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Original

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Integrase strand transfer inhibitors resistance-associated mutations in HIV-infected pregnant women

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ABSTRACT

Objective. To date, no data exist regarding the prevalence of integrase inhibitor (INSTI) resistance-associated mutations (HIVDRM) in HIV-infected pregnant women (HPW) in Latin America. We describe the prevalence and transmissibility of integrase HIVDRM in a historical cohort of INSTI-naïve HPW from Argentina (n=56) with Next Generation Sequencing (NGS).

Material and methods. Bioinformatics analysis was performed by HyDRA software for 20%, 10%, 5%, 2%, and 1% sensitivity thresholds. We calculated the mutational viral load for each INSTI-HIVDRM, considering those with >1000 c/mL as of high risk of transmissibility.

Results. The predominant HIV subtype was BF (78.5%). Major HIVDRM were not detected with the population sequencing 20% filter. With a 1% threshold, the prevalence increased to 8.9%; Y143C/S, E92G, E138K, and T66I mutations were found. The median (range) mutational load (expressed in c/mL) was: 355 (50.2-11705); with only 1 case >1000 c/mL Accessory mutations (G163R/K, T97A) were detected mostly with a 20% sensitivity threshold with an overall prevalence of 23.2%; the median (IQR) mutational load was: 23929 (4009-63158) c/mL; all of them above 1000 c/mL.

Conclusion. Our results show evidence of the presence of major INSTI-HIVDRM as aleatory mutations and a high frequency of accessory mutations with potential transmissibility in HPW.

Keywords: mutations; prevalence; pregnant women; HIV; integrase inhibitors

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Resistencia a los inhibidores de integrasa en embarazadas con VIH

RESUMEN

Objetivo. Hasta la fecha, no existen datos sobre la prevalencia de mutaciones asociadas a la resistencia (HIVDRM) a los inhibidores de la integrasa (INSTI) en mujeres embarazadas infectadas por VIH (HPW) en América Latina. Describimos la prevalencia y la transmisibilidad de las HIVDRM de la integrasa en una cohorte histórica de HPW naive de INSTI de Argentina (n=56) mediante Next Generation Sequencing (NGS).

Material y métodos. Se realizó un análisis bioinformático mediante el software HyDRA para umbrales de sensibilidad del 20%, 10%, 5%, 2% y 1%. Calculamos la carga viral mutacional para cada INSTI-HIVDRM, considerando aquellas con >1000 c/mL como de alto riesgo de transmisibilidad.

Resultados. El subtipo de VIH predominante fue BF (78,5%). No se detectaron HIVDRM principales con el filtro de secuenciación poblacional del 20%. Con un umbral del 1%, la prevalencia aumentó al 8,9%; se encontraron las mutaciones Y143C/S, E92G, E138K y T66I. La mediana (rango) de la carga mutacional (expresada en c/mL) fue: 355 (50,2-11705); con solo 1 caso >1000 c/mL. Las mutaciones accesorias (G163R/K, T97A) se detectaron principalmente con un umbral de sensibilidad del 20%, con una prevalencia general del 23,2%; la mediana (RIQ) de la carga mutacional fue: 23929 (4009-63158) c/mL; todas ellas por encima de 1000 c/mL.

Conclusión. Nuestros resultados muestran evidencia de la presencia de INSTI-HIVDRM principales como mutaciones aleatorias y una alta frecuencia de mutaciones accesorias con potencial transmisibilidad en HPW.

Palabras clave: mutaciones; prevalencia; embarazadas; VIH; inhibidores de integrasa

INTRODUCTION

Despite massive public health efforts, rates of mother-to-child transmission of HIV remains high in Argentina and in several countries in Latin America [1]. Antiretroviral therapy (ART) is mandatory for pregnant women to suppress HIV replication to prevent perinatal transmission. However, perinatal transmission is mostly driven by late-presenting pregnant women and those with a high peripartum viral load due to lack of access and HIV testing, nonadherence to ART, and virologic failure [2].

The emergence of primary mutations in the viral genome is a significant cause of drug resistance, which can lead to treatment failure [3]. Thus, drug resistance- associated mutations (HIVDRM) in HIV-infected pregnant women (HPW) may increase the risk of perinatal transmission with impact in women and children´s future treatment options [4]. According to published reports, levels of transmitted drug resistance are high in Argentina in the general population, HPW, and newborns, mostly for first-generation non-nucleoside reverse transcriptase inhibitors (NNRTI) such as efavirenz and nevirapine (NVP) [5-7].

