# Correspondence

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## Functional analysis of HIV-1 subtypes B and C HIV-1 Tat exons and RGD/QGD motifs with respect to Tat-mediated transactivation and apoptosis

HIV-1 Tat is known to influence several intracellular functions including its ability to activate long terminal repeat (LTR) promoter-mediated transactivation and cause apoptosis [1–3]. Although a number of studies have been performed with subtype B gene products, relatively little information is available for subtype C, which is responsible for causing more than 50% infections worldwide, including India where it is the major subtype. In the present study, we constructed several recombinant clones of Tat-B (derived from pNL4-3) [4] and Tat-C (derived from an Indian isolate which is ~95% similar to the consensus subtype C based on its amino acid sequence [5] (clone 93IN905, GenBank Accession no. AF067158) [6] with respect to the two exons for fine functional domain analysis. It is worth noting that most subtype C isolates possess QGD compared with RGD in the second exon of Tat gene in the same position (78–80 amino acids) (a known cell adhesion motif), which is associated with integrin-mediated signaling and cell adhesion, etc., besides other changes throughout the gene. We reasoned that these changes might modulate its ability to transactivate LTR promoters and apoptosis. Therefore, we made constructs of subtypes B and C Tat that consisted of either the RGD or QGD motif and also swapped the first and second exons of the Tat gene. Precise gene fusion technology was used to generate such chimeric constructs as described by one of us earlier [7] and confirmed by sequencing. The various constructs made are indicated at the bottom of Fig. 1a with Tat-B and Tat-C with RGD/QGD domains. An internal reporter gene control (pSV-β-gal; Promega, Madison, Wisconsin, USA) was always included to ensure uniform transfection efficiency.

The various constructs were tested for their ability to activate either LTR-B-mediated or LTR-C-mediated transactivation in human Jurkat T cells as described earlier [8,9] and the results from three independent experiments (mean ± SD) are shown in Fig. 1. Lane 1 shows the background activity from cells only with LTR-B reporter plasmid DNA; an approximately 17-fold increase with wild-type Tat-B (lane 2); but an additional almost three-fold increase with Tat-B construct, which possessed QGD motif in place of RGD (hereafter called Mt-1 construct) (lane 3). The wild-type subtype C construct showed similar activity as obtained with wild-type B construct (compare lanes 2 and 4). The Tat construct possessing wild-type subtype C exon but the RGD motif (found in the second exon of subtype-B, hereafter referred to as Mt-2), showed significant reduction compared with wild-type B or wild-type C constructs. Lanes 6–10 represent the same experiment that was repeated with LTR-C-Luciferase DNA, which essentially gave the same pattern of promoter activation. An exactly similar pattern was observed when the same experiment was carried out on human 293 cells (data not shown).

We next evaluated the extent of apoptosis in PMA-stimulated THP-1cells (transformed human macrophages) caused by various wild-type and chimeric (B/C) Tat constructs (indicated at the top of each panel) including the possible role of RGD/QGD motifs only and the representative results from three independent experiments are shown in Fig. 1b. As expected, control cells showed almost 11% apoptosis (panel I); construct with both wild-type Tat-B exons, showed approximately 29% apoptosis (panel II); with Mt-1 construct, it remained the same as with wild-type B (panel III); wild-type Tat-C showed approximately 14% (panel V) apoptosis, which is about 15% less than what was observed with wild-type Tat-B in panel II. Interestingly, in the construct where first exon of Tat-B was fused with second exon of Tat-C, a much reduced cell death was observed (panel IV). The most interesting results were obtained with Mt-2 construct, which showed approximately 10% more cell death (panel VI) when compared with the wild-type Tat-C. A chimeric Tat construct with first exon fused with second exon of Tat-B (both wild type) showed intermediate levels of cell death (panel VII).

The following important conclusions can be drawn from this study. Replacing RGD motif with QGD alone in the second exon of Tat-B resulted in 2.5–3-fold additional increase in LTR promoter activity. Furthermore, when QGD motif in the second exon of Tat-C is replaced with RGD motif, a significant reduction in LTR-mediated promoter activation was observed. We conclude that RGD motif negatively regulates the Tat-mediated LTR-promoter activation in subtype B and C Tat. Wild-type subtype B Tat caused more cell death than wild-type subtype C. When the QGD motif present in the second exon of Tat-C was replaced with RGD motif of Tat-B, increased apoptosis was observed. Thus, RGD motif alone contributes substantially to Tat-C-mediated apoptosis. These findings are important with respect to further understanding the molecular basis of pathogenesis.

