Abstracts presented at the
International Workshop on HIV & Hepatitis
Virus Drug Resistance and Curative Strategies
June 5–9 2012, Melia Sitges, Sitges, Spain
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Plenary abstracts
ABSTRACT P1
Antiviral Therapy 2012; 17 Suppl 1:A3
Eradication of HBV: can we eliminate cccDNA?
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Persistent suppression of HBV replication and control of HBV-related liver disease can be achieved in >95% of chronic carriers treated with third generation highly potent and high resistant barrier nucleotide analogues. Eradication of HBV would, however, require the complete elimination of the nuclear replicative intermediate HBV cccDNA from patient livers. The HBV cccDNA is the template for transcription of all viral mRNAs and it accumulates in the nucleus of infected cells as a stable episome organized into minichromosomes by histones and non-histone viral and cellular proteins. The accumulation and the maintenance of the cccDNA pool is ensured in infected hepatocytes by newly synthesized nucleocapsids which are not enveloped and secreted into the blood, but are transported into the nucleus. In quiescent cells the cccDNA is very stable and can persist throughout the life span of the hepatocytes without affecting its viability. A reduction of the cccDNA pool in the infected liver can be achieved either by cell death or through cccDNA dilution during cell division. Very low levels of cccDNA can persist indefinitely, possibly explaining lifelong immune responses to HBV despite clinical resolution of HBV infection. As HBV polymerase inhibitors do not directly affect the cccDNA, a decrease in cccDNA levels in patients treated with nucleos(t)ide analogues is supposed to derive from the lack of sufficient recycling of viral nucleocapsids to the nucleus, due to the strong inhibition of viral DNA synthesis in the cytoplasm, and less incoming viruses from the blood. Under this scenario, cccDNA depletion requires many years of nucleos(t)ide drug administration. Loss of HBsAg is thought to reflect cccDNA depletion and/or inactivation. Only a simultaneous treatment with antiviral agents acting through different mechanisms or capable of affecting cccDNA stability will hopefully lead to the eradicating of the cccDNA from the liver and achieve clearance of HBV infection.

We have developed a chromatin immuno-precipitation (ChIP)-based quantitative technique to study the recruitment in vivo of cellular and viral proteins onto the HBV minichromosome. Using an anti-acetylated-H3 or -H4 cccDNA ChIP assay we found that HBV replication is regulated, both in HBV replicating cells and in the liver of HBV chronically infected patients by the acetylation status of H3/H4 histones bound to the viral cccDNA. Accordingly, cellular chromatin modifying enzymes (cellular histone acetyltransferases [HATs], histone deacetylases [HDACs] and histone methyltransferases [HMTs]) are recruited in vivo onto the cccDNA. We also found that the HBx regulatory protein produced in HBV-replicating cells is recruited onto the cccDNA minichromosome to prevent cccDNA-bound histones deacetylation and to increase pgRNA transcription and HBV replication. We have also shown that IL6 affects HBV replication by inhibiting viral transcription without affecting cccDNA levels. IL6 treatment upregulates miR-34a and miR24 levels, which in turn target HNF1α and HNF4α transcripts. As a result, IL6 prevents HNF1α and HNF4α recruitment onto the HBV cccDNA and inhibits cccDNA transcription and viral replication. Finally, we found that IFN-α treatment leads to a rapid and strong decrease in both cccDNA-bound histone acetylation and pgRNA transcription from cccDNA that are paralleled by the recruitment of the transcriptional corepressors HDAC1, Hsir1, YY1 and the Polycomb protein Ezh2. These observations provide a molecular mechanism for IFN-α repression of HBV transcription and identify Ezh2 as a novel target for persistent HBV suppression. Altogether our results support the existence of a complex network of epigenetic events that influence cccDNA function and HBV replication but none of the pathways investigated, so far, directly affects cccDNA stability and half-life.
ABSTRACT P2

Antiviral Therapy 2012; 17 Suppl 1:A4

Resistance to direct antiviral agents in hepatitis C treatment: importance now and in the future

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HCV replicates with a short half-life and high viral turnover. The non-proofreading HCV NS5B RNA-dependent RNA-polymerase hereby generates a large number of closely related but different viral isolates (termed quasispecies). HCV NS3/4A protease inhibitors boceprevir and telaprevir have been approved for treatment of chronic hepatitis C genotype 1 infected patients. Both compounds showed a medium-to-strong antiviral activity in Phase I clinical studies but cannot be used as monotherapies because of a rapid selection of pre-existent resistant variants followed by viral breakthrough.