Local and international guidelines currently recommend an integrase-inhibitor (INSTI) based ART for HPW [4]. Dolutegravir (DTG) or, alternatively, raltegravir (RAL) as anchor drugs are recommended with a backbone of two nucleoside analogs (emtricitabine/tenofovir; lamivudine/tenofovir; abacavir/lamivudine). For those mothers with a high risk of transmission (those with peripartum viral loads >1000 c/mL) there is indication of zidovudine infusion during delivery and performance of cesarean section when possible. In addition, neonatal prophylaxis is an essential component of prevention for those high-risk newborns. DHHS guidelines recommend presumptive HIV therapy with lamivudine, zidovudine plus either RAL or NVP in this clinical scenario [4]. Guidelines from Argentina prefer RAL to NVP in this setting, due to the high levels of NNRTI resistance reported [8].

Despite the fundamental role of INSTI in maternal and neonatal therapy, there is no data regarding the prevalence of primary resistance to INSTI in Argentina in general or in pregnant women populations. In addition, INSTI HIVDRM transmissibility in the mother-child pair is unknown. This study constitutes a baseline epidemiological survey for INSTI resistance in pregnant women in Argentina using Next Generation Sequencing (NGS).

MATERIAL AND METHODS

Study design. Cross-sectional investigation. We included stored baseline plasma samples from a previous study that evaluated transmitted and acquired drug resistance in a cohort of HIV-infected pregnant women in Argentina [6].

Study population. HPW were enrolled in a reference public Hospital in Buenos Aires City from 2008 until 2014. All women enrolled had their blood drawn, processed, and stored for the determination of HIV resistance. For the present analysis, 89 plasma samples were available: 40 were from treatment-naïve and 49 from treatment-experienced patients. None of them had exposure to INSTI, as these drugs were not routinely available and not recommended in local guidelines during the collection period. The clinical and epidemiological profile of this cohort has been described previously [6].

Sample amplification. RT-PCR was performed on 10 µl of each extract of clinical samples using Superscript™ III One-Step RT-PCR Platinum® Hi-Fidelity Taq System (Thermo Fisher Scientific, Canada), and the primers IN-out-F1 5'- CACAYAARGGRATTGGAGGAAATG-3' (HXB2 loci 4162-4185) and IN-out-R1 5'- TARTGGRATGTGTACTTCTGAAC-3' (HXB2 loci 5195-5217). RT-PCR conditions were as follows: 50 °C for 30 minutes, 94 °C for 2 minutes, 40 cycles of 94 °C for 20 seconds, 54 °C for 30 seconds, and 68 °C for 60 seconds, and a final extension at 68 °C for 5 minutes. Following RT-PCR, a 5 µl aliquot was transferred to a nested- PCR reaction, with Phusion® Hot Start II Hi-Fidelity DNA Polymerase (Thermo Fisher Scientific, Canada) and the primers IN-nest-F1 5'- AACARGTAGATAAATTAGTHAGT-3' (HXB2 loci 4186 - 4208) and IN-nest-R1 5'-ATACATATGRTGYTTTACTARACT-3' (HXB2 loci 5107 - 5130). Nested-PCR conditions were performed as follows: 95 °C for 10 minutes, 40 cycles at 94 °C for 20 seconds, 52 °C for 30 seconds, and 72 °C for 60 seconds, followed by a final extension at 72 °C for 5 minutes.

MiSeq library prep and ultra-deep sequencing. Samples were sequenced for the integrase (IN) region using an ultra-deep sequencing protocol published by Taylor et al for genotyping of protease and retrotranscriptase region [9]. Libraries were prepared using Nextera® XT DNA Library Preparation Kit (Illumina, USA) and amplicon final concentration 0.2 ng/ul. Tagmentation, indexing, and purification were all performed according to the manufacturer's detailed Reference Guide. Libraries normalization was performed using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen). Libraries were pooled in equal fractions and the mixture was diluted to 2 nM. PhiX control library (20 pM, v3, Illumina, USA) was spiked in at 10%, to measure quality and to elevate the diversity of the amplicons libraries. Finally, the product was sequenced on the MiSeq using a v2 300-cycle MiSeq reagent kit (Illumina, USA) [9-10].