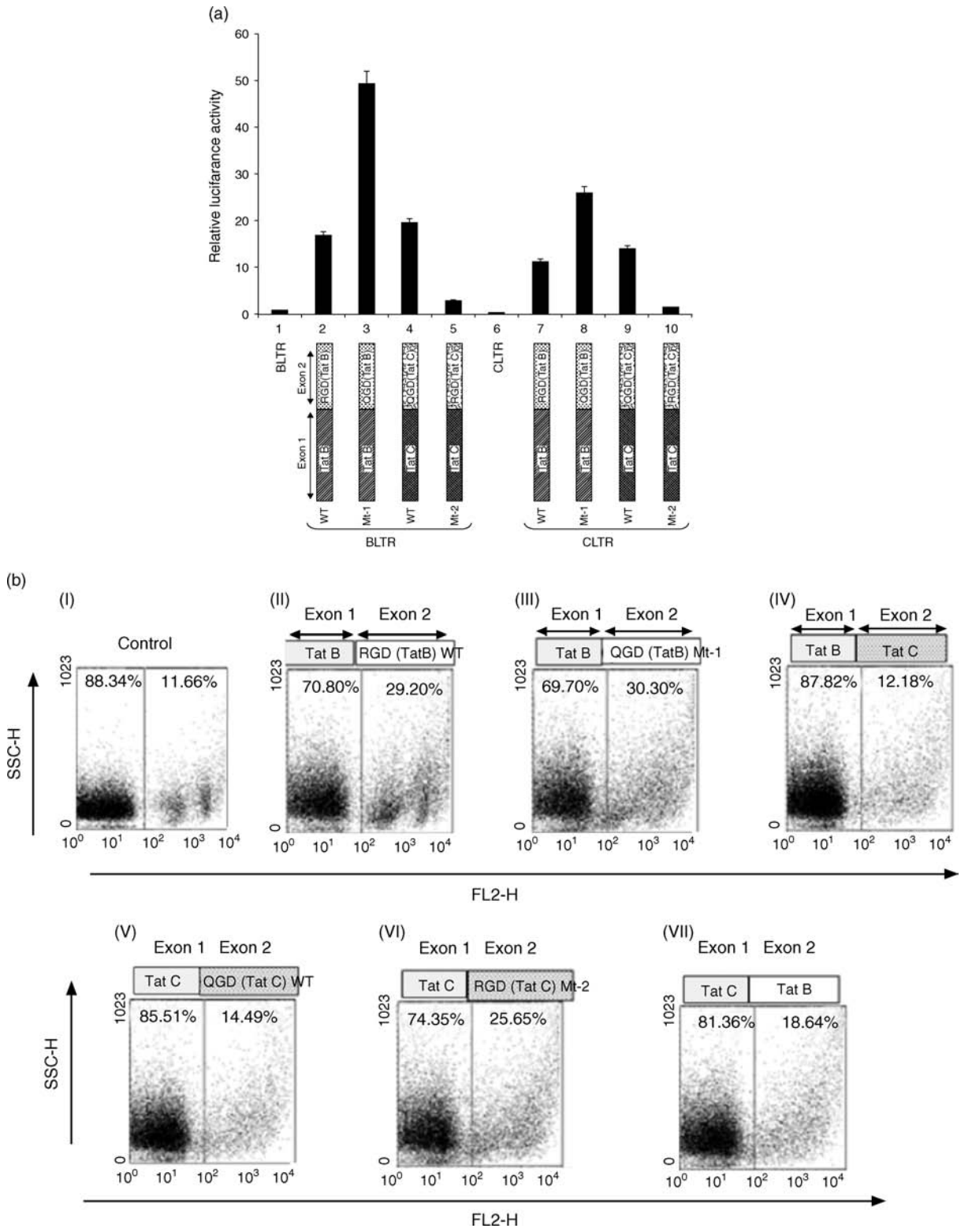


**Fig. 1. HIV-1 Tat B and C constructs mediated long terminal repeat promoter activation and their ability to cause apoptosis.**

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## Rituximab failure in fulminant multicentric HIV/human herpesvirus 8-associated Castleman's disease with multiorgan failure: report of two cases

Multicentric Castleman's disease is a lymphoproliferative disorder manifesting with fever, lymphadenopathy, splenomegaly, raised inflammatory markers, and cytopenias. The clinical course ranges from indolent to fulminant [1,2]. The proliferation of polyclonal but often monotypic lymphoplasmocytoid cells is thought to be driven by human herpesvirus 8 [2–5]. Multicentric Castleman's disease can result in death by complex immune dysregulation leading to sepsis-induced and/or cytokine-induced multiorgan failure [2,6]. An optimal treatment algorithm for fulminant multicentric Castleman's disease is unknown because of the absence of controlled trials, and the outcome is usually fatal. We report on two patients who developed multicentric Castleman's disease-associated multiorgan failure. One of them achieved complete remission on treatment with corticosteroids and chemotherapy after failing to respond to rituximab monotherapy.

## Case 1

A 36-year-old man presented with a 4-month history of night sweats and fever. He had been diagnosed with HIV infection 3 years earlier and had been on highly active antiretroviral therapy (HAART); [most recently emtricitabine (200 mg), tenofovir (300 mg), and efavirenz (600 mg) daily] since diagnosis.

Examination revealed generalized lymphadenopathy and hepatosplenomegaly. Left inguinal lymph node excision biopsy showed plasma cell multicentric Castleman's disease (MCD) associated with perifollicular HHV8-positive cells. His HIV viral load on presentation was less than 50 copies/ml, the white blood cell count (WBC) was $8.4 \times 10^9$/l, and the CD4 count $280 \times 10^6$/l. HHV8 plasma viral DNA load was 26 400 copies/ml but it increased to 104 000 copies/ml in another measurement performed 7 days later.

He was commenced on rituximab 375 mg/m$^2$. Four days later, he developed ascites, acute renal failure, cholestatic liver impairment, bilateral chest infiltrates with type I respiratory failure, and pancytopaenia. Haemofiltration was initiated, and the next day he suffered two asystolic cardiac arrests. He was successfully resuscitated but remained intubated and dependent on inotropic support. HAART medications were stopped due to worsening liver function and lactic acidosis. During the acute episode, the WBC and CD4 counts decreased to $1.3 \times 10^9$/l and $120 \times 10^6$/l, respectively, and the HIV viral load increased moderately to 2900 copies/ml. Treatment with prednisolone (100 mg daily) and

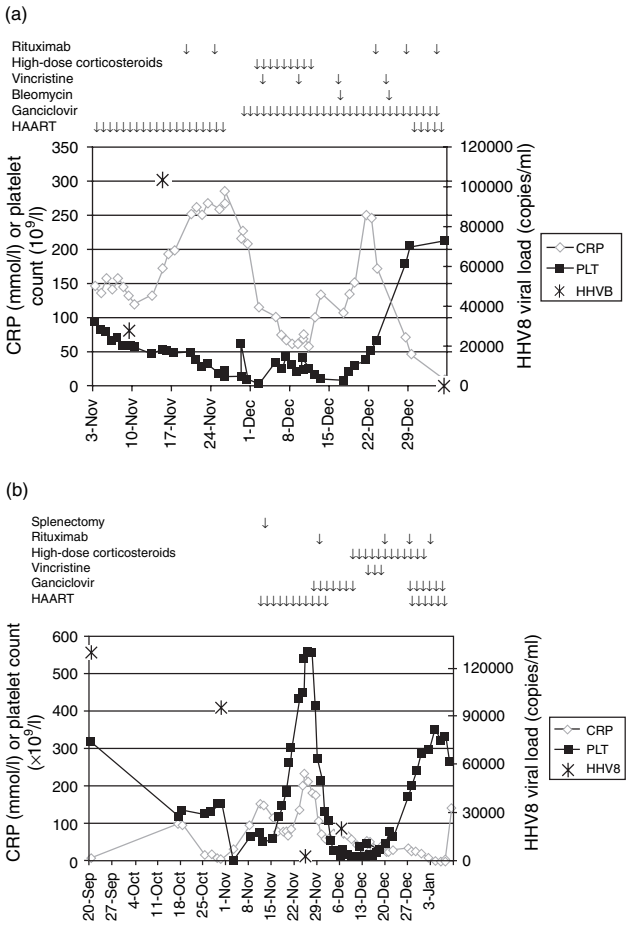


**Fig. 1. Clinical course and the treatment of fulminant multicentric Castleman's disease.** Clinical course as reflected by C-reactive protein (CRP) levels, platelet counts, and serum human herpesvirus 8 (HHV8) viral load and the treatment of fulminant multicentric Castleman's disease (MCD) in patient 1 (a) and patient 2 (b). HAART, highly active antiretroviral therapy; PLT, platelet.

reduced-dose ganciclovir (3 mg/kg daily) was started and the patient also received a single dose of vincristine (0.5 mg). As there was a marked improvement, he was extubated and discharged from intensive care unit. He continued chemotherapy with vincristine, bleomycin, rituximab, and ganciclovir (Fig. 1a). After recommencement of HAART [Kaletra (lopinavir/ritonavir) four capsules once daily], the CD4 count recovered to $490 \times 10^6$/l, the HIV viral load decreased to less than 50 copies/ml, and HHV8 viral load became undetectable. The patient remained well and without any MCD-related symptoms with a follow-up of 11 months.

## Case 2

A 32-year-old man diagnosed with HIV infection 6 years earlier presented with generalized body aches, lethargy, anorexia, and intermittent high fever. There was palpable hepatomegaly, splenomegaly, and generalized lymphadenopathy.

He had never been on HAART as his CD4 count had always been greater than $300 \times 10^6$/l despite a very high plasma HIV load in excess of 500 000 copies/ml. In the previous year, the CD4 count had been decreasing steadily from $680 \times 10^6$/l to $360 \times 10^6$/l, with WBC within the normal range.

Histology of a lymph node revealed the presence of HHV8-positive Kaposi sarcoma but this was not deemed to be a sufficient explanation for his problems and he went on to have a diagnostic splenectomy. Histological examination of the spleen and a hilar lymph node showed HHV8-positive plasma-cell MCD. His HHV8 load at this time was 96 000 copies/ml.

