FAVORABLE THERAPEUTIC RESPONSE WITH AN ANTIRETROVIRAL SALVAGE REGIMEN IN A HIV-1 POSITIVE SUBJECT INFECTED WITH A CRF11-CPX VIRUS

Complete List of Authors:
- Tau, Pamela; University of Milan, DIBIC Luigi Sacco
- Mancon, Alessandro; Hospital "Luigi Sacco", Clinical Microbiology/Virology/Bio-emergency Unit
- Mileto, Davide; Hospital "Luigi Sacco", Clinical Microbiology/Virology/Bio-emergency Unit
- Di Nardo Stuppiro, Silvia; University of Milan, DIBIC Luigi Sacco
- Bottani, Giulia Maria; University of Milan, DIBIC Luigi Sacco
- Gismondo, Maria Rita; Hospital "Luigi Sacco", Clinical Microbiology/Virology/Bio-emergency Unit
- Galli, Massimo; University of Milan, DIBIC Luigi Sacco
- Micheli, Valeria; Hospital "Luigi Sacco", Clinical Microbiology/Virology/Bio-emergency Unit
- Rusconi, Stefano; University of Milan, DIBIC Luigi Sacco

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Running head: Therapeutic response in a HIV-1+ patient

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¹ 3rd Infectious Diseases Division and ² Clinical Microbiology/Virology/Bio-emergency Unit, DIBIC “Luigi Sacco”, University of Milan, Italy.

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Corresponding author: Pamela Tau, Divisione Clinicizzata di Malattie Infettive, DIBIC “Luigi Sacco”, Università degli Studi di Milano, via G.B. Grassi 74, 20157 Milan, Italy; tel. +390250319773; fax +390250319768; pamela.tau@unimi.it
**ABSTRACT:** HIV drug resistance still represents a crucial problem in antiretroviral therapy. We report a case of a naive patient, harboring a CRF11-cpx virus, which showed drug resistance mutations in the reverse transcriptase. Drug resistance genotyping test was performed for *pol* (protease, reverse transcriptase, and integrase) and V3 regions. The initial clinical parameters results showed a 4 logs level of HIV-RNA (12,090 cp/mL) and a very low CD4+ cell count (35 cells/µL). We designed an initial HAART regimen including 3TC+ABC+DRV/r. The virus resulted highly resistant to all NRTIs and NNRTIs except to ABC, TDF, and EFV and susceptible to all PIs and INIs. A salvage regimen including RAL+DRV/r was started. Ten months after, the immuno-virological status shows CD4+ 142/µL and HIV-RNA <37 cp/mL. Our results demonstrate the effectiveness of a treatment combination that includes RAL+DRV/r in a patient infected with a complex X4-tropic CRF11-cpx virus.
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Based on phylogenetic analysis of gene sequences, HIV-1 strains were classified into four distinct groups: M (Major), O (Outlier), N (non-M, non-O) and P. Group M strains represents the majority of HIV infections in the global pandemic and are divided into nine subtypes (A-D, F-H, J and K), 2 sets of subtypes (A1, A2, A3, A4 and F1 and F2), circulating recombinant forms (CRFs) and a single recombinant (URFs), which results from the recombination of two or more subtypes. (2)

In the context of African countries, HIV-1 group O (HIV-1O) virus is one of the most frequent groups of HIV-1, which includes also groups M, N e P. Most of the identified cases of HIV-1O worldwide have been identified in Cameroon and Central America with a percentage of approximately 1% of all HIV-1 infections. Other cities in Africa and in Europe have reported the presence of HIV-10 mostly in low proportions. HIV-1O variants are genotypically divergent from HIV-1 group M viruses and a significant genetic variation, connected to important consequences in diagnosis and monitoring, is observed within group O. (3)

Group M is responsible for the pandemic and can be divided into subtypes, subsubtypes, and circulating recombinant forms (CRFs). (4) Specific distributions of the various subtypes are seen in the different continents and the greatest genetic diversity of HIV-1 has been found in Africa. (5,6) A mixture of HIV-1 variants results in an increasing likelihood of generating new recombinant viruses. Differences related to efficiency of antiretroviral drugs and diagnostic assays have been reported among the various HIV-1 variants and preliminary data suggest differences in pathogenesis and transmissibility. (5,6) Therefore, it remains important to track the molecular epidemiology of HIV-1 and to genetically characterize prevalent HIV-1 strains.