MiSeq data analysis. Reference-based mapping and variant calling against the HXB2 pol gene (loci 2253– 5096, GenBank Accession number: K03455) was performed with HyDRA Web (*http://hydra.canada.ca*) with the default settings. Drug resistance mutations detected at 1%, 2%, 5%, 10%, and 20% thresholds of sensitivity were identified based on the current Stanford list. In addition, the list of INSTI-mutations for the surveillance of transmitted drug resistance according to Tzou et al was considered [11-12].

Mutational viral load. Mutational load was estimated from maternal viral load and the frequency of each mutation within viral quasispecies. The baseline viral load per patient was the starting point to calculate the mutational load (for exam-

ple, a mutational load was 100 if a patient has a viral load of 10% corresponding to a specific mutation over a baseline viral load of 1000 c/mL). Those HIVDRM with mutational load >1000 c/mL were considered at high risk of being transmitted perinatally considering the reference value for cesarean section indication [13].

RESULTS

RT-PCR was performed on 89 samples from INSTI-naïve HPW. Of these, 56 (38 ART naïve; 18 exposed to other drug classes) were satisfactorily amplified and sequenced as shown in Figure 1. The predominant HIV subtype was BF (78.5%). Median (interquartile range, IQR) viral load was 15545 (5228-47688) c/mL.

Major mutations were not detected with the population sequencing 20% threshold neither in the overall sample (0/56) nor ART-naive population (0/38). With a 5% threshold, the prevalence was 1.7% (1/56) in the overall population and 2.6% (1/38) in the naive population. The 2% threshold increased the prevalence to 7.1% (4/56) in the overall population and 7.9% (3/38) in naive. With a 1% threshold, the overall prevalence was 8.9% (5/56) and 10.5% (4/38) in the naive population. Accessory mutations were detected mostly with a 20% sensitivity threshold with a prevalence of 23.2% (13/56). Such prevalence did not increase significantly with the 5, 2, and 1% thresholds. The viral quasispecies harboring major HIVDRM were not predominant within the overall viral population. In contrast, those harboring accessory mutations, were. A summary of these findings is described in Table 1. The proportion of quasispecies harboring HIVDRM among individual samples of experienced and naive patients is shown in Tables 2 and 3, respectively.

Considering major mutations, the median (range) mutational load was 355 c/mL (50.2-11705); with only 1 case >1000 c/mL (1/56; 1.7%), at expenses of a high baseline maternal viral load (487732 c/mL). Mutational load for Y143C, Y143S, E92G, E138K, and T66I mutations were: 63.5, 11705, 50.2, 761.7, and 355 c/mL, respectively. Considering accessory mutations T97A and G163K/R median (IQR) mutational load was 23929 (4009-63158) c/mL, all of them above the 1000 c/mL threshold.

DISCUSSION

To our knowledge, this study is the first report of primary resistance to INSTI in HIV– infected pregnant women in Latin America. We describe baseline data before its general use in our region, which may allow a better future understanding of evolving patterns of INSTI-resistance. Recently, Brazil reported