After the splenectomy, his HHV8 viral load decreased to 3600 copies/ml but he continued to have fever and fatigue. We started treatment with rituximab (375 mg/m$^2$) weekly, ganciclovir (5 mg/kg) twice daily, and Kaletra (lopinavir/ritonavir) capsules once daily (Fig. 1b). However, his condition continued to deteriorate with thrombocytopenia and hypoalbuminaemia, and we decided to start vincristine (0.5 mg) and dexamethasone (16 mg) daily. On this therapy, he became afebrile, and peripheral blood cell counts and inflammatory markers normalized. However, the CD4 count continued to decrease to $130 \times 10^6$/l. The patient developed severe cachexia, became completely bed-bound, and died 1 month after the onset of fulminant MCD. Autopsy was not permitted by the family.

## Discussion

Rituximab has recently emerged as a promising agent for the treatment of MCD and is recommended as the first-line treatment in many centres. Despite its activity in relapsing MCD, rituximab alone may not be sufficient in fulminant MCD. In a report by Marcelin *et al.* [7], the two patients who developed haematological failure died on treatment with rituximab alone. Our experience shows that the combination of nonmyelotoxic chemotherapy and high-dose corticosteroids together with intensive care support is capable of reversing MCD-associated multiorgan failure even in patients progressing on rituximab and should be considered as the next step in the treatment algorithm for fulminant MCD.

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## The impact of the journalist-to-journalist program on worldwide HIV awareness

Journalists, more commonly than other professionals, may be able to transmit complex scientific information about HIV/AIDS in a language that is understood by the general public [1,2]. This premise led to establishment of a journalist-to-journalist (J2J) HIV/AIDS training program as a component of the International AIDS Conference in Barcelona in 2002.

Prevention of HIV infection, accessible healthcare, and HIV public policy are all issues that can be highlighted through journalism. Programs on HIV prevention, stigma, the healthcare needs of those infected by HIV/AIDS, and advocating for government intervention can all be directly affected by what journalists choose to report.

The J2J program was conceived and implemented by the National Press Foundation (NPF) as a satellite meeting in advance of the main conference. The purpose was 'preparing selected journalists to cover the International AIDS Conferences, and then to continue to cover the subject at a higher level than previously imagined'. Journalists accepted into the program did not have specialized scientific training. Following Barcelona, the program was held in Bangkok, Toronto, and Sydney in 2004, 2006, and 2007, respectively. Fellows were invited to participate on the basis of journalistic competence after submitting a successful application. Preference was given to journalists from developing countries, who are often least able to afford the costs involved or to be supported by an organization (Fig. 1). Such areas are, of course, also most at risk in regard to new infections. Financial assistance was offered by the program through a grant to the NFP by the Bill and Melinda Gates Foundation.

We were asked to evaluate the J2J program in order to assess the suitability of the curriculum content and didactic quality (process evaluation) and explore the effects of the program on journalists' reporting of HIV/AIDS (outcome evaluation). We also wished to assess journalists' perceptions as to how their training impacted coverage of HIV/AIDS, and determine whether the program had helped to better inform communities about truths (and nontruths) regarding HIV/AIDS.

Material available to us included:

(1) data accessible online from slide presentations at each of the J2J programs;

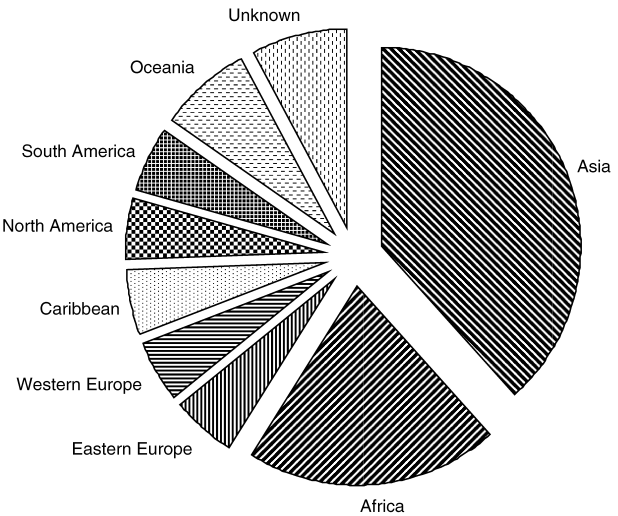


**Fig. 1. Proportion of journalist reports according to regions from which journalist-to-journalist program attendees filed.** Reports were collected through an online survey. 'Unknown' indicates reports posted online, but with no information about the region from which the report originated.

(2) evaluations completed by journalists of the program held in Sydney;
(3) contact information of all journalist fellows who participated;
(4) evaluations of the Barcelona and Bangkok programs by the National Press Foundation;
(5) a large sampling of news stories on HIV/AIDS written by participating journalists.

To establish benefits, two types of analyses were performed. First, a random sample of 39 news reports completed by journalists who participated in any of the J2J programs was examined for relevance and accuracy. Second, a short survey in the form of a questionnaire was distributed to participating journalists to assess overall perceived benefits.

We concluded that the program fully met its main purpose of enabling journalists to effectively transmit medical, epidemiological and scientific information to the general public in lay language. We believe that this raised interest and awareness in developing countries reallocation of resources aimed at both reducing rates of transmission of HIV and the treatment of those living with HIV/AIDS.

However, there appeared to be a shortage of information as to what journalists should be doing at a local level. Should they be querying their own local communities in regard to practices and the role of local health promotion authorities? This subject is complex, and, in some countries, journalists may feel intimidated in regard to the types of questions they might ask.

The need for education of communities is evident. Several reports have documented insufficient knowledge in populations at risk of acquiring HIV infection [3–5]. The media can help guide prevention efforts by promoting voluntary HIV testing as well as discussions of HIV/AIDS with partners regarding awareness that consistent condom use reduces HIV risk [6–9]. The World Health Organization has stated that comprehensive mass media programs are valuable in helping to change HIV/AIDS-related behavior among young people in developing countries [1]. Education of journalists is essential toward meeting these goals.

A continuous effort to promote education of communities through written publications or radio programs, or both, might now be established using the broad human resource represented by the J2J program. The creation of material based on J2J presentations could be encouraged and carried out in other languages. Ongoing feedback from such efforts could then be used to improve the overall effort, which could be implemented and locally tailored to meet regional needs for use in subsequent initiatives.

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## HIV immune reconstitution syndrome in sub-Saharan Africa

We wish to respond to the editorial comment 'HIV immune reconstitution syndrome in sub-Saharan Africa' by Dr Easterbrook [1]. Although our prospective study [2] suggested that the immune reconstitution inflammatory syndrome (IRIS) may have less impact on antiretroviral treatment (ART) programmes in resource-limited settings than previously suggested by retrospective studies, Dr Easterbrook questions whether our experience from Johannesburg is representative for sub-Saharan Africa and whether our study has underestimated the IRIS burden. In order to accurately draw conclusions from our study, we wish to clarify a few of our findings.

First, Dr Easterbrook correctly states that a number of factors may influence the incident estimate of a disease or outcome, and no single ART programme is representative of all patients across sub-Saharan Africa. We believe our estimate of IRIS is accurate for a number of reasons. First, outpatient clinics are the most representative setting for ART programmes. Few inpatients initiate ART because of eligibility screening, adherence counseling, and the urgent need for diagnosis and treatment for their inpatient condition. Although such patients are theoretically at increased risk for IRIS, the overall number of such patients is few.

