials and methods section. A) The presence of HERV-K specific B-cells was detected at three time points from the same patient OP-115: d116 and d352 post infection while the patient presents a high and increased viremia and still naïve for treatment and d383 post-infection, when the treatment suppressed the viremia. Plots show the double population CD19+tetramer+ and the graphics represent the specific B-cells sub-populations. B) The study of the B-cell subset was based on CD27 and IgD extracellular expression. Memory cells (CD27+) were discriminated by their IgD expression; CD27+ IgD+: unswitched memory; CD27+ IgD-: switched memory. A Plasmablast is defined by the absence of IgD expression and a high expression of CD27.

Figure 5.Humoral response against HERV-K Env TM and SU.

The detection of anti-TM and anti-SU antibodies was performed for 40 healthy uninfected donors and 80 HIV-1 chronically infected patients comprising of 40 elite controllers and 40 viremic-non-controllers. ELISA was performed as described in the material and methods section. The results are expressed using optical density (OD) after twice the background has been subtracted from the raw data. The sera were diluted at 1:200 for the anti-SU detection and at 1:400 for the anti-TM detection. AB) Comparison of the anti-SU (A) or the anti-TM (B) response between healthy donors (HD) and HIV-1 infected patients (HIV+). HD n=40 HIV+ n=80. (C) Plots represent the two antibody responses (SU and TM) for one HIV-1 infected individual patient. The detection was done on the same plate for the both epitopes with sera diluted at 1:200. N=80 (D) Detection of the anti-TM response in elite controllers (EC) and viremic non-controllers (VNC). HD n=80; EC n=40; VNC n=40. EF) Comparison of the anti-SU (E) or the anti-TM (F) response between HIV-1 infected patients on or off HAART. On HAART n=40; Off HAART n=80.

Detection of anti-SU antibodies. Sera were used at 1:200. OD were normalized with serum from a high responder in a standard curve. The STDEV intra experiment is less than 7%. Detection of anti-TM antibodies. Sera were used at 1:400. OD were normalized with serum from a high responder in a standard curve. The STDEV intra experiment is less than 4%.

The statistical significance of data between the different groups was established using the Mann & Whitney t test for A, B, E and F and a Kruskal-Wallis and Dunn's

Multiple Comparison Test for D. The figure shows the representative results of three independent experiments. A p value <0.05 considered as significant. *p<0.05, **p<0.01, ***p<0.001

Fig. 6. Anti-TM response correlates HIV-1 activity.

A) The viremia for 40 VNC was measured by real time PCR and expressed in log of copy/ml of blood. B) The anti-TM response for 80 HIV-1 infected untreated patients is inversely correlated to the CD4+ T cell count (cells/mm^3). The statistical analysis was performed with the Spearman test or with a Mann & Whitney t test, with a p value <0.05 considered as significant. The figure shows the representative results of three independent experiments. **p<0.01, ***p<0.001

Fig. 7.Epitope mapping and homologies.

AB) Identification of the residues recognized by sera on SU and TM peptides. A set of peptides with single mutation X to A was used in a peptide-based ELISA to determine which amino acids (aa) are recognized. The graphics represent the % of binding of the sera on the mutated peptide compared to the original peptide [(OD mutated peptide/OD original peptide)*100]. The original peptide corresponds to the peptide that gave the better signal for the mapping (see method section). The precise AA sequence was determined by reduction of binding. For both epitopes, sera from infected and healthy donor were used (6 for SU and 9 for TM). The results show that the sequences recognized are PKEIPKE for SU epitope and DWNTS for TM epitope. C) Comparison of the sequence of SU and TM peptides among the different main HERV-K (HML-2) families. (˙) represents same aa as the original sequence; (-) represents the absence of the aa; a letter represents a mutation. On the left are the names of the HERV-K (HML-2) families and their corresponding PubMed access numbers. D) Major homology of sequence between SU and TM sequence with other viruses. Underscored letters are the epitopes determined in the figure 7 A; homologies are represented in bold. The homologies were determined by using Blast tool from PubMed website.

Supplemental fig. 1.

HERV-K (HML-2) 108, SU and TM sequences.

The supplemental figure 1 represents the complete HERV-K (HML-2) 108 sequence used to design the set of 172 15mer peptides. Underlined are represented the peptides that gave the highest signal during the mapping: SU-56 for the epitope on the surface unit protein and TM-137 for the epitope on the trans-membrane protein. Dark green: Signal peptide; Purple: Surface Unit; Blue: Furin cleavage site; Green: Fusion peptide; Black: Extraviral part of transviral unit; Black/bold: ectodomain; Red: Transviral part; Brown: Intraviral part;

Supplemental fig. 2.Anti-TM response in HCV positive patients.

Results from the cross sectional study were analyzed regarding the HCV status of the 120 HIV-1 infected patients. 47 were identified as HCV positives and 69 as HCV negatives. This clinical information was not available for 4 patients. The statistical significance of data between the two groups was established using the Mann & Whitney t test.

Supplemental fig. 3.Ratio anti-HERV-K (HML-2) Env antibodies/total IgG.

The total IgG level was assayed using a human IgG ELISA kit (Mabtech) according to the provider protocol. The sera were diluted at 1/4000000. A ratio was calculated as following: anti-SU or anti-TM titer (OD)/total IgG (OD). n=40 for each groups. The difference of ratio shows the humoral responses against both SU and TM do not result from an unspecific polyclonal expansion. The statistical significance of data between the different groups was established using ANOVA Kruskal-Wallis and Dunn's Multiple Comparison. The figure shows the representative results of three independent experiments. A p value <0.05 considered as significant. *p<0.05, **p<0.01, ***p<0.001

Table 1. Characteristics of the cross-sectional study subjects

Participant category (n)	Median age (yr [IQR])	Gender [a] M	F	Median CD4+ T cell count (cells/mm3 [IQR])	Median HIV-1 viral load (copy/ml [IQR]
Elite controllers (40)	50 [44.25-55.75]	24	15*	771 [500-1108]	<50
HAART-suppressed (40)	50.50 [43.75-54.75]	31	8*	609 [452-807]	<50
Viremic non controllers (40)	39.50 [32.25-49]	35	3*+	444 [362-625]	40,474 [21,322-83,318]
HIV-1 negative (80)[b]	18-30 (18) 31-50 (24) 51-70 (34) >70 (4)	40	40	*n/a*	

a F, female; M, male

b Some information about the CD4+ T cell count was not available (n/a). Only a range of age was available.

* One patient in this cohort was transgender male to female

\+ One patient in this cohort was intersexe

Table2. Titration of the humoral response against HERV-K (HML-2).

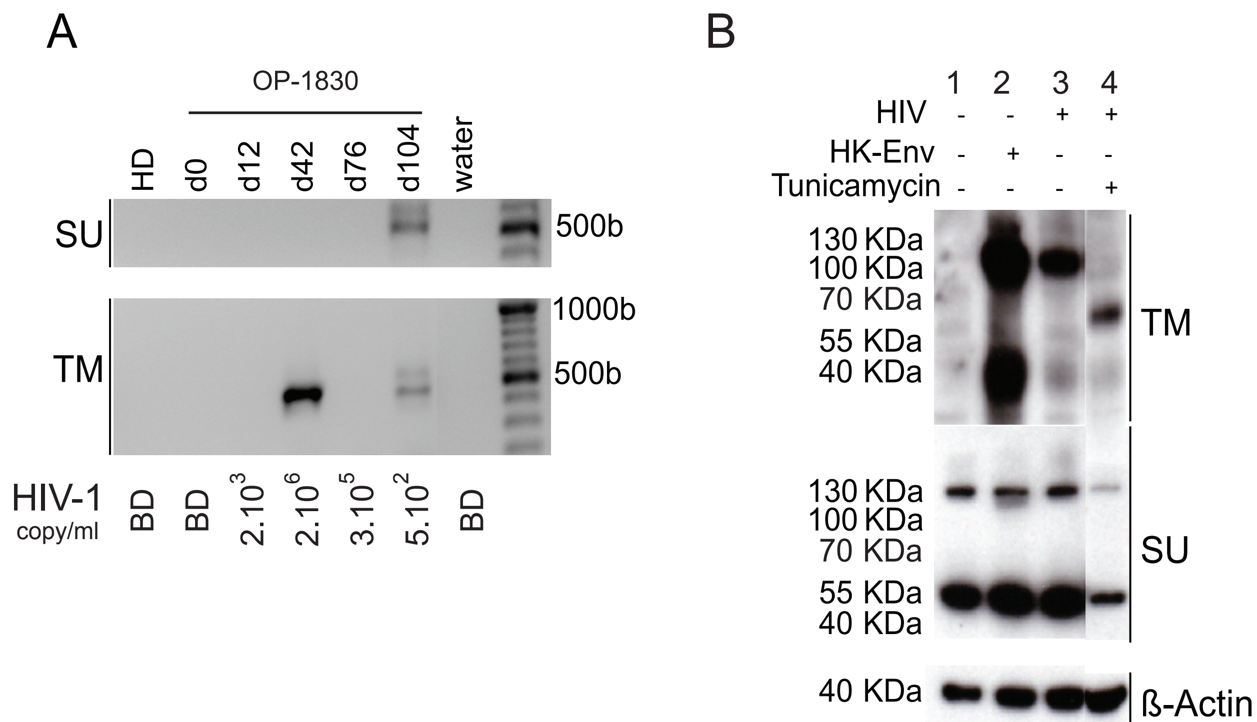
A: Anti-SU response

	HD	HIV+	EC	VNC	HAART
BD	85	88.25	91.5	85	77.5
1:200	10	10.25	5.5	15	10
1:400	2.5	1.5	3	0	10
1:800	2.5	0	0	0	2.5
1:1200	0	0	0	0	0
Total	15	11.75	8.5	15	22.5

B: Anti-TM response

	HD	HIV +	EC	VNC	HAART
BD	35	10.5	18	3	35
1/200	47.5	14.5	17	12	47.5
1/400	15	41.25	47.5	35	15
1/800	2.5	17.5	15	20	2.5
1/1600		13.75	2.5	25	0
1/3200		2.5	0	5	
Total	65	89.5	82	97	65

Figure 1. Evidence of TM trans-activation and post-transcriptional modifications



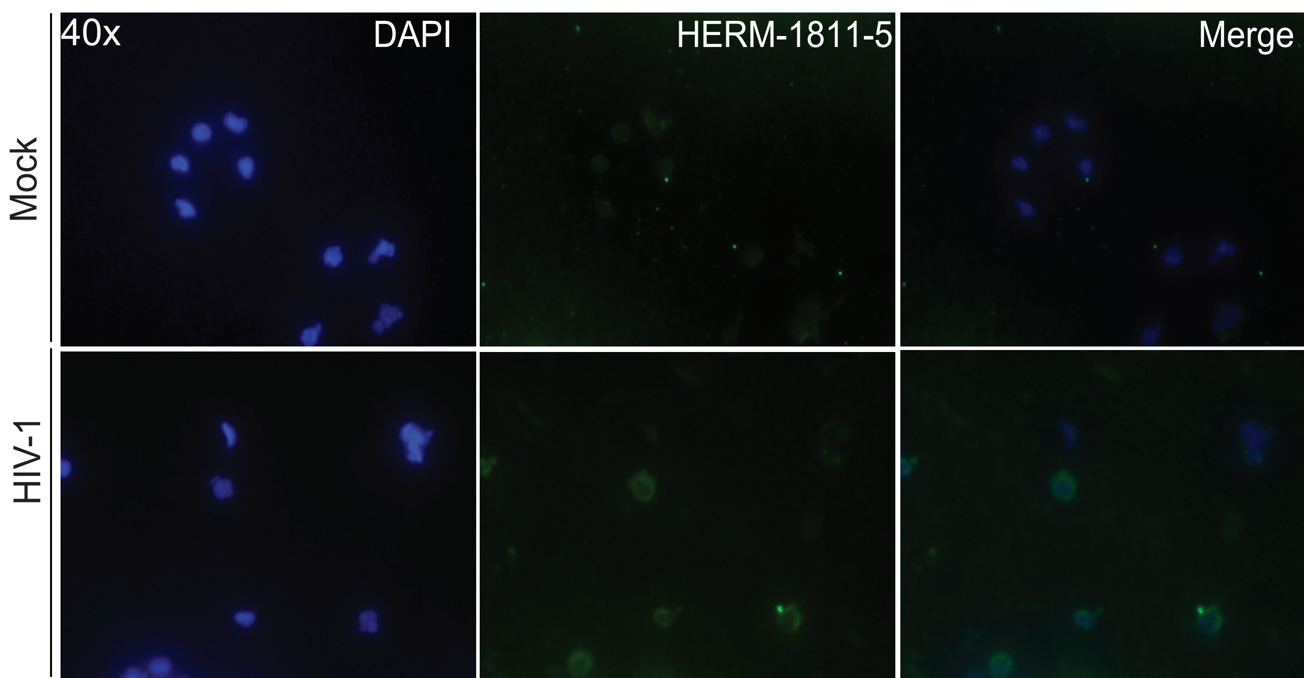
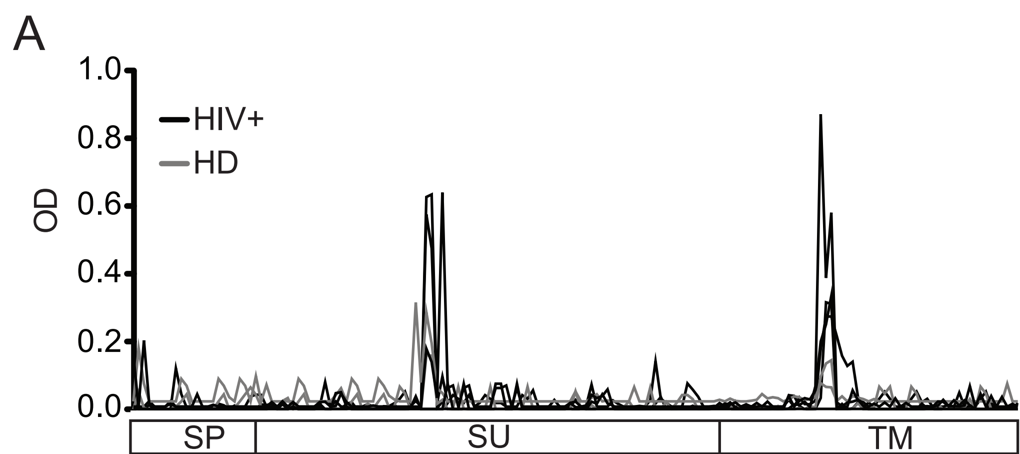


Figure 2. Identification of two linear antibody epitopes in HERV-K Env.



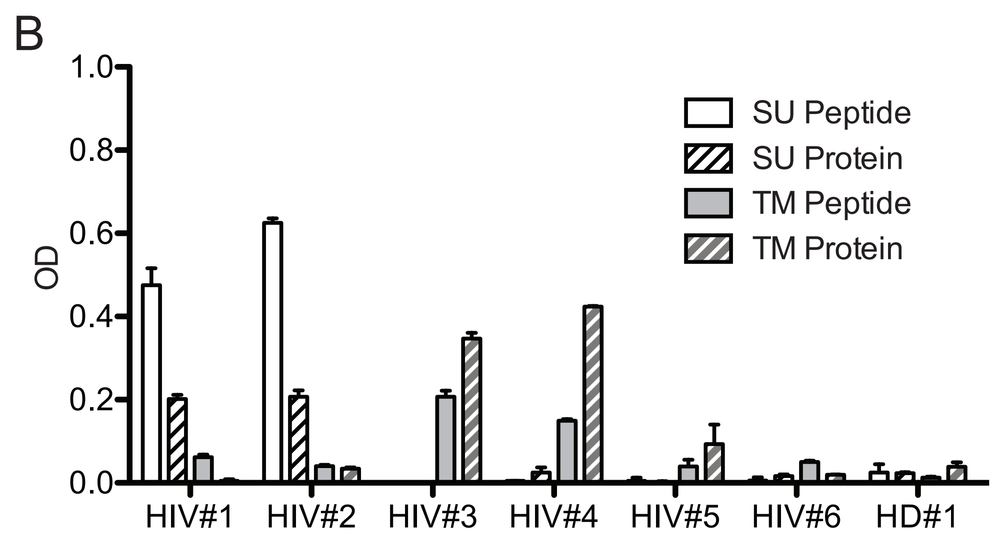
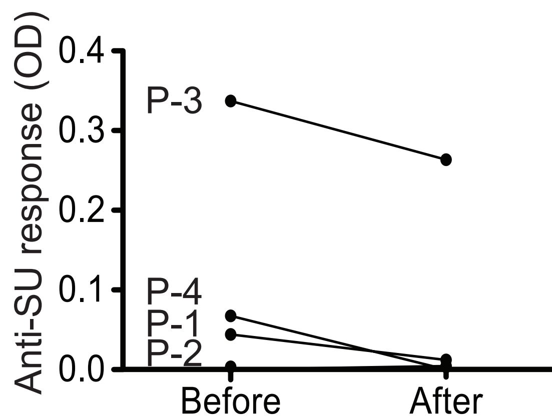


Figure 3. Kinetics of anti-HERV-K Env antibodies after HIV-1 infection.

A

CD4
Viral load
anti-TM
anti-SU
HAART
Anti-HK Env response (OD)
Log(viral load)
CD4 count (cells/ml)
0.6
0.4
0.2
0
8
6
4
2
0
1500
1000
500
0
0 15 30 45 60 75 90 105

B



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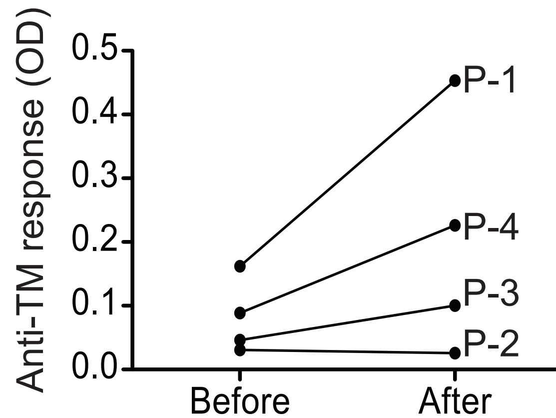

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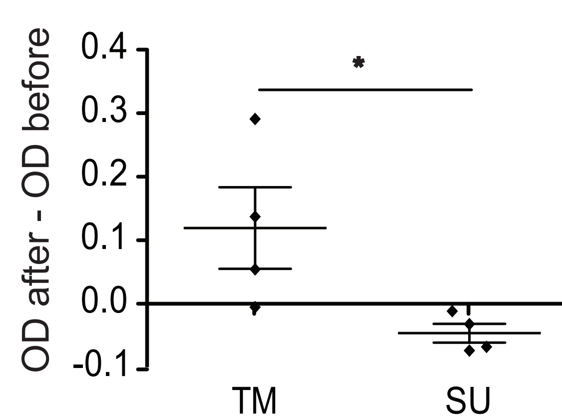


Figure 4. Anti-HERV-K B-cell response.

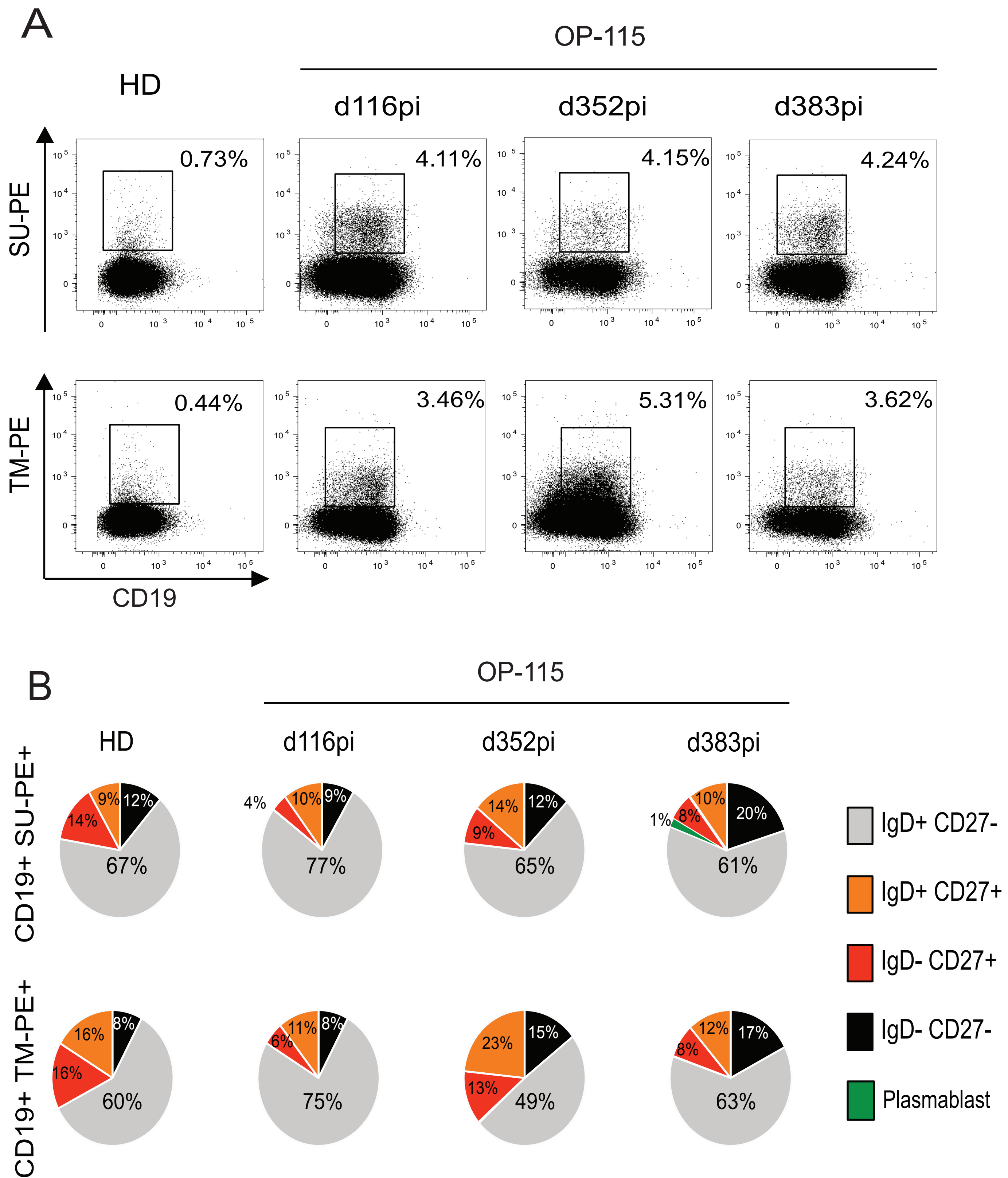
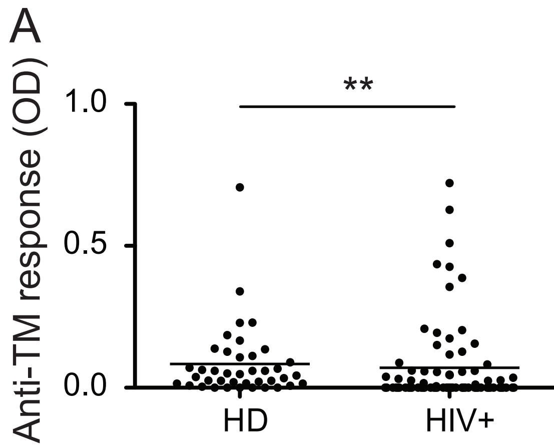
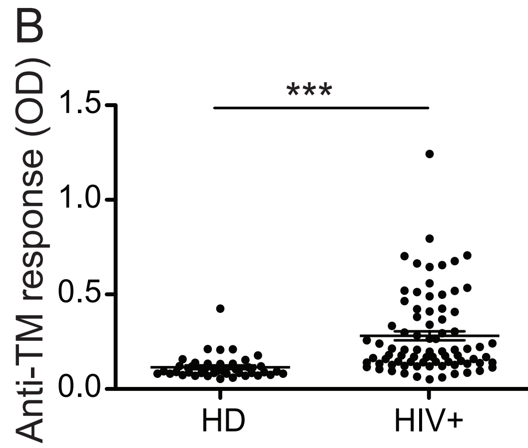
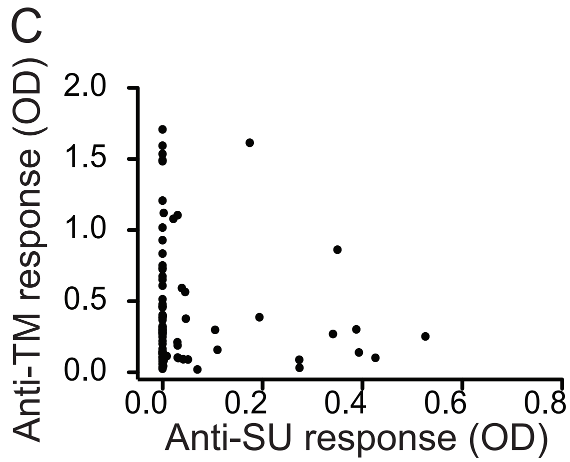
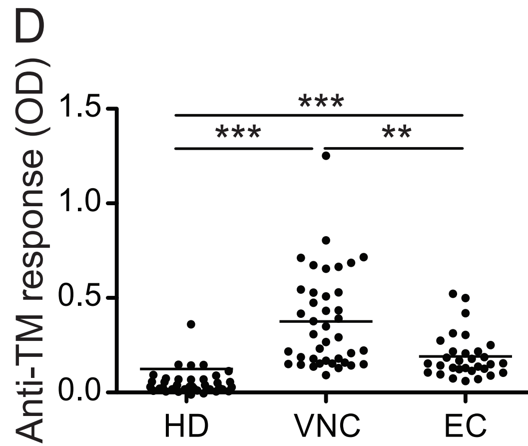


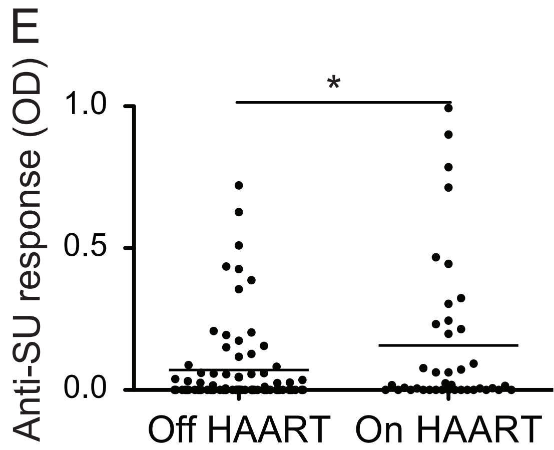
Figure 5. Humoral response against HERV-K Env TM and SU











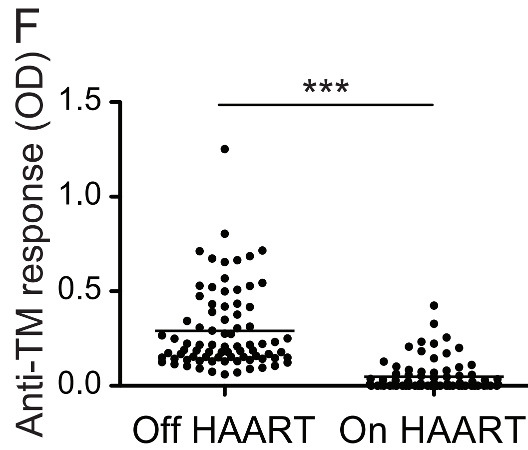
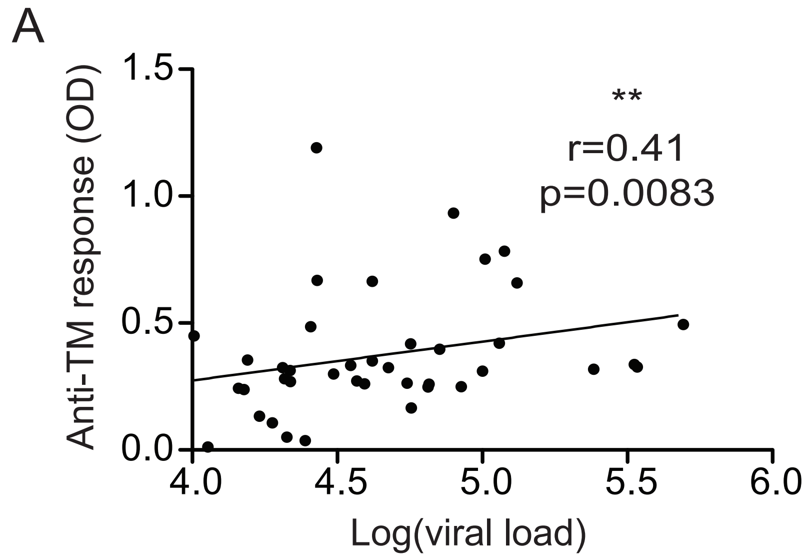


Figure 6. Anti-TM response correlates with HIV-1 activity

A



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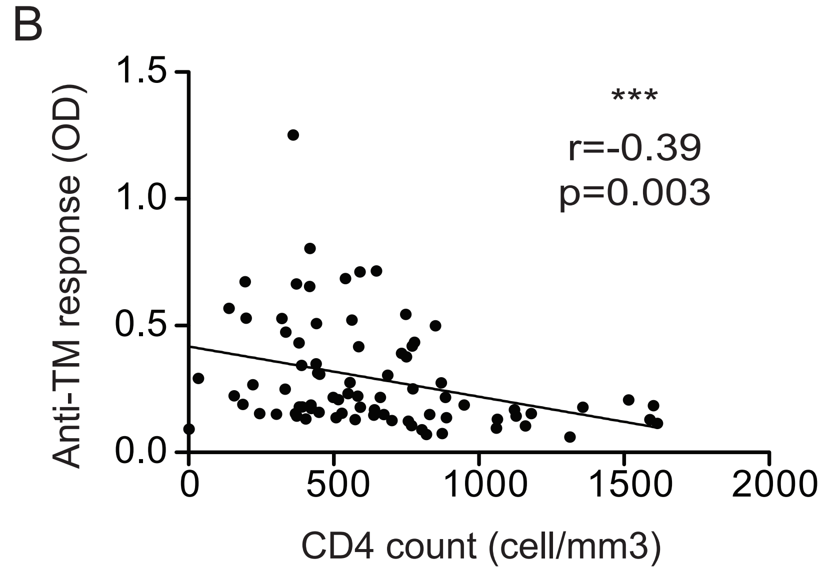
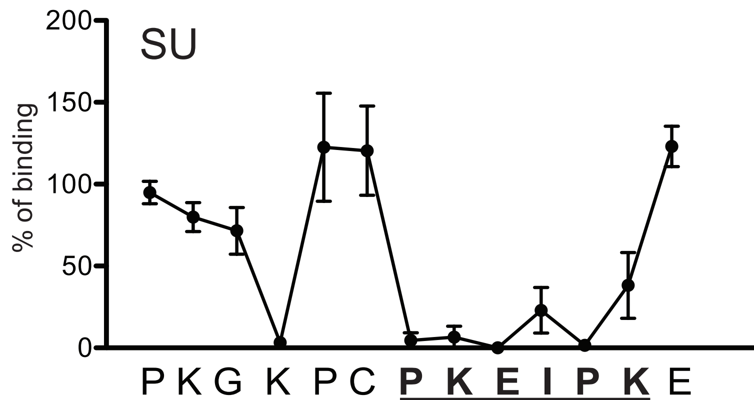


Figure 7. Epitope mapping and homologies.