So far, six genomes of CRF11-cpx have been reported: GR17 (AF179368) by Paraskevis et al. in 2000; MP818 (AJ291718), MP1298 (AJ291719), and MP1307 (AJ291720) by Montavon et al. in 2002; and 96CM4496 (AF492623) and 95CM1816 (AF492624) by Wilbe et al. in 2002. In the nef/LTR region, both the A and E segments appear to be derived from CRF01_AE, while the other
A segments are not. The segments labeled U were regions where the sequence was equidistant between G and J. (7,8,9) Thereby, we report the case of a HIV-1-infected naive patient, harboring a CRF-11cpx virus, which showed several drug resistance mutations in the RT.

Our patient, a 41-year-old men, was evaluated from 2007 to 2010 in Bangui, Central African Repubblic, where he performed several HIV-1-Ab tests with negative results, with the exception of a positive test in 2007, which was not confirmed at later time points.

RNA was extracted from plasma using QIAmp Viral RNA Mini Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer’s protocol.

EDTA whole blood was collected to perform CD4 T cell count. The viral load was assessed using the kPCR Real Time-PCR method (Siemens Healthcare Diagnostic Inc., Deerfield, IL, USA) with a cut-off of 37 cp/ml. Drug resistance genotyping test was performed on plasma samples by means of the TRUGENE HIV-1 kit (Siemens Healthcare Diagnostic Inc., Deerfield, IL, USA) for pol (protease, reverse transcriptase and integrase). V3 regions were sequenced according to a protocol used in the OSCAR study, which was adapted for the Siemens system. (10)

To determine the subtype of 6337257_pt, the pol sequence was aligned with HIV-1 reference sequences the CLUSTAL W method (MegAlign, Lasergene v. 7.2.1, DNastar, Inc., Madison, WI). Three reference sequences of subtype CFR11-cpx were included in the alignment. Gaps were stripped from the alignment using BioEdit (v.7.0.4.1, T.A. Hall, 1999, North Carolina State University, Raleigh, NC). Phylogenetic analysis was performed using PHYLIP (v. 3.67). Evolutionary distances were estimated with DNADIST (Kimura two-parameter method) and phylogenetic relationship were determined by NEIGHBOR (neighbour-joining method). Reproducibility of trees was evaluated using SEQBOOT (1000 replicates) and CONSENSE. Trees were constructed using FigTree. Programs were run with default parameters.

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In regards to the presentation of this patient, in April 2012 the initial clinical parameters showed a 4 logs level of HIV-RNA (12,090 copies/mL), a very low CD4+ cell count (35 cells/µL; 2.1%) and an A3 CDC clinical stage. We designed an initial HAART regimen including was abacavir (ABC)/lamivudine (3TC), darunavir (800 mg QD) and booster ritonavir (DRV/r) before the drug resistance results were available, due to the serious immunological status.

The resistance report showed a number of drug resistance mutations and possible resistance mutations to Nucleoside and Nucleotide RT Inhibitors and Non Nucleoside RT Inhibitors except to ABC, tenofovir (TDF), and efavirenz (EFV), which showed an intermediate resistance, and susceptible to all protease inhibitors (PIs) and integrase inhibitors (INIs), according to the Stanford algorithm. This mutation pattern included M41L, A62V, T69N, K70R, V75I/S, Y181I, M184V, T215F/S in the RT region, no relevant mutations were detected in the protease region according to the Stanford algorithm. The Geno2Pheno analysis of the gp120 V3 region showed a CXCR4 virus (FPR: 5.3). (Fig1)

After considering these results, the antiretroviral therapy was changed: ABC/3TC were substituted with raltegravir (RAL) 400 mg BID. The new treatment was accompanied by monthly clinical assessments and quarterly biological monitoring including CD4 T cells count and HIV-RNA quantification.

In June 2012, he was tested for HBsAg with a positive result, up to 125,000 copies/ml (68 CD4 cells/µL; 3.5%) . In July 2012, he had a viral load referred as HBV-DNA equal to 720,000 copies/mL (82 CD4 cells/µL; 3.0%) and tenofovir was added to the other antiretrovirals. In September 2012, the HBV-DNA decreased to 139,445 copies/mL (114 cells/µL; 4.4%). In November 2012, the HBsAg load was 65079,59 copies/mL and HBV-DNA was 19,604 copies/mL (154 CD4 cells/µL; 6.1%). In February 2013, HBV-DNA was 627 copies/mL (142 CD4 cells/µL; 8.4%).
Thus, thirteen months after starting the salvage regimen the patient’s clinical condition has been excellent without side effects; his current immuno-virological status shows CD4 302/µL (10.9%) and HIV-RNA <37 cp/mL.