At baseline, dominant variants associated with resistance to HCV protease inhibitors telaprevir and boceprevir are rarely detectable in treatment-naive patients (<1%) and the presence of such variants does not exclude virological response to triple-therapy. This is explained by a lack of cross-resistance of these variants to pegylated interferon-α/ribavirin. Thus, resistance analysis at baseline is not required for the current standard of care.

Overall, the likeliness of virological on-treatment failure is associated with the individual sensitivity to pegylated interferon/ribavirin. As the majority of treatment-naive patients is sensitive to interferon-based treatment, treatment failure on triple-therapy has to be expected in only approx. 10% while in previous null-responders more than 50% will fail to respond to triple-therapy. Stopping rules are important because continuous dosing of protease inhibitors in the presence of persistent viral replication should be strongly avoided, as this might induce selection of compensatory mutations with enhanced replicative fitness and long half-life.

Mutations within the NS3 protease associated with resistance to boceprevir and telaprevir are largely overlapping (positions V36, T54, V55, R155, A156 and V170 of the NS3 protease) and this excludes a switch between the two drugs in case of viral breakthrough.

After the end of treatment, the frequency of resistant variants within the viral quasispecies in patients with treatment failure declines. After approx. 2 years in the vast majority of patients (>80%) no resistant variants are detectable anymore by population-based sequencing. Whether residual variants may influence virological response to future direct antiviral therapies has to be explored in future studies.

For future triple therapies with one direct antiviral agent (DAA), together with pegylated interferon and ribavirin, the probabilities of pre-existing variants conferring resistance in certain HCV isolates, subtypes or genotypes may vary. Data of Phase I/IIa studies show that NS5A and non-nucleoside NS5B inhibitors show significant differences for the barrier to resistance already in different HCV subtypes while pre-existing resistant variants to nucleoside analogue inhibitors of the NS5B polymerase are rarely detectable. For several next wave NS3 protease inhibitors (that is, simeprevir) a common variant in HCV subtype 1a isolates (Q80K) may significantly influence rates of viral breakthrough and sustained virological response.

Finally, for interferon-free all oral regimens most likely pre-existing resistant variants will influence treatment outcome. Thus, resistance testing may be of importance in the future for selection of the most effective interferon-free treatment option. Interestingly, available data also point to an importance of IL28B genotype for virological response in interferon-free treatment regimens.
ABSTRACT P3

Antiviral Therapy 2012; 17 Suppl 1:A5

Finding a cure for HIV - can it ever be achieved?

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Combination antiretroviral therapy (cART) has led to a major reduction in HIV-related mortality and morbidity, but HIV can still not be cured and treatment must continue lifelong. Finding a cure for HIV has become a top research priority given the significant costs of maintaining lifelong treatment for all who need it. In addition, ongoing morbidity persists in patients on long-term ART.

There are multiple significant barriers to achieving a cure for HIV. These include persistence of long-lived latently infected cells, low level persistent virus replication and anatomical reservoirs. In addition, residual immune activation and impaired penetration of antiretrovirals in tissue compartments may play a role.

Achieving either a functional cure (long-term control of HIV in the absence of cART) or a sterilizing cure (elimination of all HIV-infected cells) remains a major challenge. Several studies have demonstrated that treatment intensification with additional antiretrovirals has little impact on latent reservoirs. Very early initiation of cART reduces the number of latently infected cells and there have been infrequent reports of control of viral replication after stopping cART (a functional cure) in this setting, although this is rare. An alternative approach is to use agents that reverse latency in vitro when used either alone or in combination. A proof of concept trial of vorinostat, an HDACi that activates latent HIV was recently shown in a small observational study to be well tolerated and to induce virus production in resting CD4+ T-cells. Further clinical trials of vorinostat, other HDACi, IL-7 and disulfiram are currently underway. Gene therapy with zinc finger nucleases has been shown to effectively reduce expression of the HIV coreceptor CCR5 in both mouse models and human studies. Interventions that reduce immune activation and/or boost HIV-specific immunity may also play a role. It is likely that a combination strategy will be required.