A



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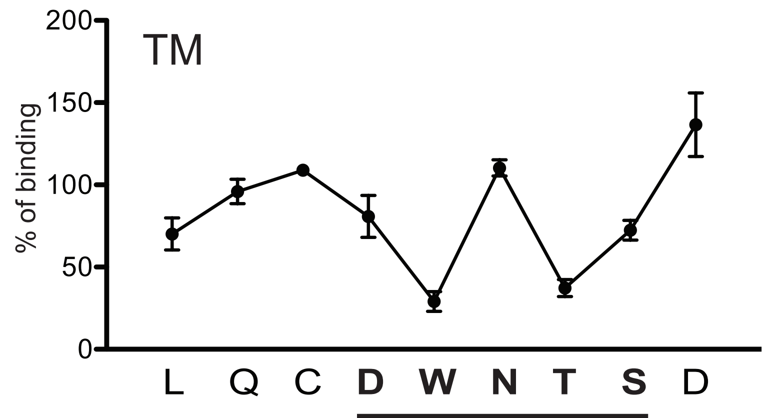


C

SU

Accession	Isolate															
gi|5802809|gb|AF164609.1|	K101	K	P	C	P	K	E	I	P	K	E	S	K	N	T	E
gi|5802811|gb|AF164610.1|	K102	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
gi|5802813|gb|AF164611.1|	K103	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.
gi|5802815|gb|AF164612.1|	K104	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
gi|5802818|gb|AF164613.1|	K107	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
gi|5802820|gb|AF164614.1|	K108	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
gi|5802823|gb|AF164615.1|	K109	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
gi|16507981|gb|AY037928.1|	K113	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
gi|16507983|gb|AY037929.1|	K115	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

TM

Accession	Isolate															
gi|5802809|gb|AF164609.1|	K101	E	H	R	F	Q	L	Q	C	D	W	N	T	S	D	F
gi|5802811|gb|AF164610.1|	K102	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
gi|5802813|gb|AF164611.1|	K103	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.
gi|5802815|gb|AF164612.1|	K104	.	.	.	.	.	.	.	.	.	.	.	M	.	.	.
gi|5802818|gb|AF164613.1|	K107	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
gi|5802820|gb|AF164614.1|	K108	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
gi|5802823|gb|AF164615.1|	K109	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
gi|16507981|gb|AY037928.1|	K113	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
gi|16507983|gb|AY037929.1|	K115	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

D

SU	KGKPCPKEI**PKESK**NT
HIV1 TAT	QPRGDPTG**PKESK**KK
TM	**LEHRFQ**LQ**C**D**W**NT**SDF**C
HIV-1 POL	D**EH**EKYHSNWRAMA**SDF**N
HCV Polyprotein	**LEHRFQ**AACNWTRGDPCN

Supplemental figures 1. Herv-K 108, SU and TM peptides sequences

1 MNPSEMQRKA PPRRRRHRNR APLTHKMNKM VTSEEQMKLP STKKAEPPTW AQLKKLTQLA

61 TKYLENTKVT QTPESMLLAA LMIVSMVVSL PMPAGAAAAN YTYWAYVPFP PLIRAVTWMD

121 NPTEVYVNDS VWVPGPIDDR CPAKPEEEGM MINISIGYHY PPICLGRAPG CLMPAVQNWL

181 VEVPTVSPIC RFTYHMVSGM SLRPRVNYLQ DFSYQRSLKF RPKGKPCPKE IPKESKNTEV
SU-56

241 LVWEECVANS AVILQNNEFG TIIDWAPRGQ FYHNCSGQTQ SCPSAQVSPA VDSDLTESLD

301 KHKHKKLQSF YPWEWGEKGI STPRPKIVSP VSGPEHPELW RLTVASHHIR IWSGNQTLET

361 RDRKPFYTID LNSSLTVPLQ SCVKPPYMLV VGNIVIKPDS QTITCENCRL LTCIDSTFNW

421 QHRILLVRAR EGVWIPVSMD RPWEASPSVH ILTEVLKGVL NRSKRFIFTL IAVIMGLIAV

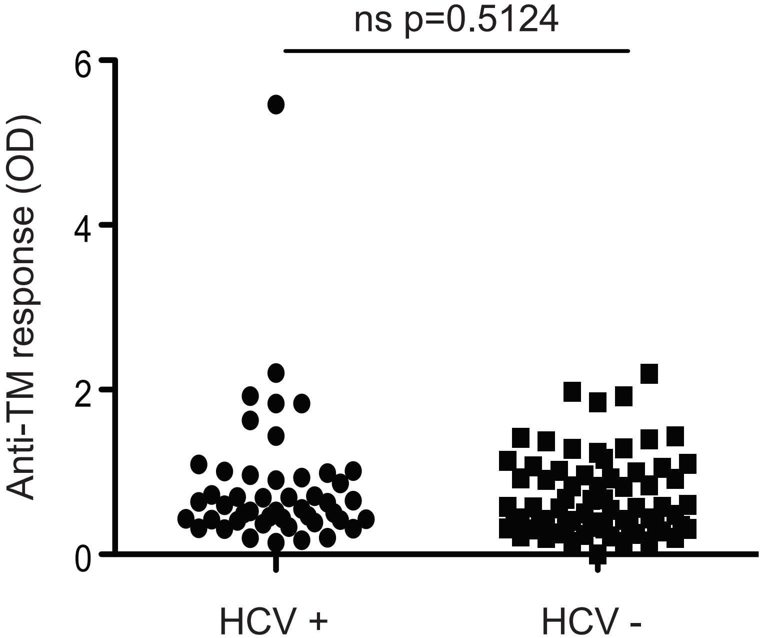
481 TATAAVAGVA LHSSVQSVNF VNDWQKNSTR LWNSQSSIDQ KLANQINDLR QTVIWMGDRL

541 MSLEHRFQLQ CDWNTSDFCI TPQIYNESEH HWDMVRRHLQ GREDNLTLDI SKLKEQIFEA
TM-137

601 SKAHLNLVPG TEAIAGVADG LANLNPVTWV KTIGSTTIIN LILILVCLFC LLLVCRCTQQ

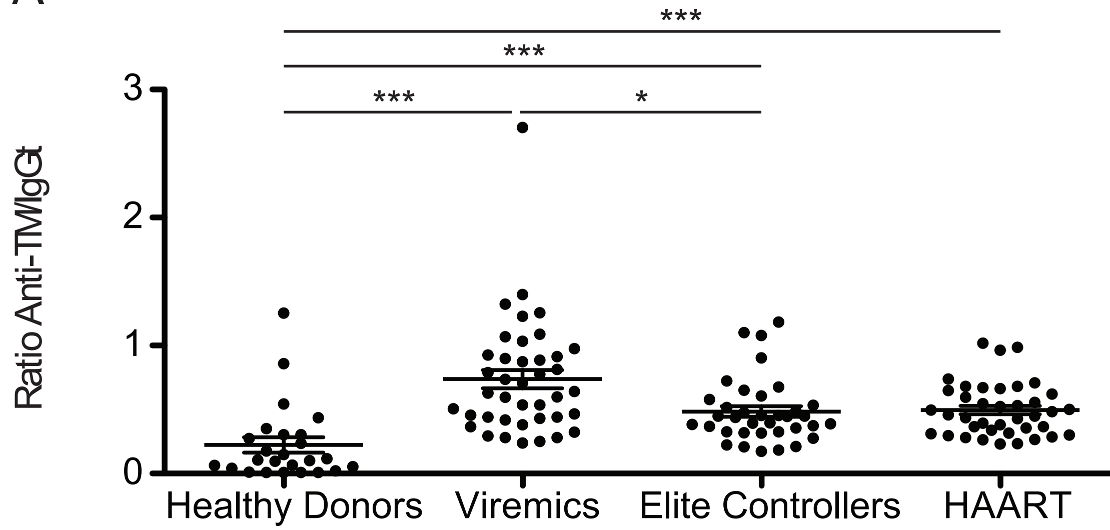
661 LRRDSDHRER AMMTMAVLSK RKGGNVGKSK RDQIVTVSV

Supplemental figure 2 Anti-TM response in HCV patients



Suplemental figure 3. Ratio anti-HERV-K Env/IgG total

A



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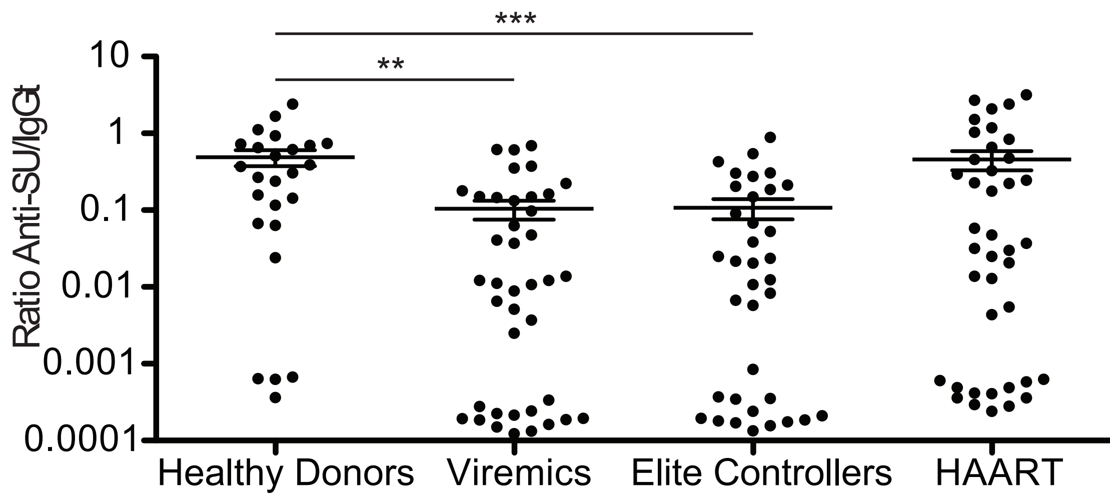

Description of HIV-1 Group M Molecular Epidemiology and Drug Resistance Prevalence in Equatorial Guinea from Migrants in Spain

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Short Title: HIV-1M strains and resistance in Equatorial Guinea.

Keywords: HIV-1 group M; Equatorial Guinea; transmitted drug resistance; CRF02_AG; Spain; Bayesian MCMC.

ABSTRACT

Background. The HIV epidemic is increasing in Equatorial Guinea (GQ), West Central Africa, but few studies have reported its HIV molecular epidemiology. We aimed to describe the HIV-1 group M (HIV-1M) variants and drug-resistance mutations in GQ using sequences sampled in this country and in Spain, a frequent destination of Equatoguinean migrants.

Methods. We collected 195 HIV-1M *pol* sequences from Equatoguinean subjects attending Spanish clinics during 1997-2011, and 83 additional sequences sampled in GQ in 1997 and 2008 from GenBank. All (n=278) were re-classified using phylogeny and tested for drug-resistance mutations. To evaluate the origin of CRF02_AG in GQ, we analyzed 2562 CRF02_AG sequences and applied Bayesian MCMC inference (BEAST program).

Results. Most Equatoguinean patients recruited in Spain were women (61.1%) or heterosexuals (87.7%). In the 278 sequences, the variants found were: CRF02_AG (47.8%), A (13.7%), B (7.2%), C (5.8%), G (5.4%) and others (20.1%). We found 6 CRF02_AG clusters emerged from 1983.9 to 2002.5 with origin in GQ (5.5 sequences/cluster). Transmitted drug-resistance (TDR) rate among naïve patients attended in Spain (n=144) was 4.7%: 3.4% for PI (all with M46IL), 1.8% for NRTI (all with M184V) and 0.9% for NNRTI (Y188L). Among pre-treated patients, 9/31 (29%) presented any resistance, mainly affecting NNRTI (27.8%).

Conclusions. We report a low (<5%) TDR rate among naïve, with PI as the most affected class. Pre-treated patients also showed a low drug-resistance prevalence (29%) maybe related to the insufficient treatment coverage in GQ. CRF02_AG was the prevalent HIV-1M variant and entered GQ through independent introductions at least since the early 1980s.

INTRODUCTION

Human immunodeficiency virus type 1 group M (HIV-1M), responsible for the HIV pandemic, originated from a zoonotic transmission from chimpanzees into humans in Cameroon [1]. For decades, the virus evolved and diversified into different lineages in the Congo River basin, where the highest viral diversity circulates [2]. Today, HIV-1M is sub-divided into 9 pure subtypes (A-D, F-H, J and K) and recombinants between them (http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html): 58 circulating recombinant forms (CRFs) and multiple unique recombinant forms (URFs).

Equatorial Guinea (GQ), a Spanish colony until 1968, is a small country located in West Central Africa between Cameroon and Gabon. The estimated number of people living with HIV in GQ (20,000 in 2009) increases annually, and antiretroviral treatment (ART) only reached 48% of them in 2011 [3]. Despite its location, close to the epicenter of HIV-1 pandemic, only two local studies [4,5] have so far reported the HIV molecular epidemiology in GQ. Ortiz et al. included 76 HIV-1M *env* gene (gp41) sequences obtained in 1999 from general HIV-infected population. Djoko et al. evaluated 41 HIV-1M *pol* sequences sampled in 2008 from military personnel in Malabo −the capital city. Both studies revealed CRF02_AG as the prevalent HIV-1M variant, as happens in the neighboring countries [6].

The latter also reported a rate of transmitted antiretroviral drug resistance (TDR) of 4.2%. This rate represents the percentage of naïve patients infected with HIV variants carrying drug resistance mutations, and threatens the effectiveness of ART. Recent studies report an increasing TDR prevalence in low- and middle-income countries following ART scale-up [7]. This rise especially affects sub-Saharan Africa, driven by the resistance to non-nucleoside reverse transcriptase inhibitors (NNRTI) [8,9]. The TDR rate in Cameroon is around 8-9% [10,11] and lower than 5% in Gabon [12]. HIV molecular epidemiology studies are crucial in these areas to prevent the widespread transmission of drug resistance, especially those lacking systematic surveillance as GQ.

We aimed to describe the circulating HIV-1M variants and the presence of antiretroviral drug resistance mutations in Equatorial Guinea. To achieve this, we applied phylogenetic methods to a combination of viral sequences sampled in Spain (frequent destination of Equatoguinean migration) and publicly available sequences sampled in the sub-Saharan country.

MATERIAL AND METHODS

Study population. All the HIV-infected subjects coming from Equatorial Guinea with available HIV-1M *pol* sequences, and attended in Spanish HIV/AIDS clinics (mainly in Madrid) were collected. They were 195 adult and pediatric patients sampled between 1997 and 2011. Most sequences were previously reported [13-16], while 37 were unpublished. In most cases (136, 69.7%) they included the complete protease (PR, 297nt) and partial reverse transcriptase (RT, 860nt of mean length). In 58 (29.7%) and 1 (0.5%) cases, only the PR or the RT sequence was available, respectively. In addition, all the HIV-1 *pol* sequences (n=83) sampled in Equatorial Guinea and available in the GenBank (www.ncbi.nlm.nih.gov/genbank) were retrieved. They belonged to two sequences batches sampled in 1997 (not published) and 2008 [5].

HIV-1M subtyping. HIV-1M variants were re-classified by phylogenetic analysis of the 278 *pol* sequences, including recombinants not available at publishing time. Representative sequences of the HIV-1M 9 subtypes and 49 CRFs downloaded from Los Alamos HIV sequence database (LANL-DB, http://www.hiv.lanl.gov) were used as references. Sequences were aligned manually, and neighbor-joining trees were built using MEGA5 (http://www.megasoftware.net) under the Kimura two-parameter model with 1,000 bootstrap re-sampling. In sequences not ascribed to any known subtype or CRF, recombination analyses were performed using SimPlot v3.5.1 and Recombination Detection Program (RDP, v3alpha44).

In the CRF02_AG subset, we searched monophyletic clusters that involved only sequences from GQ in approximately maximum-likelihood (ML) phylogenetic trees that included all CRF02_AG sequences from LANL-DB (n=2,652). ML trees were constructed under the general time reversible model of nucleotide substitutions with gamma-distributed heterogeneity rate (GTR+Γ) using FastTree v2.1.3 (http://www.microbesonline.org/fasttree). The topology robustness was tested by likelihood-based local branch support.

The time to the most recent common ancestor (TMRCA) of these Equatoguinean CRF02_AG clusters was estimated using Bayesian Markov chain Monte Carlo (MCMC) inference with the program BEAST v1.6.1 (http://beast.bio.ed.ac.uk). A lognormal distributed prior was placed on the estimated number of nucleotide substitution per site per year (mean=2.5×10^{-3}; standard deviation=0.5). The MCMC chain ran for 10^8 generations, sampling estimates every 10,000th generation. The uncorrelated lognormal molecular clock model and the GTR+Γ nucleotide substitution were selected. These parameters were chosen based on previous estimates for CRF02_AG datasets

[17]. Tree samples were used to generate a maximum clade credibility (MCC) tree, after a 10% burn-in, using TreeAnnotator v1.6.1. Those Equatoguinean CRF02_AG clusters with a high clade support in both ML and Bayesian trees (≥95%) were chosen as definitive.

Drug resistance. For HIV sequences from antiretroviral-naïve patients, the TDR prevalence was defined following the World Health Organization mutation list [18] using the Calibrated Population Resistance tool (http://cpr.stanford.edu). For antiretroviral-exposed patients, substitutions considered in the 2011 International AIDS Society-USA (IAS-USA) mutation list [19] were manually identified.

Ethics Statement. This study was part of a project approved by the review board of the Hospital Ramón y Cajal Clinical Research Ethical Committee. It was designed to protect the rights of all subjects involved under the appropriate local regulations. To maintain subject confidentiality, a unique ID number was assigned to each specimen, and written consent obtained for each patient by clinicians.

RESULTS

Equatoguinean HIV-infected patients in Spain. The main characteristics of these patients are shown in **Table 1**. Most subjects (61.1%) of this cohort were women, and were infected with HIV due to heterosexual risk practices, in concordance with the general situation in sub-Saharan Africa [20]. This information was not available in the 83 sequences retrieved from GenBank and sampled in Equatorial Guinea.

HIV-1M variants circulating in Equatorial Guinea. Table 2 shows the distribution of HIV-1M variants among the 278 *pol* sequences from Equatoguinean patients, where the recombinant CRF02_AG (47.8%) was predominant. Pure subtypes infected 122 (43.9%) patients, and other CRFs were found in 19 (6.8%) cases. Four sequences were classified as URF (URF_JU, URF_BH, URF_02D and URF_BG) using RDP and SimPlot. Of note, the frequency of subtype B was significantly higher in Equatoguinean subjects sampled in Spain than in those sampled in EQ (9.7% vs. 1.2%, P=0.012).

The new analysis of the sequences permitted to update their classification, especially among recombinants. We re-classified as CRF02_AG 21 sequences initially assigned to subtype G when they were sampled [21,22], 1 CRF11_cpx reported as subtype J [22], 1 CRF13_cpx and 1 CRF18_cpx also reported as subtype G [21] and 1 CRF22_01A1 described as subtype A [16]. The recombination analyses revealed that 2 sequences isolated in 1999 and 2000 and reported as pure subtypes G and J [21] were URF_BG and URF_JU, respectively.

CRF02_AG cluster analysis. In the general ML tree that included the 133 CRF02_AG sequences of the study population and 2,652 worldwide CRF02_AG sequences (**Figure 1**), we found 7 monophyletic clusters including exclusively Equatoguinean CRF02_AG sequences with >95% of statistical support. The geographic origin of the 2652 CRF02_AG sequences from LANL-DB is described in the legend of **Figure 1**. The further analysis with BEAST included a 93 sequence alignment: the CRF02_AG involved in the putative Equatoguinean clusters (n=37), their closest relatives among the CRF02_AG from LANL-DB according to the ML tree (n=28), and reference CRF02_AG sequences with diverse sampling dates (n=28) in order to include at least one sequence per year from 1990 when available.

The Bayesian MCC tree showed that 6 of the 7 putative clusters were supported by a posterior probability of ≥0.95 (**Table 3**). Therefore, these 6 clusters were considered as definitive due to their high statistical support. They included 33 Equatoguinean sequences (mean=5.5 sequences/cluster). All but one (cluster VI) clusters included sequences sampled in both Spain and GQ. The dates of their most recent common ancestors (MRCA) ranged from 1983.9 to 2002.5. Five of the 6 clusters were included among sequences sampled in Western Africa, and one (cluster V) was related to sequences sampled in Central Africa (**Figure 1**).

HIV-1M drug resistance. Among the 195 sequences collected in Spain from Equatoguinean subjects, 148 (75.9%) corresponded to antiretroviral-naïve patients and 31 (15.9%) to treatment-experienced subjects, with unknown treatment experience in 16 (8.2%) patients. We found at least one transmitted drug-resistance mutation (TDR) in 7 of the 148 (4.7%) naïve patients following the World Health Organization mutations list [18]. **Table 4** shows the TDR rate according to the antiretroviral class affected. In 5 (3.4%) subjects, the mutations affected PR inhibitors (PI), with M46I/L substitution in all cases. Another 2 (1.8%) presented the M184V mutation to nucleoside reverse transcriptase inhibitors (NRTI). One of the latter also presented a drug-resistance mutation to non-nucleoside reverse transcriptase inhibitors (NNRTI). No triple-class resistance was found. Among the 31 ART-experienced patients, 9 (29%) presented any drug-resistance mutation according to the IAS-USA list [19]. The highest rate (27.8%) was found for NNRTI-resistance due to mutation K103N in 3 of the 7 cases. The PI-resistance mutations found were M46L (4 cases), and L90M and I84V (3 cases each). For NRTI-resistance, the mutation M184V was found in all 3 cases.

Table 5 shows the main epidemiological and virological features of the 7 drug-naïve and the 9 drug-experienced patients carrying viruses harboring resistance mutations. Of note, most naïve (6/7; 85.7%) and pre-treated (6/9 cases; 66.7%) patients carrying

drug-resistant viruses were adult women. They were mainly infected by CRF02_AG (2/7 and 4/9, respectively) and subtype B (2/7 and 3/9 cases, respectively).

Regarding the sequences retrieved from GenBank and obtained in Equatorial Guinea, the antiretroviral status was only available for the 41 samples described in Djoko et al., which were obtained from naïve HIV-infected population in Malabo. Among them, TDR was 4.9% according to the WHO mutations list.

DISCUSSION

Despite being located in the River Congo basin, where the HIV epidemics originated [1], only two works [4,5] have provided data about the circulation of different HIV strains and drug resistance in Equatorial Guinea. Here, we expand and complete the information available about the HIV Equatoguinean epidemics combining the use of HIV-1M *pol* sequences from immigrants in Spain (the main host developed country for Equatoguinean migration) and from patients sampled in Equatorial Guinea. This is the larger study covering transmitted drug resistance in this country and the first one analyzing general HIV-infected population and dating the introduction of CRF02_AG.

CRF02_AG recombinant is prevalent in Equatorial Guinea. We confirm that recombinant CRF02_AG, the prevalent HIV-1M variant in Western Africa [6], accounts for a half of infections in Equatorial Guinea (**Table 2**). This is also the most frequent non-B variant in Spain among adult [23] and pediatric [15] HIV-infected population. The sequences' re-analyses identified CRF02_AG isolates classified as subtype G when they were described using only protease sequences [21]. We also found CRF11_cpx, CRF13_cpx, CRF18_cpx and CRF22_01A1 sequences initially described as pure subtypes [16,22]. These complex variants are found in Spain among immigrants and rarely in autochthonous population [14,23]. Recombinants might be underestimated in studies performed in periods that lacked reference sequences published afterwards. Thus, periodic re-assessments of HIV classification with updated CRFs might improve the subtyping. In addition, modern computer tools permit to identify previously unnoticed unique recombinant forms when phylogenetic analyses are inconclusive.

CRF02_AG introduction in Equatorial Guinea. The Bayesian MCMC analysis using BEAST revealed 6 monophyletic, highly statistically supported clusters of Equatoguinean CRF02_AG sequences. This could indicate that this variant entered Equatorial Guinea through several independent introductions occurred at least since the early 1980s, and not through a single introduction event. A previous study proposed that CRF02_AG was introduced in Equatorial Guinea from Cameroon [24], but our results showed that only one of the 6 fell within Central African clades, being

the rest related to Western African clades. In many cases, sequences from both regions were interspersed in the analysis (**Figure 1**), which suggests a frequent circulation of CRF02_AG between countries, as others have also indicated [25]. In both Western and Central Africa the presence of this recombinant, originated at least in the early 1970s [24], has increased in the last years [6].

Low rate of resistance among HIV-1M-infected Equatoguineans. We observed a TDR rate of 4.7% among the 148 antiretroviral-naïve Equatoguineans attending Spanish HIV/AIDS clinics, a figure close to the limit between low and moderate drug-resistance (5%) according to the World Health Organization [26]. This rate is very similar to that found in the only TDR study performed in Equatorial Guinea (4.9%), which screened military personnel in Malabo [5]. Both are lower than in other sub-Saharan countries, including the neighboring Cameroon, with a TDR around 9% [10,11]. Since ART still reaches only 48% of the HIV-infected population in need of therapy in GQ [3], a lower emergence of resistance than in countries with an older ART scale-up is expected [7].

The highest TDR rate was found for PI (3.4%), led by the presence of the substitution M46I/L in all cases. This was also the most frequent PI-resistance mutation among pre-treated patients, and it was previously reported among antiretroviral-naïve military personnel living in Equatorial Guinea [5]. This high prevalence contrasts with the rare administration of PI in Equatorial Guinea, where the first line treatment includes the NNRTI efavirenz [3]. For transmitted NRTI-resistance, the low presence found (with the predominance of change M184V) agrees with the study of Djoko et al., although they only found the substitution D67N as transmitted NRTI-resistance mutation [5].

Studies in Uganda [7,27] have related the expanded use of NNRTI as peripartum prophylaxis among HIV-infected pregnant women to a higher population-wide prevalence of NNRTI-resistance mutations. Thus, the low presence of transmitted NNRTI-resistance mutations in Equatoguineans compared with other sub-Saharan populations [8] could result from the infrequent administration of this prophylaxis in Equatorial Guinea (only in 19% of HIV-infected pregnant women in 2010) [3].

In treated Equatoguinean patients attended in Spain, we found a presence of drug-resistance mutations rate of 29%, with NNRTI as the most affected drug class (27.8%). This lower prevalence than in other sub-Saharan antiretroviral-exposed cohorts [28], could reflect a poor adherence as observed among sub-Saharans living in Spain [29], who present great rates of follow-up losses. This has also been reported in Africa [30],

caused by the low accessibility of antiretroviral drugs, as happens in Equatorial Guinea, with an antiretroviral coverage of 24% in 2010. Unfortunately, in most cases the information on specific drug exposure for the pre-treated patients was unavailable, which prevent us from a further interpretation of these data.

HIV infection place. The phylogenetic analyses showed that HIV-1M sequences sampled in Spain and/or Equatorial Guinea were interspersed, which would suggest that most of the 195 Equatoguinean patients with clinical follow up in Spain were infected in their country of origin. However, we cannot rule out that some of them could have been infected in Spain. This hypothesis would be supported by the overrepresentation of subtype B (the predominant variant in Spain [23] but infrequent in Central Africa) among samples taken in this host country versus those taken in Equatorial Guinea; and by the higher prevalence of transmitted PI-resistance in these subtype B-infected Equatoguineans sampled in Spain (where treatment is universally available) than in other variants of the study cohort.

Conversely, an evidence for an infection happened in Africa would be the different mutation pattern found in Equatoguineans sampled in Spain than in a study of general, naïve patients [14] (autochthonous and African and non-African foreigners) living in Spain between 1996 and 2010. Thus, the PI-resistance mutation M46L, present in all subtype B viruses with TDR (**Table 5**), was rare (<1%) among the subtype B-infected population living in Spain, where L90M was predominant. In addition, if the Equatoguinean patients had been infected in Spain, higher rates of NRTI- and, especially, NNRTI-resistance would have been expected.

In conclusion, the rising HIV drug-resistance transmission in sub-Saharan Africa following antiretroviral rollout highlights the need of periodical surveillance studies to monitor and prevent the resistance emergence, essential information to design or continue the implementation of ART programmes. These studies should be performed among infected people living in the study region. However, in countries lacking surveillance reports (such as Equatorial Guinea), our approach describing the HIV molecular epidemiology of low-income regions from a developed host country can provide any knowledge about the HIV epidemic in specific areas.

ACCESSION NUMBERS

Sequences sampled in Spain (n=195): AF125283(PR)/AF188336(RT), AF125287, AF125292(PR)/AF188342(RT), AF125293, AF125294(PR)/AF455646(RT), AF188349(PR)/AF188345(RT), AF247009, AF247012, AF247014, AF247017, AF247018 to AF247021, AF247023(PR)/AF455664(RT), AF247024, AF247025, AF247027, AF247028, AF247030, AF247033, AF354007(PR)/AF455638(RT), AF354012, AF354013, AF354025, AF354034(PR)/AF455634(RT),

AF354038(PR)/AF455651(RT), AF354043(PR)/AF455647(RT), AF394957(PR)/AY050184(RT), AF455609(PR)/AF479601(RT), AF455620(PR)/AF479604(RT), AF455631, AF455665(PR)/AF455636(RT), AF455666(PR)/AF455637(RT), AY248292, AY248299, AY248309, AY248311, AY248313, AY248317, AY248325, AY248329, AY248331, AY248332, AY248431, AY642099, AY642101, AY642103, AY642106, AY642112, AY647440, AY647441-AY647443, AY647445-AY647447, AY647449, DQ009056, DQ009057, DQ157799, DQ157806, DQ157807, DQ157810, DQ157812, DQ157813, DQ157825, DQ157833, EF380366, EF380367, EF380381, EF421986, EU255306, EU255307, EU255309, EU255346, EU255349, EU255354, EU255355, EU255358, EU255360, EU255363, EU255373-EU255388, EU255465, EU255468, EU255485, EU255489, EU255507, EU255513, EU255522-EU255525, EU342758, EU342760, EU342761, EU342767, EU342772-EU342775, EU342778, EU342781, EU342784, EU342785, EU342795, EU342819, EU342825, EU362920, EU362923, EU362924, EU362926, EU545186, EU545187, EU545188, EU545190, EU545191, EU545193, EU545194, EU552227, FJ481668, FJ481693, FJ481701, GQ240994, GQ241042, GQ241050, GQ241052, GU264338, HM460493, HM460496, HQ426896, HQ426901, HQ426902, HQ426905, HQ426906, HQ426908, JF929046, JF929114, JF929133, JF929142, JQ351953, JQ351955, JQ351990 and JX428539-JX428575.

Sequences sampled in GQ (n=83): AF529922-AF529954, AY580058-AY580065, DQ157832 and FN557303-FN557343.

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Table 1. Epidemiologic characteristics at sampling time of the 195 Equatoguinean HIV-infected patients followed in Spain (1997-2011).

Characteristic	**n**	**% (95% CI)**
Gender		
Male	70	38.9 (32.1-46.2)
Female	110	61.1 (53.8-67.9)
Unknown	15	-
Population		
Adult	186	95.4 (91.5-97.5)
Pediatric	9	4.6 (2.4-8.5)
Risk practice		
Heterosexual	128	87.7 (81.3-92.1)
MSM	1	0.7 (0.1-3.8)
IDU	2	1.4 (0.4-4.9)
Vertical	9	6.2 (3.3-11.3)
Transfusion/others	6	4.1 (1.9-8.7)
Unknown	49	-
ART experience		
Naïve	148	82.7 (76.5-87.5)
Pre-treated	31	17.3 (12.5-23.5)
Unknown	16	-
Sampling period		
1997-2001	44	22.6 (17.3-28.9)
2002-2006	105	53.8 (46.8-60.7)
2007-2011	46	23.6 (18.2-30.0)
Mean sampling year (± SD)	2003.96 ± 3.26	-
Mean HIV viral load (log ± SD) [a]	4.3 ± 1.0	-

CI, confidence interval; MSM, men who have sex with men; IDU, injecting drug users; ART, antiretroviral treatment; SD, standard deviation. For naïve and pre-treated patients, the mutations lists from the World Health Organization [18] and from the International AIDS Society-USA [19] were considered, respectively. This information was not available for the 83 HIV-1M sequences retrieved from GenBank and sampled in Equatorial Guinea.

[a] Viral load was only available in 126 cases.

Table 2. HIV-1 group M variants infecting the 278 patients from Equatorial Guinea.

HIV-1M variant	Total (%)	Sampled in Spain (%)	Sampled in GQ (%)
Pure subtypes	122 (43.9)	84 (43.1)	38 (45.8%)
A	38 (13.7)	24 (12.3)	14 (16.9)
B [a]	20 (7.2)	19 (9.7)	1 (1.2)
C	16 (5.7)	10 (5.1)	6 (7.2)
D	14 (5.0)	9 (4.6)	5 (6.0)
F	11 (4.0)	7 (3.6)	4 (4.8)
G	15 (5.4)	9 (4.6)	6 (7.2)
H	8 (2.9)	6 (3.1)	2 (2.4)
Recombinants	156 (56.1)	111 (56.9)	45 (54.2)
CRF02_AG	133 (47.8)	92 (47.2)	41 (49.4)
CRF06_cpx	4 (1.4)	3 (1.5)	1 (1.2)
CRF09_cpx	1 (0.4)	0 (0.0)	1 (1.2)
CRF11_cpx	7 (2.5)	6 (3.1)	1 (1.2)
CRF13_cpx	3 (1.1)	3 (1.5)	0 (0.0)
CRF18_cpx	1 (0.4)	1 (0.5)	0 (0.0)
CRF22_01A1	3 (1.1)	2 (1.0)	1 (1.2)
URF [b]	4 (1.4)	4 (2.1)	0 (0.0)
All	278	195	83

GQ, Equatorial Guinea; CRF, circulating recombinant form; URF, unique recombinant form. Sequences obtained in Spain were sampled between 1997 and 2011, and those obtained in Equatorial Guinea in 1997 (n = 35) (not published) or 2008 (n = 48; [5]).

[a] Subtype B prevalence was significantly higher among patients sampled in GQ than in Spain (*P*=0.012, chi-square test).

[b] The four URF were URF_JU, URF_BH, URF_02D and URF_BG.

Table 3. Characteristics of the monophyletic clusters of HIV-1M CRF02_AG *pol* sequences observed in the Equatoguinean study population.

Cluster	Sequences (ES; GQ)	Sampling year range	MRCA date (95% HPD)	Support (LBS; PP)
I	11 (8; 3)	1997-2006	1983.89 (1979.90-1989.76)	0.95; 0.99
II	6 (5; 1)	1997-2010	1991.73 (1987.38-1995.26)	0.99; 1
III	6 (3; 3)	1997-2008	1992.31 (1988.02-1996.19)	0.99; 1
IV	4 (3; 1)	2007-2009	1993.25 (1983.95-2000.89)	0.96; 1
V	3 (2; 1)	2001-2008	1994.52 (1990.26-1998.71)	0.98; 1
VI	3 (3; 0)	2004-2005	2002.52 (2000.32-2004.21)	0.98; 1

ES, sequences sampled in Spain; GQ, sequences sampled in Equatorial Guinea; MRCA, most recent common ancestor; HPD, highest posterior density interval; LBS, local branch support in the maximum likelihood tree; PP, posterior probability in the Bayesian dated tree.

Table 4. Prevalence of drug resistance mutations in 148 antiretroviral-naïve and 31 pre-treated HIV-1-infected patients from Equatorial Guinea and followed in Spain.

	Naïve patients (148)			Pre-treated patients (31)		
ART class	**TDR seqs.**	**No. seqs.**	**% (95% CI)**	**DRM seqs.**	**No. seqs.**	**% (95% CI)**
Any	7	148	4.7 (2.3-9.4)	9	31	29.0 (16.1-46.6)
PI	5	148	3.4 (1.4-7.7)	5	31	16.1 (7.1-32.6)
NRTI	2	108	1.8 (0.5-6.5)	3	18	16.7 (5.8-39.2)
NNRTI	1	108	0.9 (0.2-5.1)	5	18	27.8 (12.5-50.9)

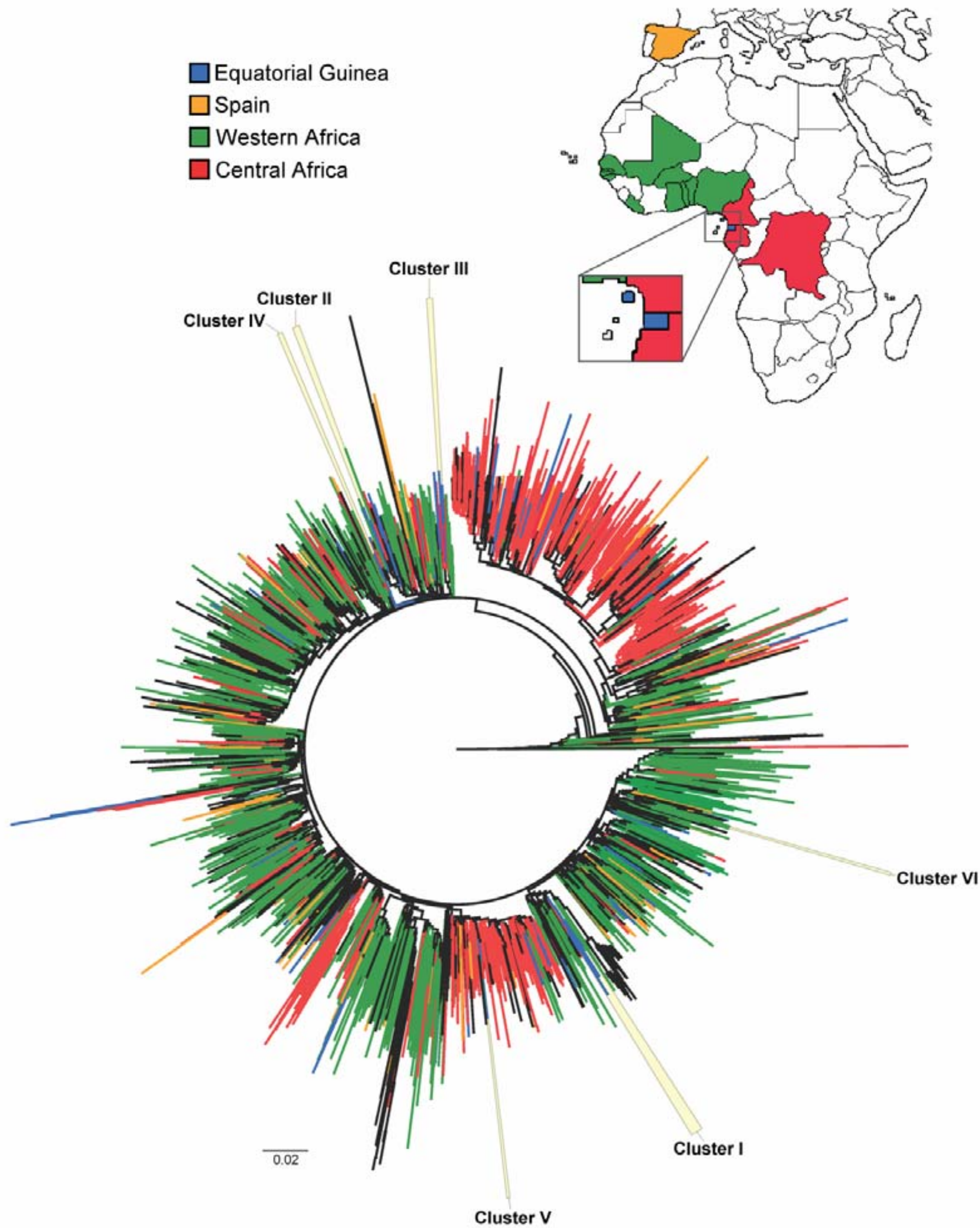
ART, antiretroviral; No, number; seqs., sequences; DRM, drug resistance mutations; CI, confidence interval; PI, protease inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors. For naïve and pre-treated patients, the mutations lists from the World Health Organization [18] and from the International AIDS Society-USA [19] were considered, respectively.

Table 5. Epidemiological and virological characteristics of the 7 antiretroviral-naïve and the 9 antiretroviral-experienced patients infected by HIV-1M variants harboring drug resistance mutations.