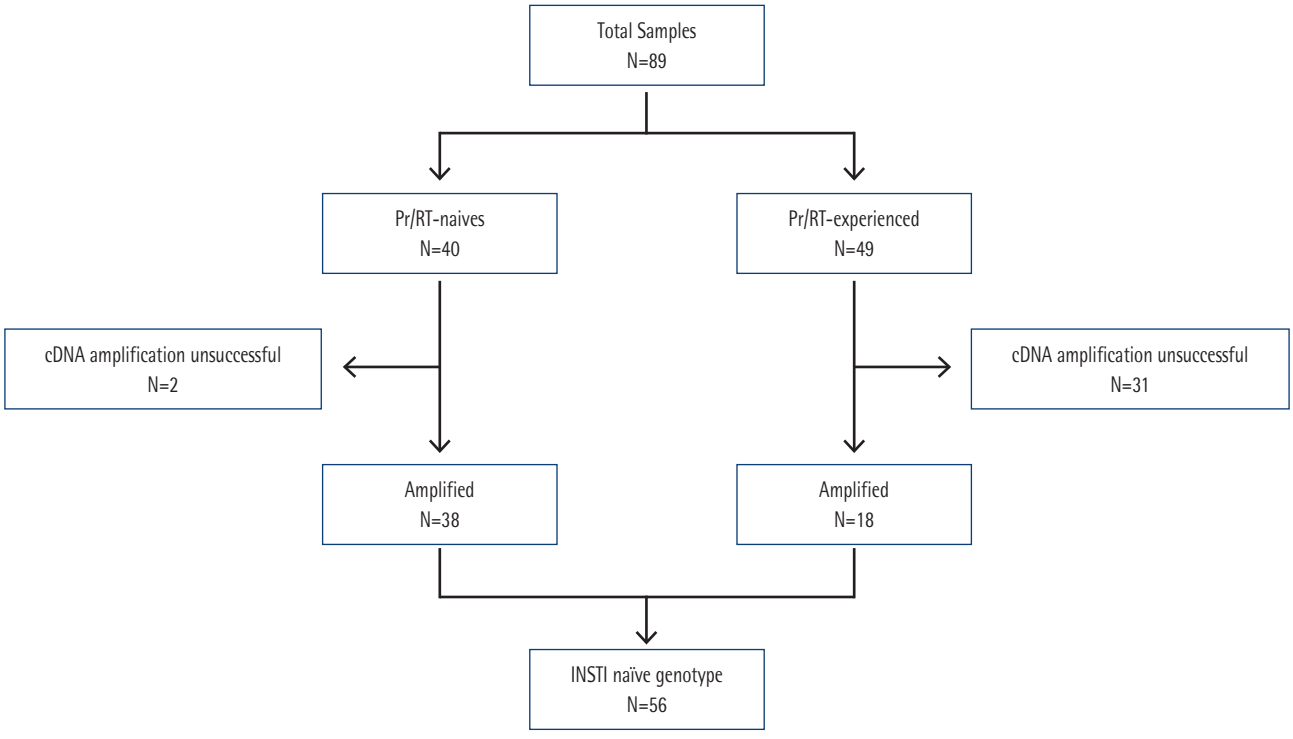

Figure 1 **Flowchart describing the inclusion of stored samples (period 2008-2014) from HIV-infected pregnant women for a baseline survey of resistance to integrase inhibitors (INSTI) by next-generation sequencing in Argentina.**

Pr/RT: protease inhibitor/reverse transcriptase inhibitor; cDNA: complementary DNA

Table 1 Prevalence of major, accessory, and other mutations (Stanford algorithm) in the integrase coding region of INSTI-naive pregnant women living with HIV in Argentina.

NGS threshold	20%	10%	5%	2%	1%
Major mutations *					
Y143C	--	--	1.7%	1.7%	1.7%
Y143S	--	--	--	1.7%	1.7%
T66I	--	--	--	1.7%	1.7%
E138K	--	--	--	--	1.7%
E92G	--	--	--	1.7%	1.7%
Accessory mutations					
T97A	3.5%	3.5%	3.5%	3.5%	3.5%
G163R	12.5%	12.5%	12.5%	12.5%	12.5%
G163K	7.1%	7.1%	8.9%	8.9%	8.9%
Other					
V151I	5.3%	7.1%	8.9%	8.9%	10.7%
L74I	1.7%	1.7%	1.7%	1.7%	1.7%

NGS: Next-generation sequencing; INSTI: integrase strand transfer inhibitor; *mutations included in the list of INSTI-mutations for the surveillance of transmitted drug resistance

Table 2 INSTI resistance-associated mutations detected in individual baseline samples of ART-experienced/INSTI-naive pregnant women in Argentina by Next Generation Sequencing (NGS) considering different thresholds. Each line represents a unique patient sample; the percentage in brackets represents the prevalence of viral quasispecies harboring each mutation and the mutational load. The first detection (threshold) of a major mutation appears in bold.