For phylogenetics analysis, the 2010 HIV-1/SIVcpz complete genome alignment was downloaded from the Los Alamos HIV sequence database. (2) The alignment was modified by restricting it to group M subtypes and CRF 11cpx. Patient’s sequence was added to this alignment and adjusted manually, then gap-striped using BioEdit. Phylogenetic analysis showed that patient’s sequence branched with the CFR11-cpx with a bootstrap value of 99%. Horizontal branch lengths were drawn to scale with the bar at the bottom indicating 0.02 nucleotide substitution per site. (Fig. 2) Bootscan analysis using SimPlot (v. 3.5, S.C. Ray, John Hopkins University, Baltimore, MD) was performed to compare the overall genomic composition of patient’s sequence to consensus sequences for group M reference strains (A1, A2, A3, A4, B, C, D, F1, F2, G, H, J, and K) and the three consensus sequences of CRF11-cpx. The patient’s sequence branched with CRF11cpx consensus sequences.

Our results indicate that the salvage treatment was successful in this patient despite his background of resistance to almost all NRTIs and NNRTIs.

As for the DRV/r plus RAL combination, Akil Jackson et al. carried out a study which analyzed DRV/r and RAL plasma and intracellular concentrations in HIV-infected individuals during co-administration. In this study, HIV-infected patients on antiretroviral therapy received RAL 400 mg twice daily for 21 days; DRV/r 800/100 mg once daily was added for 14 days and patients were randomized to continue RAL twice daily or to switch to 800 mg once daily, then they all stopped RAL intake and continued DRV/r once daily for 14 days. Drug concentrations in plasma and peripheral blood mononuclear cells were measured. No remarkable interactions between DRV/r and RAL in plasma or cells were seen. Raltegravir intracellular concentrations were assessed for the once-daily 800 mg dose for the first time. (11) More recently, once-daily DRV/r
has received approval in treatment-naïve patients and represents an excellent option for first-line therapy. (12)

In regards to the addition of TDF, its administration in HIV/HBV-coinfected patients for up to 6 years led to a significant decrease in HBsAg in the HBeAg-positive population. Early HBsAg kinetics during treatment were predictive of HBsAg seroclearance and correlated with an increased CD4 cell count, underlining the importance of immune restoration in HBV clearance. (13)

Our patient’s was from the Central African Republic. First data on HIV-1 resistance mutations to antiretroviral drugs in Central African Republic was performed by Moussa et al. In this study, they present sequence data of non-B HIV-1 isolates obtained in 2008-2009 from Central African patients failing a stavudine (d4T)/zidovudine (AZT)-3TC- nevirapine (NVP)/EFV regimen, together with two patients at failure of a regimen, including a protease inhibitor (indinavir, IDV) The resistance mutations observed are those which are expected in HIV-1 subtype B. These data confirm the high diversity of HIV-1, which reflects the different non-B subtypes/CRFs and the various drugs used and do not provide evidence of new mutation profiles compared to subtype B. (14)

Notwithstanding the difficult virological presentation, complicated by HBV co-infection, we designed an effective treatment combination that included RAL+DRV/r+TDF with the aid of precise molecular tools. Our patient presented a sustained immunological and virological response overcoming difficulties posed by a complex CXCR4-tropic CRF11-cpx virus.

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254x190mm (96 x 96 DPI)
**Fig. 1** Profile of Drug Resistance Mutations at Salvage Therapy Initiation.

**Fig. 2** Neighbour joining tree of 2010 HIV-1/SIVcpz complete genome and the patient’s sequence (pt_6337257) that are marked. Bootstrap support is shown for key nodes. Horizontal branch lengths are drawn to scale with the bar at the bottom indicating 0.02 nucleotide substitution per site.
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Dear Editor,

we submit a revised version of the manuscript entitled “FAVORABLE THERAPEUTIC RESPONSE WITH AN ANTIRETROVIRAL SALVAGE REGIMEN IN A HIV-1 POSITIVE SUBJECT INFECTED WITH A CRF11-CPX VIRUS”. We have responded to the queries posed by the reviewer as described in the e-mail of October 28.

1) It is not clear how the authors concluded that the majority of virions in this patient use the CXCR4 coreceptor. Most HIV-1 isolates use the CCR5 coreceptor, and there is some correlation between V3 sequence and coreceptor use, but the sequence with accession number KF381391 has so many "R", "Y" and other ambiguity codes in it, that it is not possible for the Geno2Pheno or similar programs to make an accurate prediction of coreceptor use.