#	ART	Population	HIV-1M variant	Gender	Risk practice	Sampling year	HIV VL (log)	TDR-PI	TDR-NRTI	TDR-NNRTI
1	Naïve	Adult	B	F	Htsex	1999	2.5	M46L, L90M	NA	NA
2	Naïve	Adult	B	F	NA	1999	4.3	M46L, I84V	NA	NA
3	Naïve	Adult	C	F	Htsex	1999	1.8	M46I	NA	NA
4	Naïve	Adult	CRF02_AG	F	NA	2005	3.6	-	M184V	-
5	Naïve	Adult	CRF11_cpx	F	Htsex	2006	2.9	-	M184V	Y188L
6	Naïve	Adult	A1	F	Htsex	2007	2.7	M46L	-	-
7	Naïve	Pediatric	CRF02_AG	M	Vertical	2007	NA	M46L	-	-
8	Treated	Adult	B	F	Htsex	1999	2.3	M46L	NA	NA
9	Treated	Adult	B	F	Htsex	1999	2.3	M46L, L90M	NA	NA
10	Treated	Adult	B	F	Htsex	1999	2.3	M46L, I84V, L90M	NA	NA
11	Treated	Adult	CRF02_AG	F	Htsex	2003	4.0	F53L, I84V, L90M	M184V	K103N
12	Treated	Adult	F2	M	Htsex	2006	NA	-	-	V106I
13	Treated	Adult	D	M	Htsex	2008	5.0	-	-	V90I
14	Treated	Pediatric	CRF02_AG	F	Vertical	2009	NA	-	M184V	-
15	Treated	Adult	CRF02_AG	M	Htsex	2009	6.0	-	-	K103N
16	Treated	Pediatric	CRF02_AG	F	Vertical	2010	NA	M46L, I84V	L74V, Y115F, M184V	K103N, G190A

#, number of patient; ART, antiretroviral treatment; VL, viral load; TDR, transmitted drug resistance mutations; PI, protease inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; F, female; M, male; Htsex, heterosexual; NA, data not available; Dash, no mutation found.

Figure 1. Maximum likelihood tree of the 133 CRF02_AG HIV-1 *pol* sequences with 2652 CRF02_AG sequences retrieved from Los Alamos Database.



Tree constructed under the GTR+Γ model of nucleotide substitution using FastTree v2.1.3 (see Methods) from an 1130nt alignment. Branch colors indicate the origin of the sequences (with the same color code in the map). In blue, sequences from Equatoguinean patients (n=133). In orange, CRF02_AG sequences from Spain (n=158). In green, CRF02_AG sequences from Western Africa: Nigeria (n=391), Mali (n=235), Senegal (n=185), Ghana (n=180), Burkina Faso (n=97), Benin (n=77), Togo (n=58), Liberia (n=4), the Gambia (n=2) and Guinea-Bissau (n=2). In red, CRF02_AG sequences from Central Africa: Cameroon (n=711), Gabon (n=72), and Democratic Republic of the Congo (n=7). In black, CRF02_AG sequences from other regions. The 6 monophyletic clusters from Equatorial Guinea further assessed using BEAST are highlighted in yellow and labeled in accordance to **Table 3**.

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Most HIV Type 1 Non-B Infections in the Spanish Cohort of Antiretroviral Treatment-Naïve HIV-Infected Patients (CoRIS) Are Due to Recombinant Viruses

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HIV-1 group M is classified into 9 subtypes, as well as recombinants favored by coinfection and superinfection events with different variants. Although HIV-1 subtype B is predominant in Europe, intersubtype recombinants are increasing in prevalence and complexity. In this study, phylogenetic analyses of *pol* sequences were performed to detect the HIV-1 circulating and unique recombinant forms (CRFs and URFs, respectively) in a Spanish cohort of antiretroviral treatment-naïve HIV-infected patients included in the Research Network on HIV/AIDS (CoRIS). Bootscanning and other methods were used to define complex recombinants not assigned to any subtype or CRF. A total of 670 available HIV-1 *pol* sequences from different patients were collected, of which 588 (87.8%) were assigned to HIV-1 subtype B and 82 (12.2%) to HIV-1 non-B variants. Recombinants caused the majority (71.9%) of HIV-1 non-B infections and were found in 8.8% of CoRIS patients. Eleven URFs (accounting for 13.4% of HIV-1 non-B infections), presenting complex mosaic patterns, were detected. Among them, 10 harbored subtype B fragments. Four of the 11 URFs were found in Spanish natives. A cluster of three B/CRF02_AG recombinants was detected. We conclude that complex variants, including unique recombinant forms, are being introduced into Spain through both immigrants and natives. An increase in the frequency of mosaic viruses, reflecting the increasing heterogeneity of the HIV epidemic in our country, is expected.

Human immunodeficiency virus type 1 (HIV-1) shows great genetic diversity due to its high replication rate, the error-prone reverse transcriptase, and recombination events that may occur during virus replication (57). On the basis of genetic homology, HIV-1 has been classified in four groups: M (main), O (outlier), N (non-M, non-O), and the recently identified group P (36). HIV-1 group M is subdivided into 9 subtypes (A to D, F to H, J, and K), at least 49 circulating recombinant forms (CRFs) (http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html), and multiple unique recombinant forms (URFs). CRFs are defined as intersubtype recombinants for which at least three epidemiologically unlinked variants are monophyletic, sharing an identical genetic structure along their full genomes. URF variants are widely distributed worldwide, with recombination breakpoints different from those found in CRFs. Genetic complexity is not always detected, mainly due to the subtyping of only one genetic region and not of the full genome. Consequently, specimens previously considered "pure" variants may be classified as recombinants when additional viral genes are analyzed. Therefore, the frequency of recombinant variants is underestimated in the pandemic. Recombination, in addition to purifying selection, is involved in the evolution of HIV (42), adaptation to its host, and escape from antiviral treatments (37). Recombination can also increase HIV fitness (50).

HIV-1 subtype B is the prevalent variant in developed areas, such as North America and Western Europe, including Spain (16, 23). However, subtypes other than subtype B and recombinants (HIV-1 non-B variants) are responsible for 90% of the 33 million infections worldwide (20, 46). These variants are increasing in prevalence and heterogeneity in developed countries (3, 15, 23, 45, 49, 54), mainly due to immigration and the movement of populations from areas of endemicity. The coexistence of multiple variants in the same region favors recombination between them after coinfection and/or superinfection events. In Spain, an increase in the frequency of HIV-1 non-B subtypes has been found among native Spaniards and immigrants newly diagnosed with HIV-1 in recent years (23), and the presence of different recombinants has been published (11, 14, 16, 21, 23, 24, 34, 52, 53).

The increasing prevalence of HIV-1 non-B variants could have implications for diagnosis (5), vaccine design (56), and the clinical management of HIV infection (39). HIV-1 non-B variants present clade-specific substitutions in positions related to drug resistance (26, 52). They could accelerate the emergence of drug-resistant viruses, change or induce alternative pathways of resistance (17, 19), influence viral replicative capacity *in vitro* (25), impair the interpretation of genotypic resistance algorithms (9, 43, 52), reduce the genetic barrier of certain protease inhibitors (47), and affect drug-binding affinity (27). Additionally, patients infected by certain HIV-1 non-B subtypes present accelerated disease pro-

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gression (2, 48) and higher cognitive impairment (40). Thus, the proper detection and description of HIV-1 variants in representative cohorts is essential for further studies.

CoRIS, the cohort of the Spanish Research Network of Excellence on HIV/AIDS, has recently reported a prevalence of 15.2% for HIV-1 non-B subtypes (16). To further explore the molecular epidemiology of HIV in Spain, the objective of the present study was to characterize the HIV-1 recombinant variants detected in CoRIS by using phylogenetic analysis (phy), the gold-standard method for subtyping and discrimination between subtypes and/or CRFs. We also defined the complex mosaic patterns in variants classified as unique recombinants (not assigned to any known subtype or circulating recombinant form), as well as the phylogenetic clusters including such variants.

METHODS

Study population. CoRIS is an open, multicenter, prospective cohort of HIV-positive, antiretroviral (ARV)-naïve subjects more than 13 years old seen at 31 HIV units of the 18 Autonomous Regions in Spain from January 2004 on. Ethics approval was obtained from participating sites. The study was designed to protect the rights of all subjects involved under the appropriate local regulations. Written informed consent was obtained at the respective sites from every patient included in the study. A detailed description of the cohort has been published previously (7).

Of the 3,351 subjects included from 2004 to 2008, 670 patients provided a FASTA sequence while naïve to antiretroviral treatment (ART) and were included in this study. The alignment including these sequences is available as file S1 in the supplemental material. Overall, 375 patients (56%) were Spanish, 181 (27%) were immigrants (118 Central and South Americans, 17 sub-Saharan Africans, 16 Western Europeans, 11 Eastern Europeans, 10 North Africans, 6 North Americans, and 3 Asians), and 114 were of unknown origin. A *pol* sequence including the complete protease (codons 1 to 99) and part of the reverse transcriptase (codons 38 to 260 or 1 to 335) was collected from each of the 670 patients included in CoRIS for whom a sequence was deposited in the database. *pol* sequences were obtained in the course of the clinical routine for drug resistance mutation analysis, before any anti-HIV therapy. Most sequences (69.5%) were obtained within the period of 2007 to 2008.

Phylogenetic analysis. HIV-1 subtypes and CRFs were identified by phylogenetic analysis (phy) of the 670 *pol* sequences. The 2008 version of the subtype reference data set provided by the Los Alamos National Laboratory (http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html) was used. It was updated to include more sequences of CRFs that had been absent or scarcely represented (26_AU, 30_0206, 32_06A1, 34_01B, 38_BF, 41_CD, and 42_BF). Therefore, at least 2 representative sequences of each of the 9 subtypes and 43 CRFs of HIV-1 group M available in GenBank at the moment of the analysis were taken as references. DNA sequences were aligned using the ClustalX program, version 2.0.11. The tree topology was obtained using the neighbor-joining method. The pairwise distance matrix was estimated using the Kimura two-parameter model within the DNAdist program, as implemented in the PHYLIP software package. Bootstrap resampling (1,000 data sets) of the multiple alignments was performed to test the statistical robustness of the tree. We considered the associations of *pol* sequences showing a bootstrap value higher than 700 in the phylogenetic tree to be "clusters."

URF characterization. Recombination analyses of sequences not assigned to any known subtype or CRF by phy were performed using several methods: SimPlot, version 3.5.1, the Recombination Detection Program (RDP; version 3alpha44), and the jumping-profile hidden Markov model (jpHMM) (http://jphmm.gobics.de/jphmm.html). In the SimPlot analysis, which applies the bootscanning method, windows of 300 nucleotides moving in 10-nucleotide increments were used, as recommended by Zhang et al. (55). Sequences obtained from GenBank with the same geographical origin as the sequences analyzed were used as references when possible. After the different recombination analyses, the definitive breakpoints were those confirmed by constructing phylogenetic trees of the subsegments in order to assign them to the parental subtypes involved in the specific recombination event.

RESULTS

HIV-1 subtypes defined by phy in CoRIS: high frequency of recombinants. A total of 670 available *pol* sequences from different patients included in CoRIS were collected. As expected, 588 (87.8%) were assigned to subtype B and 82 (12.2%) to HIV-1 non-B variants. The majority ($n = 59$ [71.9%]) of HIV-1 non-B sequences were, in fact, viruses recombinant at *pol* (Fig. 1). Forty-eight viruses (58.5% of HIV-1 non-B variants) were 12 different CRFs (2 CRF01_AE, 31 CRF02_AG, 2 CRF03_AB, 1 CRF06_cpx, 1 CRF11_cpx, 1 CRF12_BF, 4 CRF14_BG, 1 CRF15_01B, 2 CRF19_cpx, 1 CRF20_BG, 1 CRF28_BF, and 1 CRF42_BF). Eleven viruses were unique recombinants (URFs), i.e., not assigned to any subtype or CRF. CRFs and URFs caused 7.2% and 1.6% of total infections, respectively. URF sequences did not cluster with any other known HIV-1 subtype or CRF after phylogenetic analyses of *pol* sequences, and they presented complex mosaic patterns due to recombination events between different subtypes (Fig. 1). In summary, among the 82 HIV-1 non-B *pol* sequences, 59 (71.9%) were shown by phy to be recombinants (81.4% CRFs and 18.6% URFs). Recombinant form CRF02_AG and URFs accounted for one-third and one-fifth, respectively, of HIV-1 non-B infections. The data showed that HIV-1 recombinants caused 8.8% of HIV-1 infections (this proportion rose to 14% when only the year 2008 was considered) and represented 71.9% of the HIV-1 non-B variants identified by phy.

HIV-1 non-B variants found in native Spanish individuals. Of the 375 native Spaniards included in the study, 31 (8.3%) carried HIV-1 non-B variants. Among these, only 9 harbored pure HIV-1 non-B subtypes; 22 were infected with recombinants (18 CRF and 4 URF). It is remarkable that 1 out of 2 CRF01_AE viruses, the CRF12_BF virus, 3 out of 4 CRF14_BG viruses, 1 out of 2 CRF19_cpx viruses, the CRF20_BG virus, and the CRF42_BF virus were found in Spanish individuals. Although the frequency of these variants among native Spaniards was low, native patients accounted for more than one-third (37.8%) of the total number of patients infected with HIV-1 non-B variants.

Origins of CoRIS patients infected by HIV-1 recombinants. The origins of subjects infected by each HIV-1 recombinant variant are shown in Fig. 2. According to the origins of patients, the prevalences of HIV-1 recombinants were as follows, in descending order: 64.7% (11/17) for sub-Saharan Africans, 60% (6/10) for North Africans, 33.3% (1/3) for Asians, 18.2% (2/11) for Eastern Europeans, 5.9% (7/118) for South and Central Americans, 5.9% (22/375) for Spaniards, and 0% for North Americans (0/6) and Western Europeans other than Spaniards (0/16). Among patients of unknown origin, this prevalence was 8.8% (10/114). Surprisingly, a CRF02_AG variant was isolated from an Asian patient, and CRF03_AB recombinants, first described in Russia, were isolated from South American patients.

Complex variants in CoRIS and epidemiological data. The 11 URFs presented mosaic patterns due to recombination events in the *pol* region between different subtypes and/or CRFs (Fig. 1). Recombinants presented fragments from 7 different pure subtypes (A, B, C, F, G, J, and K) and CRF02_AG. Some carried B/CRF02_AG (4/11), B/F1 (2/11), and B/A1 (2/11) sequences, and

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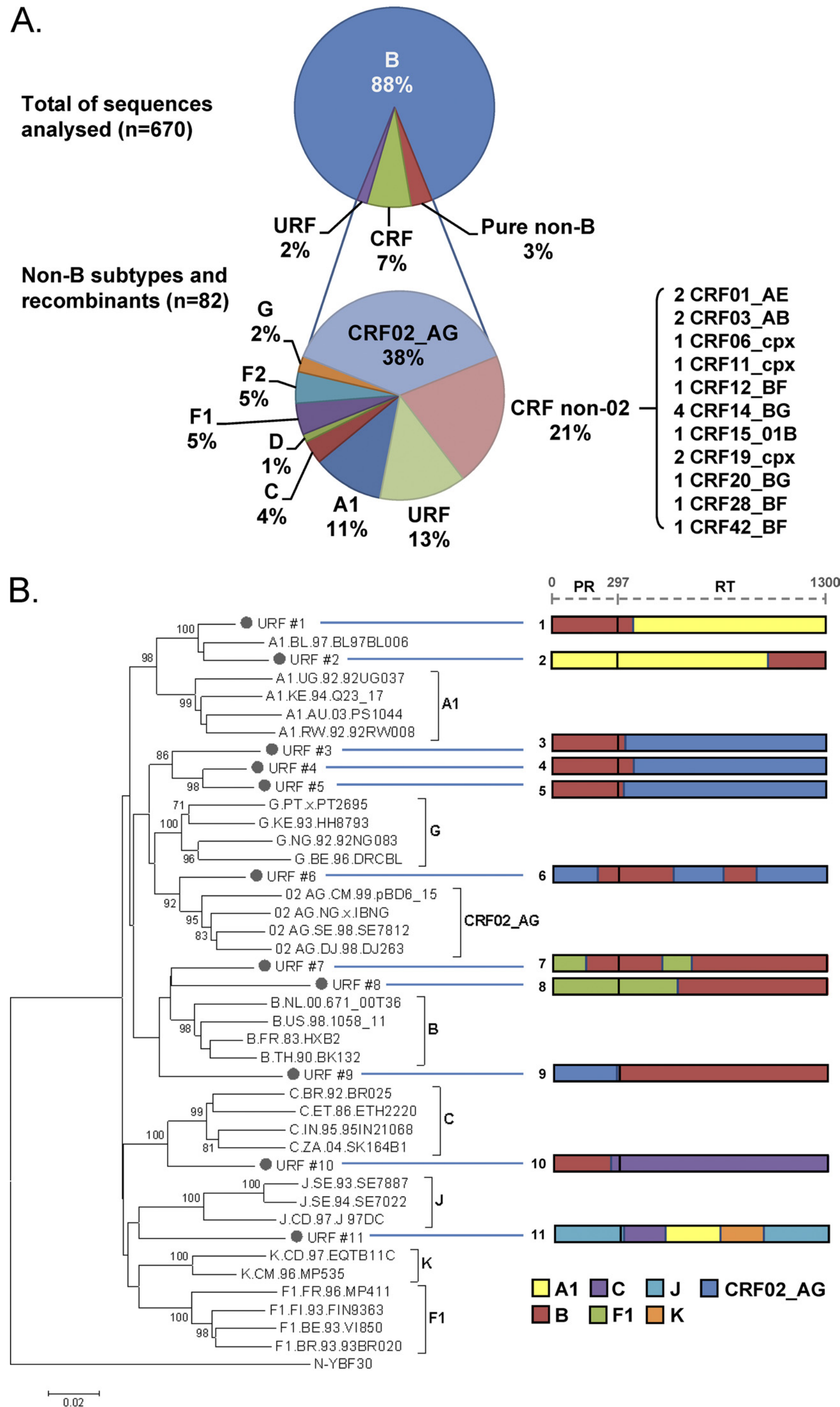

FIG 1 HIV-1 subtypes in the Spanish Cohort (CoRIS). (A) Distribution of HIV-1 variants in CoRIS according to phylogenetic analyses of the 670 *pol* sequences. (B) Neighbor-joining phylogenetic tree including the 11 HIV-1 unique recombinant forms found in the study population. The URFs are bulleted and are connected by blue horizontal lines to diagrams of the mosaic patterns of their *pol* sequences (length, 1,300 nucleotides). PR, protease; RT, reverse transcriptase. Bootstrap values of 1,000 repetitions are expressed as percentages. The original tree included all CRFs described at the moment of this work.

one showed a complex pattern including regions of subtypes A1, C, J, and K. Interestingly, 10 of the 11 URFs contained subtype B regions.

Table 1 records the epidemiological features of 11 HIV-1 URF-infected CoRIS patients defined by phy (Fig. 2) and confirmed by bootscanning and other methods (Fig. 1). Seven of these patients came from sub-Saharan Africa ($n = 3$), Eastern Europe or Russia ($n = 2$), South or Central America ($n = 1$), or North Africa ($n = 1$). Of note, the remaining four were native Spaniards (36.4% of cases), demonstrating the increasing heterogeneity of HIV-1 even in native Western Europeans. The modes of transmission for the URF-infected patients were as follows: 6 and 2 acquired HIV in-

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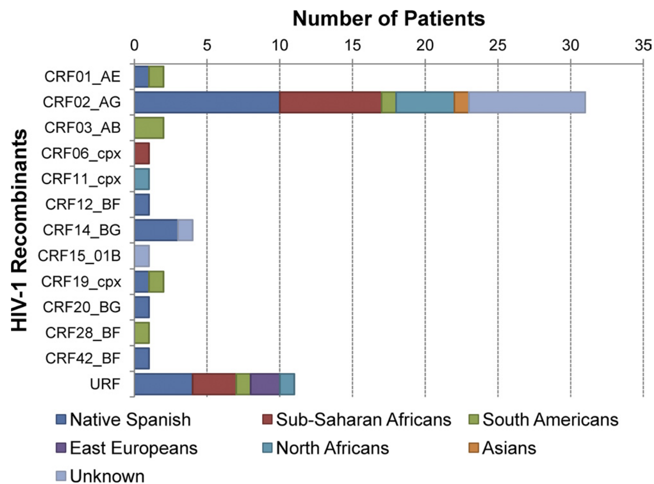

FIG 2 Distribution of patients infected by HIV-1 recombinants in CoRIS according to their geographic origins.

fection through heterosexual or homosexual routes, respectively; 2 were injecting drug users (IDU); and for 1 patient, viral transmission took place through a contaminated-blood transfusion. In other words, URF variants in the CoRIS cohort were transmitted mostly through heterosexual contacts.

Clusters of CoRIS patients carrying complex recombinants. One cluster was found in the phylogenetic analysis of the 11 sequences assigned to HIV-1 URF variants (Fig. 1). This cluster consisted of three samples harboring sequences from HIV-1 subtype B, mainly at the protease, and CRF02_AG sequences at the reverse transcriptase (Fig. 1, URFs 3 to 5), with very similar breakpoint estimates. The three patients were diagnosed in Madrid, Spain, and were infected by heterosexual transmission. Two of them were from sub-Saharan Africa (Table 1), which could explain the presence of CRF02_AG sequences, given the predominance of this strain in their country of origin (Equatorial Guinea). The third was a North African. The remaining eight URFs did not cluster with any other sample in the study population.

DISCUSSION

One of 10 HIV-1-infections in Spain could represent recombinant variants. This study was performed within a large and representative Spanish cohort of ART-naïve HIV-infected patients included in the Research Network on HIV/AIDS (CoRIS). CoRIS collects data on patients from different areas of Spain, a country with one of the highest HIV prevalences in the European Union (13, 46). The results presented confirmed that subtype B is still the main HIV-1 variant in Spain, as reported previously for the same sample of subjects from CoRIS (16), and are concordant with data from other Spanish studies (23, 31, 34). What our study adds is the finding that 8.8% of the 670 CoRIS patients (14% of the 164 patients assessed in 2008) were infected with HIV-1 recombinant variants (CRFs and URFs) and the description of the genetic nature of the complex recombinants. The recombinant variants represented almost three-quarters (72%) of the 82 HIV-1 non-B variants found. The highest prevalence of recombinants was found in patients from sub-Saharan Africa, followed by Eastern Europeans. As previously reported in the initial CoRIS description (16) and in other studies (23, 24, 31, 34), recombinant CRF02_AG was the most frequently found HIV-1 non-B variant in our country, accounting for more than one-third of HIV-1 non-B infections. This is due to the high prevalence of this recombinant in Central and West Africa, the most common origin of infected sub-Saharan Africans residing in Spain. Of note, we detected CRF02_AG in an Asian patient, as well as CRF03_AB in two South American patients. To our knowledge, this was the first time that these two variants were reported for patients from those regions in Spain, suggesting that transmission occurred in Spain.

Introduction of complex HIV-1 variants into the native population. In recent years, increasing prevalences of HIV-1 non-B subtypes and recombinants have been reported across Western Europe (23, 51, 54). The rising prevalence of HIV-1 non-B variants has traditionally been attributed to the growing number of immigrants from developing regions where these variants are prevalent. In fact, one-third of patients newly diagnosed with HIV in Spain in 2008 were immigrants (8, 38). However, the current heterogeneity of the HIV epidemic in Spain caused by recombinants can be only partially explained by immigration. Among the 23 and 59 subjects infected by HIV-1 non-B pure subtypes or recombinants, 9 (39.1%) and 22 (37.3%), respectively, were native patients. Among the 22 native subjects infected by recombinants, 18 and 4 patients carried CRF and URF variants, respectively. The detection in native Spaniards of 37.3% of the recombinant variants found in this study is evidence of the intro-

TABLE 1 Epidemiological features of the 23 HIV-1-infected patients included in CoRIS carrying URF sequences at *pol*

URF no.[a]	Patient ID[b]	Sex[c]	Origin	Parental strains	Exposure category[d]	City (region) of sampling	Yr of sampling
1	21	F	Eastern Europe	B/A1	IDU	Granada (East Andalusia)	2005
2	41	M	Eastern Europe	A1/B	IDU	Elche (Valencia)	2008
3	46	M	Equatorial Guinea	B/CRF02	Heterosex.	Madrid	2007
4	52	M	Equatorial Guinea	B/CRF02	Heterosex.	Madrid	2005
5	29	F	North Africa	B/CRF02	Heterosex.	Madrid	2005
6	50	M	Spain	B/CRF02	Homosex.	Malaga (South Andalusia)	2008
7	1	U	Spain	B/F1	Heterosex.	Seville (West Andalusia)	2008
8	76	M	Spain	B/F1	Homosex.	Madrid	2006
9	39	M	South America	CRF02/B	Other	Madrid	2006
10	34	M	Spain	B/C	Heterosex.	Madrid	2008
11	66	F	Sub-Saharan Africa	J/C/A1/K	Heterosex.	Terrassa (Catalonia)	2008

[a] Numbered as in Fig. 1.
[b] Patient number according to the sequence alignment provided as File S1 in the supplemental material.
[c] M, male; F, female; U, unknown.
[d] Heterosex., heterosexual risk behavior; Homosex., homo/bisexual risk behavior; IDU, injecting drug user.

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duction of complex HIV-1 variants into the native population of Spain, which also happens in other developed countries. Thus, the attribution of the increasing HIV-1 heterogeneity exclusively to immigrant populations is a prejudgment that only partially explains this phenomenon.

Origin and transmission of URFs in Spain. Interestingly, 13% of infections with HIV-1 non-B variants were caused by URFs. These are very frequent in regions where multiple clades cocirculate, such as sub-Saharan Africa (10), and are increasingly present in developed countries (15). However, whether the complex recombinant variants were transmitted directly from the immigrant community to the native population or whether the recombination events took place in native Spaniards cannot be ascertained with the current data.

URF sequences from heterosexually infected sub-Saharan Africans that form a cluster (URFs 3 to 5) resulted from very similar recombination events involving CRF02_AG and subtype B, a phenomenon previously reported in both Spain and France (24, 29). Two of these patients came from Equatorial Guinea, where CRF02_AG is highly prevalent (12), but the third patient was from North Africa, where subtype B is prevalent (1). The different origins of the patients suggest that the infection and subsequent spread occurred in Spain. Of note, URFs 6 and 9 also were B/CRF02_AG recombinants, although these recombinants did not share a common origin with those included in the cluster. In addition, other recombinants, including sequences typically found in Eastern European (URFs 1 and 2, including subsubtype A1) and South American (URFs 7 and 8, including subtypes B and F) countries, were found, reflecting the wide variety of geographical origins of immigrants in Spain.

Despite the pandemic spread of HIV-1 recombinants, their times of origin are not well understood. A recent paper suggests that recombination was common in the early evolutionary history of HIV-1 (44). In this cohort, the oldest URF was sampled in 2005, but complex recombinants have been reported in our country at least since the end of the 1990s (22–24, 30, 33), including the description of the first CRF that originated in Western Europe (CRF14_BG) (11). More epidemiological data about the infection date, risk behavior, and the geographical regions in which these patients have resided or traveled, as well as additional sequencing of longer genetic regions and specific computer programs to study the viruses' genetic evolution, would be necessary to confirm these findings and to define the origin of complex recombinants circulating in Spain.

Possible underestimation of the frequency of HIV-1 URFs in molecular epidemiology studies. This work reveals the circulation and spread of complex HIV-1 variants in a large and representative cohort of HIV-infected persons in Spain. However, the detection of URFs could be even higher if more viral regions were analyzed. For instance, more than one-third of HIV-1 sequences described in the Los Alamos HIV database to date might be found to be recombinant forms if different genes or full-length sequences were analyzed (41), and therefore, complex recombinants could be more frequent than expected in the HIV-1 pandemic (35). Another limitation of our study lies in the representativeness of the data, given the unequal distribution of the patients across the 18 centers and the territory of Spain. Thus, due to the scarcity of data from certain regions and/or hospitals, our findings could not be representative of the HIV-1 epidemic in our country. Nevertheless, this is the largest HIV-1 molecular epidemiology study performed in Spain using phylogeny to determinate the distribution of HIV-1 variants.

Biological consequences of recombination in HIV-1 evolution. Not only is the frequency of recombinants likely underestimated, but an increasing presence of URFs in developed countries is expected in the coming years due to the movement of populations between countries where different HIV-1 variants are prevalent. In fact, decreasing numbers of pure subtype B viruses and increases in the numbers of unique recombinants including subtype B sequences among HIV-1-seropositive patients have also been reported recently in neighboring countries (15). It has also been suggested that the HIV-1 epidemic could be evolving toward a more complex epidemiological landscape (18). Recombination seems to be very important in the evolution of HIV-1 (42), since it can provide a biological advantage versus parental viruses (28), promoting biological adaptation and enhancing fitness (6). It can also facilitate drug resistance and may allow superinfecting HIV-1 strains to evade preexisting immune responses (32). Thus, the continuous spread of HIV-1 recombinants may have serious implications for efforts to control the AIDS pandemic (including future vaccination trials) and could represent one of the highest barriers to HIV-1 eradication (32). However, despite some cases where URFs are described as highly pathogenic (4), the clinical implications of the presence of URFs for the AIDS pandemic remain to be clarified.

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Increase of Transmitted Drug Resistance among HIV-Infected Sub-Saharan Africans Residing in Spain in Contrast to the Native Population

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Abstract

Background: The prevalence of transmitted HIV drug resistance (TDR) is stabilizing or decreasing in developed countries. However, this trend is not specifically evaluated among immigrants from regions without well-implemented antiretroviral strategies.

Methods: TDR trends during 1996–2010 were analyzed among naïve HIV-infected patients in Spain, considering their origin and other factors. TDR mutations were defined according to the World Health Organization list.

Results: *Pol* sequence was available for 732 HIV-infected patients: 292 native Spanish, 226 sub-Saharan Africans (SSA), 114 Central-South Americans (CSA) and 100 from other regions. Global TDR prevalence was 9.7% (10.6% for Spanish, 8.4% for SSA and 7.9% for CSA). The highest prevalences were found for protease inhibitors (PI) in Spanish (3.1%), for non-nucleoside reverse transcriptase inhibitors (NNRTI) in SSA (6.5%) and for nucleoside reverse transcriptase inhibitors (NRTI) in both Spanish and SSA (6.5%). The global TDR rate decreased from 11.3% in 2004–2006 to 8.4% in 2007–2010. Characteristics related to a decreasing TDR trend in 2007-10 were Spanish and CSA origin, NRTI- and NNRTI-resistance, HIV-1 subtype B, male sex and infection through injection drug use. TDR remained stable for PI-resistance, in patients infected through sexual intercourse and in those carrying non-B variants. However, TDR increased among SSA and females. K103N was the predominant mutation in all groups and periods.

Conclusion: TDR prevalence tended to decrease among HIV-infected native Spanish and Central-South Americans, but it increased up to 13% in sub-Saharan immigrants in 2007–2010. These results highlight the importance of a specific TDR surveillance among immigrants to prevent future therapeutic failures, especially when administering NNRTIs.

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Introduction

The presence of transmitted drug resistance mutations (TDR) in patients unexposed to highly active antiretroviral treatment (HAART) is a major problem in the management of HIV-1 infection. Several studies have described a high risk of virological failure to first therapy in patients harbouring resistance mutations conferring resistance to any of the drugs received. Nevertheless, first-treatment guided by initial resistance testing achieves similar efficacy in patients with primary drug resistance as in patients with wild-type virus [1–4]. Therefore, international guidelines recommend that initial treatment choice should depend on the HIV resistant test results prior to starting HAART [5–7].

Numerous works have analysed the TDR prevalence in Western Europe and the United States. After several years with a continuous increase in the TDR rate [8–10], the efficacy of HAART and the development of both new antiretroviral drugs and classes have led to stable [2,11–14] or decreasing trends [15,16] of TDR. However, the TDR trends could be intimately related to the antiretroviral programmes implemented in each region, but only few studies have reported TDR prevalences according to the origin of the patients [11,17]. Given that immigrant patients account for a growing portion of the HIV-infected population in developed countries, the presence and trends of TDR among this subgroup should be explored in detail. In Spain, a third of newly HIV-diagnosed patients are immigrants [18–19], most of them coming from Central and South America or sub-Saharan Africa. The special socio-cultural characteristics of these populations as well as the antiretroviral policies established in their regions of origin may affect the transmission of resistant variants. Therefore, we

analyze here the changes in the TDR rate through the last 15 years in a large set of HIV-infected naïve patients taking into account their origin.

Materials and Methods

Study population

A total of 732 HIV-1-infected patients diagnosed between 1996 and 2010 with at least one *pol* sequence prior to any antiretroviral treatment were included. Most (>98%) were under follow-up in different HIV/AIDS clinics in Madrid, Spain. The origin of the patients (i.e., self-reported place of birth) was: 292 native Spanish, 226 sub-Saharan Africans (SSA), 114 Central and South Americans (CSA), 26 East Europeans (including Russia), 20 West Europeans and North Americans, 3 North Africans, 2 Asians and 49 of unknown origin. The most frequent countries of origin for SSA were Equatorial Guinea (111 patients), Nigeria (28), Sierra Leone (10) and Liberia (7). For CSA, Ecuador (20), Argentina (15), Colombia (14), Brazil (13) and Cuba (13). For East Europeans Romania (10) and Russia (7). Finally, for West Europeans, the most frequent origins were France and Portugal (6 each). Both protease (PR) and reverse transcriptase (RT) sequences were available for 641 patients, only PR sequence for 89 patients and only RT for 2 patients. Most (495, 67.6% [CI: 64.2−71]) of the HIV-1 sequences included had been previously published [20–21]. The remaining were collected from Hospital Ramón y Cajal (n = 218) and Hospital Doce de Octubre (n = 19) in Madrid, Spain. This study was part of a project approved by the review board of the Hospital Ramón y Cajal Clinical Research Ethical Committee. It was designed to protect the rights of all subjects involved under the appropriate local regulations. To maintain subject confidentiality, a unique ID number was assigned to each specimen, and written consent obtained for each patient by clinicians.

Drug resistance

The prevalence of transmitted drug resistance was defined according to the list of mutations for TDR surveillance as recommended by the World Health Organization [22] using the Calibrated Population Resistance tool [23]. Genotypic interpretation of these resistance mutations was evaluated using the Stanford HIVdb Algorithm [24], version 6.0.11. Resistance was normalized in three levels: susceptible (S), intermediate (I), and resistant (R).

HIV-1 subtyping

HIV-1 subtypes and circulating recombinant forms (CRF) were identified by phylogenetic analysis of the *pol* sequences. The 2008 version of the subtype reference dataset provided by Los Alamos National Laboratory was used. At least two representative sequences of each 9 subtypes and the 43 CRF of HIV-1 group M available at the moment of the analysis were taken as references. DNA sequences were aligned using the ClustalX 2.0.11 program. The tree topology was obtained using the Neighbour-Joining method. The pairwise distance matrix was estimated using the Kimura two-parameter model within the DNAdist program, as implemented in the PHYLIP software package. Bootstrap resampling (1,000 data sets) of the multiple alignments was performed, with the bootstrap cut-off set at 700.

Statistical analysis

Prevalences were expressed in percentage and 95% confidence interval (CI). Continuous variables were compared using the *t*-test. Categorical variables were compared using the chi-square test or Fisher's exact test if appropriate. Association between epidemiological, clinical and virological factors was analysed by univariate and multivariate logistic regression. The maximum model included the variables of origin of the patient, route of HIV infection, HIV subtype and time period of infection. Changes over time in the resistance prevalences were analysed using a chi-square test for trends (linear-by-linear association). Significance was set at $p<0.05$.