Clinical trials that test strategies for HIV eradication pose multiple unique challenges. These include the need for more accurate and standardized assays to quantify persistent virus in patients on cART; identification of the most appropriate clinical end points for eradication studies, including the role of treatment interruption; very careful consideration of the risk benefit for any intervention given that most patients have an excellent quality of life on cART; and finally ensuring that any approach to cure can ultimately be scaled up and delivered in low-income settings.
Oral abstracts
ABSTRACT 1

Identification and profiling of a novel HIV nucleotide competing RT inhibitor series

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OBJECTIVES: Several groups have recently reported on the identification of nucleotide competing reverse transcriptase inhibitors (NcRTIs), a new class of RT inhibitors. NcRTIs reversibly inhibit binding of the incoming nucleotide to the RT active site but do not act as chain terminators, unlike the nucleos(t)ide reverse transcriptase inhibitors (NRTIs) class. Our objective was to identify, optimize and characterize a novel NcRTI chemical series which combines increased potency over previously reported NcRTI (Tibotec/Valeant) with minimal NRTI or NNRTI cross-resistance.

METHODS: Through high throughput screening (primer extension assay), we identified a novel benzo[4,5]furo[3,2,d]pyrimidin-2-one NcRTI chemical series. SAR evaluation of this series using both RT and viral replication assays led to the identification of Compound A, a potent NcRTI. The activity of Compound A against wild-type viruses and site-directed mutants encoding NRTI- and NNRTI-resistance mutations was assessed. Viruses resistant to Compound A were selected by in vitro passage.

SUMMARY OF RESULTS: Compound A potently inhibits HIV-1 RT in a primer extension assay (IC₅₀=2.6 nM) but has no measurable activity against human DNA polymerase γ (selectivity >4,000-fold). It potently inhibits HIV-1 replication in vitro (EC₅₀=1.5 nM), which is 54-fold more potent than previously described NcRTI-1 (EC₅₀=81 nM). Notably, viruses encoding K65R are hypersusceptible (sevenfold) to inhibition by Compound A. It retains activity against viruses encoding either M184V or M41L/D67N/K70R/T215F/K219E, with fold-change (FC) values of 1 in both cases. The antiviral potency of Compound A was also unaffected by the presence of NNRTI mutations tested (K103N/Y181C, L100I, V106A or Y188L). In vitro viral resistance studies performed with Compound A led to the selection of a single RT mutation: W153L. A recombinant virus encoding the W153L RT mutation was highly resistant to Compound A (FC>140). W153 is a highly conserved residue in HIV RT and not previously associated with drug resistance.

CONCLUSIONS: We discovered a novel NcRTI series with optimized antiviral activity, minimal cross-resistance to existing RT inhibitor classes and a distinct resistance profile. Our results further establish NcRTIs as an emerging RT inhibitor class which may provide novel treatment options for HIV patients.
ABSTRACT 2

*Antiviral Therapy* 2012; 17 Suppl 1:A10

**The in vitro cross-resistance profile of the NRTI BMS-986001 against known NRTI resistance mutations**

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**BACKGROUND:** BMS-986001 (4ʹ-ED4T) is a thymidine analogue currently in Phase IIb clinical trials. However, little is known about the resistance and cross-resistance profile of BMS-986001.

**METHODS:** A replication competent proviral clone containing a *Renilla* luciferase gene was used to make site-directed mutants in RT. Viruses were generated by transfection of 293T cells and drug susceptibility to BMS-986001 and other NRTIs (TDF, ABC, d4T, AZT and FTC) were determined in MT-2 cells. Drug susceptibility (fold change versus wild-type virus) was calculated for each experiment and averaged. In addition, RT genes from 19 clinical isolates with various RT mutations were examined in the Monogram Phenosense® HIV assay.