To determine the virus tropism we used a FPR significance level of 10%, according to the Recommendations from the European Consensus Group on clinical management of HIV-1 tropism testing (LPR Vandekerckhove et al.; European guidelines on the clinical management of HIV-1 tropism testing; The Lancet Infectious Diseases, Volume 11, Issue 5, Pages 394 - 407, May 2011), while the reviewer used a FPR cut-off of 1%: for this reason we concluded that patient harbours a CXCR4-tropic virus. Moreover, the reviewer changed the sequence with accession number KF381391: all ambiguity codes were substituted with N code (as reported in the geno2pheno report attached by the reviewers). These changes resulted in several aminoacids substitutions, that affected and worsened the geno2pheno interpretation. Ambiguity are usually present in HIV sequences (especially in the gp120 hypervariable domains), owing to the high heterogeneity and variability of viral population. In our opinion, we would not modify the referenced sequence, thus introducing aminoacids changes not present in our original sequence and indeed in the sample (i.e.: in our sequence the 11th codon is AGY, coding for Ser for both AGT and AGC; in the reviewers sequence at the same position it is reported a AGN codon, that could encode Ser for AGT and AGC and Arg for AGA and AGG).

2) The drug resistance profile reported for this patient does not match the results I get using the Stanford drug resistance database (http://sierra2.stanford.edu/sierra/servlet/JSierra?action=sequenceInput):

KF381391 result:
NNRTI Resistance Mutations: Y181I
Nucleoside RTI
lamivudine (3TC) High-level resistance
abacavir (ABC) High-level resistance
zidovudine (AZT) High-level resistance
stavudine (D4T) High-level resistance
didanosine (DDI) High-level resistance
emtricitabine (FTC) High-level resistance
tenofovir (TDF) Intermediate resistance

Non-Nucleoside RTI
efavirenz (EFV) Intermediate resistance
etravirine (ETR) High-level resistance
nevirapine (NVP) High-level resistance
rilpivirine (RPV) High-level resistance

Our drug resistance profile reported for this patient was carried out in May 2013, with Stanford version 6.2.0 that has been active from 29.05.2012 up to 05.06.2013. After this version, there was the version 6.3.0 up to 19.09.2013; and then until today the version 6.3.1 (that I think you have used). This explains the slightly different profiles that we got.

3) Please carefully check for typos in the paper. For example, please change "...41-year-old men, was evaluated from 2007 to 2010 in Bangui, Central African Repubblic, where he performed..." to "...41-year-old man, was evaluated from 2007 to 2010 in Bangui, Central African Republic, where he was tested with...

We agree with the reviewer.

4) It is very highly unusual for a HIV-seronegative man (the paper says he repeatedly tested negative, with just one positive test: ELISA only, not western blot?? in 2007?) to harbor an X4 drug resistant virus. Was the 2007 ELISA very strongly positive? What was the reason for this man being repeatedly tested? What is his risk for infection?

The transmission of resistant variants is well documented: for this reason all guidelines suggest to test patients before starting therapy to identify drug resistance transmission. On CXCR4 variants transmission there are not confirmed data yet, but, in contrast with previous studies, recent reports suggest that CXCR4 viruses are likely to be transmitted as well (reviewed in: C. Hedskog et al.; Transmission of the X4 Phenotype of HIV-1: Is There Evidence Against the ‘‘Random Transmission’’ Hypothesis?; JID 2012, 205:163-165).

In 2007, our patient was tested only for HIV-1 Ab Elisa in Bangui, Central African Republic, whereby we have only the qualitative data (positive / negative), that is positive according to the Abbott Elisa test. We do not have a clear explanation for the repetition of tests, we can only say that both tests resulted negative in 2009 and 2010. His risk factors for HIV-1 infection were blood transfusions and unprotected heterosexual intercourse.
5) In the paragraph pointed out in comment 3 above, the paper says the patient had only one positive test, in 2007, never another. But on the next page the paper says that the patient has a positive ELISA and western blot in 2012. Combining the statements into one paragraph explaining that the patient had negative results in Bangui but positive tests in Milan might reduce confusion. We agree with the reviewer.

6) The authors should use the "Subtype Reference Alignment" with nonrecombinants plus CRF11_cpx sequences. The paper states that the complete genome alignment was used, but there are hundreds of complete genomes in the LANL HIV genome alignment, and a very few have been carefully chosen for "references".  
http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html

The phylogenetic tree is acceptable as it is, this paper is not primarily about the phylogeny, so just clarifying that the subtype reference alignment was used, should be sufficient. We agree with the reviewer.

Best wishes,
Pamela Tau and Stefano Rusconi