Results

TDR prevalence (i.e., to any antiretroviral drug class) among the 732 patients diagnosed in Madrid, Spain, between 1996 and 2010 was 9.7% (CI: 7.6−11.8) (**Figure 1**). **Table 1** shows the main characteristics of the population comparing patients infected with wild-type viruses with those harbouring TDR. Regarding the antiretroviral drug class, TDR prevalence among patients in our study was 2.9% (CI: 1.7−4.1) for PR inhibitors (PI) (3.1% [CI: 1.1−5.1] in Spanish, 1.8% [CI: 0.1−3.5] in SSA and 1.7% [CI: −0.7−4.2] in CSA), 6.1% (CI: 4.2−7.9) for nucleoside reverse-transcriptase inhibitors (NRTI) (6.4% [CI: 3.6−9.3] in Spanish, 6.5% [CI: 2.8–10.2] in SSA and 5% [CI: 0.7−9.4] in CSA), and 5.4% (CI: 3.7−7.2) for non-nucleoside RT inhibitors (NNRTI) (5% [CI: 2.5−7.6] in Spanish, 6.5% [CI: 2.8–10.2] in SSA and 4% [CI: 0.2–7.9] in CSA). To assess the TDR prevalence according to the calendar year, sequences were grouped in three periods (2000-03, 2004-06 and 2007-10) according to the observed resistance trend by calendar year. Sequences obtained before year 2000 were included in the global estimates but excluded in the analyses due to their low number (n = 32) and representativeness since all were sequences from immigrant patients. In the whole study population, the TDR prevalence to any antiretroviral drug was 10.6% (CI: 5.3−15.9), 11.3% (CI: 7.8−14.8) and 8.4% (CI: 5−11.8) in those periods, respectively (p trend = 0.35). In the following paragraphs, the TDR prevalences and temporal trends in these periods are detailed according to different factors.

According to drug class

As observed in the global TDR prevalence, the presence of transmitted PI-resistance mutations reached a maximum in 2004-06 (4.1% [CI: 1.9−6.3]), and declined to its minimum value (1.6% [CI: 0−3.2]) in the last period (2007-10) (p trend = 0.23). On the other hand, rates of both NRTI- and NNRTI-resistance declined progressively and significantly along the three periods: 13.2% (CI: 5.2−21.3), 6.1% (CI: 3.4−8.7) and 4.4% (CI: 1.9−6.9), for NRTI (p trend = 0.03) and 10.3% (CI: 3.1−17.5), 5.8% (CI: 3.2−8.4) and 3.6% (CI: 1.3−5.9), for NNRTI (p trend = 0.04). This decline was more pronounced for NRTI-resistance mutations, getting closer to the NNRTI-resistance mutations rate in the last period (**Figure 2a**). Thus, transmitted resistance to RT inhibitors was two or threefold higher than that for PI in 2007-10 (p = 0.06).

According to HIV-1 variant

Subtype B was found in 383 (52.3%, [CI: 48.7–55.9]) patients, meanwhile non-B subtypes and recombinants infected 349 (47.7% [CI: 44.1–51.3]) patients. Among them, the most frequent variants were: CRF02_AG (128 patients), subtype A (36), subtype G (35), CRF12_BF (23) and subtype C (22). Although global TDR prevalence was similar in subtype B and in non-B variants (10.2% [CI: 7.1–13.2] *vs.* 9.2% [CI: 6.1–12.2], p = 0.64) their TDR temporal trend was different (**Figure 2b**): in subtype B, TDR declined from 12.5% (CI: 3.1−21.9) in 2000-03 to 6.8% (CI: 2.3–11.4) in 2007-10 (p trend = 0.16), but in non-B variants this prevalence remained stable (9.5% [CI: 3.2–15.8] and 9.8% [CI: 4.7–14.8], respectively [p trend = 0.99]). Of note, TDR was higher

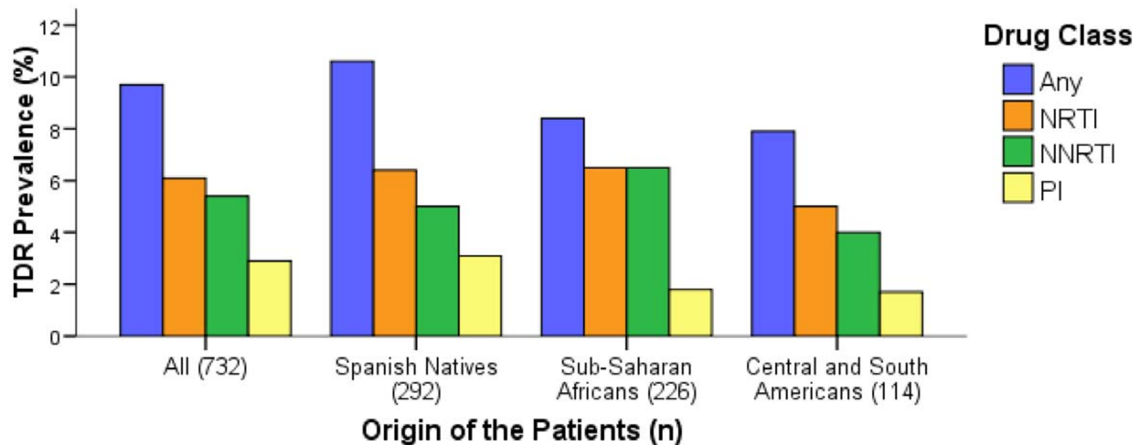

Figure 1. Prevalence of TDR to each drug class according to the origin of the patients. TDR, transmitted drug resistance; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; PI, protease inhibitors; n, number of patients.
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in non-B than in subtype B (9.8% [CI: 4.7–14.8] *vs.* 6.8% [CI: 2.3–11.4], $p = 0.54$) in the last period.

According to the origin of patients

Global TDR prevalences were 10.6% (CI: 7.1–14.1) in native Spanish, 8.4% (CI: 4.8–12) in SSA and 7.9% (CI: 2.9–12.8) in CSA (**Figure 1**). Regarding the temporal trends in each population (**Figure 2c**), TDR declined constantly in Spanish natives: 13.2% (CI: 2.41–23.9) in 2000-03, 11.8% (6.7–17) in 2004-06 and 8% (CI: 2.7–13.3) in 2007-10 (p trend = 0.28). In CSA, no TDR mutations were detected in 2000-03, but TDR reached similar rates as in natives in the following periods: 10.2%

Table 1. Comparison of characteristics between patients infected with virus harbouring TDR and patients infected with wild-type virus.

	Total	With TDR	Wild-type	*p* value	OR (95% CI) in univariate analysis
Patients (n)	732	71 (9.7)	661 (90.3)	-	
Origin [n (%)]					
Spain	292 (39.9)	31 (43.7)	261 (39.5)	0.5	1
SSA	226 (30.9)	19 (26.8)	207 (31.3)	0.4	0.77 (0.42–1.40)
CSA	114 (15.6)	9 (12.7)	105 (15.9)	0.47	0.73 (0.33–1.58)
Other	100 (13.7)	12 (16.9)	88 (13.3)	0.38	1.15 (0.57–2.33)
Sex [n (%)][a]					
Male	469 (68.6)	47 (69.1)	422 (68.5)	0.92	1
Female	215 (31.4)	21 (30.9)	194 (31.5)	0.92	1.67 (0.50–5.58)
Route of transmission [n (%)][b]					
Heterosexual contact	269 (49.4)	23 (44.2)	246 (50.0)	0.43	1
Homo/bisexual contact	196 (36.0)	18 (34.6)	178 (36.2)	0.82	0.90 (0.46–1.78)
Injection drug use	68 (12.5)	9 (17.3)	59 (12.0)	0.27	1.58 (0.73–3.41)
Other	11 (2.0)	2 (3.8)	9 (1.8)	0.33	1.22 (0.70–2.12)
HIV-1 Subtype [n (%)]					
B	383 (52.3)	39 (54.9)	344 (52.0)	0.64	1
Non-B	349 (47.7)	32 (45.1)	317 (48.0)	0.64	0.89 (0.54–1.46)
Year of HIV-1 Infection [n (%)]					
<2000	32 (4.4)	0 (0)	32 (4.8)	-	0
2000-03	132 (18.0)	14 (19.7)	118 (17.8)	0.82	1
2004-06	318 (43.4)	36 (50.7)	282 (42.7)	0.19	1.08 (0.56–2.07)
2007-10	250 (34.1)	21 (29.6)	229 (34.6)	0.39	0.78 (0.38–1.58)

TDR, transmitted drug resistance; OR, odds ratio; CI, confidence interval; n, number of patients; SSA, sub-Saharan Africa; CSA, Central and South America.
[a]Data available for 684 patients.
[b]Data available for 544 patients.
[c]Data available for 541 patients.
doi:10.1371/journal.pone.0026757.t001

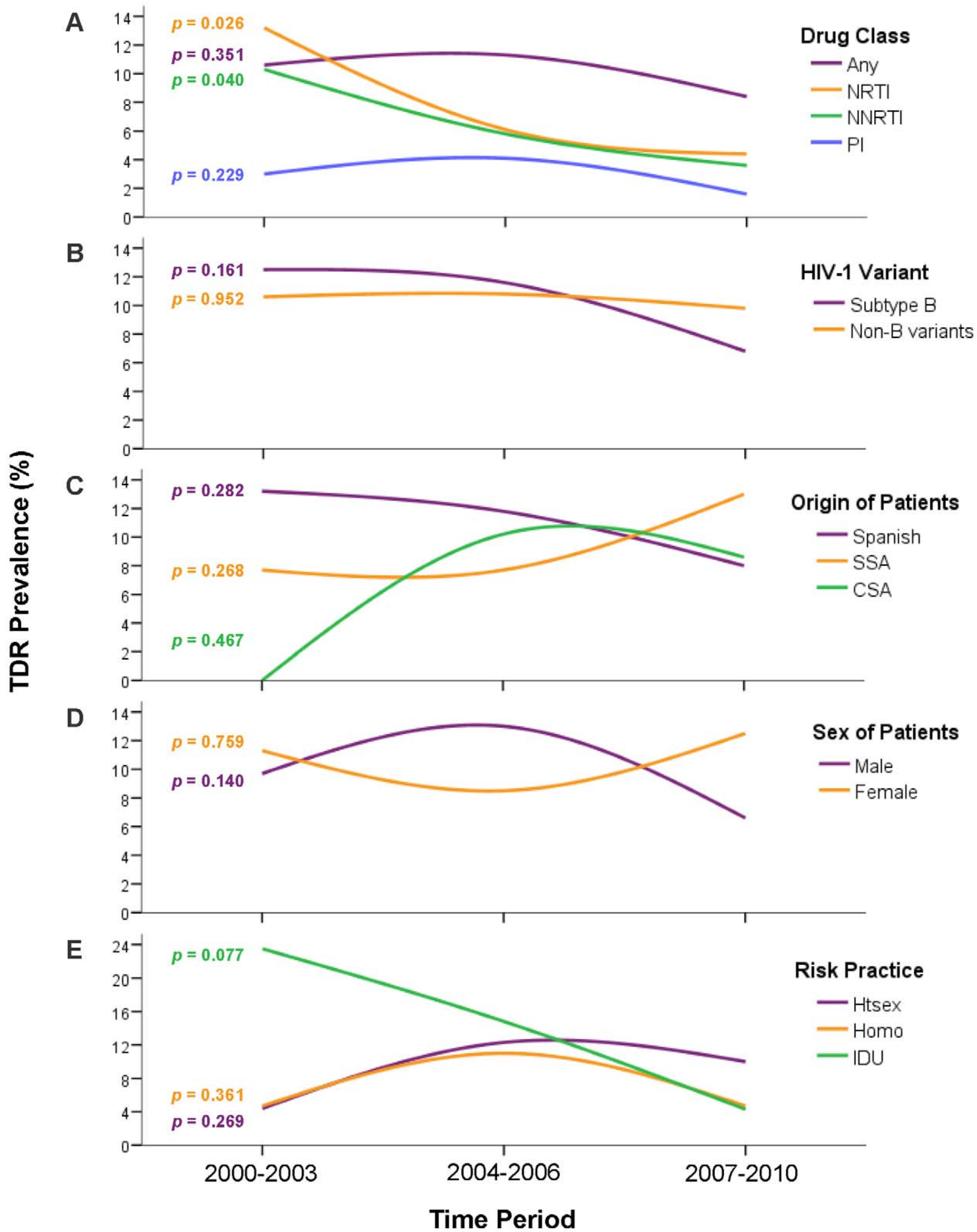

Figure 2. Temporal trends of TDR prevalence. The prevalences are detached according to A) drug class; B) HIV-1 variant; C) origin of the patients (as self-reported birth place); D) sex of the patients; and E) risk practice. TDR, transmitted drug resistance; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; PI, protease inhibitors; SSA, sub-Saharan Africa; CSA, Central and South America; Htsex, heterosexual contact; Homo, homo/bisexual contact; IDU, injection drug use. The *p*-values shown next to the lines and sharing color code correspond to the chi-square test for trend in each case.
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(CI: 2.5–17.9) in 2004-06 and 8.6% (CI: −0.7–17.8) in 2007-10 (p trend = 0.47). In contrast, among SSA the TDR prevalence remained stable in 2000-03 and 2004-06 (7.7% [CI: 1.2–14.2] in both cases) and rose up to 13% (CI: 5.1–21) in 2007-10 (p trend = 0.27). Thus SSA presented the highest TDR frequency in the last period. Finally, TDR prevalence was very high among East Europeans (5/26, 19.2% [CI: 4.1–34.4]) and West Europeans (3/20, 15% [CI: −0.6–30.6]), although the low number of patients did not allow to differentiate the prevalence according to temporal trend.

According to sex of the patients

Most (68.6% [CI: 65.1–72]) of the subjects with available data were males (**Table 1**). Global TDR prevalence was similar between them and females (10% [CI: 7.3–12.7] *vs.* 9.8% [CI: 5.8–13.7], respectively, p = 0.97). Although this similarity was observed in 2000-03 (9.7% [CI: 2.3–17] *vs.* 11.3% [CI: 3.4–19.2]), TDR increased among male patients but decreased among females in 2004-06 (13% [CI: 8.7–17.4] *vs.* 8.5% [CI: 1.4–15.6], $p = 0.46$) and vice versa in 2007-10 (6.6% [CI: 2.8–10.3] *vs.* 12.5% [CI: 4.9–20.1], $p = 0.21$).

According to route of HIV infection of the patients

Half (49.4% [CI: 45.2–53.6]) of the patients with known route of transmission had been infected through heterosexual intercourse (**Table 1**). TDR prevalence was higher (13.2% [CI: 5.2–21.3]) in injection drug users (IDU) and similar in those infected through homo/bisexual (9.2% [CI: 5.1–13.2]) and heterosexual (8.5% [CI: 5.2–11.9]) intercourse. Regarding the temporal trend, there was a drastic decrease in the TDR rate among IDU (n = 68), the most common risk practice in Spain until the late 90 s. This rate was 23.5% (CI: 3.4–43.7; 4/17 patients) in 2000-03, 14.8% (CI: 1.4–28.2; 4/27) in 2004-06 and 4.3% (CI: −4–12.7; 1/23) in 2007-10 (p trend = 0.08). Among homo/bisexuals, the prevalence rose from 2000-03 (4.7% [CI: −4.3–13.9]) to 2004-06 (12.6% [CI: 6.44–18.8]) but decreased to the initial level in the last period (4.7% [CI: −0.5–10]) (p trend = 0.36). Finally, the TDR rate in heterosexuals seemed to stabilize after an increase from 2000-03 (4.4% [CI: −0.5–9.3]) to 2004-06 (12.3% [CI: 4.8–19.9]), and was the risk practice with the highest TDR prevalence in 2007-10 (10% [CI: 4.1–15.9]) (p trend = 0.32).

Patients infected with viruses harbouring TDR mutations

Among the 71 patients harbouring TDR mutations (**Table 1**), 31 were Spanish, 19 SSA, 9 CSA, 5 East Europeans, 3 West Europeans, 1 North African and 3 of unknown origin. In three quarters (53, 74.6% [CI: 64.2–84.8]) of the cases, TDR affected only a single drug class. However, in 6 patients (4 Spanish, 1 SSA and 1 CSA) a triple-class resistance was found. The pattern of TDR mutations was different for NRTI- and PI-resistance according to the origin (**Figure 3**). Among SSA, RT-M184I/V and PR-M46L were the most frequent mutations, respectively, meanwhile in both Spanish natives and CSA, RT-T215rev and PR-V82A were the most prevalent substitutions. For NNRTI-resistance, K103N was the most frequent mutation in all groups, although its prevalence was higher in SSA (4.7% [CI: 1.5–7.8]). In fact, K103N was the most prevalent mutation in all periods, and it was the mutation found in a third of the cases (15/45) where a single TDR mutation was found. The temporal trend of specific mutation prevalences showed significant reductions from 2000-03 to 2007-10 for tymidine-analogue mutations together (8.8% [CI: 2.1–15.6] to 2.4% [CI: 0.5–4.3], p trend = 0.03) and K103N (10.3% [CI: 3.1–17.5] to 3.2% [CI: 1–5.4], p trend = 0.02) and non-significant reduction for M184I/V (4.4% [CI: −0.5–9.3] to 2.4% [CI: 0.5–4.3], p trend = 0.67). In the logistic model (**Table 1**), there was no significant difference in the prevalence of TDR according to sex, year or route of HIV-1 infection or origin of the patients.

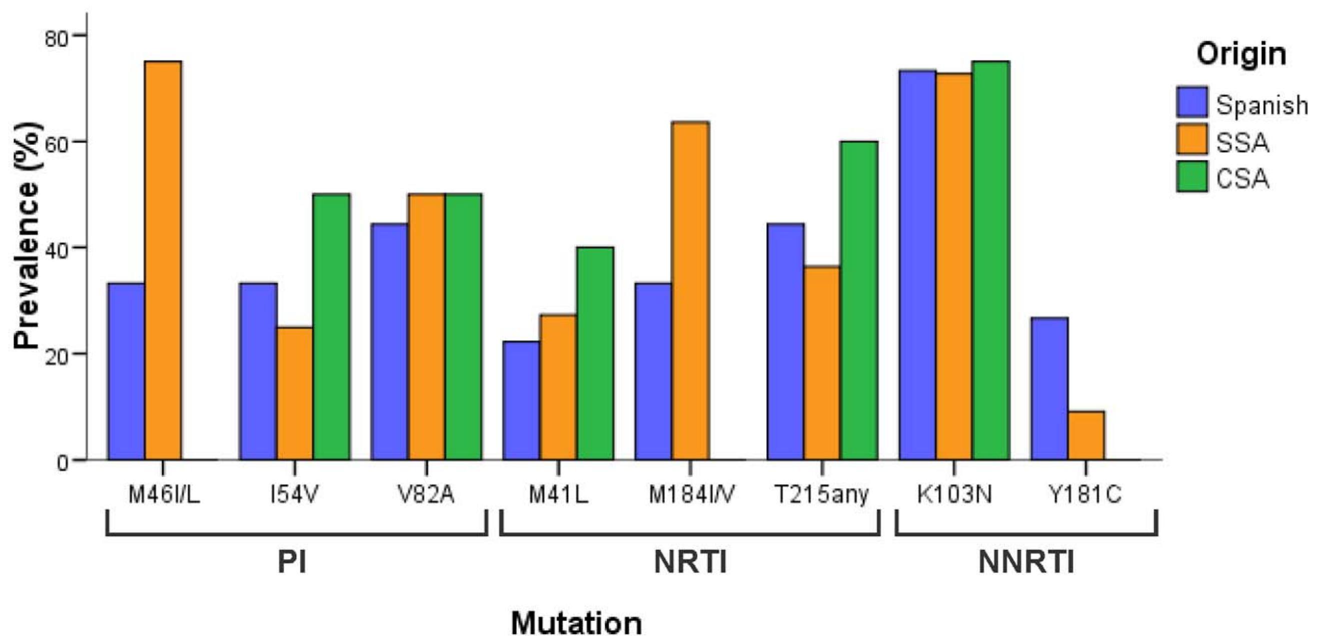

Figure 3. Prevalence of individual mutations among the patients infected with HIV-1 variants harbouring any TDR. SSA, sub-Saharan Africa; CSA, Central and South America; PI, protease inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors.
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Clinical implications of TDR

The analysis of the genotypic resistance interpretation of the 71 *pol* sequences harbouring TDR (**Figure 4**) revealed that especially in the case of PI there are options to select a fully active drug even in patients carrying PI-resistance mutations if the treatment choice is guided by the resistance test. Actually, none of the 21 patients with PI-resistance mutations presented high resistance levels to all PI tested due to their susceptibility to new-generation drugs like darunavir or tipranavir. Among the 39 patients with NRTI-resistance mutations, only 5 (12.8% [CI: 2.3–23.3]) presented some level of resistance to all NRTI included. Furthermore, 14 (35.9% [CI: 20.8–50.9]) and 16 (41% [CI: 25.6–56.5]) were fully susceptible to the most used NRTI combinations, emtricitabine/tenofovir and lamivudine/abacavir. Regarding NNRTI-resistance, none of the 34 patients with resistance mutations was fully susceptible to efavirenz and nevirapine, the most used drugs of this class. Only etravirine would have some level of activity in these patients.

Differences in HIV viral load

Patients infected with HIV variants harbouring TDR presented a significantly lower mean viral load compared to those infected with wild-type HIV (4.1 log [SD: 0.83] *vs.* 4.4 log [SD: 0.83] RNA-HIV-1 copies/ml, $p = 0.03$). This result was also obtained when comparing patients infected with viruses carrying or not the mutation M184I/V (3.9 log [SD: 0.86] *vs.* 4.4 log [SD: 0.83], $p = 0.04$). In addition, according to sex, male patients presented a significantly higher viral load than female patients (4.5 log [SD: 0.79] *vs.* 4.2 log [SD: 0.89], $p < 0.001$) at diagnosis time. There were no differences in plasma HIV viraemia according to origin, risk practice, HIV infection year or HIV subtype in the study population.

Transmission clusters

Phylogenetic analyses only revealed a cluster of drug-resistance mutations transmission. This involved a native Spanish and a Chilean diagnosed in 2006 in the same clinic and infected with three-drug class resistant subtype B viruses. Both specimens harboured mutations F53L and L90M at PR and M41L, K103N, L210W and T215D at RT.

Discussion

This study explores the evolution of TDR rates through 15 years (1996–2010) in a large set of HIV-infected naïve patients diagnosed in Spain taking into account different factors: drug class, viral variant, origin of the patient, route of infection and sex. The global TDR prevalence reported in this work (9.7%) is in agreement with the most representative European studies, where this rate has stabilized around 10% [1,11,12,15]. In the USA, the TDR prevalence has traditionally been higher, but it also has started to stabilize [4,25]. According to our results, the rate of transmitted NRTI-resistance is clearly dropping in Spain until reaching the levels of NNRTI-resistance, as already reported in other developed countries [11,15]. In addition, in three quarters of the cases the transmission of resistance is limited to a single class.

The stabilization of TDR rates is partially due to the high efficacy of the HAART in Europe, where there is universal access to this treatment. However, in other regions where treatment is not available to all HIV-infected people on a regular basis, emergence and transmission of HIV resistant variants is likely to happen. Thus, it could be assumed that the TDR trend previously reported in developed countries will be observed in developing regions as treatment programmes are implemented. According to this, low TDR prevalences found in the last years in regions with shorter HAART tradition [26–27] are expected to be followed by a peak as seen in Europe around years 2002-03. This has been reported in African regions with an older treatment scale-up [28–30]. Here, we describe a growing TDR prevalence among sub-Saharan patients (SSA) in contrast to the native HIV-infected population and the general trends reported in West Europe. A high rate of losses to follow-up has been described among SSA under treatment in Spain probably due to a low educational level and their high geographic mobility looking for work [31]. Due to an ineffective follow-up, drug-resistance variants can be emerging and circulating among treated SSA and eventually producing TDR events within this subgroup. Given that SSA patients

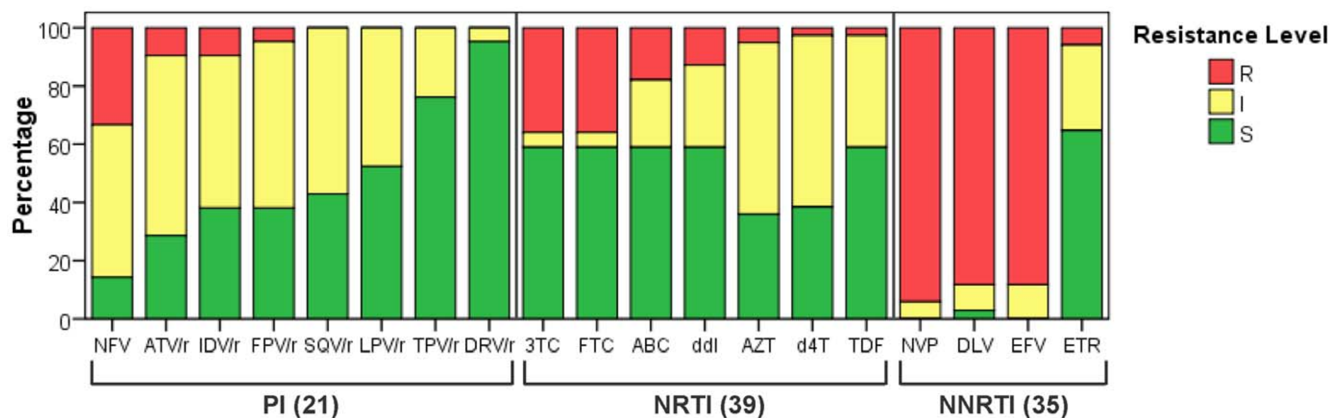

Figure 4. Predicted susceptibility to antiretroviral drugs of the 71 viruses carrying TDR mutations. Susceptibility was estimated according to the HIVdb Interpretation Algorithm (Stanford University, Palo Alto, CA, USA). TDR, transmitted drug resistance; PI, protease inhibitors: nelfinavir (NFV); atazanavir/r (ATV/r), indinavir/r (IDV/r), fosamprenavir/r (FPV/r), saquinavir/r (SQV/r), lopinavir/r (LPV/r), tipranavir/r (TPV/r) and darunavir/r (DRV/r), where "/r" indicates co-administration with low-dose ritonavir (RTV) for pharmacological "boosting". NRTI, nucleoside reverse transcriptase inhibitors: lamivudine (3TC), emtricitabine (FTC), abacavir (ABC), didanosine (ddI), zidovudine (AZT), stavudine (d4T), tenofovir (TDF). NNRTI, non-nucleoside reverse transcriptase inhibitors: nevirapine (NVP), delavirdine (DLV), efavirenz (EFV), etravirine (ETR); R, high level resistance; I, intermediate resistant; S, susceptible.
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occasionally come to Spain to receive antiretroviral treatment and return afterwards to Africa, transmission of HIV resistant variants within this collective could be happening either in Africa or in Spain. Thus, the observed increasing TDR trend among infected SSA in our study population, the high prevalence of HIV-1 non-B variants in that collective, and the different NRTI-resistance TDR pattern found in SSA than in native Spanish, would strongly suggest that SSA in Spain could have acquired the infection in their countries of origin or in Spain through infected Africans carrying resistant viruses.

On the other hand, we observed an absence of TDR in 2000-03 among the 18 CSA in our cohort. However, TDR rates reached similar levels as in native Spanish in the following periods. This could be due to the low number of CSA patients included in the first period or to the limited scale up access to treatment in these regions. Nowadays, TDR trends in CSA patients are expected to be similar to trends among Spanish because antiretroviral therapy programmes have been working in most of these countries for at least a decade [32]. Also the presence of non-B variants typical from certain regions of Central and South America (CRF12_BF in Argentina and Chile and CRF23_BG in Cuba) among CSA patients harbouring resistant strains suggests that these infections probably occurred before arriving to Spain. Despite their low representation, both East and West Europeans (26 and 20 patients, respectively) showed TDR prevalences beyond 15%. Nevertheless, these patients infected with resistance variants were diagnosed before 2006, and were IDU in most cases, both characteristics related to a higher TDR prevalence according to our results.

Focusing on the HIV subtype, we describe a higher TDR prevalence in the last study period (2007-10) in patients carrying non-B variants *versus* subtype B. This was due to the TDR decrease among subtype B in contrast to its stabilization among non-B variants. This is in agreement with other studies reporting growing TDR rates in non-B *versus* subtype B [20,33]. However, most studies [11–13,15] report higher TDR rates in subtype B, although all of them describe the absolute prevalences in the entire study period and not the temporal trends according to the subtype. Our results could be misleading due to the high prevalence of non-B subtypes and recombinants among SSA patients. Actually, excluding SSA patients, non-B variants showed a decreasing TDR prevalence from 15.8% to 6.1% in the first and last period, respectively (results not shown). Therefore the increase was probably due to the origin, not to the subtype. On the other hand, the correlation between HIV subtype and birth place of the patients could also have confused the different mutational pattern described: the preferential presence of NRTI-resistance mutation M184I/V in SSA compared to T215revertants in Spanish and CSA could appear to be associated to the infection by non-B variants and subtype B, respectively, as reported in other countries [34,35]. However, the different antiretroviral strategies implemented in these regions could also be a probable cause of this different mutational pattern in the studied naive HIV-1-infected patients carrying resistant viruses.

Our observation of a lower viral load among patients infected with variants carrying M184I/V has previously been described [36] due to the fitness reduction related to its acquisition. In addition, the lower viral load in female *versus* male at baseline has already been observed [37]. Regarding the analysis according to the route of infection, the decrease of the TDR rate among IDU across the years is a consequence of the evolution of HIV epidemics in Spain and a better treatment compliance among the IDU population. In the late 90 s the use of injecting drugs was by far the first route of HIV infection, but clearly decreased while HIV transmission due to unprotected sexual contacts increased [19].

According to the genotypic resistance interpretation, the TDR mutations found in 71 patients mainly affect the NNRTI-based therapy, the first choice in Spain for naïve patients [7], due to the presence of K103N, the most frequent mutation in the study especially among SSA. Despite its transmission as a singleton mutation, K103N seriously compromises the use of efavirenz or nevirapine. Only etravirine, the second-generation NNRTI approved solely for salvage regimens, could have some level of activity in these patients. Therefore, resistance testing prior to first-line treatment choice is strongly recommended. In developed countries there are therapeutic options as new generation NNRTI, PI or even new classes as integrase or entry inhibitors. Unfortunately, all these options are not available in developing regions where NNRTI-resistance among naïve patients is rising [28]. Interestingly, the highest NNRTI-resistance prevalence in our study was found among SSA, although in some cases this could possibly be explained by unrecorded, prophylactic use of nevirapine in Africa. Our results also suggest that PI could be active in most naïve patients carrying PI-resistance mutations if the treatment choice were guided by the resistance test.

The main limitation of this study is the restricted number of patients which prevents a solid statistical support of the TDR trends, for instance analyzing East and West Europeans living in Spain. The delimitation of the study periods could also be a source of bias despite the fact they were selected to reflect the trend by calendar year as accurately as possible. In addition, sub-Saharan patients might be overrepresented due to the special care they receive in the Tropical Medicine Units ascribed to the clinics included in the work, especially patients from Equatorial Guinea, a former Spanish colony located between Cameroon and Gabon. Nevertheless, since the year 2000 the immigration in Spain has increased exponentially, particularly in Madrid, where 17% of the general population were foreigners in 2010. Finally, the surprisingly high rate in native Spanish of RT-M184I/V (4.4%), a mutation rapidly cleared due to its cost in terms of viral fitness [38], might reflect an unrecorded previous treatment exposure in some patients.

In summary, TDR prevalence in Spain follows the general trend in Western Europe. The TDR rate is decreasing especially for NRTI-resistance. However, the TDR trends during 2000–2010 differed according to the origin of the patients. Whereas in native Spanish and Central-South Americans the TDR rate is decreasing, we report for the first time an increasing TDR prevalence among sub-Saharan patients diagnosed in Spain, who form the collective with the highest TDR prevalence in Spain since 2007. This could be due to the special socio-cultural characteristics of these patients, which may compromise antiretroviral treatment compliance and follow-up. Since TDR is expected to increase in developing regions as treatment is implemented, the presented results highlight the importance of a specific TDR surveillance among immigrants living in high-income countries to prevent future therapeutic failures, especially when administering NNRTI due to the high K103N prevalence.

Author Contributions

Conceived and designed the experiments: ÁH GY. Performed the experiments: GY MdM. Analyzed the data: GY MdM JAPM. Contributed reagents/materials/analysis tools: MJPE JAPM JCG JLG SM. Wrote the paper: ÁH GY. Revised and contributed to the manuscript: ÁH GY MdM MJPE JAPM JCG JLG SM.

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Sensitivity of seven HIV subtyping tools differs among subtypes/recombinants in the Spanish cohort of naïve HIV-infected patients (CoRIS)

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ABSTRACT

Background: HIV-1 group M is classified into 9 subtypes and recombinants (CRFs/URFs). Variants other than subtype B (non-B) cause 90% of infections worldwide. HIV is often subtyped using automated tools instead of the gold-standard phylogenetic analysis. We evaluated the reliability of subtyping tools *vs.* phylogeny in a panel of HIV-1 *pol* sequences from the cohort of naïve patients of the HIV/AIDS Spanish Research Network (CoRIS).
Methods: HIV-1 subtyping was performed using seven automated subtyping tools (Stanford, Geno2pheno, Rega, NCBI, EuResist, STAR, TherapyEdge) in HIV-1 *pol* sequences from 670 CoRIS patients previously subtyped by phylogeny (587 subtype B/83 non-B). Sensitivity with respect to phylogeny was assessed.
Results: Most tools correctly classified subtype B, although up to 15% of non-B sequences were wrongly identified as B depending on the tool. For subtype B and CRF02_AG identification, Stanford/NCBI and Geno2pheno/Rega presented the highest/lowest sensitivities, respectively. EuResist and Geno2pheno correctly classified all 13 non-B "pure"subtypes at *pol*. The efficacy of all subtyping tools dropped clearly when identifying recombinants different from CRF02_AG. Only NCBI05, Rega and STAR identified URF, but with very low sensitivities. NCBI classified the highest number of subtypes B as non-B, and overestimated recombinants, especially when including references of 2009.
Conclusions: Automated tools are useful for subtype B identification, although they present serious limitations in classifying variants uncommon in developed regions, especially recombinants. Their sensitivity depends on the prevalence of non-B variants in the population, and decreases drastically when the frequency of recombinants increases. Furthermore, HIV-1 variant distribution differs according to the tool used.

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1. Introduction

Human immunodeficiency virus type 1 (HIV-1) has been divided in four groups: M (main), O (outlier), N (non-M, non-O) and the recently identified P (Plantier et al., 2009). Most variants are included in group M, which is subdivided into 9 subtypes (A–D, F–H, J, K), at least 45 circulating recombinant forms (CRFs) (http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html) and multiple unique recombinant forms (URFs). URFs are widely spread throughout the world, with different recombination breakpoints from those found in CRFs. The global distribution of HIV-1 clades is unequal. Subtype B only accounts for around 10% of the total infections worldwide (Hemelaar et al., 2006), but is prevalent in developed countries, including Western Europe and North America. Thus, most clinical and biological studies are based on this clade. The remaining subtypes and the recombinant forms (grouped as "non-B" variants) have been studied less, even though they cause about 90% of the estimated 33 million HIV

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infections. Among them, subtypes C and A and recombinants CRF01_AE and CRF02_AG are responsible for nearly 70% of all HIV infections. HIV-1 molecular epidemiology studies have revealed an increasing prevalence of non-B subtypes and recombinants in developed countries in the last decade (Wensing et al., 2005; Sagir et al., 2007; Yerly et al., 2007; Frange et al., 2008; Holguín et al., 2008a). In Spain, non-B variants have been increasing among newly diagnosed HIV-1 natives and immigrants in the last years, and their current prevalence is about 15% (Holguín et al., 2008a; Cuevas et al., 2009; De Mendoza et al., 2009), although it is higher when the surveillance studies include a larger immigrant population.