**RESULTS:** M184V- and P119S/T165R/M184V-containing viruses (reportedly selected *in vitro* by BMS-986001) had modest decreases in susceptibility (two- and fivefold) to BMS-986001. BMS-986001 virus was hypersusceptible (0.43-fold change) to the K65R site-directed mutant and exhibited good coverage of L74V (0.65-fold change), while addition of M184V to either virus reverted these to near wild-type levels. Thymidine analogue mutation pattern 1 (TAM1) pathway viruses (including M41L, L210W, T215Y) exhibited increasing fold changes with increasing numbers of mutations, while TAM2 pathway viruses (including D67N, K70R, T215F) exhibited a 6–8-fold change, regardless of the number of TAMs. BMS-986001 also exhibited hypersusceptibility to Q151M containing viruses (0.17–1.24-fold changes, increasing with increasing numbers of mutations), while the addition of M184V to this constellation (A62V/V75I/F77L/F116Y/Q151M/M184V) reduced susceptibility (>40 FC). Finally, the T69SS/T210W/K215Y virus exhibited a high fold change to all NRTIs. Clinical isolates with various NRTI resistance mutations examined in the Monogram assay exhibited concordance with the site-specific mutant data.

**CONCLUSIONS:** BMS-986001 appears to offer a unique susceptibility profile to known NRTI resistance mutations. Hypersusceptibility was observed for K65R, Q151M (without M184) and possibly L74V viruses. High fold changes were observed with multiple TAM1 mutations, T69SSS and a Q151M virus that also contained M184V, while relatively low fold changes were observed for most other viruses, including most M184V-containing viruses. Clinical data from trials of subjects with NRTI resistance mutations will be needed to determine the clinically relevant resistance threshold.
ABSTRACT 3

Antiviral Therapy 2012; 17 Suppl 1:A11

Effect of primary elvitegravir resistance mutations in HIV-1 integrase on drug susceptibility and viral replicative fitness

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BACKGROUND: Elvitegravir (EVG) is a potent HIV-1 integrase strand transfer inhibitor (INSTI) currently undergoing advanced clinical development. Six primary INSTI resistance mutations have been identified in treatment-naive and -experienced patients failing regimens containing EVG: T66I/A/K, E92Q/G, T97A, S147G, Q148R/H/K, N155H. Virological failure may involve a mixture of 1–4 primary mutations. In this study we have characterized the impact of single and multiple primary mutations on susceptibility to the INSTIs (EVG, raltegravir [RAL] and dolutegravir [DTG]), integrase enzyme activities and viral replication fitness.

METHODS: Site-directed mutants with primary INSTI resistance were constructed. Drug susceptibilities were determined in acutely infected MT-2 cells using 5-day cell viability and PhenoSense IN assays (Monogram Biosciences). HIV-1 replicative fitness was evaluated in growth competition experiments using allele-specific real-time RT-PCR. INSTI susceptibilities and activities (3’end processing and strand transfer) of integrase proteins were evaluated using a homogenous time-resolved FRET (HTRF) assay.

RESULTS: Viruses containing primary INSTI mutations observed in EVG clinical trials exhibited a range of reduced susceptibility to EVG: Q148R (108-fold) >E92Q>N155H (35–39-fold) >T66I (15-fold) >S147G (10-fold) >T97A (2-fold), which were confirmed in biochemical assays. These residues mapped to the structural space of the metal chelating core and halobenzyl groups of EVG. Less commonly observed mutations also yielded reduced EVG susceptibilities: T66K, E92G and Q148K (~40–50-fold) and T66A and Q148H (~5–10-fold). Some primary mutations conferred resistance to EVG but retained susceptibility to RAL and DTG (T66I/A, E92G, S147G), while others reduced susceptibility to both EVG and RAL, but not DTG (T66K, E92Q, Q148R/H/K, N155H). Nearly all primary mutations exhibited significantly reduced viral fitness: WT ≈ T66I ≈ S147G > E92Q > N155H > Q148R/K/H.

Viruses containing two primary mutations displayed larger reductions in EVG and RAL susceptibilities and fitness than viruses containing a single mutation.

CONCLUSIONS: EVG primary mutations with reduced susceptibility to EVG have reduced viral replication fitness. There is broad cross-resistance between EVG and RAL primary mutations, but susceptibility to DTG remains in most cases. While multiple primary mutations confer greater EVG resistance, further reduced viral fitness may force mutual exclusion and genotypic switching between resistance pathways.