VL (cop/ml)	NGS 20%	NGS 10%	NGS 5%	NGS 2%	NGS 1%	Subtype
2018	-	-	V151I (5.33%, 107)	V151I (5.33%, 107)	V151I (5.33%, 107)	BF
60507	-	-	G163K (5.5%, 3327)	G163K (5.5%, 3327)	G163K (5.5%, 3327)	B
45184	G163R (99.9%, 45138)	G163R (99.9%, 45138)	G163R (99.9%, 45138)	G163R (99.9%, 45138)	G163R (99.9%, 45138)	BF
5529	V151I (41.1%, 2272)	V151I (41.1%, 2272)	V151I (41.1%, 2272)	V151I (41.1%, 2272)	V151I (41.1%, 2272)	B
55617	G163R (99.9%, 55561)	G163R (99.9%, 55561)	G163R (99.9%, 55561)	G163R (99.9%, 55561)	G163R (99.9%, 55561)	BF
70826	G163K (99.9%, 70755)	G163K (99.9%, 70755)	G163K (99.9%, 70755)	G163K (99.9%, 70755)	G163K (99.9%, 70755)	BF
12103	G163R (99.9%,12090)	G163R (99.9%, 12090)	G163R (99.9%, 12090)	G163R (99.9%, 12090)	G163R (99.9%, 12090)	BF
487732	S230N, (99.5%,485293)	S230N (99.5%, 485293)	S230N (99.5%, 485293)	**Y143S (2,4%, 11705),** S230N (99.5%, 485293)	Y143S (2.4%, 11705), S230N (99.5%, 485293)	B
4706	G163K (99.7%,4691)	G163K (99.7%, 4691)	G163K (99.7%, 4691)	G163K (99.76%,4691)	G163K (99.76%, 4691)	BF
33147	L74I (99.2%, 32881), S230N (99.3%, 32914)	L74I (99.2%,32881), S230N (99.3%, 32914)	L74I (99.2%, 32881), S230N (99.3%, 32914)	L74I (99.21%, 32881), S230N (99.31%,32914)	L74I (99.2%, 32881), S230N (99.3%, 32914)	BF

INSTI: integrase strand transfer inhibitor; PR/TR: protease/reverse transcriptase viral subtype; VL: viral load

increasing INSTI resistance in naive populations in the context of the use of these drugs in the general population [14].

In our historical cohort, major INSTI mutations were detected only by thresholds <2%. This is in concordance with other publications in which these mutations were infrequent or not reported at all [15-17]. Therefore, the 8.9% prevalence of HIVDRM to INSTI detected at the 1% threshold warrants careful interpretation. It´s important to note that this preva-

Table 3 **INSTI resistance-associated mutations detected in individual baseline samples of ART-naive pregnant women in Argentina by Next Generation Sequencing (NGS) considering different thresholds. Each line represents a unique patient sample; the percentage in brackets represents the prevalence of viral quasispecies harboring each mutation and the mutational load. The first detection (threshold) of a major mutation appears in bold.**

VL (cop/ml)	NGS 20%	NGS 10%	NGS 5%	NGS 2%	NGS 1%	Subtype
214738	T97A (99,48%, 213621)	T97A (99,48%, 213621)	T97A (99.4%, 213449)	T97A (99.4%, 213449)	T97A (99.4%, 213449)	BF
13851	-	V151I (14%, 1939)	V151I (14%, 1939)	V151I (14%, 1939)	V151I (14%, 1939)	BF
69941	V151I (100%, 69941)	V151I (100%, 69941)	V151I (100%, 69941)	V151I (100%, 69941)	V151I (100%, 69941)	B
1095	-	-	**Y143C (5.8%, 63)**	**E92G (4.5%, 49),** Y143C (5.8%, 63)	E92G (4.5%, 49), Y143C (5.8%, 63)	BF
18700	T97A (93.3%, 17447)	T97A (93.3%, 17447)	T97A (93.3%, 17447)	T97A (93.3%, 17447)	T97A (93.3%, 17447)	BF
111000	G163R (99.7%, 110667)	G163R (99.7%, 110667)	G163R (99.7%, 110667)	G163R (99.7%, 110667)	G163R (99.7%, 110667)	BF
3152	G163R (99.6%, 3139)	G163R (99.6%, 3139)	G163R (99.6%, 3139)	G163R (99.6%, 3139)	G163R (99.6%, 3139), Q164P (1.8%, 56)	BF
242473	G163R (63.8%, 154697)	G163R (63.8%, 54697)	G163R (63.8%, 154697)	G163R (63.8%, 154697)	G163R (63.8%, 154697), V151I (1.1%, 2667)	BF
3036	G163R (43.4%, 1317)	G163R (43.4%, 1317)	G163R (43.4%, 1317)	G163R (43,4%, 1317)	G163R (43.4%, 1317)	BF
26967	V151I (99.5%, 26832)	V151I (99.5%, 26832)	V151I (99.5%, 26832)	V151I (99,5%, 26832)	V151I (99.5%, 26832)	BF
24074	G163K (99.4%, 23929)	G163K (99.4%, 23929)	G163K (99.4%, 23929)	G163K (99.4%, 23929)	G163K (99.4%, 23929)	BF
17000	-	-	-	**T66I (2.09%, 355)**	T66I (2.09%, 355)	BF
55200	G163K (99.8%, 55089)	G163K (99.8%,55089)	G163K (99.8%,55089)	G163K (99.8%, 55089)	G163K (98.2%, 55089), **E138K (1.38%, 761)**	BF