In addition to the epidemiological impact, the presence of non-B subtypes and recombinants has implications for the diagnosis (Candotti et al., 2000), the viral load quantification (Gottesman et al., 2004; Kim et al., 2007; Rouet et al., 2007, 2010; Steegen et al., 2007; Holguín et al., 2008b; Korn et al., 2009; Wirden et al., 2009), the vaccine design (Zhang et al., 2010), the progression to AIDS (Vasan et al., 2006; Baeten et al., 2007) and the cognitive impairment (Sacktor et al., 2009). The genetic peculiarities of non-B variants (Kantor and Katzenstein, 2003; Yebra et al., 2010) could affect the emergence of resistance (Grossman et al., 2004; Gonzalez et al., 2008), the viral replicative capacity (Holguín et al., 2006), the genetic barrier of certain drugs (Van de Vijver et al., 2005), the drug-binding affinity (Kinomoto et al., 2005) and the reliability of algorithms of genetic resistance interpretation (Snoeck et al., 2006; Champenois et al., 2008; Yebra et al., 2010). Thus, a proper detection and description of HIV-1 variants in representative cohorts are of clinical importance. Phylogenetic analysis (phy) is the gold standard method for subtyping and discrimination between subtypes and/or CRFs. However, it is not widely implemented in clinical settings because of its complexity. Several automated subtyping tools have been developed for HIV-1 classification in the clinical routine. They are fast and easy to use, and most are free-of-charge. However, they have considerable limitations *vs.* phy that confound their results especially in the analysis of non-B variants (Smith et al., 2005; Holguín et al., 2008c,d; Ntemgwa et al., 2008; Galán et al., 2009; Wilkinson and Engelbrecht, 2009; Yebra et al., 2010). Furthermore, the results of different tools are usually in disagreement (Gifford et al., 2006; Loveday and MacRae, 2006), especially in the analysis of recombinants.

The objective of the present study was to assess the sensitivity and specificity of seven subtyping tools (Stanford, Rega, Geno2pheno, NCBI, STAR, EuResist and TherapyEdge) in classifying a panel of HIV-1 *pol* sequences (587 subtype B/83 non-B) subtyped by phy from 670 different patients included in CoRIS, a large cohort of HIV-infected treatment-naïve patients in Spain.

2. Materials and methods

2.1. Study population

The Spanish cohort of ARV-naïve HIV-infected patients included in the Research Network on HIV/AIDS (CoRIS) is a multicenter, hospital-based prospective cohort of subjects over 13-years-old seen at 31 hospitals of the 19 Autonomous Regions in Spain from January 2004. This study was approved by a review board and Ethical Committee of the CoRIS Cohort. It was designed to protect the rights of all subjects involved under the appropriate local regulations. About 60% of these patients have started ARV therapy and more than 30% of subjects are immigrants (Caro-Murillo et al., 2007). Out of the 670 patients included in this study, 375 (56%) were Spanish, 181 (27%) were immigrants (118 Central and South Americans, 17 Sub-Saharan Africans, 16 West Europeans, 11 East Europeans, 10 North Africans, 6 North Americans and 3 Asians) and 114 were of unknown origin. Basal *pol* sequences in fasta format were collected including the complete protease (PR) (codons 1–99) and part of the reverse transcriptase (RT) (codons 38–260 or 1–335) from the 670 naïve patients with available sequence.

2.2. HIV-1 variants identified by phy

HIV-1 subtypes and CRF were identified by phylogenetic analysis (phy) of the 670 *pol* sequences. The 2008 version of the subtype reference dataset provided by Los Alamos National Laboratory (available at: http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html) was used. It was updated including more sequences of CRF absent or scarcely represented (26_AU, 30_0206, 32_06A1, 34_01B, 38_BF, 41_CD and 42_BF). Therefore, at least two representative sequences of each 9 subtypes and the 43 CRF of HIV-1 group M available at the moment of the analysis were taken as references. The tree topology was obtained using the Neighbor-Joining method. DNA sequences were aligned using the ClustalX 2.0.11 program. The pairwise distance matrix was estimated using the Kimura two-parameter model within the DNADIST program, as implemented in the PHYLIP software package. Bootstrap re-sampling (1000 data sets) of the multiple alignments was performed, with the bootstrap cut-off set at 700. Out of the 670 sequences, 587 (87.6%) were subtype B, and 83 (12.4%) non-B variants. Only 13 (15.7%) were "pure" non-B subtypes: 1 A1, 3C, 4 F1, 4 F2, and 1 G. The remaining 70 (84.3%) non-B were recombinant. Forty-seven (67.1%) clustered with 12 different CRFs (1 CRF01_AE, 31 CRF02_AG, 2 CRF03_AB, 1 CRF06_cpx, 1 CRF11_cpx, 1 CRF12_BF, 4 CRF14_BG, 1 CRF15_01B, 2 CRF19_cpx, 1 CRF20_BG, 1 CRF28_BF, 1 CRF42_BF) and 23 were URF. They did not cluster to any known subtype/CRF, and presented complex mosaic patterns, including fragments from 8 different subtypes (A, B, C, D, F, G, J and K) and 3 different CRF (01_AE, 02_AG and 03_AB). In more detail, they carried B/CRF02_AG sequences (17.4%), A1/CRF03_AB (13%), B/A1/CRF03_AB (13%), B/F1 (8.7%), B/A1 (8.7%) or others (39.1%).

2.3. Automated HIV-1 subtyping tools

HIV-1 subtyping by seven automated tools was also assessed using all sequences. Six were free-of-charge and available online: Stanford HIVdb 6.0.5, Geno2pheno 3.0, Rega 2.0, NCBI (including both 2005 and 2009 reference sets), EuResist 2009, STAR 2006; however TherapyEdge-HIV 2009 was a commercial tool. They were available at: http://hivdb.stanford.edu/pages/algs/sierra_sequence.html (HIV-1 Drug Resistance Database; Stanford University, Palo Alto, CA); http://www.geno2pheno.org (Max Planck Institute for Informatics, Saarbrücken, Germany); http://www.bioafrica.net/subtypetool/html (Rega Institute for Medical Research, Leuven, Belgium); http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi (National Center for Biotechnology Information, Bethesda, MD); http://engine.euresist.org/data_analysis/viral_sequence/new (EuResist Project, Rome, Italy); http://www.vgb.ucl.ac.uk/starn.shtml (UCL Division of Infection and Immunity, Royal Free & University College Medical School, London, UK) and http://www.therapyedge.com (TherapyEdge-HIV, ABL, Luxembourg). A discrepancy was considered when the tool assigned a different HIV-1 subtype/CRF than phy. For NCBI tool, we used two different reference sequences' sets (2005 and 2009) in the analysis, because NCBI09 included 22 more CRFs than NCBI05.

Table 1
Sensitivity of the automated subtyping tools *vs.* phy in the 670 HIV-1 *pol* sequences classification.

HIV-1 variants (no.)	% Sensitivity (95% CI)							
	Stanford 6.0.5	Rega 2.0	Geno2pheno 3.0	TherapyEdge 2009	STAR 2006	EuResist 2009	NCBI 2005	NCBI 2009
Subtype B (587)	98.6 (98;99)	85.9 (83;89)	95.9 (94;97)	95.6 (94;97)	95.1 (93;97)	97.6 (96;99)	81.3 (78;84)	48.7 (45;53)
Non-B variants (83)	47 (36;58)	48.2 (37;59)	59 (48;70)	55.4 (45;66)	50.6 (40;61)	57.8 (47;68)	54.2 (43;65)	16.9 (9;53)
Pure non-B subtypes (13)	76.9 (54;100)	92.3 (78;100)	100	92.3 (78;100)	84.6 (65;100)	100	76.9 (54;100)	53.8 (27;81)
CRF02_AG (31)	90.3 (80;100)	71 (55;87)	93.5 (85;100)	83.9 (71;97)	83.9 (71;97)	87.1 (75;99)	77.4 (63;92)	9.7 (−1;20)
CRF non-02_AG (16)	6.2 (−6;18)	12.5 (−4;29)	43.75 (19;68)	50 (25;74)	6.2 (−6;18)	50 (25;74)	31.2 (8;54)	25 (4;46)
URF (23)	0	17.4[a] (2;33)	0	0	17.4[a] (2;33)	0	26.1 (8;44)	0
Total (670)	77.3 (74;80)	81.2 (78;84)	91.3 (89;93)	90.6 (88;93)	89.5 (87;92)	92.7 (91;95)	77.9 (75;81)	44.8 (41;48)

No., number of sequences; CI, confidence interval; CRF, circulating recombinant form; URF, unique recombinant form; Phy, phylogenetic analysis. We considered as correct classification when the subtyping tool assigned the same HIV-1 subtype or CRF as that provided by phylogenetic analysis for each *pol* sequence. Pure non-B subtypes included: 1 A1, 3C, 4 F1, 4 F2 and 1 G. CRF non-02_AG included: 1 CRF01_AE, 2 CRF03_AB, 1 CRF06_cpx, 1 CRF11_cpx, 1 CRF12_BF, 4 CRF14_BG, 1 CRF15_01B, 2 CRF19_cpx, 1 CRF20_BG, 1 CRF28_BF and 1 CRF42_BF (see Table 2). Complex recombinants or URFs not ascribed to any HIV-1 subtype or circulating recombinant (CRF) were defined using a bootscanning method (Simplot) (Yebra et al., unpublished data).

[a] All the URFs whose mosaic patterns were correctly identified by Rega and STAR (4/23 each) were, however, unassigned by each tool.

2.4. Statistical analysis

Sensitivity and specificity were obtained using a calculator available at: http://www.hrc.es/investigacion/bioest/otras_calculadoras.html.

3. Results

3.1. Different sensitivities of automated subtyping tools for HIV-1 variant identification

3.1.1. In the complete cohort

The sensitivity of the seven automated tools for HIV-1 identification when compared to phy differed among HIV-1 variants and tools. Including our entire study cohort (n = 670) with predominance of clade B (87.6%), sensitivity exceeded 90% for Geno2pheno, EuResist and TherapyEdge. However, when only the 83 HIV-1 non-B subtypes and recombinants were included, Geno2pheno, EuResist, TherapyEdge and NCBI05 only identified about 55% of them (Table 1). The remaining tools identified a half at best. Of note, a high rate (9.3% and 17.2%, respectively) of the 670 *pol* sequences was not assigned to any subtype/recombinant using STAR and Rega. STAR unassigned 33 (40%) non-B variants and 83 (14.1%) subtypes B and Rega 32 (38.5%) non-B variants and 29 (4.9%) subtypes B. Stanford and TherapyEdge provided the subtype at PR and RT separately, even when the complete pol (PR and RT) in one file was used. Stanford provided very similar sensitivities for HIV-1 variant detection in PR/RT meanwhile TherapyEdge subtyped RT slightly better than PR (data not shown).

3.1.2. In HIV-1 pure subtypes

For subtype B (n = 587) identification, Stanford presented the highest sensitivity (98.6%) and only misclassified 6 subtypes B (Table 1), but only in PR and not in RT. The specificity of subtyping tools was also high, ranging from 84.3% (Geno2pheno) to 100% (Rega). However, we observed misclassifications. In fact, all rapid subtyping tools, except Rega and STAR, identified as non-B variants from 1.4% (Stanford) to 51.3% (NCBI09) of the 587 subtype B sequences defined by phy. NCBI presented the highest overestimation of non-B variants, since it only correctly classified 71.3% or 48.7% of the subtype B sequences defined by phy when the 2005 or 2009 versions were used, respectively (Table 1). In the remaining cases, NCBI included regions of other subtypes or CRF besides subtype B, especially in the case of NCBI09.

For non-B pure subtypes (n = 13, including A1, C, F1, F2 and G), the rate of correct identifications was high as well. EuResist and Geno2pheno correctly classified all of them. The sensitivity was 77–92% in the remaining tools (Table 1). The specificity was also high (>99%) in most cases. Of note, among the 24 sequences misclassified by Geno2pheno, 19 were assigned to subtype D. TherapyEdge provided 4.4% of false non-B variants, 11 of them subtyped as D^{PR}/B^{RT}. Thus, Geno2pheno and TherapyEdge could overestimate subtype D, which has been demonstrated to have a faster progression to AIDS and a higher pathogenicity (Vasan et al., 2006; Baeten et al., 2007; Sacktor et al., 2009).

3.1.3. In HIV-1 recombinant forms: CRFs and URFs

All subtyping tools presented a high sensitivity for CRF01_AE and CRF02_AG detection, although 7–29% of the 31 CRF02_AG were underestimated using subtyping tools excluding NCBI09, which presented important limitations in detecting both variants (Table 1). The specificity for CRF01_AE and CRF02_AG was very high, ranging from 98.4% (EuResist) to 100% (NCBI'05) and 99.7% (EuResist) to 100% (Rega and NCBI09), respectively.

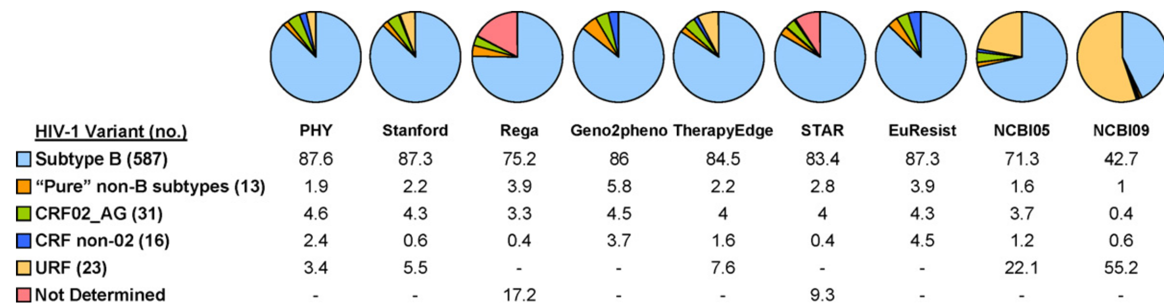
However, the efficacy of all subtyping tools clearly dropped when identifying recombinants different from CRF02_AG (Table 2). Among the 15 *pol* sequences ascribed by phy to 10 different CRFs non-CRF01_AE, non-CRF02_AG, automated tools failed to detect most of them, although EuResist, TherapyEdge, and Geno2pheno provided the best results (Table 1).

Regarding the identification of the complex recombinant mosaics or URF (n = 23) previously characterized in our cohort by phylogenetic analysis and bootscanning, only NCBI05, Rega and

Table 2
Subtyping of HIV-1 recombinant forms by automated tools.

HIV-1 recombinants by phy (no.)	Correct identifications (no.) by each subtyping tool *vs.* phy
CRF01_AE (1)	EuR, G2p, NCBI05, Rega, Stanf, STAR, and TE (1)
CRF02_AG (31)	G2p (29); Stanf (28); EuR (27); STAR and TE (26); NCBI05 (24); Rega (22); NCBI09 (3)
CRF03_AB (2)	None
CRF06_cpx (1)	EuR, G2p and TE (1)
CRF11_cpx (1)	TE (1)
CRF12_BF (1)	G2p, NCBI05, Rega and TE (1)
CRF14_BG (4)	EuR, G2p (4); NCBI05, NCBI09 and TE (3)
CRF15_01B (1)	NCBI05 (1)
CRF19_cpx (2)	EuR (2); TE (1)
CRF20_BG (1)	NCBI09 (1)
CRF28_BF (1)	None
CRF42_BF (1)	None
URF (23)	NCBI05 (6); Rega and STAR (4)

No., number of sequences; CRF, Circulating Recombinant Form; URF, Unique Recombinant Form; EuR, EuResist 2009; G2p, Geno2pheno 3.0; Stanf, Stanford-HIVdb 6.0.5; TE, TherapyEdge-HIV 2009; NCBI05 and NCBI09, versions with reference datasets of 2005 and 2009 respectively; Rega, Rega version 2.0; STAR, STAR version 2006.



HIV-1 Variant (no.)	PHY	Stanford	Rega	Geno2pheno	TherapyEdge	STAR	EuResist	NCBI05	NCBI09
Subtype B (587)	87.6	87.3	75.2	86	84.5	83.4	87.3	71.3	42.7
"Pure" non-B subtypes (13)	1.9	2.2	3.9	5.8	2.2	2.8	3.9	1.6	1
CRF02_AG (31)	4.6	4.3	3.3	4.5	4	4	4.3	3.7	0.4
CRF non-02 (16)	2.4	0.6	0.4	3.7	1.6	0.4	4.5	1.2	0.6
URF (23)	3.4	5.5	-	-	7.6	-	-	22.1	55.2
Not Determined	-	-	17.2	-	-	9.3	-	-	-

Fig. 1. Distribution of HIV-1 variants in the CoRIS cohort according to each subtyping method. PHY, phylogenetic analysis considered the gold standard method to classify HIV-1; no., number of sequences; CRF, circulating recombinant forms; URF, unique recombinant forms. The automated tools included were: Stanford HIVdb 6.0.5, Rega 2.0, Geno2pheno 3.0, TherapyEdge-HIV 2009, STAR 2006, EuResist 2009 and NCBI (including both set of references from 2005 and 2009). Stanford and TherapyEdge provided independent results for protease and reverse transcriptase, and the cases with different results in the two regions were considered as URF. For NCBI, the *pol* sequences including regions ascribed to different HIV-1 subtypes and/or CRF were considered as URF.

STAR were capable of identifying URF, but with very low sensitivities (Table 1). Rega and STAR unassigned 15 and 16 of the 23 URF, respectively, and the remaining URF were wrongly classified as pure subtypes or CRF using both tools. Nevertheless, Rega correctly showed the subtypes involved in the recombinants in 4 unassigned URF (2 URF_BF1, 1 BA1, 1 BC) and so did STAR in 4 other unassigned URF (3 URF_B02, 1 BC). On the other hand, NCBI05 correctly detected 6 URF (3 URF_02B, 1 03B, 1 BG02 and 1 BC). The URF_BC was the only one correctly identified by the three tools.

3.2. HIV-1 non-B variants identified as subtype B by automated subtyping tools

A considerable rate (1–16%) of the 83 non-B variants by phy was wrongly identified as subtype B by automated subtyping tools, with the exception of NCBI09 and Rega (Table 2). Among the 83 non-B variants, Geno2pheno identified as subtype B 16% of cases, EuResist 14%, TherapyEdge 6%, Stanford 5% and STAR and NCBI05, 1%. Therefore, Geno2pheno and EuResist presented the highest overestimation of subtype B, and NCBI and Rega the lowest.

3.3. HIV-1 variant distribution in CoRIS according to the subtyping tool

The distribution of HIV-1 subtypes and recombinants among the 670 patients from CoRIS was different according to each tool (Fig. 1). In STAR and Rega, a high rate (9 and 17%, respectively) of sequences was not assigned to any subtype/CRF. The differences between tools were especially pronounced in CRFs non-02_AG: present in 2.4% of patients according to phy, but $\leq$0.6% for STAR, Stanford and Rega, and almost fivefold for EuResist. Finally, although URF represented only 3.4% of cohort by phy, this estimation was 55.2% for NCBI09 and 22.1% for NCBI05. For TherapyEdge and Stanford, different results in PR and RT were considered as URF (7.6% and 5.5%, respectively).

4. Discussion

4.1. Reliability of automated subtyping tools

HIV-1 subtyping is of clinical importance, as previously mentioned. The correct identification of non-B variants is important due to their high prevalence in pandemics and their increasing rate in industrialized countries by population movements from areas where non-B variants are epidemic (Wensing et al., 2005; Yerly et al., 2007; Holguín et al., 2008a). In fact, a third of newly HIV-diagnosed cases in Spain are immigrants (Caro-Murillo et al., 2009). Since the HIV/AIDS pandemic tends to constantly grow in complexity (Zhang et al., 2010) by the increasing spread of CRF and URF favored by coinfections and/or superinfections, the expansion of recombinant viruses has complicated the HIV-1 subtyping. Thus, an improvement in complex recombinant variants detection is strongly recommended. Phylogeny (phy) is considered the gold-standard method for HIV subtyping. However, since it can be laborious and complex, automated tools have been developed. Although useful for subtype B identification, they present limitations (Smith et al., 2005; Gifford et al., 2006; Loveday and MacRae, 2006; Ntemgwa et al., 2008; Galán et al., 2009; Wilkinson and Engelbrecht, 2009; Yebra et al., 2010).

This study compared the sensitivity of seven widely used HIV-1 subtyping tools [Stanford, Geno2pheno, Rega, NCBI (2005 and 2009 versions), EuResist, STAR and TherapyEdge] with respect to phy in the identification of subtypes and recombinants in a large and representative Spanish HIV-infected Cohort (CoRIS). To our knowledge, this work includes the highest numbers of tools, HIV-1 sequences and different CRFs among the studies which compare results of automated tools with phylogeny (Table 3).

We have shown that the sensitivity of certain automated subtyping tools (especially EuResist and Geno2pheno) in large cohorts of HIV-1 infected patients can be very high if subtype B is predominant, as in our cohort. This good sensitivity is due to the overrepresentation of subtype B in the databases because of its dominance in the HIV epidemic in developed countries, where more *pol* sequences are routinely available for resistance testing (Table 3). However, we observed important failures in the subtype B identification for specific tools. Most of the automated subtyping tools provided, to a greater or lesser extent, false subtype B among the non-B sequences, overestimating the rate of subtype B isolates in a given population. In more detail, between 10 and 15% of non-B sequences were identified as subtype B when using Geno2pheno, EuResist, and the separate analysis of PR and RT by Stanford and TherapyEdge.

For pure non-B subtypes identification, the best tools were also Geno2pheno and EuResist (sensitivity 100%). Previous reports have also described specific limitations in pure non-B subtypes identification using online subtyping tools. For instance, subtype D was usually underestimated using Rega (Gifford et al., 2006; Holguín et al., 2008c) but overestimated using Geno2pheno, Stanford (Galán et al., 2009) and TherapyEdge due to its confusion with subtype B especially in the PR, as our study confirmed. The misclassification of subtype D is of special relevance because this variant seems to be more pathogenic than others (Vasan et al., 2006; Baeten et al., 2007; Sacktor et al., 2009). An inadequate detection of subtype G by STAR (Gifford et al., 2006), subtype J by Rega (Gifford et al., 2006; Holguín et al., 2008c) and subtype A by Stanford (Gifford et al., 2006) has also been published.

Focusing on recombinant variants, our data demonstrate that the sensitivity of the automated tools decreases drastically when there is an increase in non-B recombinants other than CRF01_AE

Table 3
Studies of the limitations of automated subtyping tools which include sequences of HIV-1 non-B variants.

Author	Country	Number of HIV-1 sequences analyzed (number of different variants)	Viral region	Subtyping method included										
				Phy	Stanford	Rega	Geno2pheno	Therapy Edge	STAR	EuResist	NCBI	jpHMM	LANL	Virco
Present study	Spain	83 non-B sequences (5 subtypes, 12 CRF) 587 subtype B	*Pol*	✓	✓	✓	✓	✓	✓	✓	✓			
Yebra et al. (2010)	Spain	128 non-B (6 subtypes, 11 CRF) 226 subtype B	*Pol*	✓	✓	✓	✓			✓	✓			
Wilkinson and Engelbrecht (2009)	South Africa	10 non-B (3 subtypes, 7 URF) 1 subtype B	Nearly full genome	✓		✓						✓		
Galán et al. (2009)	Spain	56 non-B (5 subtypes, 8 CRF, 11 URF, 3 U) 14 subtype B	*Pol*	✓	✓	✓	✓				✓			✓
Holguín et al. (2008c)	Spain	277 PR/171 RT non-B (8 subtypes, 9 CRF) 33 subtype B	*Pol*	✓	✓	✓					✓			
Holguín et al. (2008d)	Spain	5 non-B (1 CRF, 4 URF)	Nearly full genome	✓	✓	✓					✓			
Ntemgwa et al. (2008)	Canada	4 sequences of the same URF	*Pol*	✓	✓	✓	✓		✓			✓		✓
Loveday and MacRae (2006)	UK	1,002 non-B and B	*Pol*		✓	✓			✓		✓		✓	
Gifford et al. (2006)	UK	10,503 PR/10,476 RT non-B and B	*Pol*		✓	✓			✓					
Smith et al. (2005)	UK	81 non-B (5 subtypes, 2 CRF) 19 subtype B	*Env*	✓							✓		✓	

UK, United Kingdom; PR, protease; RT, reverse transcriptase; Pol, region including complete PR and partial RT; U, unclassified; CRF, circulating recombinant form; URF, unique recombinant form; Ref, corresponding reference; Phy, phylogenetic analysis (the gold standard method to classify HIV variants); jpHMM, jumping profile Hidden Markov Model (available at: http://jphmm.gobics.de); LANL, Los Alamos National Laboratory RIP or BLAST tools (available at: http://www.hiv.lanl.gov). The "tick" symbol indicates the inclusion of the corresponding subtyping method in each study.

and CRF02_AG, as they are absent or scarcely represented in the databases of these tools. For instance, neither STAR nor Stanford could subtype any single CRF other than 01_AE and 02_AG (Table 2), reflecting the lack of sufficient sequences in their databases. Regarding URFs, subtyping tools were inefficient in their detection. Only NCBI05, Rega and STAR were capable of identify URF, but with very low sensitivity. Our study presents a low number of non-01, non-02 CRFs ($n = 15$), due to their low prevalence in Spain, which is probably insufficient to extract a conclusion with statistical support. However, our results agree with other studies (Holguín et al., 2008c; Galán et al., 2009; Yebra et al., 2010), and the presented data could be very useful in countries where complex recombinants are more frequent.

In addition, our data reveals that the HIV-1 subtype distribution in any study cohort is different according to the automated subtyping tool used. In other words, the results of a molecular epidemiology study would change depending on the tool used and on the prevalence of non-B variants, especially recombinants (Fig. 1).

4.2. Technical differences among subtyping tools

The described discrepancies between various automated tools *vs.* phy could be explained by differences in both the subtyping methods applied and the reference datasets included. HIV-1 variants were defined by phy using the Neighbor-Joining (NJ) method. Among the automated tools assessed here, only Rega was based on phylogeny, also applying NJ combined with bootscanning. However, this tool has a threshold which prevents the assignation of a subtype/CRF when it does not obtain enough statistical support. This restricts its efficacy despite using phylogeny. It is remarkable that the highest sensitivity was obtained by tools which performed a simple BLAST search, assigning to the query sequence the subtype/CRF of the most similar reference in their databases (Geno2pheno, EuResist, TherapyEdge, Stanford and NCBI). In particular, NCBI uses a sliding-window along the query sequence and each window is compared to the references by BLAST. However, this sliding-window causes an overestimation of recombination, magnified when more reference sequences are included as we reported comparing 2005 *vs.* 2009 versions. With this method, the inclusion of multiple references which share similarity confounded the results instead of improving them. In several cases when NCBI05 assigned a specific subtype/CRF, NCBI09 provided a mixture of subtypes/CRFs with common regions. Some CRFs are very close and difficult to discriminate in the studied region and it is thus extremely difficult to obtain reliable results with systems based on Blast analysis. In contrast, STAR is a statistical method that uses position-specific scoring matrices of each subtype to perform profile subtype alignments. But, as well as Rega, there are many cases where STAR does not assign any subtype if the assignment's *P*-score does not reach the threshold. The different reference sets used by each tool are also important in the discrepancies. Meanwhile STAR and Stanford excluded any CRF different from CRF01_AE and CRF02_AG, Rega and Geno2pheno included up to CRF14_BG and TherapyEdge up to CRF19_cpx. Only NCBI09 includes references of the CRFs as they are described (at the moment of the analyses, there were 45 different CRFs).

4.3. Important considerations

Several main considerations should be considered for understanding the incorrect HIV-1 subtyping using automated subtyping tools in a given population. First, the rate of non-B variants in the study cohort; second, the rate of recombinants other than CRF01_AE and CRF02_AG among these non-B variants; third, the automated tool used for subtyping; fourth, the rate of non-B variants wrongly identified as subtype B and *vice versa*, which underestimates the

prevalence of non-B or B variants; fifth, the high rate of sequences (especially non-B) unassigned in the cases of STAR and Rega.

Thus, our data reveals that subtyping tools should be regularly updated as is done for resistance mutations before their use in routine clinical settings by increasing the number of non-B/CRF sequences to improve their detection. In light of our results, when the use of phylogenetic analysis is not available we would suggest EuResist, Geno2pheno and/or TherapyEdge as the best subtyping tools for cohorts with predominance of clade B as in our cohort. Despite the great specificity of Rega and STAR for subtype B, too many sequences were unassigned. On the other hand, in areas where non-B variants and especially recombinant forms are prevalent, none of the tools evaluated here would be sufficiently reliable. In these cases, the use of several subtyping tools instead of just one is recommended in order to compare their results. HIV-1 subtypes and predominant recombinants need to be identified with tools providing high specificities and sensitivities and subsequent phylogenetic analysis is recommended on samples that cannot be classified.

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HIV-1 non-B subtypes: High transmitted NNRTI-resistance in Spain and impaired genotypic resistance interpretation due to variability

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ABSTRACT

Genotypic resistance algorithms interpret drug-resistance mutations, but are mainly developed for HIV-1 subtype B, meanwhile non-B subtypes cause 90% of worldwide infections. They include clade-specific amino acid at drug-resistance positions different than subtype B.

This study explores: (i) the variability at resistance-related positions in 128 non-B and 226 B sequences from 354 treatment-naïve patients diagnosed in Spain (1999–2007); (ii) the discordances between five resistance interpretation algorithms (ANRS, Stanford, Rega, Geno2pheno, RIS); and (iii) the reliability of five subtyping tools (Stanford, Geno2pheno, Rega, NCBI, EuResist) for each HIV-1 variant.

Primary drug-resistance prevalence was 13.6%, although higher in non-B *vs.* B subtypes (18.7% *vs.* 10.6%), due to a twofold higher NNRTI-resistance prevalence (15.7% *vs.* 7.6%). Most secondary PI-resistances, more frequent in non-B, were in fact clade-specific residues. Most sequences were interpreted as susceptible to all antiretrovirals by the five resistance algorithms, except for tipranavir by ANRS in non-B clades. Interalgorithm discordances were significantly higher in non-B variants for specific drugs. The agreement with phylogenetic analysis differed among subtyping tools testing non-B variants.

We found a higher prevalence of NNRTI-resistance mutations in non-B subtypes. Certain algorithms overestimate the resistance in non-B subtypes due to natural patterns of mutations. Subtyping tools should be optimised for non-B variants.

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1. Introduction

The expanding use of antiretroviral drugs for the treatment against human immunodeficiency virus type-1 (HIV-1) favours the emergence of virus harbouring resistance mutations. This can generate an increasing prevalence of primary resistance mutations in viruses from treatment-naïve patients who have been infected by pre-treated subjects, compromising the effectiveness of the first antiretroviral therapy. Transmission of drug resistant viruses has been widely reported in Europe and the USA, with a prevalence ranging from 5% to 15% (Booth and Geretti, 2007; Sagir et al., 2007; Wensing et al., 2005; Wheeler et al., 2007). In Spain, the rate of primary resistance mutations differs among regions and time periods, but these mutations are present in around 10% of treatment-naïve patients (de Mendoza et al., 2005; Martínez-Picado et al., 2005; Palacios et al., 2008; Sanchez-Oñoro et al., 2007). The rate rarely reaches 10% in treatment-naïve patients from developing countries (Geretti, 2007; Nyombi et al., 2008; Ojesina et al., 2006), and is mainly limited to a few reverse transcriptase inhibitors (RTI), which are the most available drugs in these countries.

International guidelines recommend routine HIV resistance testing for the selection of an optimal antiretroviral therapy selection. Genotypic resistance tests are used more than phenotypic tests, due to their lower costs and easier implementation. Several online algorithms have been developed by correlating genotypic patterns with clinical or phenotypic data. Recent reports have demonstrated their utility to predict virological response in the clinical settings (Rhee et al., 2009). Furthermore, they are inexpensive and widely used for detection and interpretation of resistance mutations using *pol* (protease, PR and reverse transcriptase, RT) sequences.

Both genotypic drug-resistance interpretation algorithms and resistance prevalence studies have been mainly based on results derived from patients infected by subtype B. This is the most prevalent HIV-1 variant in industrialized countries where all antiretroviral drugs are available. However, the remaining HIV-1 variants (non-B subtypes and recombinants), traditionally ignored in the studies, are responsible for 90% of the 33 million infections worldwide (Hemelaar et al., 2006; UNAIDS, 2009). They are prevalent in developing regions and are continuously increasing among new infections in Western countries, including Spain (Holguín et

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al., 2008a). HIV-1 non-B subtypes and recombinant variants have a high genetic variability when the consensus of subtype B is used as reference for the definition of resistance mutations. HIV-1 variants show clade-specific polymorphisms (substitutions present in more than 10% of samples from treatment-naïve patients). Non-B subtypes can also present different wild-type amino acids than subtype B in positions related to drug-resistance to RTI and protease inhibitors (PIs) (Kantor and Katzenstein, 2003). This can be misinterpreted by algorithms. Moreover, scored drug-resistance mutations still vary among different algorithms (Champenois et al., 2008), which complicates the comparison of genotypic resistance among them, mostly when HIV-1 non-B variants are studied and secondary or minor resistance mutations are analyzed. The influence of this clade-specific pattern in the antiretroviral long-term response for each drug in each HIV-1 subtype and recombinant is still unknown. Thus, it is important to clarify the nature and frequency of clade-specific residues in viral positions related to drug-resistance in treatment-naïve patients infected by different HIV-1 subtypes and recombinants. Also it is important to study their influence in interalgorithm discordances.

Hence it is essential to identify HIV-1 subtypes and circulating recombinant forms (CRF). Phylogenetic analysis (phy) is the gold standard method for subtyping, although it is not widely implemented in clinical settings because of its complexity. Most clinicians use online subtyping tools, despite their limitations for the correct classification of some non-B subtypes and most recombinants different to CRF02_AG (Holguín et al., 2008b). This study reports: (i) the prevalence of primary and secondary drug-resistance mutations in 128 HIV-1 non-B and recombinant variants *vs*. 226 clade B sequences from 354 treatment-naïve patients diagnosed in Spain from 1999 through 2007; (ii) the reliability of interpretation of drug-resistance by five algorithms free of charge and available online used in clinical practice in all analyzed sequences belonging to different HIV-1 subtypes and recombinants; and (iii) the reliability of five rapid subtyping tools in detecting HIV-1 subtypes and circulating recombinant forms (CRF) compared to phylogenetic analysis.

2. Patients and methods

2.1. Study population

Pol sequences from plasma samples from 354 HIV-1-infected patients unexposed to antiretroviral therapy, according to the clinical reports, were collected. Patients were diagnosed from 1999 through 2007 in four Spanish HIV/AIDS clinics: 210 in Centro Sanitario Sandoval (Madrid), 130 in Hospital Carlos III (Madrid), 5 in Hospital de Móstoles (Madrid), 5 in Hospital Doctor Negrín (Las Palmas, Canary Islands) and 4 in NGO Medicus Mundi facilities (Madrid). The patients were native Spaniards (45.8%), South-Americans (20.6%), Africans (18.6%), other Europeans (5.9%), Asians (0.3%), and of unknown origin (8.8%). Epidemiological data suggested that most of the migrants had probably been infected overseas.

PR and RT sequences were available from 210 of 226 subtype B sequences and 108 of 128 non-B subtypes. In the remaining cases only PR was available. Of the 354 sequences, 64 non-B and 67 subtype B *pol* sequences were previously published (Gutiérrez et al., 2004; Holguín et al., 2007, 2008a). The new HIV-1 sequences were from patients under follow up in Centro Sanitario Sandoval ($n = 191$), Hospital Carlos III ($n = 29$), Hospital de Móstoles ($n = 3$), and NGO Medicus Mundi ($n = 4$), all located in Madrid, Spain.

This study was part of a project approved by a review board and Ethical Committee of our institution. It was designed to protect the rights of all subjects involved under the appropriate local regulations. To maintain subject confidentiality, a unique ID number was assigned to each specimen.