ACKNOWLEDGEMENTS: Gregg Jones, Manuel Tsiang, Elaine Kan, Dharmaraj Samuel, Magdeleine Hung, Debi Jin, Xiaohong Liu.
ABSTRACT 4
Antiviral Therapy 2012; 17 Suppl 1:A12
Integrated analysis of emergent drug resistance from the HIV-1 Phase 3 QUAD studies through week 48
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BACKGROUND: Two Phase 3 studies of QUAD (elvitegravir [EVG]/cobicistat/emtricitabine [FTC]/tenofovir disoproxil fumarate [TDF]) in treatment-naive subjects are ongoing (GS-US-236-0102 and 236-0103). The week 48 responses were robust and non-inferior for QUAD versus efavirenz ([EFV]/FTC/TDF; ATR; 88% versus 84%) and QUAD versus ritonavir-boosted atazanavir (ATV/r+FTC/TDF (ATV/r+TDF; 90% versus 87%). Resistance analyses through week 48 are presented.

METHODS: Genotypic analyses of HIV-1 protease (PR) and reverse transcriptase (RT) were performed at screening (GeneSeq, Monogram Biosciences); subjects with resistance to study drugs were excluded. Subjects in the post-baseline resistance analysis population had genotypic/phenotypic analyses at failure and baseline for PR, RT and integrase (IN) using PhenoSense GT, IN GeneSeq, IN PhenoSense (Monogram), or IN GenoSure (Labcorp).

RESULTS: Of 1,408 randomized and treated subjects in the two studies, 94.5% had HIV-1 subtype B. At baseline for QUAD subjects, all were sensitive to FTC/TDF but pre-existing primary resistance mutations were found to NNRTIs (14%; 95/701), PIs (2.6%; 18/701), and NRTIs (7.4%; 52/701) and their presence did not lower treatment response. In the QUAD group through week 48, resistance development was analysed for 26 subjects (3.7%; 26/701), with data available for 25. None had IN resistance at baseline. Thirteen (1.9%; 13/701) developed primary IN or NRTI resistance mutations (IN T66I [N=2], E92Q [N=8], Q148R [N=3] and N155H [N=3]; RT M184V/I [N=12], K65R [N=4]) and phenotypic resistance to ≥1 component of QUAD. In the ATR group, 17 subjects were analysed (4.8%; 17/352). Seven (2.0%; 7/352) developed RT resistance to ≥1 component of ATR, most commonly K103N [N=7] and M184V/I plus K65R (N=2). In the ATV/r+TVD group, 8 subjects were analysed (2.3%; 8/355) and none developed resistance. At virological failure within the QUAD group, the mean fold change in susceptibility to EVG for the 11 subjects with EVG resistance mutations was >67-fold, and all were cross-resistant to raltegravir. Eight subjects with follow-up data who developed QUAD resistance initiated standard 2nd line regimens.

CONCLUSIONS: Treatment with QUAD achieved high rates of virological suppression in HIV-1 treatment-naive subjects. Resistance development to ≥1 components of QUAD occurred infrequently (1.9% of treated subjects). The primary emergent HIV-1 integrase resistance mutations in the QUAD group were T66I, E92Q, Q148R and N155H and had cross-resistance to raltegravir.
ABSTRACT 5

Antiviral Therapy 2012; 17 Suppl 1:A13

Dolutegravir treatment of HIV subjects with raltegravir resistance: integrase resistance evolution in cohort II of the VIKING study

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BACKGROUND: The integrase inhibitor (INI) dolutegravir (DTG) has low fold change (FC) in IC50 against HIV with raltegravir (RAL)-resistant Y143 and N155 pathway and Q148H/K/R single mutants; FC generally increases for Q148H/K/R as RAL-associated mutations increase. The VIKING study enrolled two sequential cohorts of ARV-experienced adults harbouring RAL-resistant HIV. Subjects received DTG 50 mg once daily (cohort I) or twice daily (BID; cohort II). Cohort II also required at least one fully active drug (PSS ≥ 1) in the optimized background regimen (OBR).

METHODS: Integrase genotypic and phenotypic results, minor variant analyses and plasma HIV-1 RNA were assessed.