Second, although the patient population consisted of an urban cohort in a tertiary referral ART clinic, baseline data were comparable with other reported cohorts [3–5]. Sixty-three percent had a history of one or more opportunistic infections, and 92 of 423 (21.8%) had one or more opportunistic infections within 30 days of ART initiation, most commonly tuberculosis (TB), oral candidiasis, and pneumonia. Cotrimoxazole was prescribed in over 86% of patients. 'Comorbid illnesses' referred to chronic, noninfectious conditions, such as hypertension and diabetes, and is reflective of the young and predominantly female characteristics of the cohort.

Third, although Dr Easterbrook questions the rigor of baseline screening, our data demonstrate baseline screening is relatively comprehensive. Symptom-based screening for TB at baseline included 179 of 423 (43%) of patients having at least two AFB smears and/or a BACTEC culture obtained prior to ART. Of the 16 'unmasking' TB-IRIS cases, 12 (75%) were screened with AFB sputa, BACTEC cultures, and/or chest radiographs. Of the four unscreened patients, three were asymptomatic at baseline and demonstrated abdominal or lymphadenitis manifestations, likely indicating subclinical disseminated TB at ART initiation. The remaining patient completed TB therapy 18 months prior to ART and was asymptomatic at initiation. These patients manifested abrupt onset of symptoms 22–34 days after ART initiation, supporting the IRIS diagnosis. Although the systematic screening of all patients initiating ART, independent of symptoms, using sputum and chest radiographs could influence the proportion of 'unmasking' versus paradoxical IRIS, the reality of employing such a strategy in an endemic TB setting would be unsustainable in a resource-limited healthcare system.

Fourth, we employed an active case finder to identify patients with missed visits, and loss to follow-up (12.6%) was similar to other Southern African cohorts (0–15%) [6]. Even assuming IRIS was present in these patients and associated with a significant mortality, the effect of these patients on the overall incidence rate would be modest.

Finally, case finding was active. In addition to scheduled clinic visits, patients were also evaluated at patient-initiated unscheduled visits, whether there were IRIS-related symptoms or not. According to South African guidelines, patients procured their ART medications monthly in the pharmacy clinic, affording additional opportunities to identify problems related to ART.

To date, no objective measure of immune reconstitution is readily available to the practicing clinician. A reduction in viral load of at least 1 log was included in our IRIS case definition in order to provide as much objective immunological evidence as possible to exclude ART noncompliance and HIV viral resistance at the time of IRIS evaluation. Our study is the first to prospectively apply some, albeit imperfect, measure of immune reconstitution. We agree that it is not likely to be predictive of IRIS events, but included it in the analysis to address findings of previous studies [7]. What is sorely needed is a practical, objective measure of immune reconstitution to support IRIS case definitions. Until such a measurement is available, IRIS definitions that rely solely on clinical signs and symptoms in developed or resource-limited settings will be plagued by the lack of immunological validation and the subjectivity of their application across heterogeneous patient populations.

We hope these data provide insight into the clinical spectrum of this difficult complication of ART therapy and will aid clinicians involved in the treatment of HIV-infected patients in sub-Saharan Africa. Future prospective studies should focus on pathogen-specific IRIS in diverse populations to characterize and measure its clinical spectrum, elucidate the underlying immunopathology, and identify methods for its prevention.

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## A new CRF01_AE/B recombinant structure of HIV type 1 found in Heilongjiang province, China

The HIV-1 epidemic in China has demonstrated both a complicated diversity and geographic characteristics. The second National HIV Molecular Epidemiology Survey revealed that HIV-1 subtypes A, B, C, CRF01_AE, CRF-BC and other unusual subtypes have been found in China. HIV-1 CRF_BC is the main epidemic component (50.2%), and subtype B (31.66%) and CRF01_AE (15.54%) take second and third places, respectively (http://shgy.jhgl.org/shownews.asp?newsid=893).

Heilongjiang province is located in the northeast of China, neighbored with the far eastern part of the Russian Federation. Since the first AIDS case was officially reported in 1993, the spread of HIV/AIDS in Heilongjiang province has been comparatively slow. By the end of 2006, 385 HIV-1-infected individuals with 106 AIDS cases have been reported (www.hlj.gov.cn).

To clarify the molecular epidemiology and the genetic diversity of HIV-1 in Heilongjiang province, we analyzed the sequence features of the *gag*, *pol* and *env* gene regions of HIV-1 isolates circulating locally.

Blood samples from 30 HIV-1-infected or AIDS cases were obtained from Heilongjiang province Center for Disease Control and Prevention, where the individuals presented from 2004 to 2006. Twenty-nine patients were from eight different regions of Heilongjiang province, whereas the location of the other patient was unknown. Genomic DNA was extracted from the peripheral blood mononuclear cells. Nested PCR assays were used for the individual amplifications of the *gag* (672 bp, nt 836–1507, HXB2 numbering), *pol* (835 bp, nt 2592–3426, HXB2 numbering) and full-length *env* regions. The PCR products of *gag* and *pol* were purified and sequenced directly. The full-length *env* gene was cloned and sequenced. The GenBank accession numbers of the HIV-1 sequences reported here are EU131787-EU131871 and AY905493-AY905497.

The sequences of *gag*, *pol* and *C2-V3* (nt 6828–7372, HXB2 numbering) were compiled, aligned and adjusted with the reference sequences from Los Alamos database (http://www.hiv.lanl.gov/content/hiv-db/SUBTYPE_REF/align.html and [1]) using the CLUSTAL W program [2]. The Neighbour-joining trees were made using DNAMAN version 4.0 (Lynnon BioSoft, Quebec, Canada). The reliability was estimated by 1000 bootstrap replications [3]. The intergenetic distances of *gag* as well as the *env* region among Heilongjiang subtype B strains and mean pairwise nucleotide distances of the *C2-V3* region between Heilongjiang B strains and the reference strains of other provinces were measured using the Kimura two-parameter model supplemented in MEGA 3.1 [4].

A total of 90 gene sequences including 30 each of *gag*, *pol* and *env* regions were obtained. Phylogenetic analysis based on *gag, pol and C2-V3* sequences showed that 29 (96.7%) of the 30 samples had concordant subtypes in the three gene regions. Among them, 28 sequences showed subtype B (96.6%), whereas one (3.4%), CNHLJ_H06054, proved to be a intersubtype recombinant, CRF07-BC. However, one sample, CNHLJSH06059, was grouped in the same cluster with subtype CRF01_AE in the *gag* region and in the same cluster with subtype B in the *pol* and *env* (*C2-V3*) regions (Fig. 1).

The nucleotide divergence of the *env* region (mean, $6.9 \pm 0.3\%$) was significantly higher than that of the *gag* region (mean $= 4.1 \pm 0.5\%$) among Heilongjiang B strains.