2.2. HIV-1 subtyping

Direct sequencing of nested PCR purified products from viral RNA was performed in the HIV-1 *pol* coding region. *Pol* sequences included the complete protease (codons 1–99) and part of the reverse transcriptase (codons 1–247 or 1–335) using Trugene (Siemens, Barcelona, Spain) or Viroseq (Celera Diagnostics, Alameda, CA, USA) assays, respectively. In some specimens with amplification difficulties, *PR* and/or *RT* amplification was made using primers and conditions reported elsewhere, utilizing an automatic sequencer for sequencing (ABI Prism, Applied Biosystems, Foster City, CA, USA) (Holguín et al., 2005, 2006a).

All 354 *pol* sequences were subtyped by phylogenetic analysis. At least two representative sequences of each subtype/CRF within HIV-1 group M available at the moment of the analysis were taken as references. DNA sequences were aligned using the ClustalW program. The tree topology was obtained using the Neighbour-Joining method. The pairwise distance matrix was estimated using the Kimura two-parameter model within the DNADIST program, as implemented in the PHYLIP software package. Bootstrap re-sampling (1000 data sets) of the multiple alignments was performed to test the statistical robustness of the tree. Bootstrap cut-off was set at 700.

Besides using phylogenetic analysis, HIV-1 subtyping was also assessed in all *pol* sequences by five online rapid subtyping tools: Stanford 4.3.7, Geno2pheno 3.0, Rega 2.0, NCBI 2008 and EuResist 2008. They were available at: http://hivdb.stanford.edu (HIV-1 Drug Resistance Database; Stanford University, Palo Alto, CA); http://www.geno2pheno.org (Max Planck Institute for Informatics, Saarbrücken, Germany); http://www.bioafrica.net/subtypetool/html (Rega University, Leuven, Belgium); http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi (National Center for Biotechnology Information, Bethesda, MD, USA) and http://engine.euresist.org/data_analysis/viral_sequence/new (EuResist Project, Roma, Italy). A discrepancy was considered when the subtyping tool assigned a different HIV-1 subtype or CRF than that provided by phy.

2.3. Genotypic drug-resistance interpretation algorithms

Drug-resistance mutations defined by the International AIDS Society-USA (IAS) (Johnson et al., 2008) were manually located in the *pol* sequences. Resistance mutations at PR gene were classified as primary (major) or secondary (minor) following the IAS-USA nomenclature. We considered primary or major mutations at PR: D30N, V32I, L33F, M46I/L, I47V/A, G48V, I50L/V, I54M/L, Q58E, T74P, L76V, V82A/T/F/S/L, I84V, N88S, L90M; as secondary mutations at PR: L10V/I/R/F/C, V11I, I13V, G16E, K20R/M/I/T/V, L24I, L33I/V/F, E34Q, E35G, M36I/L/V, K43T, F53L/Y, I54V/T/A/S, D60E, I62V, L63P, I64L/M/V, H69K, A71V/I/T/L, G73C/S/T/A, V77I, V82I, N83D, I85V, N88D, L89V, I93L/M. Resistance mutations at RT: M41L, A62V, K65R, D67N, T69 insertion, K70R/E, L74V, V75I, F77L, V90I, A98G, L100I, K101E/H/P, K103N, V106M/A/I, V108I, Y115F, F116Y, E138A, Q151M, V179D/F/T, Y181C/I/V, M184V/I, Y188C/L/H, G190S/A, L210W, T215Y/F, T215rev, K219Q/E, P225H and M230L. The objective was to study their prevalence among each HIV-1 subtype and CRF from naïve subjects, as well as their influence in the prediction of resistance to each PR or RT inhibitor in current therapeutic use.

The concordance between five different genotypic resistance algorithms (ANRS 2008.07, Stanford 4.3.7, Rega 7.1.1, Geno2pheno 3.0, and RIS-2008) was also studied. For that purpose, all 354 *pol* sequences were introduced in the corresponding web-

Table 1
Substitutions in PR (a) and RT (b) associated with drug-resistance in sequences from 354 treatment-naïve patients carrying different HIV-1 variants.

(a) Substitutions at PR	HIV-1 variants (no.)																	% of HIV-1 variants		p
	Subtypes								CRF											
	A (9)	B (226)	C (8)	D (1)	F (8)	G (15)	02 (41)	06 (5)	10 (3)	11 (2)	12 (16)	13 (1)	14 (9)	19 (1)	22 (1)	23 (6)	31 (2)	Non-B (128)	B (226)	
Primary																				
V32I	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	0.8	0	NS
L33F	–	1	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	0.8	0.4	NS
M46I/L	1	3	–	–	–	–	1	–	1	–	–	–	1	–	–	–	–	3.1	1.3	NS
Q58E	–	–	–	–	–	–	–	–	–	–	3	–	–	–	–	–	–	2.3	0	NS
I50L/V	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	0.4	NS
V82A/T/F/S/L	–	3	–	–	–	–	–	–	1	–	3	–	–	–	–	–	–	3.1	1.3	NS
L90M	–	4	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	0.8	1.8	NS
Secondary																				
L10V/I/R/F/C	3	25	–	–	3	2	8	–	–	1	5	–	1	–	–	–	1	18.7	11.1	<0.05
V11I	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.8	0	NS
I13V	**7**	42	2	–	1	**13**	**33**	**5**	**2**	1	3	**1**	**9**	–	**1**	6	**1**	66.4	18.6	<0.05
G16E	2	1	–	–	2	1	6	1	–	1	3	–	–	–	–	–	2	14.1	0.4	<0.05
K20R/M/I/T/V	3	8	1	–	3	**15**	**38**	**5**	–	–	5	1	**9**	–	–	**6**	2	68.7	3.5	<0.05
L24I	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	0.4	NS
L33I/V	1	11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.8	4.9	<0.05
E34Q	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	0.8	0	NS
E35G	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	0.8	0	NS
M36I/L/V	**8**	38	**7**	**1**	**6**	**15**	**38**	**5**	**3**	**1**	**16**	**1**	**9**	**1**	**1**	**6**	**1**	93	16.8	<0.05
K43T	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	0.4	NS
F53L/Y	–	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	1.8	NS
I54V/T/A/S	–	3	–	–	–	–	–	–	–	–	3	–	–	–	–	–	–	2.3	1.3	NS
D60E	–	5	–	–	–	–	–	–	1	2	–	–	–	–	–	–	–	2.3	2.2	NS
I62V	–	49	–	–	1	1	1	1	1	–	1	–	1	–	–	–	–	5.5	21.7	<0.05
L63P	–	119	3	1	2	4	4	–	1	–	–	–	–	–	1	–	1	13.3	52.6	<0.05
I64L/M/V	2	59	–	–	–	1	5	3	1	–	1	–	–	–	–	3	–	12.5	26.1	<0.05
H69K	**9**	1	**8**	–	–	**15**	**40**	**4**	**2**	**2**	**2**	**1**	**9**	–	**1**	**6**	**2**	78.9	0.4	<0.05
A71V/I/T/L	–	36	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	15.9	<0.05
V77I	–	74	1	–	1	2	1	–	–	1	–	1	–	–	–	–	–	5.5	32.7	<0.05
V82I	–	1	–	–	–	**12**	1	–	–	–	–	–	**9**	–	–	**6**	–	21.9	0.4	<0.05
I93L/M	1	80	**6**	–	–	1	1	–	–	–	4	–	–	–	–	1	**1**	11.7	35.4	<0.05

(b) Substitutions at RT	HIV-1 variants (n)														% of HIV-1 variants		p
	Subtypes						CRF										
	A (8)	B (210)	C (8)	D (1)	F (7)	G (6)	02 (38)	06 (3)	10 (3)	11 (2)	12 (16)	14 (8)	23 (6)	31 (2)	Non-B (108)	B (210)	
NRTI-resistance																	
M41L	–	4	–	–	–	–	–	–	1	–	–	–	–	–	0.9	1.9	NS
A62V	–	3	–	–	–	–	–	–	–	–	–	–	–	–	0	1.4	NS
K65R	–	1	–	–	–	–	–	–	–	–	–	–	–	–	0	0.5	NS
D67N	–	3	–	–	–	–	–	–	1	–	–	–	–	–	0.9	1.4	NS
K70R/E	–	2	–	–	–	–	–	–	–	–	–	–	–	–	0	0.9	NS
L74V	–	1	–	–	–	–	1	–	–	–	–	–	–	–	0.9	0.5	NS
F77L	–	–	–	–	1	–	–	–	–	–	–	–	–	–	0.9	–	NS
M184V/I	–	1	–	–	–	–	2	–	–	–	–	–	–	–	1.8	0.5	NS
L210W	–	3	–	–	–	–	–	–	1	–	–	–	–	–	0.9	1.4	NS
T215Y/F	–	1	–	–	–	–	–	–	–	–	–	–	–	–	0	0.5	NS
T215rev	1	2	–	–	–	–	–	–	1	–	–	–	–	–	1.8	2.9	NS
K219Q/E	–	2	–	–	–	–	–	–	–	–	–	–	1	–	0.9	0.9	NS

Table 1 (*Continued*)

(b) Substitutions at RT	HIV-1 variants (*n*)														% of HIV-1 variants		*p*
	Subtypes						CRF										
	A (8)	B (210)	C (8)	D (1)	F (7)	G (6)	02 (38)	06 (3)	10 (3)	11 (2)	12 (16)	14 (8)	23 (6)	31 (2)	Non-B (108)	B (210)	
NNRTI-resistance																	
V90I	–	1	–	–	–	–	6	–	–	–	–	–	–	–	5.6	0.5	<0.05
K101E/P	–	1	–	–	–	–	1	–	–	–	–	–	–	–	0.9	0.5	NS
K103N	1	9	–	–	–	–	1	–	1	–	2	–	–	–	5.6	4.3	NS
V106M/A/I	–	–	–	–	2	–	1	–	–	–	–	–	1	–	3.7	–	NS
V179D/F/T	–	4	–	–	–	–	–	–	–	–	–	–	–	–	0	1.9	NS
Y181C/I/V	–	1	–	–	–	–	1	–	–	–	–	–	–	–	0.9	0.5	NS
G190S/A	1	–	–	–	–	–	1	–	–	–	–	–	–	–	1.8	–	NS
P225H	–	–	–	–	–	–	1	–	–	–	–	–	–	–	0.9	–	NS

No., number; PR, protease; RT, reverse transcriptase; NRTI, nucleoside RT inhibitors; NNRTI, non-nucleoside RT inhibitors; CRF, circulating recombinant form (the numbers indicate the specific CRF: CRF02_AG, CRF06_cpx, CRF10_CD, CRF11_cpx, CRF12_BF, CRF13_cpx, CRF14_BG, CRF19_cpx, CRF22_01A1, CRF23_BG, and CRF31_BC); NS, not significant (*p*-value >0.05); dash means no changes. In bold, those amino acids that are, in fact, the wild-type amino acid in the corresponding HIV-1 subtype/CRF according to Los Alamos HIV Database (www.hiv.lanl.gov). Drug-resistance mutations are listed according to IAS-USA (Johnson et al., 2008). At the PR no primary mutations D30N, I47V/A, G48V, I54M/L, T74P, L76V, I84V and N88S were found. At the RT, neither T69insertion nor changes V75I, A98G, L100I, V108I, Y115F, F116Y, E138A, Q151M, Y188C/L/H and M230L were observed. T215rev in RT means T215 revertants including changes T215A/C/D/E/G/H/I/L/N/S/V. Some of the sequences presented more than one change.

sites: ANRS (http://www.hivfrenchresistance.org), Stanford HIVDB (http://hivdb6.stanford.edu/asi/deployed/hiv_central.pl?program=hivdb&action=showSequenceForm), Rega (http://www.rega.kuleuven.be/cev), Geno2pheno (http://www.geno2pheno.org) and RIS (Spanish AIDS Research Network, http://www.retic-ris.net/default_principal.asp?idx=&cidioma=2). All algorithms provided different resistance levels. Thus, they were normalized for a better comparison in three resistance levels: susceptible (S), intermediate (I), and resistant (R), as in the HIValg Program in the Stanford website (http://hivdb.stanford.edu/pages/algs/HIValg.html).

Interpretations were considered concordant when all algorithms assigned the same level of resistance (S, I or R) to a specific sequence for a particular drug. We considered full discordances when one of the algorithms scored a sequence as S for a particular drug and another one as R. Partial discordances were considered when: (1) one algorithm scored a specific sequence as S for a particular drug and another one as I, or (2) when one algorithm scored a sequence as I for a particular drug and another one as R.

2.4. Statistical analysis

Chi-square test was performed with Epi Info 3.5 program (Center for Disease Control and Prevention, Atlanta, GA, USA). Significance was set at $p < 0.05$.

3. Results

3.1. Phylogenetic characterization of HIV-1 variants

Among the 354 HIV-1 *pol* sequences, 226 (63.8%) were ascribed to clade B by phylogenetic analysis and 128 (36.2%) to non-B subtypes and recombinants: 9 A, 8 C, 1 D, 8 F, 15 G, 41 CRF02_AG, 5 CRF06_cpx, 3 CRF10_CD, 2 CRF11_cpx, 16 CRF12_BF, 1 CRF13_cpx, 9 CRF14_BG, 1 CRF19_cpx, 1 CRF22_01A1, 6 CRF23_BG and 2 CRF31_BC. Patients infected by non-B variants came from Spain (16.4%), sub-Saharan Africa (50%), South-America (16.4%), other European countries (6.2%), or were of unknown origin (11%). Subjects carrying subtype B came from Western Europe (68.1%), South-America (23%), North-Africa (0.9%), Asia (0.4%), or were of unknown origin (7.6%). New sequences were submitted to GenBank: from FJ481650 to FJ481713 (64 non-B variants) and from FJ481714 to FJ481872 (159 subtype B).

3.2. Twofold higher prevalence of global primary and NNRTI-resistance mutations in non-B vs. B subtypes

Global primary drug-resistance prevalence (i.e., to any antiretroviral drug class) in viruses from 354 treatment-naïve patients from Spain collected during 1999–2007 was 13.6% (Fig. 1). All carried primary or major resistance mutations in PR or/and RT. Considering the HIV-1 variant, prevalence was almost twofold higher in non-B subtypes than in clade B viruses (18.7% *vs.* 10.6%, $p < 0.05$) due to a twofold higher prevalence of NNRTI-resistances (13.1% *vs.* 7.1%, $p < 0.05$). Prevalence of drug-resistance mutations was similar in non-B *vs.* B subtype for PI (6.2% *vs.* 4.4%) and NRTI (4.6% *vs.* 5.2%) (Fig. 1). We observed a similar rate of non-B *vs.* B viruses showing resistance to 2 and 3 drug classes, ranging from 2 to 4%.

The nature and frequency of primary and secondary (or minor) drug-resistances found in each HIV-1 variant is recorded in Table 1. Most frequent primary PI-resistance mutations were M46L (3.1%) and V82A (3.1%) in non-B and M46L/I (3.1%) and L90M (1.8%) in subtype B. For NRTI-resistance substitutions, M184V (1.8%) and M41L (1.9%) were the most common changes in non-B and B, respectively. Surprisingly, the frequency of T215 revertants, normally the most

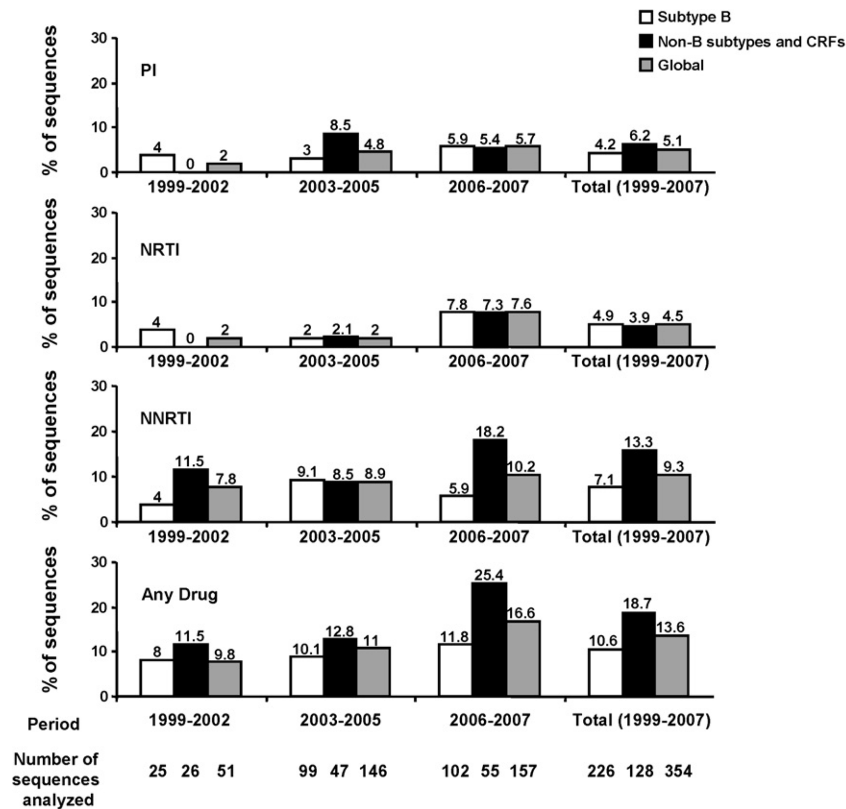

Fig. 1. Prevalence of sequences with primary drug-resistance mutations across HIV-1 subtypes from 354 treatment-naïve patients in Spain across years (1999–2007). PI, protease inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; CRFs, circulating recombinant forms. Figures on the top of the bars indicate the percentage of sequences with primary resistance mutations.

commonly transmitted drug-resistance, was very low. Finally, the most frequent NNRTI-resistance mutations were V90I and K103N (5.6% each one) in non-B and K103N (4.3%) in clade B variants (Table 1).

3.3. Most secondary PI-resistance mutations are clade-specific amino acids

Most (100% non-B, 93% B) of 354 PR sequences presented secondary or minor PI-resistance mutations, different in frequency and nature across variants since some of them were the wild-type amino acid in some subtypes and recombinants (Table 1). This could explain the higher frequency of amino acid related to secondary resistance in residues 10, 13, 16, 20, 36, 69, and 82 in specific non-B clades and in residues 62, 63, 64, 71, 77, and 93 at clade B variants (Table 1). For instance, substitutions M36I (related to atazanavir, indinavir, nelfinavir and tipranavir-resistance) and H69K (related to tipranavir-resistance) were detected in most analyzed non-B specimens. Additionally, change V82I (related to atazanavir-resistance) appeared in all CRF14_BG, CRF23_BG, and 80% of subtype G specimens in our country.

3.4. High interalgorithm discrepancies in non-B subtypes for specific drugs

Drug susceptibility to 19 drugs (PI, NRTI and NNRTI) was predicted in all 354 non-B and B sequences using five different algorithms: French ANRS 2008.07, Stanford 4.3.7, Rega 7.1.1,

Table 2
Percentage of HIV-1 sequences from 354 treatment-naïve subjects with genotypic drug-resistance interpretation to 19 different drugs provided by five online genotypic resistance algorithms.

Drugs	HIV-1 variants (no.)	Genotypic resistance algorithms (% of sequences scored to each level of resistance)														
		ANRS 2008.07			Stanford 4.3.7			Rega 7.1.1			Geno2pheno 3.0			RIS 2008		
		S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
PI	Non-B (128)	9.4	14.8	75.8	94.4	5.5	0.8	94.4	3.1	3.1	94.4	3.1	3.1	94.4	3.1	3.1
	B (226)	92.5	2.6	4.9	94.7	4.9	0.4	95.6	2.6	1.8	95.6	2.2	2.2	96	3.1	0.9
NRTI	Non-B (108)	95.4	1.8	2.8	92.6	4.6	2.8	94.4	2.8	2.8	78.7	14.8	6.5	95.4	1.8	2.8
	B (210)	93.8	2.4	3.8	93.3	5.2	1.4	93.3	3.8	2.9	81	16.7	2.4	93.8	3.8	2.4
NNRTI	Non-B (108)	81.5	0	18.5	91.7	0.9	7.4	91.7	0.9	7.4	91.7	0	8.3	92.6	0	7.4
	B (210)	95.7	0	4.3	94.3	1.4	4.3	95.7	0	4.3	94.8	0	5.2	95.2	0	4.8

No., number of sequences; PI, protease inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; S, percentage of sequences interpreted as susceptible by the respective algorithm for a drug family; I, intermediate resistant; R, resistant. ANRS 2008.07 (http://www.hivfrenchresistance.org), Stanford HIVDB 4.3.7 (http://hivdb6.stanford.edu/asi/deployed/hiv_central.pl?program=hivdb&action=showSequenceForm), Rega 7.1.1 (http://www.rega.kuleuven.be), Geno2pheno 3.0 (http://www.geno2pheno.org) and RIS 2008 (http://www.retic-ris.net/default_principal.asp?idx=&cidioma=2).

Table 3
Discordances between five genotypic drug-resistance interpretation algorithms testing 128 non-B and recombinants *vs*. 226 subtype B *pol* sequences.

Drugs	Full discordances			Partial discordances			Concordances non-B/B
	Non-B/B (%)	*p*	Variants with discordances	Non-B/B (%)	*p*	Variants with discordances	
Any PI	76.6/4.4	**<0.01**	99 non-B, 17B	10.9/6.6	NS		22.3/92.5
Atazanavir	2.3/1.3	NS		2.3/2.6	NS		95.3/96.0
Darunavir	0/0	–		0.8/0.9	NS		99.2/99.1
Fosamprenavir	3.1/0.4	**0.04**	3CRF12_BF, 1CRF10_CD, 1B	0/2.2	NS		96.9/97.3
Indinavir	2.3/1.8	NS		3.1/1.3	NS		94.5/96.0
Lopinavir	2.3/1.3	NS		0.8/0.9	NS		96.9/97.8
Saquinavir	0/0	–		4.7/3.1	NS		95.3/96.9
Nelfinavir	0/0.4	NS		6.2/3.5	NS		94.5/96.0
Tipranavir	73.4/0.9	**<0.01**	94 non-B, 2B	10.9/1.8	**<0.01**	14 non-B, 4B	12.5/97.3
Any NRTI	5.6/3.8	NS		16.7/13.8	NS		83.3/85.2
Lamivudine	0.9/0	NS		0.9/1.9	NS		98.1/98.1
Abacavir	0.9/0.4	NS		2.8/2.9	NS		95.4/96.7
Zidovudine	0.9/0.9	NS		1.8/4.3	NS		97.2/94.8
Estavudine	0/0.9	NS		9.3/12.6	NS		90.7/86.2
Didanosine	6.5/1.9	**0.03**	4CRF02_AG, 1A, 1CRF10_CD, 1CRF14_BG, 4B	6.5/3.8	NS		87.0/94.3
Emtricitabine	0/0	–		0.9/1.4	NS		99.1/98.6
Tenofovir	0.9/1.4	NS		1.8/7.6	**0.03**	16B, 1A, 1CRF10_CD	97.2/90.9
Any NNRTI	11.1/1.9	**<0.01**	8CRF14_BG, 2CRF23_BG, 1C, 1CRF10_CD, 4B	0.9/0.9	NS		87.0/97.1
Delavirdine	0/0	–		0/0.5	NS		100/99.5
Efavirenz	0.9/0	NS		0/0	–		99.1/100
Etravirine	0/0.5	NS		0.9/0.5	NS		99.1/99.0
Nevirapine	11/1.4	**<0.01**	8CRF14_BG, 2C, 2CRF23_BG, 3B	0/0.5	NS		88.9/98.1
Any RTI	15.7/5.7	**<0.01**	8CRF14_BG, 4CRF02_AG, 2C, 2CRF23_BG, 1A, 12B	17.6/14.8	NS		73.2/83.3

Drug susceptibilities to protease inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI) were predicted using five different algorithms: ANRS 2008.07, Stanford 4.3.7, Rega 7.1.1, Geno2pheno 3.0, and RIS 2008. In bold, specific drugs showing significant ($p < 0.05$) differences in full or partial discordances comparing non-B *vs*. B sequences (128/108 non-B and 226/210 subtype B PR/RT sequences from different antiretroviral-naïve patients). The 354 analyzed sequences were: 9A, 226B, 8C, 1D, 8F, 15G, 41CRF02_AG, 5CRF06_cpx, 3CRF10_CD, 2CRF11_cpx, 16CRF12_BF, 1CRF13_cpx, 9CRF14_BG, 1CRF19_cpx, 1CRF22_01A1, 6CRF23_BG and 2CRF31_BC. Full discordances when one of the algorithms scored a sequence as S for a particular drug and another one as R. Partial discordance when one of the algorithms scored the sequences as S for a particular drug and another one scored it as I, or when one of the algorithms scored the sequences as I for a particular drug and another one scored it as R. The five algorithms analyzed the susceptibility to all the drugs shown except for darunavir, tipranavir and etravirine (not provided by Geno2pheno), nelfinavir (not provided by RIS) and delavirdine (not provided by ANRS and RIS algorithms). NS, Chi-square test showed non-significant differences ($p > 0.05$). Zalcitabine resistance was not included because it could only be tested by the Geno2pheno algorithm.

Geno2pheno 3.0, and Spanish RIS 2008. Their interpretations are shown in Table 2. Most sequences were interpreted as susceptible (S) to all antiretrovirals by the five algorithms, except for tipranavir by ANRS in non-B. On the other hand about 4–8% of the 354 sequences from treatment-naïve patients were intermediate (I) or resistant (R) to specific drugs by all algorithms. ANRS ascribed as I or R to a significantly higher number of non-B *vs*. B sequences for PI (90.6% *vs*. 7.5%, $p < 0.05$) and NNRTI (18.5% *vs*. 4.3%, $p < 0.05$). The other algorithms ascribed as I or R to PI or NNRTI were 6–7% or 8% of non-B sequences, respectively. Stanford was the algorithm that provided the lowest number of HIV-1 sequences as R to PI (Table 2). Geno2pheno ascribed as not susceptible (I or R) to NRTI a higher number of non-B and B sequences than other algorithms (around 20% *vs*. 5–7%).

Considering HIV-1 variants, interalgorithm discordances (including both full and partial) were significantly higher in non-B *vs*. B variants for NNRTIs (13% *vs*. 2.9%, $p < 0.05$), and similar for NRTI (16.7% *vs*. 14.8%, p = NS) and for PIs excluding tipranavir (8.6% *vs*. 5.7%, p = NS) (Table 3). Regarding drug families, interalgorithm discordances (full and partial) were significantly more frequent for NRTI *vs*. NNRTI or PI excluding tipranavir in all variants (15.4% *vs*. 6% or 6.8%, respectively; $p < 0.01$) but were similar among drug families when only full discordances were considered (4.4%, 5%, and 4.2%, respectively). Considering specific drugs, non-B variants displayed significantly more interalgorithm full discordances than subtype B for tipranavir (73.4% *vs*. 0.9%, $p < 0.05$), fosamprenavir (3.1% *vs*. 0.4%, $p < 0.05$), nevirapine (11% *vs*. 1.4%, $p < 0.05$) and didanosine (6.5% *vs*. 1.9%, $p < 0.05$), and lower partial discrepancies than clade B for tenofovir (1.8% *vs*. 8.6%, $p < 0.05$) (Table 3).

Sequences with changes in residues M36, H69 and L89, highly common in non-B viruses (Table 1), were considered as R to tipranavir by ANRS and as I when two of these changes appeared. For ANRS, all non-B sequences were susceptible to didanosine, since this algorithm, in contrast to the remaining tools, did not include L74V as a didanosine-resistance mutation. Geno2pheno considered sequences with I135T, in combination with V60I or T200A, as I to tenofovir, although none of them were included in the IAS-USA list. ANRS interpreted a higher number of nevirapine-resistant non-B sequences, due to the inclusion of the A98S change. This is actually the wild-type residue in CRF14_BG (www.hiv.lanl.gov), first described in Spain (Delgado et al., 2002), and it is also a natural polymorphism in CRF24_BG.

3.5. *Low reliability of five online rapid subtyping tools in the assignment of non-B subtypes and recombinants*

The 354 *pol* HIV-1 sequences were introduced in five online rapid subtyping tools (Stanford, Geno2pheno, Rega, NCBI and EuResist) to test their agreement with phy (Table 4). Most subtype B sequences, prevalent in developed countries, were correctly assigned (87.2–99.6%), the worst was NCBI and the best was Stanford. The five tools showed a low agreement with phy ascribing non-B variants and differed among tools. Only three quarters of the sequences were correctly assigned by Geno2pheno (75.8%) and EuResist (71.9%), and results were even lower for NCBI (60.9%), Rega

Table 4
Agreement of five online subtyping tools vs. phylogenetic analysis in 354 sequences from different HIV-1 variants.

HIV-1 variant[a] (no.)	HIV-1 subtyping method					
	Phy *vs.* Stanford[b]	Phy *vs.* Geno2pheno	Phy *vs.* Rega[c]	Phy *vs.* Rega[d]	Phy *vs.* NCBI	Phy *vs.* EuResist
A (9)	22.2	44.4	88.9	100	11.1	33.3
C (8)	87.5	100	87.5	100	87.5	100
D (1)	100	100	100	100	0	0
F (8)	87.5	87.5	100	100	25	100
G (15)	93.3	93.3	73.3	100	53.3	86.7
CRF02_AG (41)	82.9	95.1	63.4	84.4	92.7	92.7
CRF06_cpx (5)	0	100	60	100	60	100
CRF10_CD (3)	0	33.3	0	0	0	0
CRF11_cpx (2)	0	50	100	100	100	50
CRF12_BF (16)	0	43.7	43.7	70	43.7	37.5
CRF13_cpx (1)	0	100	0	–	100	100
CRF14_BG (9)	0	100	0	0	100	100
CRF19_cpx (1)	0	0	0	–	0	0
CRF22_01A1 (1)	0	0	0	–	0	0
CRF23_BG (6)	0	0	0	–	0	0
CRF31_BC (2)	0	0	0	0	0	0
Pure non-B subtypes (41)	75.6	82.9	85.4	100	43.9	78
Recombinants non-CRF02_AG (46)	0	52.2	26.1	50	47.8	47.8
Total Recombinants (87)	39.1	72.4	43.7	69.6	69	69
Total Non-B (128)	50.8	75.8	57	81.3	60.9	71.9
Subtype B (226)	99.6	95.1	90.7	100	87.2	97.8

No., number of sequences; Phy, phylogenetic analysis considered as the gold standard subtyping method; CRF, circulating recombinant forms; dash indicates those cases when Rega did not assign any of the sequences of the subtype/CRF. Websites: Stanford HIVDB 4.3.7, (http://hivdb6.stanford.edu/asi/deployed/hiv_central.pl?program=hivdb&action=showSequenceForm), Geno2pheno 3.0 (http://www.geno2pheno.org), Rega 2.0 (http://www.bioafrica.net/subtypetool/html), NCBI 2008 (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) and EuResist 2008 (http://engine.euresist.org).
[a]Subtypes in the *pol* coding region.
[b]Stanford differenced protease and reverse transcriptase in the analysis. Out of 63 failures, 6 occurred in a PR with RT correctly subtyped and 4 vice versa.
[c]Rega analysis including or [d]excluding all the sequences not assigned to a subtype by the Rega tool (6 "pure" non-B subtypes, 31 CRF and 21 clade B).

(57%) and Stanford (50.8%) (Table 4). Rega was unable to assign any subtype in 21 (9.3%) B sequences and 37 (29%) non-B variants, and 84% of them were CRF. Rega's agreement increased up to 81.3% when only assigned subtypes were considered.

Results were better for pure non-B subtypes than for CRF, except for NCBI (Table 4). The agreement with phy testing pure non-B subtypes was higher for Geno2pheno (82.9%) and Rega (85.4%), and lower for EuResist (78%), Stanford (75.6%) and NCBI (43.9%). CRF02_AG was the best assigned, reaching 90% in some tools. However, only 50% of the 46 specimens adscribed as CRF by phy other than CRF02_AG, were correctly assigned by all online tools except Stanford, which did not identify any of them. None of the online tools could correctly subtype any CRF19_cpx, CRF22_01A1, CRF23_BG or CRF31_BC (Table 4). In summary, none of the five subtyping tools showed a complete reliability in the assignment of non-B subtypes and recombinants, although Geno2pheno, EuResist and Rega showed the best global results. Rega showed the best results subtyping pure non-B subtypes and NCBI the worst. Geno2pheno had the highest agreement in recombinant assignment and Stanford the lowest.

4. Discussion

This study analyzed different aspects. Firstly, it defined the drug-resistance prevalence in a large cohort of treatment-naïve patients infected by different HIV-1 subtypes and CRF in Spain. Secondly, it reported the discordances in resistance interpretation comparing five online algorithms. Thirdly, it showed the agreement of five online tools with phylogenetic analysis (phy) for HIV-1 subtyping. The overall prevalence of drug-resistance mutations found (13.8%) was similar to other surveillance studies in Europe and Spain (Booth and Geretti, 2007; de Mendoza et al., 2005; Sanchez-Oñoro et al., 2007; Wensing et al., 2005; Wheeler et al., 2007), although it was higher in some areas (Shet et al., 2006). To our knowledge, the study cohort of treatment-naïve subjects infected by HIV-1 non-B subtypes and recombinants is one of the largest identified in Madrid and in Spain including these variants. However, the number of samples analyzed for certain subtypes and/or recombinants was very limited. It would be more appropriate to have a significant number of each HIV-1 variant instead of merging all in a single group, given that each clade has its own peculiar characteristics and specific wild-type sequence. Nevertheless, this is not always possible due to the unequal distribution of non-B subtypes in our country. In addition, the number of patients does not allow the reliable analysis of the trends of resistance over time. These trends of transmitted resistance vary depending on the time period, geography and cohort characteristics, including the country of origin. An additional source of variation is that distinct drug-resistance surveillance studies are based on different lists of drug-resistance mutations.

Nevertheless, the knowledge of primary resistance can be cost-effective during the clinical practice and treatment proposed regimen (Smith et al., 2007). Our treatment-naïve population presented a significantly higher prevalence of drug-resistance mutations in non-B *vs.* B subtypes. This was due to the higher rate of NNRTI-resistance mutations in certain variants, mainly in CRF02_AG (19.5% of sequences with NNRTI-resistance mutations *vs.* 7.1% in subtype B, $p < 0.05$) (Table 1b). In fact, CRF02_AG variants are the most frequent HIV-1 non-B viruses in Spain (Holguín et al., 2008b). NNRTIs are frequently used in the native countries of most foreign non-B infected patients in Spain. It was also recently reported that transmission of NNRTI-resistances in Europe is rising faster than for other antiretroviral families (SPREAD programme, 2008). It would be useful if non-B variants were detected among these NNRTI-resistant viruses in future European surveillance studies.

New HIV-1 diagnoses caused by non-B variants increased in Spain in the last decade (Holguín et al., 2008a), as in other industrialized countries (Aggarwal et al., 2006; Pillonel et al., 2008). Thus, HIV-1 subtype and country of infection of treatment-naïve patients should be considered in drug-resistance studies, since resistance prevalence may differ in subjects infected in areas with fewer avail-

able drugs. Accordingly, there is a low resistance prevalence in non-B infected patients living in developing countries with scarce therapy distribution (Agwale et al., 2006; Booth and Geretti, 2007; Vessière et al., 2006).

We observed a high frequency of secondary PI-resistance mutations in all variants, which showed a different nature among HIV-1 subtypes and CRF. Despite the low number of studies correlating mutations and response to therapy in non-B subtypes (Martínez-Cajas et al., 2008), it is known that some clade-specific amino acids at resistance positions may influence drug susceptibility, selection of different resistance pathways, and/or a more rapid emergence of drug-resistance (Holguín et al., 2006b). Regardless of their therapeutic and epidemiological implications, these residues have been excluded in a specific list created in a positive attempt to homogenise transmitted drug-resistances detected in treatment-naïve patients (Bennett et al., 2009). However, this list only includes two of the 43 HIV-1 circulating recombinant forms described to date (http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html), and should be improved to be applicable to all recombinants, that are increasing in global prevalence.