INSTI: integrase strand transfer inhibitor; PR/TR: protease/reverse transcriptase viral subtype

lence was observed at very low thresholds (1-2%) and not detected at the clinically relevant 20% threshold typically used in population sequencing. These low-frequency mutations likely represent random mutations rather than true transmitted drug resistance, especially considering the study period (2008-2014) when INSTI use was not widespread in our country. Detection of major HIVDRM with the highest sensitivity filters shows evidence of their existence as aleatory mutations, with minimal representation within viral quasispecies. In consequence, they have a low mutational load and risk of transmissibility in the absence of selective pressure. Therefore, the high sensitivity of NGS allows detection of viral quasispecies that may not be clinically relevant. Considering the current recommendations and the widespread use of INSTI in people living with HIV, including pregnant women, further research is needed with current samples to detect signals of increasing trends in INSTI resistance in our country. This finding underscores the importance of interpreting low-frequency mutations cautiously and in the context of their true potential clinical impact.

Conversely, we found a high overall prevalence of accessory HIVDRM in the integrase coding gene, mostly due to G163K/R mutations. Such mutations have a potential impact on susceptibility ("low-level resistance") to first-generation INSTIs (raltegravir, elvitegravir) according to the Stanford algorithm. These results were congruent with previous reports [18-19] and may be attributable to polymorphisms of circulating subtypes, with high representation within viral quasispecies. These quasispecies can be transmitted perinatally because they exceed the threshold of 1000 c/mL.

Neither Latin America nor Argentina have achieved the programmatic objective of HIV perinatal transmission <2% expected for non-breastfeeding populations, being >15% for Latin America and >4% for Argentina [1]. Despite the late diagnosis and limited access to ART remain significant determinants of mother-to-child transmission, drug-resistance is a cause of maternal virologic failure potentially contributing to neonatal infection [2-4]. The INSTIs are, to date, the most effective therapeutic strategy to reduce the viral load in pregnant women to prevent perinatal HIV transmission, even in late diagnoses [8]. Considering the recommendations of INSTI- based therapy in local and international perinatal guidelines (as well as for the general population), resistance surveillance remains crucial.

While our study reports findings at various detection thresholds, including 1%, it's crucial to emphasize the limit-

ed clinical significance of mutations detected at such low frequencies. The 20% threshold is generally considered clinically relevant for population sequencing, as it represents the limit of detection for Sanger sequencing and is more likely to impact treatment outcomes [20-21]. Therefore, mutations detected at the 1% threshold, while interesting from a research perspective, may not have clinical implications. These low-frequency variants may not persist over time due to lack of selective pressure or fitness costs, are unlikely to impact the virological response to INSTI-based regimens and may represent natural aleatory or random mutations rather than true drug resistance. Therefore, while NGS allows us to detect these low-frequency variants, their clinical relevance remains uncertain [1]. Treatment decisions should primarily be based on mutations detected at higher, clinically validated thresholds. The low-frequency mutations reported in this study should be interpreted as providing insight into viral diversity and potential resistance pathways rather than as direct predictors of potential treatment failure

Despite the intrinsic limitations and limited external validity inherent to the retrospective and local nature of our study, this first report on HIVDRM in pregnant women supports the need for surveillance of transmitted and acquired drug resistance in Argentina and other Latin American countries.

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CONFLICTS OF INTEREST

DC participated in educational activities organized by MSD (travel grant, speaker fees). Rest of authors declare no conflict of interest.

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