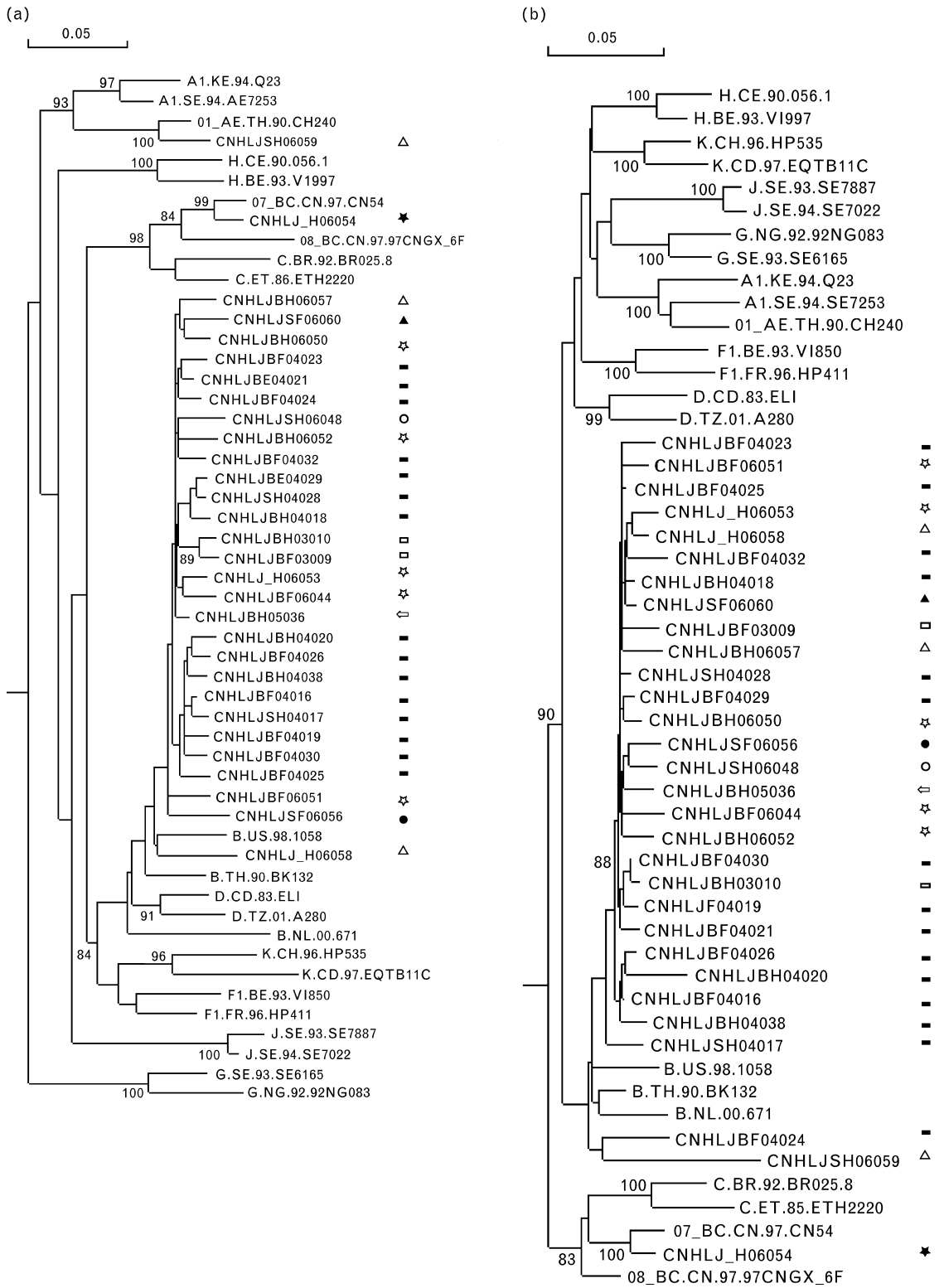


**Fig. 1. Phylogenetic analysis of HIV-1 in Heilongjiang province.** Phylogenic analysis on the HIV-1 *gag* (a), *pol* (b) and *env* (C2-V3 region) (c) genes of the strains characterized and the reference sequences (from Los Alamos database, http://www.hiv.lanl.gov/content/hiv-db/SUBTYPE_REF/align.html). In the tree, study participants from different locations are indicated, including Harbin (Δ), Beian (▬), Zhaodong (▭), Mudanjiang (○), Shuangyashan (☆), Jixi (●), Jiamusi (★), Suihua (▲) and unknown (⇦). This tree was constructed using the Neighbour-joining method. No sequence gaps were included for analysis. The analysis was corrected for multiple substitutions. Values on the branches represent the percentage of 1000 bootstrap replicates. Bootstrap values over 80% are marked in the tree.

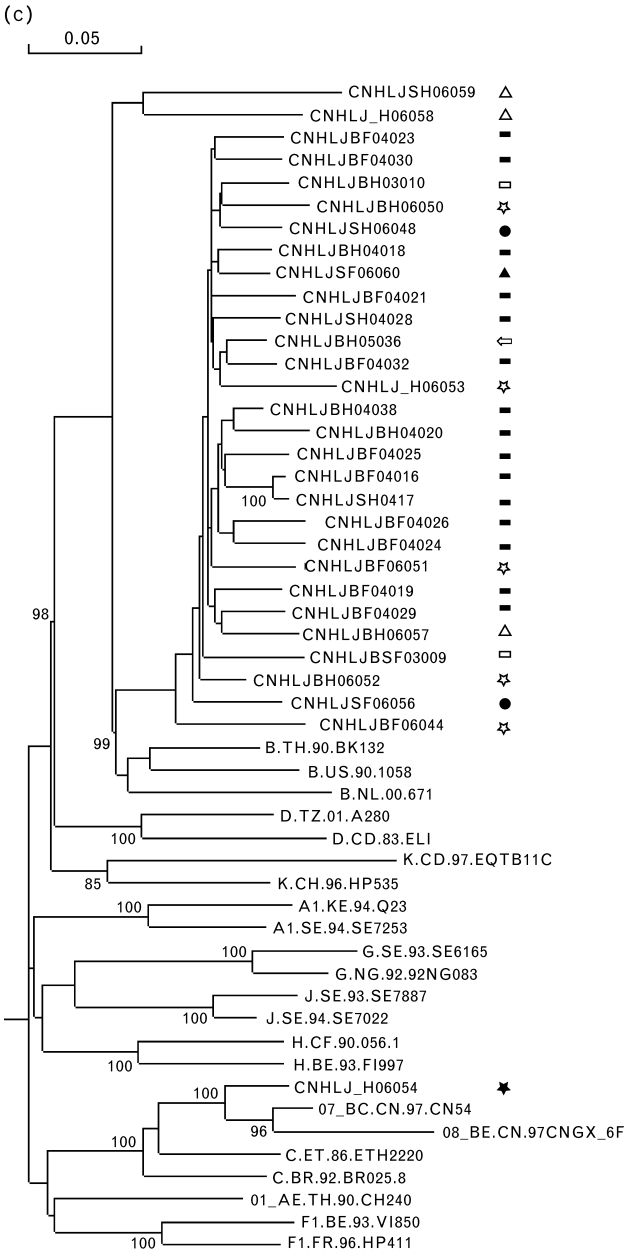


**Fig. 1.** (*Continued*).

The genetic distances of the *C2-V3* region between Heilongjiang B strains and reference strains of Guangxi, Henan and Yunnan provinces of China were $8.3 \pm 0.8\%$, $9.7 \pm 1.0\%$ and $15.3 \pm 1.4\%$, respectively, suggesting the lowest divergent between Heilongjiang and Guangxi provinces.