Some of these subtype-associated polymorphisms in positions related to drug-resistance are responsible for the discrepancies found in drug-resistance interpretation using different algorithms. Our study reports that when using five different algorithms discordances were significantly higher in non-B *vs*. B variants for didanosine, nevirapine, tipranavir, and fosamprenavir, and significantly lower for tenofovir. These discrepancies highlight that the patterns of drug-resistance mutations have not been yet completely clarified in non-B variants, especially for PI. The use of certain algorithms could lead to an overestimation of the resistance in the analysis of specific non-B subtypes because of the lack of consensus in the resistance mutations considered. Nevertheless, it does not mean that the analyzed tools are useless or that the current interpretation algorithms may be invalidated due to non-B subtypes. These algorithms are easy to use and useful in the routine practice for clinicians during the clinical follow up of their HIV-infected patients and a good concordance among them was observed for most (but not all) variants and drugs. The discrepancies between them would justify the necessity of including more samples from subtypes different from B in the databases from different algorithms to improve the excellence of the methods, reducing the potential mistakes in some non-B specimens for some drugs using specific algorithms. For instance, it would avoid interpreting as resistant a sequence which presumably is not. On the other hand, with these data we cannot affirm which of the algorithms are the most accurate. In order to clarify this aspect phenotypic assays should be performed to elucidate which algorithm is right and which is wrong.

Although discrepancies have been previously reported (Champenois et al., 2008; Kijak et al., 2003; Muñoz et al., 2005; Poonpiriya et al., 2008; Ravela et al., 2003; Snoeck et al., 2006; Vergne et al., 2006), only a few studies have compared discrepancies testing different HIV-1 subtypes and recombinants. In fact, very few studies have included different CRF ranging from 1 to 6 (Poonpiriya et al., 2008; Champenois et al., 2008; Vergne et al., 2006; Snoeck et al., 2006) whereas our work has included sequences from 11 different CRFs. Moreover, to our knowledge, no previous reports have compared as many algorithms as the present work. Some of these studies tried to correlate clade-specific substitutions and discrepancies, as previously described using ANRS algorithm for tipranavir-resistance associated with PR changes M36I, H69K and L89M (Champenois et al., 2008), which are in fact wild-type amino acid in most of the non-B variants. Our work also reports that A98S substitution, wild-type amino acid in CRF14_BG, was interpreted as a nevirapine-resistance marker by the ANRS algorithm.

Finally, HIV-1 rapid subtyping tools can be useful for clade B identification but their efficacy is lower for other variants and differs among tools, as previously reported (Holguín et al., 2008b; Ntemgwa et al., 2008). It can be due to the fact that they assign the subtype by simply applying the similarity method. Phylogenetic analysis is still the only reliable method to correctly assign HIV-1 non-B subtypes and CRF, which are growing in number and complexity. Furthermore, this analysis is complicated to perform in the routine practice. We recommend the use of several rapid subtyping tools instead of only one, in order to compare the results. Furthermore, before entering routine clinical use, rapid subtyping tools should be optimised and updated periodically, including larger numbers of different non-B subtypes and CRF sequences in reference databases. The prediction of subtyping by these tools should be improved before being used in routine clinical settings. Thus, a common global effort is needed for the databases unification in only one rapid subtyping tool to facilitate the rapid and more correct identification of HIV-1 subtypes and CRFs.

In summary, we found a higher prevalence of drug-resistance mutations in non-B *vs*. B subtypes, which reinforces the need to identify HIV-1 variants in drug-resistance surveillance studies. Databases used by online genotypic resistance algorithms and subtyping tools should be optimised and updated periodically by increasing the number of non-B and CRF sequences. This would improve their suitability for the analysis of these variants before their use in routine clinical settings.

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Clinical Differences and Viral Diversity between Newly HIV Type 1-Diagnosed African and Non-African Patients in Spain (2005–2007)

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Abstract

The diagnosis of HIV-1 is increasing in African-born persons residing in Europe. They present a high prevalence of HIV-1 non-B variant infections and of parasitic infections, both of which are infrequent in Western countries. Immigration favors their presence in nonendemic countries. In this study, all newly HIV-diagnosed individuals at an HIV/AIDS and Tropical Medicine reference center in Madrid from 2005 through 2007 were retrospectively studied. HIV-1 subtyping was performed in *gag, pol,* and *gp41* coding regions by phylogenetic analyses. The presence of other pathogens was also evaluated. Furthermore, all HIV-1-infected Africans were screened for parasitic infections. Newly diagnosed HIV-1 subjects included 90 sub-Saharan Africans and 188 non-Africans (116 Spaniards, 13 other Europeans, and 59 Latin Americans). Significantly higher numbers of HIV-1-infected Africans than non-Africans were females, acquired HIV-1 by heterosexual contact, and presented a more advanced clinical CDC stage and criteria for starting antiretroviral therapy in the first clinical visit. They predominantly carried non-B subtype infections, mainly intersubtype recombinants. Half of HIV-1-infected Africans had parasitic infections. $CD4^+$ T cell counts were lower among Africans than Europeans at the time of HIV-1 diagnosis. At 12 months of follow-up after starting antiretroviral treatment, a significantly lower proportion of Africans than non-Africans achieved undetectable viremia due to their higher loss to follow-up. However, $CD4^+$ T cell recovery and virological failure rates were similar. Therefore, the profile of African HIV-1-infected immigrants varies widely with respect to Spanish HIV-infected individuals. More advanced immunodeficiency and the coexistence of parasitic diseases and infections with a large diversity of HIV-1 non-B and recombinant variants are expected.

Introduction

One of the main entrance points of Africans into the European Union is through Spain, and in recent years there has been an increase in the number registered in our country. Most of them came from sub-Saharan Africa, which has been severely affected by the human immunodeficiency virus (HIV) pandemic and accounts for 70% of cases worldwide.[1] Furthermore, about 70% of adults and nearly 90% of children infected with HIV in the world live in this region,[1] where HIV-1 non-B subtypes and intersubtype recombinant[2,3] variants are the most frequent strains found.[4] Moreover, additional tropical disease infections are frequent in this population. In the past 15 years, the number of immigrants in Spain has increased dramatically,[5] and sub-Saharan African immigrants make up a small, but growing percentage of the people affected by HIV/AIDS in our country.[6,7] In 2006, nearly 21% of the new HIV-1-infected diagnoses involved foreigners, and 39.5% of these were sub-Saharan Africans.[7] Medical management of this population represents a clinical challenge, because of both the cultural and language barriers and the special health care needs that these patients present. Most health care professionals in Spain are unfamiliar with diseases that are common in tropical environments but infrequent in Western countries, and consequently this can result in a delay in the diagnosis of some tropical pathologies or even misdiagnosis.

As has happened in other European countries[8–11] where subtype B infections still predominate, the introduction of HIV-1 non-B subtypes and recombinant variants is expected in Spain as a result of population movements from

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countries endemic for those variants[12–16] such as sub-Saharan Africa.[4] The coexistence of multiple HIV-1 non-B clades with a native B subtype involves a more heterogeneous and complex viral population as a result of recombination between these clades and/or recombinant variants.[17] This relevant phenomenon has been suspected of having clinical implications,[18] affecting disease progression,[19] treatment efficacy,[20] viral load measurements,[21] rapid subtyping tool reliability,[22] and diagnostic tests.[23] In the present study, the epidemiology, differential clinical features, and genetic nature of HIV-1 variants were analyzed in a cohort of HIV-1-infected sub-Saharan Africans who were newly diagnosed during a 3-year period in a Spanish HIV/AIDS and Tropical Medicine reference center located in Madrid. A control group, consisting of all HIV-1-infected non-Africans newly diagnosed during the same period, was also included in order to compare both populations.

Materials and Methods

Study population

Patients were recruited and followed at Hospital Carlos III, an HIV/AIDS and Tropical Medicine reference center located in Madrid, Spain. Although the study was mainly focused on African patients, all newly diagnosed HIV-1 subjects over 18 years of age who attended at least once from January 2005 through December 2007 were included in the study. Demographic, clinical, and laboratory data extracted from patients' medical records are shown in Table 1. Eosinophilia, a surrogate marker of helminthic infection, was defined as an absolute eosinophil count $>0.7/\mu l$ and relative eosinophilia as $>7\%$ eosinophil and eosinophil count $<0.7/\mu l$. Elevated immunoglobulin E (IgE) was considered if >200 IU/ml. The patients diagnosed with HIV-1 in 2005 and 2006 who started highly active antiretroviral treatments (HAART) in their first visit to the clinic were retrospectively analyzed. CD4 counts and viral load values were evaluated at baseline and after 1 year on HAART. Finally, plasma viremia was measured using the VERSANT HIV-1 RNA bDNA v3.0 (Bayer Diagnostic, Tarrytown, NY) and $CD4^+$ lymphocyte count was performed by flow cytometry (Coulter, Barcelona, Spain).

HIV-1 and hepatitis serological diagnosis

Two enzyme-linked immunosorbent assays (ELISAs) designed with synthetic peptides or recombinant proteins were used for the detection of antibodies to HIV-1 (AxSYM, Abbot Diagnostics, Chicago, IL). Reactive samples were confirmed using an HIV-1 viral lysate Western blot (New LAV BLOT I, BIO-RAD, Marnes la Coquette, France) and a line immunoassay (LIA) able to distinguish antibodies to HIV-1 and HIV-2 (Pepti-LAV, BIO-RAD). HIV infection was considered as

Table 1. Demographic and Clinical Data for Newly HIV Diagnosed Subjects According to their Origin[a]

		Control group (n = 188)		
	Africans	*South Americans*	*Europeans*	p
No. of patients	90	59	129	—
Age (years) [median (IQR)]	41 (31.5–49.5)	34 (27–37)	38 (32–45)	<0.001
Male gender (%)	42.2	86.4	89.1	<0.001
Perceived exposure category (%)				
MSM	1.1	75.4	64.7	<0.001
Injecting drug use	1.1	0	9.2	0.004
Heterosexual	89.9	22.8	24.4	<0.001
Other/unknown	7.9	1.8	1.7	0.025
No. CD4 count (cells/ml) [median (IQR)]	270 (146–434)	266 (171–459)	375 (220–601)	0.014
CD4 [median (IQR)] (%)	13 (9–22)	14 (9–21)	20 (13.5–30)	<0.001
Patients with <200 CD4/ml (%)	37	32.7	23.2	NS
Patients with <50 CD4/ml (%)	9.9	12.7	8.8	NS
Recent HIV infection (%)	4	13.2	20.5	0.002
Viral load (log_{10} copies/ml) [median (IQR)]	4.5 (3.8–4.9)	4.6 (4.1–5.1)	4.4 (3.9–5.0)	NS
CDC stage (%)				
A	54.5	70.9	79	<0.001
B	17	12.7	10.5	NS
C	28.4	16.4	10.5	0.001
HBsAg positive (%)	5.6	3.4	4.7	NS
HCVAb positive (%)	11.1	0	10.9	0.003
Tuberculosis (%)	18.2	8.6	2.3	<0.001
Bacterial infections (%) (excluding TB)	23.8	9.1	7.2	0.001
STD (%) (excluding HPV)	10.7	21.4	14.5	NS
Syphilis (%)	7.1	14.5	12.2	NS
Protozoal or helminthic infections (%)	51.1	7	4.2	<0.001

[a]IQR, interquartile range; MSM, men who have sex with men; CDC, Centers for Disease Control and Prevention; HBsAg, hepatitis B surface antigen; HCVAb, hepatitis C antibodies; TB, tuberculosis; STD, sexually transmitted diseases; HPV, human papiloma virus; NS, not significant ($p > 0.05$).

recent if it happened within 12 months before HIV diagnosis, according to physicians' information. Diagnosis of hepatitis B and C was performed using serological tests (Architect, Abbott Diagnostics, Chicago, IL).

Diagnosis of other infectious diseases

All African-born patients, even if asymptomatic, were systematically screened for the following conditions by standardized techniques[24]: stool concentrate microscopy of three different samples, day bloods, and skin snips samples for microfilariae, filarial, *Schistosoma,* and African trypanosomiasis serology. *Strongyloides, Toxocara, Equinococcus,* and *Fasciola* serology and specific IgE to *Ascaris lumbricoides* were performed in case of total or relative eosinophilia and/or elevated IgE without a diagnosis after the first screening. The study of malaria included thick blood smear and amplification of *Plasmodium* DNA by seminested multiplex polymerase chain reaction (PCR). Other investigations were performed according to any clinical suspicion in all African or non-African patients.

HIV-1 subtyping

In the African group, RNA extraction from plasma specimens, direct sequencing, and phylogenetic analyses of sequences obtained from nested PCR purified products were performed for HIV-1 subtype characterization in *gag*, *pol*, and *gp41* coding regions. In some specimens *gag* and *pol* were amplified in one amplicon (positions from 693 to 3250 in the HXB2 isolate) with outer primers 506D (5′-GGA ACC CAC TGC TTA AGC CTC AAT AAA G-3′) and NE135 (5′-CTT ACT AAC TTC TGT ATG TCA TTG ACA GTC CAG CT-3′) and inner primers 693D (5′-CTC TCG ACG CAG GAC TCG GCT TGC TG-3′) and RT4m (5′-AGG ATG GAG YTC ATA YCC CAT CCA AAG-3′). Conditions were incubation at 94°C for 3 min, 35 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 2.30 min, with a final extension at 72°C for 10 min. The sequencing reaction used primers 693D (see above), 1309D (5′-GCA TTA TCA GAA GGA GCC ACC CCA C-3′), 1333R (5′-GTG GGG TGG CTC CTT CTG ATA ATG C-3′), RT4m (see above), and Prot4 (5′-AAT GCT TTT ATT TTT TCT TCT GTC AAT GGC-3′). When *gag* and *pol* were analyzed separately, *gag* was amplified completely (1384 bp, residues 790–2292 in HXB2) or partially (515 bp, positions 1754–2269 in HXB2) and sequenced as previously described.[25,26] The HIV-1 *pol* sequences, including complete protease and partial reverse transcriptase (codons 1–247 or 1–335), were obtained using the Trugene (Siemens, Barcelona, Spain) or ViroseqTM HIV-1 genotying System (Celera Diagnostic, Alameda, CA), respectively. Sequencing of *pol* was performed using the primers Prot3 (5′-TCA GAG CAG ACC AGA GCC AAC AGC CCC A-3′), RT4m (see above), and A35 (5′-TTG GTT GCA CTT TAA ATT TTC CCA TTA GTC CTA TT-3′). The complete *gp41* coding region (codons 1–345) was amplified and sequenced according to a previous report.[27]

Phylogenetic analyses using the *gag, pol,* and *gp41* sequences were carried out for HIV-1 subtype or intersubtype recombinant characterization as reported,[26,27] using as reference sequences those belonging to the HIV-1 group M available at the GenBank. All trees were rooted with the HIV-1 divergent YBF30 group N sequence. The tree topology was obtained using the neighbor-joining method, and the sequences were aligned using Clustal X software. The pairwise distance matrix was estimated using the Kimura two-parameter model within the DNADIST program, as implemented in the PHYLIP software package. Bootstrap resampling (1000 data sets) of the multiple alignment was performed to test the statistical robustness of the tree.

We considered as "pure" those sequences with the same nonrecombinant subtype in all the assessed regions. The recombinant variants included variants harboring the same or different CRF sequences at *gag, pol,* and *gp41,* as well as the HIV-1 strains carrying different clades in at least one of the regions.

Data analysis

Differences between continuous variables and correlations were analyzed using parametric and nonparametric tests. Differences between categorical variables were analyzed using the chi-square test or Fisher's exact test as appropriate. Significance was set at $p < 0.05$.

Results

Demographic, clinical, and immunological status

Two hundred and seventy-eight newly diagnosed HIV-1-infected patients were identified, 188 (67.6%) non-Africans and 90 (32.4%) Africans coming from eight different sub-Saharan countries, mainly (75) from Equatorial Guinea, a former Spanish colony located between Cameroon and Gabon. Among the HIV-1-infected non-Africans, considered as the control group, 129 were Europeans (116 native Spaniards) and 59 came from 14 South American countries. The median time from arrival of the HIV-1-infected African patients in Spain until their first visit to the clinic was 2 weeks (interquartile range: 0–14 weeks). This short period is due to the fact that several patients came to Spain specifically for treatment. Table 1 shows the main demographic and clinical data from all subjects according to their origin. Africans and Europeans showed a similar median age at HIV-1 diagnosis, which was significantly higher than in Latin Americans. More Africans than non-Africans were females (58% vs. 12%; $p < 0.001$), acquired HIV infection through heterosexual risk contacts (90% vs. 24%; $p < 0.001$), and presented a more advanced clinical CDC stage in the first visit to the clinic. At the time of HIV diagnosis 9.3% of the sub-Saharan women of childbearing age were pregnant. Gender, CDC stage, baseline viral load, or comorbidity did not differ between Latin Americans and Europeans.

The immunological status of HIV-1-infected Africans and Latin Americans was similar. They respectively presented a lower median $CD4^+$ T cell count (270 and 266 vs. 375 cells/ml; $p < 0.001$) and a lower CD4 percentage (13% and 14% vs. 20%) than Europeans. Thus, Europeans had the best immunological status at their first visit. The number of Africans with cell counts less than 200 CD4 cells/ml at the first visit tended to be similar to non-Africans (37% vs. 32%). It was only significantly lower in the Europeans (37% vs. 23%; $p = 0.04$), even with similar HIV-1 viremia at baseline. The highest number of recent HIV-1 infections occurred in Europeans (20.5%) and the lowest in Africans (4%). In addition, the most prevalent AIDS-defining condition was tuberculosis, accounting for 64% of all the AIDS diagnoses in Africans compared to 27% in

non-Africans ($p = 0.008$). Other AIDS-defining diseases found among Africans were Kaposi's sarcoma in four subjects, cytomegalovirus disease in three individuals, *Pneumocystis jiroveci* pneumonia, esophageal candidiasis, and cerebral toxoplasmosis, each in two patients, progressive multifocal leukoencephalopathy in one patient, and wasting syndrome due to HIV in one patient.

Concomitant infections at time of HIV-1 diagnosis

Hepatitis B virus coinfections were similar among groups (Table 1). However, no hepatitis C virus coinfection was reported among HIV-1-infected Latin Americans, probably because of the absence of intravenous drug users in this group. A similar number of Africans and Europeans presented sexually transmitted diseases (STDs) at HIV-1 diagnosis, including syphilis. However, STDs were more frequent among HIV-1-infected Latin Americans (Table 1). A higher proportion of Africans presented a bacterial infection at the time of HIV-1 diagnosis, including seven patients with urinary tract infections, four with bacterial pneumonia, three with upper respiratory tract infections, two with bacterial gastroenteritis or deep abscesses, respectively, one with acute cholangitis, and another one with acute cholecistitis.

All HIV-1-infected Africans and non-Africans with clinical suspicion of protozoal or helminthic infections were screened to rule out parasitic infections. Eosinophilia and elevated IgE were common among HIV-1-infected Africans (17% and 58%, respectively), with 29% presenting relative eosinophilia. Parasitic infections in the first visit were more frequent among HIV-1-infected Africans (51%) than non-Africans (5%) tested for a broad spectrum of parasitic infections (Table 2). Parasitic-infected Africans showed a significantly lower median $CD4^+$ count at HIV diagnosis than those without evidence of infection (238 vs. 303 cells/ml; $p = 0.028$). The $CD4^+$ cell count was inversely related to the number of concomitant parasitic infections ($r = -0.317$; $p = 0.005$) and directly related to total eosinophil number ($r = 0.359$; $p = 0.001$). The total and relative eosinophil and IgE numbers were significantly higher in those HIV-1-infected Africans with any helminthic disease ($p = 0.001$, $p = 0.049$, and $p = 0.009$, respectively), but only in those with more than 200 CD4 cells/μl.

Malaria was diagnosed in 13 HIV-1-infected Africans (14.4%): 12 infected by *P. falciparum* and one by *P. malariae*. They presented significantly higher HIV-1 viremia (4.9 vs. 4.2 logs; $p = 0.037$) and lower CD4 cells (245 vs. 285; $p = 0.1$) than those without malaria. In six patients a positive malaria diagnosis was made by a positive PCR test and negative thick blood smear, even though the patients were asymptomatic or referred only to mild unspecific symptoms. Their viral load was significantly higher (5 vs. 4.2 logs; $p = 0.002$) and their CD4 cells count tended to be lower (252 vs. 291; $p = 0.081$) than those without malaria.

Response to HAART in HIV-1-infected Africans vs. non-Africans

More Africans than non-Africans presented criteria to start HAART at the first visit (72% vs. 56%; $p = 0.024$). Eventually, in 2005 and 2006, 61 non-Africans and 29 Africans started HAART, including protease inhibitors in 60.7% and 46.5%, respectively. At 1 year of follow-up after starting HAART, a significantly lower proportion of Africans achieved a viral load below 50 HIV RNA copies/ml (41.4% vs. 82%; $p < 0.001$). A similar median $CD4^+$ count increment was observed among those Africans vs. non-Africans who achieved an undetectable viral load after 1 year of treatment (251 vs. 215 cells/ml; $p = 0.20$). Africans presented a significantly higher proportion of loss to follow-up than the non-Africans (41.4% vs. 11.5%; $p < 0.001$), but both groups showed similar rates of virological failure (18.5% vs. 6.6%; $p = 0.11$). Four out of the five virological failures observed in Africans were associated with a transient lack of antiretroviral treatment during short visits to their countries.

Table 2. Associated Protozoal and Helminthic Infections in 90 Newly HIV-1-Infected Africans

Diagnoses	*No.*	%[a]
Protozoos		
Malaria	13	14.4
Entamoeba histolytica/dispar	4	4.4
Giardia lamblia	4	4.4
Trypanosoma brucei	1	1.1
Helminths		
Schistosoma sp.	3	3.3
Ascaris lumbricoides	11	12.2
Trichuris trichiura	7	7.7
Strongyloides stercoralis	3	3.3
Hookworm	1	1.1
Toxocara sp.	1	1.1
Taenia solium/cisticercosis	1	1.1
Mansonella perstans	5	5.5
Oncocerca volvulus	3	3.3
Loa loa	2	2.2
Any parasitic infection	46	51.1

[a]Among the parasitic-infected Africans, 69% presented one pathogen, 26% two, and 5% three or more parasitic infections at HIV diagnosis.

HIV-1 variants infecting newly diagnosed HIV-1 patients

Table 3 shows the number and percentage of patients carrying different subtypes and recombinant viruses. HIV-1 subtyping could be performed in any region in 73% and 66.5% of viruses from Africans and non-Africans, respectively. The rest could not be determined because of failures of PCR amplification (probably due to genetic heterogeneity) or to a lack of plasma samples (Table 4). The nature and the GenBank accession number of all generated sequences are shown in Table 4. The rate of HIV-1 non-B subtypes and recombinants among Africans was extremely high compared to the control group (97% vs. 8.8%) (Table 3). In Africans, CRF02_AG recombinants were the most frequently found variant and unique recombinant forms (URF) caused nearly 40% of recombinant infections. Considering the three different coding regions, nearly 80% of Africans carried HIV-1 variants including recombinant sequences at *gag* (72%), *pol* (71.4%), and *gp41* (78.4%). In contrast, HIV-1 recombinants at *pol* were present only in 5.6% of HIV-1-infected non-Africans. "Pure" non-B HIV-1 viruses, carrying the same non-B subtype in all analyzed regions, infected 18.2% of Africans and 3.2% of non-Africans (Table 3). Subtype B was the most frequent HIV-1 variant found (91.2%) among non-Africans. Only 11 non-Africans (two Spaniards) carried non-B sequences at

Table 3. HIV-1 Variants among Newly HIV Diagnosed Subjects with Available Subtype According to their Origin[a]

	% of HIV-1 subtyped subjects			
	Control group (n = *125)*			
HIV-1 variant (gag/pol/env)	*Africans* (n = *66)*	*South Americans* (n = *39)*	*Europeans* (n = *86)*	p
Subtype B	3 (2/66)	92 (36/39)	91 (78/86)	<0.001
Non-B subtypes and intersubtype recombinants	97 (64/66)	8 (3/39)	9 (8/86)	<0.001
"Pure" non-B subtypes	18 (12/66)	0 (0/39)	5 (4/86)	<0.001
Recombinant forms (CRF + URF)	79 (52/66)	8 (3/39)	5 (4/86)	<0.001
CRF	61 (32/52)	100 (3/3)	75 (3/4)	NS
URF	38 (20/52)	0 (0/3)	25 (1/4)	NS

[a]Non-B subtypes and intersubtype recombinants include all the variants not ascribed to subtype B. Among them, two categories can be distinguished: pure non-B and recombinant non-B. We considered as ''pure'' non-B subtypes those presenting the same non-B subtype in all the regions analyzed. Recombinant forms are those with different subtypes or the same recombinant sequence in any region. At the same time, recombinants can be subdivided into CRF (circulating recombinant forms) and URF (unique recombinant forms); NS, not significant ($p > 0.05$).

pol (1A, 1C, 2G, 1CRF01_AE, 1CRF02_AG, 1CRF10_CD, 2CRF12_BF, and 2CRF31_BC), mostly in subjects coming from regions where those variants circulate or having risk contact with natives from those areas.[28,29] Further analysis in *gag* and *gp41* revealed the complex nature of the CRF10_CD specimen from a Spanish female infected by her Kenyan partner, and this was redefined as a URF: outgroup^{gag}/CRF10_CD^{pol}/A^{gp41}.

Discussion

In the present study we compared clinical and epidemiological characteristics and HIV-1 variants in a large cohort of newly diagnosed HIV-1-infected Africans vs. non-Africans during a 3-year period. A significantly increasing number of immigrants has continuously arrived in Spain in the last 15 years. However, their clinical characteristics have received little attention. In addition, HIV-1 subjects coming from developing countries can present endemic infections not commonly found in Western countries, thus complicating their clinical management.

In 2002, the HIV-1 prevalence among African immigrants in Spain was 5.4%.[6] Since then the diagnosis of the disease has been increasing in African-born persons in Spain, as has occurred in other European countries and in the United States.[30,31] As we confirmed in the non-African group, subtype B is the most prevalent HIV-1 variant in developed regions, including Spain.[15,16] However, new variants are being introduced through immigration,[8–18] changing the HIV-1 molecular epidemiology in the host countries. In Europe, a relatively high and increasing non-B variant prevalence (from 15% to nearly 50%) has been documented among newly diagnosed HIV-1 cases in natives and immigrants.[8,10,11] Focusing on Spain, a gate of entrance of Africans into Europe, non-B viruses are currently about 10–15% of HIV-1 infections, but they are on the rise, mainly due to the increasing number of sub-Saharan migrants in recent years.[15,16] In our study, recombinants caused 79% of 75 new non-B infections diagnosed in Spain from 2005 through 2007. Moreover, this important rate would probably be higher if other viral regions not analyzed in our study were assessed in phylogenetic analysis.

It is necessary to mention that identification of HIV-1 non-B subtypes and recombinants could have an impact on the clinical outcome. Natural polymorphisms in HIV-1 protease and reverse transcriptase, targets of antiretrovirals, are often present in these variants at positions associated with drug resistance in subtype B. These may accelerate or modify the pathways of drug resistance emergence according to the HIV-1 clade.[25] However, in our study the virological and immunological response to HAART was similar in all groups during 1 year of treatment, as previously reported.[20,32] Long-term monitoring of larger cohorts each carrying different variants will be required to find differences in response to specific drugs for each variant.

In contrast to the general European epidemic, women constituted a high proportion of newly diagnosed HIV-1 patients among African immigrants in Spain, as occurs in Africa, where almost 61% of HIV-1-infected adults in 2007 were females.[1] Moreover, a significant number of them were diagnosed with HIV-1 during pregnancy, as happened in other series,[30] highlighting the importance of antenatal HIV testing to reduce vertical transmission. HIV-1 homosexual transmission was uncommon among new HIV-1 diagnoses in Africans vs. non-Africans.

Nearly a third of foreigners (Africans and Latin Americans) presented with a count of fewer than 200 CD4 cells at diagnosis, suggesting long-term infection before HIV-1 diagnosis. We can suspect a longer infection time among Africans than Latin Americans considering the more advanced CDC stage (as previously reported[30,31]) and the lower number of observed recent HIV-1 infections. Thus, it is fundamental that health educational programs are started both in developing regions and among immigrant communities in Western countries to increase the awareness about HIV infection and treatment resources and to reduce the stigma associated with HIV or the misperception of disease risk.[31] Access to and linkage of care for persons undergoing treatment must be ensured.

Tuberculosis, the leading cause of death among people with HIV infection worldwide,[33] was the most common opportunistic infection in our series of African immigrants. Helminthic and protozoal infections were highly prevalent among newly HIV-diagnosed Africans, mainly in those with deeper immunosuppression. Other asymptomatic but relevant conditions found in HIV-infected Africans were filariasis, strongyloidiasis, and schistosomiasis. Our data support the need for evaluation of parasitic infection in HIV-1-infected African immigrants, even though absolute or relative eosinophilia is not present. Moreover, it emphasizes the necessity

Table 4. Characterization of the HIV-1 Variants Infecting the African Group and Accession Number of the Analyzed Sequences[a]

No.	Country of origin	HIV-1 subtype			GenBank accession numbers		
		gag	pol (PR/RT)	gp41	gag	pol (PR/RT)	gp41
1	EG	CRF02_AG	CRF02_AG	URF(A)	EU342785	EU255308	EU342828
2	EG	C	C/C	C	EU342786	EU255465/255476	EU342829
3	EG	A	A/A	URF(A)	EU342787	EU255375	EU342830
4	EG	CRF02_AG	CRF02_AG	CRF02_AG	EU342789	EU255376	EU342832
5	EG	CRF11_cpx	CRF11_cpx	CRF11_cpx	EU342790	EU255378	EU342833
6	EG	CRF02_AG	CRF02_AG	CRF02_AG	EU342791	EU255468	EU342834
7	EG	CRF02_AG	CRF02_AG	CRF02_AG	EU342792	EU255379	EU342835
8	EG	F2	F2/F2	F2	EU342793	EU255380	EU342836
9	EG	CRF11_cpx	CRF11_cpx	CRF11_cpx	EU342794	EU255381	EU342837
10	EG	CRF02_AG	CRF02_AG	CRF02_AG	EU342795	EU255382	EU342838
11	EG	Neg	H/H	Neg	—	EU255383	—
12	EG	A	CRF01_AE	A	EU342796	EU255384	EU342839
13	Nigeria	G	G/G	G	EU342797	EU255391	EU342840
14	Kenya	URF	CRF10_CD	A	EU342798	EU255389	EU342841
15	EG	CRF02_AG	CRF02_AG	CRF02_AG	EU342800	EU255385	EU342843
16	EG	C	C/C	C	EU342802	EU362922	EU342846
17	EG	Neg	CRF10_CD	D	—	EU342817	AY751065
18	EG	CRF02_AG	CRF02_AG	CRF02_AG	EU342803	EU255388	EU342848
19	EG	n.d.	Neg	CRF02_AG	—	—	EU342849
20	EG	CRF11_cpx	CRF11_cpx	CRF11_cpx	EU342806	EU342806	—
21	EG	n.d.	Neg	B	—	—	EU362925
22	EG	URF	C/C	CRF02_AG	EU342807	EU255386	EU342853
23	EG	CRF22_01A1	CRF22_01A1	CRF22_01A1	EU342808	EU342808	—
24	EG	Neg	CRF02_AG	CRF02_AG	—	EU342822	EU342855
25	Nigeria	n.d.	G/G	CRF14_BG	—	EU342823	EU342856
26	EG	Neg	CRF11_cpx	URF	—	EU342825	EU342858
27	EG	CRF06_cpx	CRF06_cpx	CRF02_AG	EU342809	EU255507	EU342859
28	EG	Neg	C/C	URF	—	EU255513	EU342860
29	EG	Neg	A/A	URF	—	EU342826	EU342861
30	EG	n.d.	CRF02_AG	CRF02_AG	—	EU255524	EU342862
31	EG	CRF02_AG	CRF02_AG	CRF02_AG	EU342810	EU342810	—
32	EG	CRF02_AG	CRF02_AG	CRF02_AG	EU342811	EU255522	EU342865
33	EG	CRF02_AG	CRF02_AG	CRF02_AG	EU342812	EU255530	EU342866
34	EG	Neg	URF	URF	—	EU255373	EU342867
35	EG	CRF02_AG	CRF02_AG	CRF02_AG	EU342813	EU255529	EU342868
36	EG	Neg	CRF11_cpx	URF	—	EU255525	EU342870
37	EG	A	Neg	URF	EU342815	—	EU342871
38	DRC	Neg	CRF11_cpx	URF	—	EU255510	EU342872
39	EG	n.d.	G/G	n.d.	—	EU362924	—
40	EG	n.d.	CRF02_AG	n.d.	—	EU255527	—
41	EG	n.d.	CRF02_AG	n.d.	—	EU362923	—
42	Nigeria	n.d.	CRF02_AG	n.d.	—	EU342827	—
43	EG	n.d.	CRF02_AG	n.d.	—	EU545186	—
44	EG	n.d.	CRF02_AG	n.d.	—	EU362926	—
45	EG	n.d.	URF(D)	n.d.	—	EU362927	—
46	EG	n.d.	URF(02/06)	n.d.	—	EU362928	—
47	EG	n.d.	URF(A/01)	n.d.	—	EU545187	—
48	EG	n.d.	URF(02/06)	n.d.	—	EU545188	—
49	IC	n.d.	CRF02_AG	n.d.	—	EU545189	—
50	EG	n.d.	CRF02_AG	n.d.	—	EU255355	—
51	EG	n.d.	CRF02_AG	n.d.	—	EU545190	—
52	EG	n.d.	F2	n.d.	—	EU545191	—
53	EG	n.d.	CRF02_AG	n.d.	—	EU255307	—
54	EG	n.d.	G	n.d.	—	EU545192	—
55	EG	n.d.	CRF02_AG	n.d.	—	EU255360	—
56	EG	n.d.	CRF02_AG	n.d.	—	EU255309	—
57	Cameroon	n.d.	CRF02_AG	n.d.	—	EU255528	—
58	EG	n.d.	G	n.d.	—	EU545193	—
59	EG	n.d.	A	n.d.	—	EU545194	—
60	EG	n.d.	G	n.d.	—	EU255342	—
61	EG	n.d.	CRF02_AG	n.d.	—	EU255354	—

(*Continued*)

TABLE 4. (CONTINUED)

No.	Country of origin	HIV-1 subtype			GenBank accession numbers		
		gag	pol (PR/RT)	gp41	gag	pol (PR/RT)	gp41
62	EG	n.d.	URF (G/02)	n.d.	—	EU255358	—
63	GB	n.d.	B	n.d.	—	EU552227	—
64	EG	n.d.	CRF02_AG	n.d.	—	EU552228	—
65	Nigeria	n.d.	CRF06_cpx	n.d.	—	EU529856	—
66	Nigeria	n.d.	G	n.d.	—	EU255477	—

[a]URF, unique recombinant form; CRF, circulating recombinant form; EG, Equatorial Guinea; GB, Guinea-Bissau; DRC, Democratic Republic of the Congo; IC, Ivory Coast; Neg, PCR negative; n.d., not determined due to lack of sample.

of increasing awareness among clinicians regarding the occurrence of these parasites in this population.

Malaria is, together with HIV and tuberculosis, the most important pathogen that is currently devastating the tropics. Complex bidirectional interactions between infection with *P. falciparum* and HIV have been found. Malaria has been associated with a transitory higher viral load and lower CD4 counts[34] and could increment HIV replication, accelerating the course of HIV disease.[35] In contrast, HIV infection appears to increase only modestly the risk of parasitemia and clinical malaria in semiimmune adults in regions of stable and heavy transmission.[35] A high rate of asymptomatic malaria, detectable only by molecular techniques, was found as most of our HIV-1-infected Africans came from West African regions of stable malaria transmission. Acquired immunity plays an important role in the clearance of parasites and severe immunosuppression might facilitate the persistence of malaria. As malaria is common in recent HIV-infected African immigrants, efforts should be taken for its diagnosis and treatment, particularly in the most severely immunosuppressed subjects.