BootScan analysis further confirmed that sample CNHLJSH06059 was a CRF01_AE/B recombinant. The recombination was structurally different from those reported previously as the *pol* and *env* genes of this recombinant were derived primarily from subtype B, and the *gag* region was derived from CRF01_AE. The blasting result indicated that there is the highest genetic homology in the *gag* region (CRF01_AE) between CNHLJSH06059 and one HIV-1 isolate from Khabarovsk of the Russian Federation, U93610 (97.1%).

In this study, for the first time, we found two recombinants, CNHLJ_H06054 (CRF07_BC) and CNHLJSH06059 (CRF01_AE/B), in Heilongjiang province. As the CRF07_BC strains were isolated first and distributed among injecting drug users in Xinjiang province and some HIV-1-infected individuals living in Heilongjiang province were originally from Xinjiang province, we believe that monitoring the migrated populations is extremely important for the control of the spread of HIV in this area. Furthermore, as there was active communication both economically as well as culturally between the two border regions, Heilongjiang province of China and Khabarovsk region of the Russian Federation, the fact that there is the highest homology between the sequences from the two regions might suggest there are potential transmission routes of HIV-1 between the two countries.

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## HIV treatment in times of civil strife: serious threats to antiretroviral drug access in the Kibera slum following the Kenyan elections

The turbulence in Kenya following the elections in December 2007 was broadcast across the world, but few voices were raised regarding its potential impact on access to essential healthcare including antiretroviral (ARV) drugs for HIV positive patients [1,2]. Successful ARV treatment relies on high adherence to avoid the development of drug resistance [1]. The informal settlement of Kibera with an estimated 1 million people of mixed ethnic origin, of which more than one fifth are HIV positive, was one of the areas worst hit by the postelection violence.

We studied access to ARV treatment and staff experiences during the postelection period between 1 January and 1 February 2008 at an HIV clinic run by the African Medical Research Foundation (AMREF) in the Kibera slum, where 722 patients were receiving ARV. The number of missed appointments was compared with corresponding figures for the previous year (January 2007) using patient records. A self-administered questionnaire was used to explore experiences of AMREF staff at the Kibera clinic regarding access to the clinic, safety, and alternative sources of ARV drug supply during the postelection period.

Our review of patient records from January 2008 showed that 42% of 447 scheduled appointments were missed compared with only 14% in January 2007. This corresponds to more than 25% of all currently active ARV patients at the clinic (Table 1).

Twenty-five out of 63 (40%) staff responded to our questionnaire. The most common reason for absence from the clinic was fear of ethnic violence and a feeling of insecurity, hindering both patients and staff from traveling back to Kibera after the Christmas holidays in rural homes. Several respondents stated that they had been attacked by street gangs or ethnic mobs and had feared for their lives coming to the clinic, which is centrally located in Kibera. All staff members said that they had provided ARV to patients who normally go to other clinics, so it is not unlikely that some AMREF patients also obtained drugs at other clinics during the turbulence.

**Table 1. Activity at the Kibera clinic for the months of January 2007 and 2008, by patient category, number of patients and percentage of currently active patients.**

| Category | 2007 | 2008 |
|---|---|---|
| Screened | 47 (9) | 23 (3) |
| New ARV initiated clients | 26 (5) | 9 (1) |
| Scheduled appointments | 319 (63) | 447 (62) |
| Missed appointments | 44 (9) | 186 (26) |
| Unscheduled appointments | 96 (19) | 62 (9) |
| Refused treatment | 0 (0) | 3 (0) |
| Total clients enrolled on ARV (accumulated) | 687 | 1022 |
| Current active clients on ARV (accumulated) | 507 | 722 |

Percentage values are shown in parenthesis. ARV, antiretroviral drugs.

In conclusion, more than four in 10 HIV patients are likely to have experienced treatment interruption lasting for several weeks. As many patients in this context seek care late with low CD4 cell counts, treatment interruptions may rapidly lead to AIDS symptoms and deteriorating health. Also, since 99% (data not shown) of the patients are on Lamivudine, Stavudine 30 mg, Stavudine 40 mg, Zidovudine, Didanosine 125 mg, Didanosine 200 mg (nucleoside reverse transcriptase inhibitor) and Nevirapine 200 mg, Efavirenz 600 mg (non-nucleoside reverse transcriptase inhibitor) only, even short periods of irregular drug intake may lead to the development of drug resistance [3,4], which is especially problematic when second or third line ARV are not affordable. Studies from sub-Saharan Africa have shown that adherence levels of 68–85% can be achieved [5] but weak health systems, staff shortages and stigma contribute to jeopardizing regular drug intake and patient retention in ARV programmes [6,7]. The AMREF ARV programme in Kibera is faced with fairly low adherence even under normal conditions: our unpublished data (ongoing prospective cohort study, $n = 407$) show that 27% of the HIV-positive patients on ARV treatment have a mean overall adherence to ARV below 95%, with consequent risks of developing drug resistance [5,6].

Kenya is considered to be one of Africa's most stable democracies. Our results demonstrate that a political event superimposed on an already fragile context puts HIV patients on ARV at high risk of treatment interruption and irregular drug supply that could rapidly lead to drug resistance deteriorating health. As most patients are supplied with ARV drugs for only 1 month in most ARV programmes in Africa, ARV providers and donors could be better prepared in future to prevent ARV treatment interruptions by providing patients with medication for extended periods, when civil disorder is expected. Evident drawbacks to extended supplies include the risk of medicines being destroyed, lost, sold or given away. A systematic back-up plan to handle these

situations is necessary given the shortage of human resources [8]. Tracing of patients who are lost to follow-up could be facilitated through extended peer networks and digitized record keeping indicating when drug refills are needed. Further, healthcare providers could be better prepared for times of civil unrest by developing formal collaborations with other nearby nongovernmental organizations and international nongovernmental organizations, as our findings show that HIV patients do try to access ARV drugs even during unstable situations.

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## Mutation rate in hepatitis C virus NS3 protease is not influenced by HIV-1 protease inhibitor therapy

Limited analysis by population sequencing has been reported for the selection of isolates with mutations within the NS3 protease that confer resistance to the hepatitis C virus (HCV) protease inhibitors. Little is known about the influence of anti HIV-1 protease inhibitors, through selection pressure, on the HCV protease. We noted, in a cohort of HCV-infected and HIV–HCV-coinfected patients, that the natural strains of the NS3 protease domain related to resistance to HCV-protease inhibitors were well conserved. Anti-HIV-1 protease inhibitors had no influence on the mutation rate in the NS3 protease. This finding could have implications for the future monitoring of HIV–HCV-coinfected patients receiving anti-HCV protease inhibitors.

Encouraged by the stunning success of HIV protease inhibitors in halting the progression of AIDS, researchers turned to HCV protease to treat HCV infection. Since the discovery of the first protease inhibitor, BILN, which was stopped for cardiotoxicity, new protease inhibitors have been developed in human clinical trials [1,2]. Indeed, phase II and III were conducted in HCV patients with SCH5036 (Boceprevir) and VX 950 (Telaprevir) [3,4]. Like HIV-1, HCV persists as a population of multiple, closely-related variants generated by the low-fidelity HCV RNA polymerase. The HCV NS3 protease is a chymotrypsin-like serine-protease responsible for cleavage of the nonstructural proteins of HCV that plays a pivotal role in viral life cycle [5,6]. Limited analysis by population sequencing has been reported for the selection of isolates with mutations within the NS3 protease that confer resistance to the HCV protease inhibitors [7]. Selection of drug-resistant mutants was demonstrated by in-vitro and clinical studies with HCV NS3-4A protease inhibitors [3,4]. It appeared, in in-vitro and in-vivo studies, that mutations V36M, A71T, T72I, P88L, R155Q A156T, D168V, and V170I/M were selected that confer resistance to each protease inhibitor [8–10].

Owing to common routes of transmission (i.e. intravenous drug use and transfusion), one third of the patients infected with the HIV in the USA and Europe are coinfected with HCV [10–12]. From 20% to 40% of HIV–HCV-coinfected patients may achieve a sustained virological response with combined treatment of pegylated interferon plus ribavirin [12–16]. In this therapeutic context, there is an urgent need to develop more specific antiviral drugs associated with a shorter therapy for treating HIV–HCV-coinfected persons [1]. Future therapy for HIV–HCV-coinfected patients will include HCV protease and polymerase inhibitors. Inhibition of wild-type HCV may 'select' naturally occurring drug-resistant variants. However, the influence of anti-HIV-1 protease inhibitor, through selection pressure, on the HCV protease is not still established. The aim of the present study was to describe the natural polymorphism of the NS3 sequence in different HCV 1 strains and to compare the diversity of the protease in

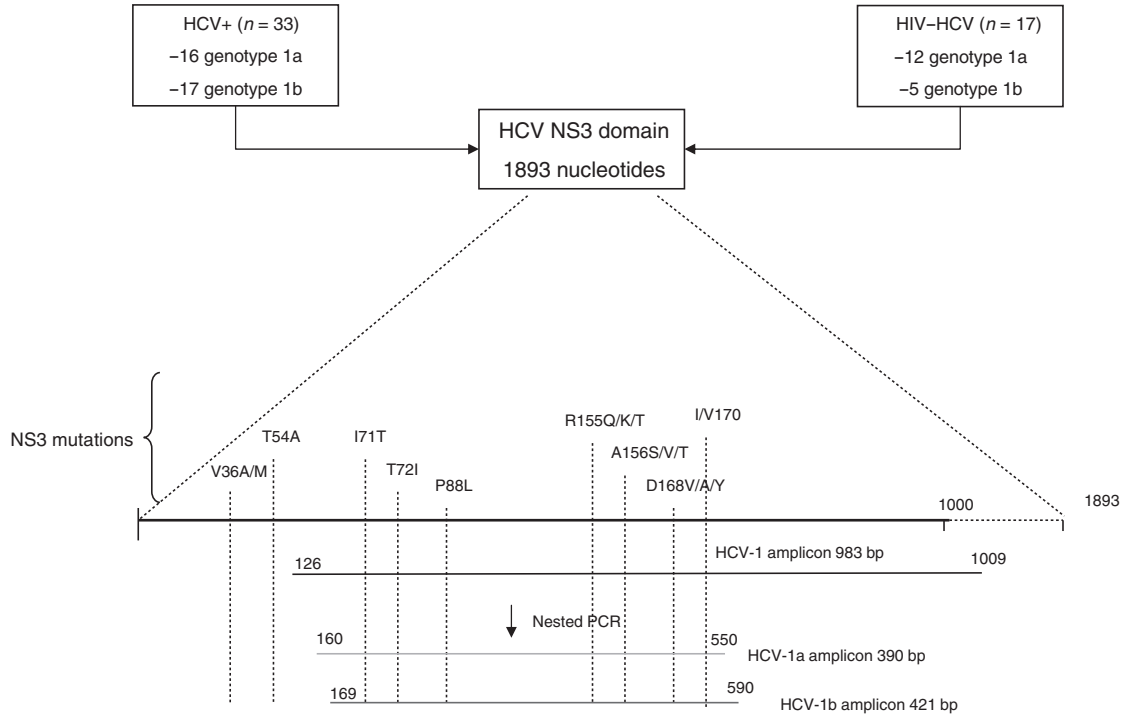


**Fig. 1. Schematic representation of the sequence analysis of the hepatitis C virus NS3 domain.**

33 HCV-monoinfected patients (16 genotype 1a and 17 genotype 1b) and in 17 HIV–HCV-coinfected patients (12 genotype 1a and five genotype 1b) receiving HIV-1 protease inhibitor therapy (Atazanavir boosted by Ritonavir in seven patients, Fosamprenavir boosted by Ritonavir in eight patients, Saquinavir boosted by Ritonavir in two patients). The NS3 protease domain (amino acids 54–197) was amplified by reverse transcriptase-PCR. PCR products were purified and directly sequenced for genotypic and phenotypic analysis of amino acid changes (Fig. 1) [17]. Multiple alignments of nucleotides and deduced amino acid sequences were inferred by Clustal X, version 1.64b. Fisher's exact test was used to compare proportions of mutation at positions 36, 54, 71, 72, 88, 155, 156, 168, and 170. The Wilcoxon rank-sum test was used to estimate clinical and virological differences between HCV-monoinfected patients and HIV–HCV-coinfected patients.

The mutation rates observed in the different positions were not different for HCV-infected and HIV–HCV-coinfected patients (19% and 18%, respectively). No differences in aminoacid sequences were found between genotype 1a and genotype 1b patients. Diversity on the protease was more frequently observed in positions 71 and 72; positions 36, 155, 156, 168, and 170 were well conserved regardless of the HCV subtype 1 and the HIV-1 coinfection status. Despite the full 534-bp NS3 protease catalytic domain not being fully analyzed in this study, the limited portion of part of the protease gene analyzed (390-bp for HCV-1a and 421 bp for HCV 1-b) involved the main relevant mutations associated with HCV protease drug resistance.

Sensitive sequencing analysis of HCV protease of patients treated with anti-HIV-1 protease inhibitor demonstrated an absence of selection pressure on the HCV protease. Data should be gathered on the selection of various, so far unknown mutations within the NS3 protease with different resistance levels and increasing frequencies [3,4]. One of the weaknesses of the present study is the low number of patients included in each group.

In conclusion, in this cohort of HCV-infected and HIV–HCV-coinfected patients, the natural strains of the NS3 protease domain related to resistance to HCV-protease inhibitor were well conserved. Anti-HIV protease inhibitor therapy had no influence on the mutation rate in the NS3 protease. This finding could have implications for future monitoring of HIV–HCV-coinfected patients receiving anti-HCV protease inhibitors. Further studies on larger cohorts should confirm this finding.

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P.H., M.B., and P.P. designed the study; P.H. wrote the paper; M.B. and V.O. recruited the patients; H.K., A.M.,

and J.C. performed the reverse transcriptase-PCR analyses; and G.P. performed the statistical analyses. All authors have read and approved the final version of the paper.

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## Noninferiority and lopinavir/ritonavir monotherapy trials

We sincerely appreciate the comments by Richert *et al*. [1] about the important methodological issues of noninferiority trials. Our answers to their comments related to the OK04 study are as follows:

(1) Richert *et al*. [1] are concerned about 'the apparent absence of a consensus regarding the choice of the primary endpoint in trials comparing different strategies of antiretroviral treatment'. However, they fail to realize that the strategy tested in OK04 [2] is completely different from other trials of triple therapy. The differences between lopinavir monotherapy and other simplification strategies still based on triple therapy are so fundamental that new methodological approaches and new endpoints are warranted.