A limitation of our study is that most of our HIV-1 Africans came from Central-Western Africa. A longer-term follow-up is also necessary. The profile of sub-Saharan HIV-infected immigrants varies widely from that of the usual HIV-infected individuals in Spain. Regarding the HIV-1 diversity, the study would be more accurate if non-Africans were further analyzed in other genetic regions. It would probably be useful to detect the heterogeneity we cannot perceive with only *pol* analysis. Finally, more advanced immunodeficiency, the coexistence of parasitic diseases, and infections with a large diversity of HIV-1 non-B and recombinant variants are expected.

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Increase of Non-B Subtypes and Recombinants Among Newly Diagnosed HIV-1 Native Spaniards and Immigrants in Spain

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Abstract: Although HIV-1 clade B variants are predominant in Western Europe, non-B subtypes are rapidly spreading, mainly due to immigration from endemic regions. All newly diagnosed HIV-1-infected individuals at a HIV/AIDS clinic in Madrid from 2000 to 2007 were identified. Subtype assignment was based on phylogenetic analysis of *pol* sequences from plasma specimens collected at first visit. A total of 1,430 newly diagnosed HIV-1 individuals were identified: 902 Spaniards, 232 South Americans, and 162 Africans, among others. The proportion of South-Americans and Africans among diagnosed HIV-1 patients increased from 2000 to 2007 (from 17% to 22% and from 4% to 21%, respectively). Half of diagnosis of HIV-1 in 2007 was in foreigners whereas in previous years Spaniards were predominant. Non-B variants were found in 157 (24%) of the 649 subjects who could be subtyped: 11A, 6C, 2D, 1F2, 13G, 4H, 1J, 3CRF01_AE, 64CRF02_AG, 2CRF03_AB, 3CRF06_cpx, 3CRF10_CD, 7CRF11_cpx, 9CRF12_BF, 9CRF14_BG, 1CRF18_cpx, 1CRF19_cpx, 2CRF31_BC, 10 URF and 5 outgroups. They represented 93%, 14% and 4% of newly-diagnosed HIV-1 Africans, South-Americans and native Spaniards, respectively. Non-B subtypes increased from 9% in 2000 to 32% in 2007, specially among South-Americans (from 11% to 20%) and native Spaniards (from 4% to 10%). Most (75%) were recombinant viruses. The highest number and diversity of HIV-1 variants among natives was observed in 2007. HIV-1 non-B subtypes are increasingly present among newly diagnosed HIV-1 individuals in Madrid, representing a third of cases in 2007, whereas 10% of newly diagnosed HIV-1 native Spaniards had non-B viruses.

Keywords: HIV-1, non-B subtypes, recombinants, new diagnosis, Spain.

INTRODUCTION

Human immunodeficiency virus (HIV) shows a wide genetic diversity, largely caused by the high viral replication rate, the error-prone reverse transcriptase and recombination events that may occur during the virus replication [1,2]. Several major genetic variants can be recognised within HIV-1 group M, including nine subtypes (A, B, C, D, F, G, H, J, K) [3], at least 43 major circulating recombinant forms (CRFs) (http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html), and multiple unique recombinant forms (URFs). CRFs are defined as intersubtype recombinants for which at least three epidemiologically unlinked variants are monophyletic with an identical genetic structure. URFs are widely distributed worldwide, with recombination breakpoints at different sites from those found in CRFs; moreover, they have been reported in only one or two individuals. Recombination results from HIV-1 coinfection and/or superinfection events. They are particularly prevalent in populations where multiple subtypes co-circulate, as in sub-Saharan Africa, where all HIV types, groups, subtypes and most recombinants are present [4].

HIV-1 non-B subtypes and recombinants play an important role in the HIV pandemic, since they cause up to 90% of the estimated 36 million persons living with HIV. Among those variants, subtype C, A, CRF01_AE and CRF02_AG are the most prevalent, and are responsible for nearly 70% of all cases [5]. HIV-1 subtype B accounts for around 10% of all HIV-1 infections worldwide [6]. However, this clade is the best characterised both biologically and clinically, since it is by far the most prevalent variant in industrialised regions, such as North America and Western Europe [7]. In the last decade, population movements, mainly from countries where non-B and recombinant variants are endemic, have contributed to a large extent to the current spreading of HIV-1 non-B variants in developed countries. In Europe, a relatively high and growing rate of new infections is caused by these variants [8-15]. In Spain, currently one of the main gates of entrance of Africans into Europe, non-B viruses now infect around 10-15% of all HIV-1 individuals but are on the rise [11,16-20]. Most sub-Saharan Africans are firstly diagnosed with HIV-1 at the time of their initial health evaluation upon arrival. The prevalence of HIV-1 infection is elevated in this population [21]; for example, nearly 10% of Africans attended for the first time at the institution where the study was performed are HIV-1 positive [22].

The extensive variability of HIV-1 has a potential impact on epidemiology, diagnosis, therapy and prevention of infection. Recognition of non-B subtypes and recombinants is important in the light of confronting potential problems using antiretroviral drugs and diagnostic tools, which have been designed based on clade B sequences [23-25]. Thus, HIV-1 subtype characterisation may be an important aspect of the clinical management of infected persons [6,24,26].

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The aim of this study was to assess the prevalence and nature of non-B subtypes among newly diagnosed HIV-1 individuals seen from 2000 through 2007 at Hospital Carlos III, a Spanish reference HIV/AIDS clinic in Madrid.

MATERIALS AND METHODS

Study Population, HIV-1 Diagnosis and HIV-1 Subtype Assignment

A total of 1,430 individuals who received a new diagnosis of HIV-1 infection by serological standard assays at Hospital Carlos III were identified during the study period (January 2000 through December 2007). Two ELISAs designed with synthetic peptides or recombinant proteins were used for the detection of antibodies to HIV-1 (AxSYM, Madrid, Spain; and BIO-RAD, Marnes la Coquette, France). Reactive samples were confirmed using an HIV-1 viral lysate Western blot (New LAV BLOT I, BIO-RAD) and a line immunoassay (LIA) able to distinguish antibodies to HIV-1 and HIV-2 (Pepti-LAV, BIO-RAD). In patients with plasma samples collected at diagnosis time, plasma aliquots had been separated from blood cells within 4 hours following blood drawl, and frozen at –80ºC until the time of analysis. Direct sequencing of nested PCR purified products from viral RNA was performed in the HIV-1 *pol* coding region. *Pol* sequences included the complete protease (codons 1-99) and part of the retrotranscriptase (codons 1-247 or 1-335) using Trugene (Siemens, Barcelona, Spain) or Viroseq™ (Celera Diagnostics, Alameda, CA), respectively. In some specimens with amplification difficulties, *pol* amplification was made using primers and conditions reported elsewhere [18,27].

For subtype or CRF characterisation, phylogenetic analyses using *pol* sequences were carried out taking as reference HIV-1 sequences belonging to HIV-1 group M variants available at the GenBank when the analysis was performed. The tree topology was obtained using the Neighbour-Joining method. Alignment of DNA sequences was performed using the ClustalW program. The pairwise distance matrix was estimated using the Kimura two-parameter model within the DNADIST program, as implemented in the PHYLIP software package. Bootstrap re-sampling (1000 data sets) of the multiple alignment was performed to test the statistical robustness of the tree. In 26 samples only protease sequences were available.

HIV-1 Subtyping Using Rapid Tools

Besides using phylogenetic analysis, HIV-1 subtyping was assessed in all *pol* sequences by two rapid tools (Stanford and NCBI), which are available at the following websites: http://hivdb.stanford.edu/ (HIV-1 Drug Resistance Database, Stanford University, Palo Alto, CA); and http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD). A bootscan analysis of outgroup sequences (not ascribed to any known subtype or CRF by phylogenetic analysis) was performed using both NCBI and REGA subtyping tools to explore whether any known HIV-1 variant could be recognised in the examined *pol* coding region.

Plasma HIV-RNA Quantitation

Plasma viraemia was measured using the second generation branched DNA (bDNA) assay (Siemens, Barcelona, Spain), which has a lower limit of detection of 50 HIV-RNA copies/ml.

RESULTS

Main Characteristics of the Study Population

A total of 1,430 individuals newly diagnosed with HIV-1 infection were identified by serological standard assays during the study period (January 2000 through December 2007). The study population represented 8.6% of all new diagnoses of HIV-1 infection in Spain until June 2007 [28]. A total of 902 (63%) individuals were native Spaniards and 528 (37%) were foreigners. Of the latter, 162 (31%) were Africans, 232 (44%) South Americans, 54 (10%) from other European countries, and 80 (15%) from other regions. The proportion of Africans and South Americans increased over time among newly diagnosed HIV-1 individuals. It shifted from 4.1% to 21.3% in Africans and from 17% to 21.9% in South Americans.

Seventy-six per cent of newly diagnosed HIV-1 persons during the study period were male. Of 1,012 with a known risk group, 65.1% had acquired HIV-1 infection through sexual relationships, either heterosexual (22.4%) or homosexual (42.7%), while 28.7% admitted intravenous drug use. The mean CD4 count at first diagnosis was 411.4 cells/µl, but 26% of subjects had CD4 counts below 200 cells/µl, suggesting that they had been infected for a long time before being diagnosed as HIV-1 positive. Interestingly, a total of 286 subjects (21.4%) showed undetectable plasma viraemia. The mean viral load value for the rest was 4.93 log HIV-RNA copies/ml. Nineteen percent of the patients were naive, while the remaining 81% had started high active antiretroviral treatment (HAART), with regimens including protease inhibitors in 45% of cases. A 32% of Africans, 17% of South-Americans, 17% of native Spaniards, and 15% of other immigrants were naive for any antiretroviral treatment. Thus, Africans were the group with the highest rate of untreated patients.

With respect to the origin of newly HIV-1 diagnosed patients in Hospital Carlos III over time, a significant higher number of diagnoses were performed in native Spaniards *vs* foreigners during 2000-2006. However, in 2007, half of HIV-1 diagnosis was in the foreign population, mainly among Africans and South-Americans (Fig. **1**).

HIV-1 Subtyping

Of the 1,430 individuals newly diagnosed with HIV-1 during the 8-year study period, subtype assignment in the *pol* coding region could be obtained in only 649 (45%) cases. In a high number of seropositive HIV-1 patients, plasma samples were not collected at diagnosis time and were not available at other time points, because of lost to follow-up of the patients. Low plasma HIV-RNA loads, primer mismatches due to genetic variability and lack of enough plasma volume did not let us run the results for the rest. Of the subtyped population, 356 were Spaniards and 293 foreigners. Overall, viral subtyping was obtained for 39.5% (356/902) of native

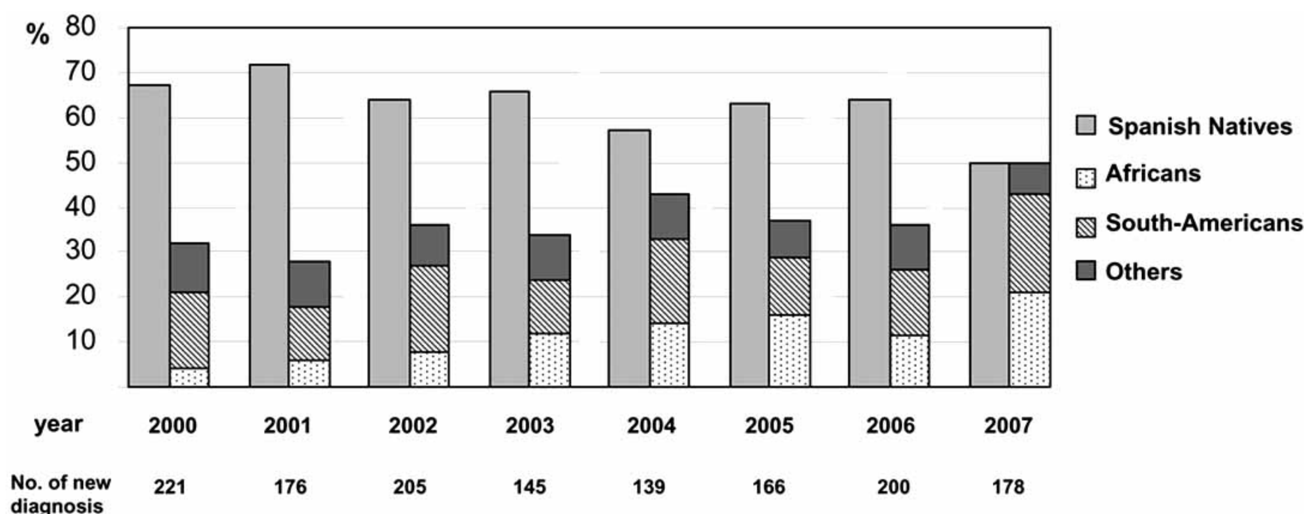

Fig. (1). Origin of newly 1,430 HIV-1 diagnosed patients from 2000 through 2007. Among the 1,430 subjects, 902 were Spaniards and 528 were foreigners. From Africa: Angola (1), Cameroon (4), Central African Republic (1), Democratic Republic of the Congo (5), Equatorial Guinea (99), Ethiopia (2), Guinea-Bissau (2), Ivory Coast (1), Kenya (2), Liberia (2), Mali (1), Morocco (5), Mozambique (3), Nigeria (11), South Africa (1), Uganda (1), unknown (22). From America: Argentina (34), Brazil (22), Bolivia (2), Chile (9), Colombia (36), Costa Rica (3), Cuba (21), Dominican Republic (6), Ecuador (44), El Salvador (1), Guatemala (1), Haiti (1), Honduras (1), Mexico (9), Paraguay (2), Peru (15), the United States of America (5), Uruguay (4), Venezuela (12) and unknown (3). From Europe: Belgium (1), Bulgaria (3), Croatia (1), Finland (1), France (7), Germany (4), Italy (3), the Netherlands (1), Portugal (18), Russia (3), Romania (1), Serbia and Montenegro (1), Switzerland (2), the Ukraine (2) and the United Kingdom (4). Other patients came from Australia (1), Indonesia (2), the Philippines (4), Eastern Timor (1), Thailand (1) and unknown (73).

subjects and 55.5% (293/558) of foreigners [80.2%, (130/162) Africans and 50% (116/232) South-Americans].

All 649 HIV-1 *pol* sequences were obtained from the first plasma sample collected at the time of HIV-1 diagnosis, and before exposure to any antiretroviral therapy. (Fig. **2**) records the proportion of individuals with newly diagnosed HIV-1 infection who could be subtyped each year.

High Rate of Inter-Subtype Recombinants Among HIV-1 Non-B Variants

As expected, subtype B was the most prevalent variant (75.8%, 492/649) during the whole study period in this population. Only the subset of African subjects had a low prevalence of clade B infections (6.9%), occurring among 3 patients from Equatorial Guinea, 2 from Morocco, and one each from Guinea-Bissau, Democratic Republic of Congo, and South Africa.

Overall, HIV-1 non-B subtypes and inter-subtype recombinant strains were recognized in 157 (24%) of 649 individuals with known HIV-1 clade (Fig. **2**). The distribution was as follows: 11A, 6C, 2D, 1F2, 13G, 4H, 1J, 3CRF01_AE, 64CRF02_AG, 2CRF03_AB, 3CRF06_cpx, 3CRF10_CD, 7CRF11_cpx, 9CRF12_BF, 9CRF14_BG, 1CRF18_cpx, 1CRF19_cpx, 2CRF31_BC, 10 URF and 5 outgroups. The latter did not cluster with any other known subtype or CRF after phylogenetic analyses of *pol* sequences. In summary, 38 (24%) of the 157 non-B sequences were ascribed to "pure" non-B subtypes and the remaining 119 (76%) to recombinants. In the last group, 104 (87%) sequences clustered within 12 of the 34 CRFs described when the study was performed. The proportion of sequences ascribed to inter-subtype recombinants was high in all years, ranging from 61% to 89% of non-B sequences examined.

A bootscanning analysis of the 10 URF *pol* sequences was carried out using the NBCI and REGA tools (data not shown). The objective was to explore in detail whether a genetic recombinant nature could be tracked by examining distinct recombination breakpoints. Viruses classified as URF included sequences which could be clustered with clade G or CRF02_AG (3 cases), and subtype A or CRF01_AE (5 cases). The remaining 2 URF sequences did not cluster in phylogenetic analyses with any known subtype or CRF sequence.

The 157 non-B subtypes and recombinants found during the study period belonged to 14 native Spaniards and 143 foreigners, including 121 coming from Africa (84.6%), 16 from America (11.2%), 5 from other European countries (3.5%) and 1 from an unknown country. In more detail, countries of origin were Equatorial Guinea (78), a former Spanish colony located between Cameroon and Gabon, Nigeria (10), Democratic Republic of Congo (3), Cameroon (2), Mozambique (2), Central African Republic (1), Ivory Coast (1), Kenya (1), Angola (1), Ethiopia (1), Liberia (1), Guinea-Bissau (1), Uganda (1), Argentina (4), Paraguay (2), Cuba (2), Ecuador (2), Brazil (1), Bolivia (1), Colombia (1), the United States of America (1), Bulgaria (2), France (1), Portugal (1) and the Ukraine (1). For 21 subjects the country was undisclosed, although they come from Africa (18) or South-America (2). Finally, among the 14 native Spaniards infected with non-B strains, 11 were infected by recombinant variants (1CRF01_AE, 2CRF02_AG, 1CRF06_cpx, 1CRF10_CD, 3CRF12_BF, 2CRF14_BG and 1URF) and 3

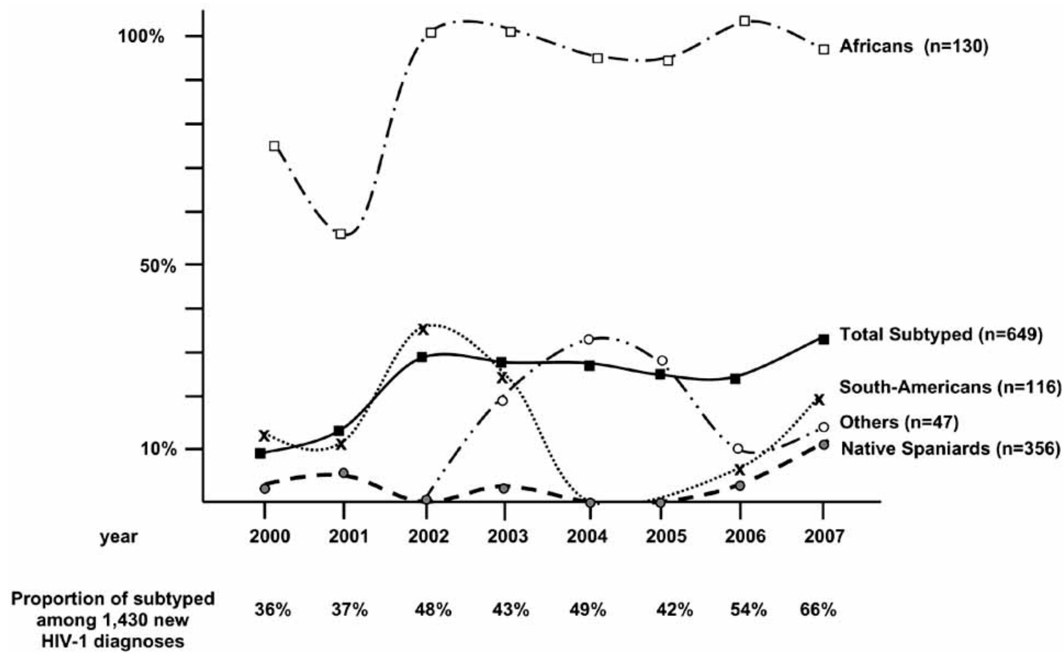

Fig. (2). Rate of HIV-1 non-B subtypes and recombinant variants among 649 newly diagnosed HIV-1 individuals with known HIV-1 subtype in Madrid. Out of the 649 subjects subtyped, 356 were Spaniards and 293 foreigners, who came from 41 different countries. From Africa: Angola (1), Cameroon (3), Central African Republic (1), Democratic Republic of the Congo (4), Equatorial Guinea (79), Ethiopia (1), Guinea-Bissau (2), Ivory coast (1), Kenya (2), Liberia (1), Morocco (2), Mozambique (3), Nigeria (10), South Africa (1), Uganda (1) and unknown (18). From America: Argentina (14), Bolivia (2), Brazil (13), Chile (5), Colombia (13), Costa Rica (1), Cuba (12), Ecuador (23), Guatemala (1), Mexico (5), Paraguay (2), Peru (8), the United States of America (2), Uruguay (2), Venezuela (5) and unknown (8). From Europe: Bulgaria (4), Finland (1), France (5), Germany (1), Italy (2), Portugal (10), Russia (1), Serbia and Montenegro (1), the Ukraine (1) and the United Kingdom (2). Others patients came from Australia (1), the Philippines (4) and unknown (14).

by non-B "pure" subtypes (1A, 1C and 1G). The Spaniard carrying CRF01_AE variant had risk behaviour in Thailand, where these recombinants were originally found [29]. The one harbouring CRF06_cpx strain was an intravenous drug user, as was the one infected by CRF12_BF and CRF14_BG. The Spanish woman with the recombinant CRF10_CD was infected by her African partner, who acquired the infection in Kenya, his country of birth. The native subjects with clade G, URF and the one with CRF12_BF acquired the HIV-1 infection through homosexual contacts. The remaining Spaniards were infected by heterosexual contacts. However, if their sexual HIV-1-infected partners came from endemic countries for non-B subtypes or if they had travelled to those regions was not reported in the clinical records.

Some of the 9CRF14_BG strains found in the study, variant initially identified among an intravenous collective in north-west Spain [16], were present in individuals who had acquired the HIV-1 infection by a heterosexual or homosexual route, blood transfusion, as well as intravenous drug abuse.

Steady Increase of HIV-1 Non-B Subtypes and Recombinants Over Time

The rate of non-B subtypes among newly diagnosed HIV-1 individuals has steadily increased since 2000, and is nearly a quarter of new cases in the whole study period and almost a third in 2007. During the whole study period the prevalence of non-B variants was 93% among Africans, 14% among South Americans, and 4% among native Spaniards. However, there was a significant increase with respect to 2000 among recently infected South Americans (shift from 11.1% to 20%) and native Spaniards (from 3.8% to 10%) (Fig. **2**). Overall, 10% of newly diagnosed HIV-1 native Spaniards in 2007 were infected by non-B strains. Considering the newly diagnosed HIV-1 population in the study period and with a known subtype, non-B variants increased from 8.9% in 2000 to 32.2% in 2007. The highest number and diversity of HIV-1 variants among natives was observed in 2007 (Table **1**).

Unique recombinant forms have been present among HIV-1+ African migrants since year 2001 and later in the analysed native HIV-1+ Spaniards (Table **1**), suggesting a higher genetic complexity of African HIV-1 isolates. Of interest, all non-B variants among Americans (but only South-Americans) were ascribed to 6 different recombinant circulating forms, previously reported in those areas [30,31], where subtype B is prevalent. Of note, three of them were CRF carrying subtype B sequences in their genome (CRF12_BF, CRF14_BG, and CRF31_BC).

Assignment of Non-B Sequences to Subtypes and Recombinants Using Rapid Tools

All *pol* sequences identified by phylogenetic analysis (phy) as belonging to non-B subtypes were tested using the Stanford and NCBI subtyping tools. Both rapid tools correctly ascribed all sequences defined as C (6), D (2), F (1), H (4), J (1), CRF02_AG (64) and CRF18_cpx (1). Regarding the remaining variants, NCBI was more reliable than Stan-

Table 1. Distribution of 157 HIV-1 non-B subtypes and recombinant variants per year of HIV-1 diagnosis among the 649 subtyped specimens according to the origin of the patients

Origin	HIV-1 non-B subtypes and recombinant specimens per year (no. total subtyped)							
	2000 (n=79)	2001 (n=65)	2002 (n=71)	2003 (n=70)	2004 (n=68)	2005 (n=70)	2006 (n=108)	2007 (n=118)
Spaniards (n=14)	1 CRF06_cpx 1 CRF12_BF	1 CRF02_AG 1 CRF14_BG	-	1 CRF12_BF	-	-	1 G 1 CRF10_CD 1 URF	1 A 1 C 1 CRF01_AE 1 CRF02_AG 1 CRF12_BF 1 CRF14_BG
Africans (n=121)	1 A 1 C 1 CRF02_AG	1 G 1 CRF02_AG 1 CRF03_cpx 2 URF	1 G 2 H 1 J 7 CRF02_AG 1 CRF03_cpx 1 CRF10_CD 1 CRF11_cpx 1 CRF18_cpx 1 outgroup	3 A 1 C 1 D 1 CRF01_AE 7 CRF02_AG 1 CRF10_CD 1 CRF12_BF 1 CRF14_BG	1 H 1 CRF01_AE 11 CRF02_AG 1 CRF14_BG 2 URF 1 outgroup	1 A 4 G 6 CRF02_AG 1 CRF11_cpx 1 CRF14_BG 3 URF 1 outgroup	2 A 2 C 1 F 3 G 1 H 9 CRF02_AG 2 CRF11_cpx 1 outgroup	2 A 1 C 1 D 2 G 13 CRF02_AG 1 CRF06_cpx 3 CRF11_cpx 2 URF 1 outgroup
Americans (n=16)	2 CRF02_AG	1 CRF06_cpx	1 CRF02_AG 3 CRF12_BF 1 CRF19_cpx	1 CRF12_BF 1 CRF14_BG	-	-	1 CRF02_AG	1 CRF02_AG 2 CRF12_BF 2 CRF31_BC
Others (n=6)	-	-	-	1 CRF14_BG	1 CRF14_BG	1 A 1 CRF14_BG	1 CRF02_AG	1 G
HIV-1 variants								
Pure (%)	28.6	12.5	19	25	5.5	31.6	38.5	24.3
Recombinant (%)	71.4	87.5	81	75	94.5	68.4	61.5	75.7

Recombinant includes Circulating Recombinant Forms (CRF) and Unique Recombinant Forms (URF). The outgroup sequences are those which could not be assigned to any subtype or recombinant form.

ford identifying A (5/11 *vs* 4/11), G (12/13 *vs* 11/13), CRF03_AB (2/2 *vs* 0/2), CRF06_cpx (2/3 *vs* 0/3), CRF11_cpx (7/7 *vs* 1/7), CRF12_BF (4/9 *vs* 0/9), and CRF14_BG (8/9 *vs* 0/9). Both tools failed with all CRF10_CD (3), CRF19_cpx (1) and CRF31_BC (2) sequences, and obtained the same results with CRF01_AE (1/3). These results highlight the limitations of currently available rapid subtyping tools, specially for the correct assignment of some pure non-B subtypes and certain CRFs other than CRF02_AG. However, NCBI showed the best concordance with phy in all cases of non-B sequences, both "pure" and inter-subtype recombinants.

Sequence Data

All 157 sequences from subjects infected with non-B viruses reported in this study have been submitted to the GenBank, including 34 previously submitted [20]. New phylogenetic analyses of those 34 *pol* sequences including sequences from all CRF described to date allowed reassignment of CRF02_AG, previously ascribed as subtype G [20]. The GenBank Accession Numbers for non-B sequences reported in this study, both pure and recombinant, are shown as follows: 10 Subtype A: AY642098, AY642109, EU255331, EU255375, EU255377, EU255503, EU342750, EU529851, EU529855, EU545194; 6 Subtype C: AF354014, AY642103, EU255374, EU255386, EU255509, EU255513; 4 Subtype D: EU255489, EU342768; 1 Subtype F (F2): EU255380; 12 Subtype G: EU255342, EU255345, EU255363, EU255390, EU255391, EU255415, EU255477, EU255518, EU342752, EU342758, EU362924, EU545193; 4 Subtype H: AF125286 (PR), AY248332 (PR), EU255383, EU342779; 1 subtype J: AY642104 (PR); 3 CRF01_AE: AY647443, EU255514, EU342782;63 CRF02_AG: AF125292 (PR), AF354007 (PR)/AF455630 (RT), AF354025 (PR) AF455665 (PR)/AF455636 (RT), AY248294, AY248298 (PR), AY248300, AY248311 (PR), from AY647440 to AY647442, AY647445, AY647447, AY647452, from EU255307 to EU255309, EU255354,

EU255355, EU255360, EU255376, EU255379, EU255382, EU255385, EU255387, EU255388, EU255442, EU255444, EU255467, EU255468, EU255485, from EU255522 to EU255524, from EU255527 to EU255531, EU255548, EU342748, EU342749, EU342753, EU342755, EU342760, EU342761, EU342767, from EU342770 to EU342778, EU342784, EU362923, EU362926, EU529852, EU545186, EU545188, EU545189; 2 CRF03_AB: AY248329, AY647446; 3 CRF06_cpx: EU255507, AF466244 (PR)/AF455661 (RT), EU342747; 3 CRF10_CD: AF188350 (PR)/ AF188347 (RT), EU255456, EU342764; 7 CRF11_cpx: AY248331(PR), EU255349, EU255378, EU255381, EU255510, EU255525, EU342825; 9 CRF12_BF: EU255542, EU255544, EU255553, EU342746, EU342754, EU342756, EU342759, EU342762, EU342765; 9 CRF14_BG: AY642100, EU255306, EU255328, EU255540, EU342751, EU342763, EU342766, EU342769, EU342783; 1 CRF18_cpx: AY248313 (PR); 1 CRF19_cpx: EU342757; 2 CRF31_BC: EU529853, EU529854; 5 Outgroups: AY642106, EU255373, EU255384, EU362927, EU342781; 9 URFs : AF455667(PR)/AF455654(RT), AY248325, AY647448, EU255310, EU255358, EU255526, EU342780, EU255346, EU362928.

DISCUSSION

No prior studies have examined the prevalence of non-B variants among newly diagnosed HIV-1 individuals in Madrid. Since 2000 we have found a steady yearly increase in the proportion of newly HIV-1-infected persons with these variants. It is estimated that the study population herein examined represented 8.6% of the total newly diagnosed HIV-1 individuals in Spain until June 2007, the last official available report at the time this study was finished [28]. The current population of Madrid is around six million inhabitants, of whom approximately 650,000 are legal immigrants. Among them, nearly 87,000 came from African countries [28,32], representing the 10.7% of Africans residing in Spain. As it is well known, in Africa a high prevalence of HIV-1 infections with non-B subtypes and recombinants have been reported [4].

The number of immigrants depends on several factors, such as country of origin, immigration control, and on the political, economical and social situation of countries of origin and destination. Consequently, immigration influx has no constant rates. Thus it is reasonable to have different values for each year, as happened with South-Americans due to the tightening of immigration rules some years ago in Spain for certain countries. Despite these fluctuations, the resultant proportion of immigrants among newly HIV-1-infected persons has grown in recent years in Spain [21], particularly due to the flow of African immigrants and because an increase in HIV-1 incidence among them [33]. In 2006, 21.2% of the new HIV-1 diagnoses in Spain were in foreigners, 39.5% of them Sub-Saharan Africans. A large number of HIV-1-infected immigrants were first informed of their HIV-1 status at the time of their first health evaluation upon arrival. This is the case for Sub-Saharans arriving in the Canary Islands, which is one of the main gates for illegal immigration to Europe. This archipelago faces the north-western coast of Africa and belongs administratively to Spain [11]. However, the number of HIV-1 infections among Africans living in Spain may be underestimated, due to illegal immigration, under-reporting and under-diagnosis, as has happened in HIV/AIDS surveillance studies in other parts of the European Union [35]. Thus, an underestimation of HIV-1 non-B subtypes and recombinant prevalence in this collective is to be expected. Different HIV-1 flow of immigrants coming from endemic regions from non-B variants may vary the proportion and nature of these variants observed across different years.

In Spain, HIV-1 diagnosis is almost always centralized in national health hospitals. Under Spanish regulations, both natives and foreigners have free access to medical care. Approximately 12% of new HIV-1 diagnosis during the period 2003-2005 was found in Sub-Saharan immigrants [28]. Most of these HIV-1 Africans residing in Spain were probably infected in their countries of origin, as strongly suggested by the recognition of HIV-1 non-B clades and recombinants among them [17,20,34]. Moreover a large number of them presented a late diagnosis of HIV-1 performed in Spain, as was supported by the high proportion showing less than 200 CD4 T-cells/µl [28]. In agreement, 31% of HIV-1-infected Africans in our study had CD4 counts below 200 cells/µl at their first visit to the clinic.

A high prevalence of recombinants among HIV-1 non-B variants previously considered as "pure" clades based on information derived from only one viral coding region has been reported. Using adequate phylogenetic analyses including reference sequences from the 9 HIV-1 subtypes and the 34 available CRFs variants when the analyses were performed, our study revealed that 75% of non-B *pol* sequences were ascribed to recombinant variants. Moreover, the examination of additional viral regions could bring even more recombinant strains among "pure" non-B clades and some specimens considered as CRF should be ascribed as URF. The performance of rapid subtyping tools, such as those provided by Stanford and NCBI, showed several limitations which may preclude their wide use in clinical facilities. They should be optimised and updated with information derived from a larger number of different non-B subtypes and CRF sequences. Thus, phylogenetic analysis must remain as the gold standard method for identifying non-B subtypes and CRFs.

HIV-1 clade B variants are predominant among all risk groups in Spain, as in most Western European countries. However, the introduction and spread of non-B subtypes in the native population can be associated with sexual transmission between local and immigrant partners. In our study, an increasing proportion of native Spaniards was newly diagnosed with HIV-1 non-B subtypes, representing 10% in 2007. Moreover, an increasing amount of non-B subtypes in the last years (2005-2007) is patent, corresponding to the political period with a larger influx of immigrants to our country. Although the high number of native patients carrying HIV-1 non-B clades and recombinants observed in new diagnoses during 2007 could just be a local punctual maximum for Spain or for our city, our results are in agreement with other HIV/AIDS surveillance studies in the rest of the continent. In fact, an even more accentuated trend has already been shown in other European countries with stronger relationships with Sub-Saharan countries, where non-B variants prevalence rises to nearly 50% [9,12,15]. A limitation of

our study is that the complete patient population could not receive a clade assignment, mainly because of a lack of plasma specimens and negative amplifications due to primer mismatches. However, this study provides, with a previous report or our group [19], the first information about the prevalence of HIV-1 non-B subtypes and recombinants among newly HIV-1 diagnosed individuals in Spain during the last decade. The results in this article complement the previously published studies monitoring the increasing prevalence of HIV-1 non-B subtypes across Europe. To our knowledge, our study includes the highest number of both subtyped sequences by phylogenetic analysis and reported HIV-1 non-B variants among those characterising *pol* subtypes by phylogenetic analysis in Europe [8,9,14, 36].

The importance of identifying HIV-1 non-B subtypes including recombinant variants may have implications which are beyond epidemiological interest. First, a different rate of progression to AIDS in subjects infected with some non-B subtypes has been reported [37]. Second, natural polymorphisms in the viral protease and retrotranscriptase, which are the most common targets of antiretroviral compounds, are often present in a subtype-dependent manner. These characteristic changes may influence drug susceptibility, as well as selection of different pathways and/or more rapid emergence of drug resistance and subsequent treatment failure [24]. Third, genetic differences between subtypes may compromise the reliability of diagnostic testing, including viral load measurements. Finally, the continuous spread of HIV-1 inter-subtype recombinants may have serious implications on efforts to control the AIDS pandemic with future vaccination trials. Thus, it remains necessary to reinforce that HIV-1 molecular epidemiology studies must be part of national HIV and AIDS surveillance programs. This study proves a rising presence of multiple HIV-1 variants in Madrid, mainly introduced by the continuous immigration influx and population movements from countries were HIV-1 non-B subtypes and recombinant variants are frequent. New HIV-1 molecular epidemiology studies are required to determine the frequency and distribution of HIV-1 non-B subtypes and recombinants among these new infections in our country in the coming years.

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