(2) Richert *et al*. [1] are somewhat surprised about our choice of primary endpoint. In particular, about our decision of not considering successful reinductions as failures. After a pilot study [3], we learned that patients with virological rebound while on monotherapy did not develop resistance, and they could be successfully resuppressed with the same nucleosides without losing therapeutic options. On the basis of the results of our pilot trial, the scientific question we have tried to answer in OK04 is, for a patient without resistance to protease inhibitors who has viral suppression while taking two nucleosides and lopinavir/ritonavir, which strategy is better: to continue triple therapy or stop the nucleosides and use them only if patient fails to maintain suppression? To answer this question our endpoint is appropriate. Not considering successful reinductions as a positive outcome would not allow us to answer our scientific question. Nevertheless, sensitivity analyses with more standard definitions were performed and

presented. We disagree with Richert *et al.*'s comparison with 'other randomized trials evaluating simplification regimens' as they mention two lopinavir/ritonavir monotherapy studies that included naïve patients (different to our study) [4,5] and another study using efavirenz for simplification [6] instead of a boosted protease inhibitor (it is well known that the consequences of virological failure with a no-nucleoside are very different to the failure with a boosted protease inhibitor).

(3) Apart from the primary analysis, we have provided six additional sensitivity analyses in our study. Richert *et al.* [1] ask for even more analyses. In particular, they mention the 'worst-case' methodology. This methodology is a way of 'torturing data until they confess'. This method could be useful when the truth is hidden. However, when reported data have provided all the information clinicians need, we consider such kind of torture unnecessary. Our sensitivity analyses give enough information to obtain a complete picture about the strengths and limitations of the monotherapy strategy. The disposition of patients has been fully detailed and allows the reader to perform other sensitivity analysis if this is considered important. It is now time to discuss from a clinical point of view how this new information has to modify or not our therapeutic approaches.

(4) We agree with Richert *et al.* [1] that the margin of noninferiority has to be rigorously chosen and justified. The noninferiority margin of OK04 was selected before the study as clinically relevant, taking into account the rates of failure in the clinical trials testing accepted strategies of simplification (with efavirenz, nevirapine, or abacavir) [7]. This margin could be even more appropriate in our trial because the benign nature of the 'failure' in the monotherapy group.

(5) OK04 is a real noninferiority trial because, even if monotherapy has a slightly lower probability of maintaining suppression, the added values of the strategy are very clear: reducing cost [8], reducing toxicity [9], and potentially increasing survival [10]. In addition, the consequences of failures do not imply losing therapeutic options [11]. We cannot count how many times we have been criticized because monotherapy does not make sense in the era of 'safe' nucleosides. We recommend that these critics look back at to what has happened with 'safe' nucleosides during the last 4 months [12].

In summary, we agree with many of the careful points raised by Richert *et al.* [1], but we have the impression that they have picked the wrong trial to exemplify the many methodological problems inherent to noninferiority trials.

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## Electrocardiographic changes in HIV-infected, drug experienced patients treated with atazanavir

After reading the case study of 75 HIV infected patients treated with atazanavir-based therapy reported by Gianotti *et al.* [1], we express concerns regarding the methodology used to measure ECG intervals in this report. In particular, we note that automated measurement by ECG recording machines was used. This is in contrast to the current industry standard for ECG interval measurements to be digitally measured with electronic calipers or confirmed by experienced reviewers. In our view, the absence of digital overreading resulted in inadequate quality control for the QRS interval measurements and a false-positive finding of QRS prolongations in patients treated with atazanavir [1].

The authors report a median increase in QRS duration of 5 ms [interquartile range (IQR) = 0–9 ms; $P < 0.0001$] on atazanavir after 51 weeks of follow-up (range = 36–89.9 weeks) compared with pretreatment values. In particular, the authors report 14 patients with pretreatment QRS 100 ms or less who experienced an end-of-study QRS interval greater than 100 ms. One of these patients (patient #1) appeared to experience a marked increase in QRS duration (baseline QRS of 88 ms compared with end-of-study QRS of 145 ms). The authors report involvement of a cardiologist in the ECG review. However, because of the possibility of unrecognized ECG machine error, we requested further information from the site. Dr Gianotti *et al.* graciously supplied photocopies of the actual baseline and end-of-treatment ECGs for the 75 patients in the original report [1].

All of the 150 paper copies (75 patient sets) were electronically scanned using a standard HP Digital Scanner at 300 dpi and then transferred to Scan ECG (AMPS, New York, New York, USA), by Cardiocore (Bethesda, Maryland, USA). Due to poor quality, 16 of the 75 sets could not be interpreted due to unsuccessful digitization. Using electronic calipers, 59 sets of ECGs were measured for QRS width. This technique, often referred to as semiautomatic overreading, is the industry standard and involves expert review.

In the set of 59 digitized samples, we found that only four patients experienced an increase in QRS duration of greater than 10 ms, compared with 15 patients as determined by machine interrogation. Furthermore, no patient experienced QRS duration greater than 116 ms. We found significant discrepancies for two patients reported to have QRS greater than 120 ms in the original report, including the one with the marked increase at end-of-study, patient #1, with corrected values, as shown in Table 1.

**Table 1. Comparison of QRS values.**

| | Machine calculation (ms) | | Digital measurement (ms) | |
|---|---|---|---|---|
| Patient no. | Baseline | End-of-study | Baseline | End-of-study |
| 1 | 88 | 145 | 83 | 84 |
| 2 | 126 | 124 | 102 | 106 |

The machine versus digital-caliper discrepancy is very likely due to random error by the machine algorithm, which can be affected by the variability in rate of change of the tracing as is commonly found with artifact. The end-of-study tracing for patient #1 shows a significant degree of artifact.

Taking the 59 digitally measured sets altogether, the median baseline to end-of-study QRS change was 1.0 ms (IQR = −2 to −4; $P = 0.246$) The corresponding machine-calculated median change was 5 ms (IQR = 1–10; $P < 0.001$) in the same set of 59 patients.

Because the QRS width is difficult to gauge with the human eye using manual calipers (with a likely error of ±15–20 ms) and because the machine QRS calculation is affected by artifact, the conclusions of Gianotti *et al.* [1] should be regarded with caution. Overreading using digital calipers represents the gold-standard technique. Although we were only able to investigate 80% of the originally reported ECG set, the uncorrected median value of 5 ms for this subset compares closely to the originally reported uncorrected median value of 5 ms for the whole cohort [1]. Furthermore, as the subset includes the extremely anomalous result for patient #1, our reanalysis is likely to be relevant. As such, we note that the corrected median QRS change of 1 ms in these 59 sample sets is likely to have very little clinical relevance.

We respectfully disagree with the QRS results and the conclusions presented by Gianotti *et al.* [1], including their recommendation for routine ECG monitoring with regard to QRS as well as their proposed association between QRS changes and bundle branch block effects. We note that one patient experienced resolution of bundle branch block findings while on atazanavir. As we show here, the study would have been better served by digital collection of ECG tracings with intervals determined by the digital caliper overread technique. We are grateful to the authors for sharing the ECG data.

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