RESULTS: A broad range of RAL-resistant genotypes and DTG FC was observed in the 24 cohort II subjects. Through week 24, 75% of subjects achieved <50 c/ml HIV-1 RNA, and 5/24 (21%) subjects experienced VF (Pt5 at day 11, Pt2 and Pt3 at week 8, Pt4 and Pt1 at week 16). Three of the 24 subjects, all with virus having Q148 + ≥1 additional integrase resistance-associated mutations at day 1, had treatment-emergent integrase resistance-associated mutations and reduced DTG susceptibility at the time of VF. All three subjects had initial HIV-1 RNA declines of 1–2 log10 c/ml with subsequent viral rebound. Virus from Pt1 added T97T/A, E138E/K, N155H with DTG FC increase from 6.23 to 93 (week 16). Virus from Pt2 added E92E/Q, T97T/A, with DTG FC increase from 6.04 to 42 (week 8). Virus from Pt3 added E138E/K, N155H with DTG increase from 4.11 to 63 (week 8). Minority species and evolutionary analyses revealed that small day 1 cluster(s) acquired additional linked integrase resistance-associated mutations and expanded as a majority of the VF population.

CONCLUSION: Few ARV-experienced subjects with RAL resistance in VIKING cohort II experience VF while receiving DTG 50 mg BID and OBR that includes at least one active drug. Treatment-emergent IN resistance with an increase in DTG FC was detected in only three subjects all harbouring virus with Q148 plus ≥3 additional integrase resistance-associated mutations at VF. These results support DTG 50 mg BID use for a larger ongoing Phase III study (VIKING-3) in this patient population.
ABSTRACT 6

Antiviral Therapy 2012; 17 Suppl 1:A14

Genotypic and phenotypic correlates of virological response to the attachment inhibitor BMS-626529 in a short-term monotherapy study with its prodrug BMS-663068

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BACKGROUND: Administered as monotherapy for 8 days, BMS-663068, the prodrug of the attachment inhibitor BMS-626529, demonstrated significant reductions in plasma HIV-1 RNA. Although baseline IC50 >100 nM to BMS-626529 correlated with a poor virological response, BMS-663068 did not appear to select for BMS-626529 resistance on population sequencing or phenotyping. This report describes the genotypic correlates of phenotypic BMS-626529 susceptibility in the non-responders in this study through population and clonal analyses.

METHODS: Genotypic population analyses of baseline samples from non-responders identified amino acid changes that could potentially encode for reduced susceptibility to BMS-626529. Reverse genetics of functional envelope clones confirmed changes responsible for this in phenotypic assays. Additional genotypic, phenotypic and reverse genetic assays were performed on samples from responders to probe the context dependence of the identified substitutions.

RESULTS: The gp120 M426L substitution was the major change associated with reduced virological response (present in 5 of 6 non-responders) and high BMS-626529 IC50 (present in virus from 6 of 7 subjects with IC50 >100 nM). The remaining non-responder virus sample contained an S375M substitution that encoded reduced susceptibility. However, the M426L substitution was also identified in two responders, one with reduced susceptibility (IC50 6,300 nM) and another with low IC50 (38 nM). A series of functional clones from 4 samples (including 2 responders with resistance mutations on population genotyping) were analysed for susceptibility to BMS-626529. Variability of susceptibility of clones (37–246-fold) was higher than variability observed with other entry inhibitors (enfuvirtide, 6–9-fold; maraviroc, 3–9-fold). In the responder subject with M246L, all functional clones contained M426L and susceptibility varied by 246-fold, suggesting that susceptibility is highly context dependent. One of the responders contained viruses of either tropism. Clones of R5- or X4-tropic viruses from this individual exhibited the same variable range of susceptibility to BMS-626529.

CONCLUSIONS: gp120 substitutions M426L and S375M were found to be strongly, albeit not exclusively, associated with low susceptibility to BMS-626529 and a lack of virological response to its prodrug, BMS-663068. Functional clones derived from single individuals exhibited 2–3 log10 variability in susceptibility to the agent, regardless of tropism, suggesting that susceptibility can be highly context dependent.
ABSTRACT 7

Antiviral Therapy 2012; 17 Suppl 1:A15

Molecular and structural analysis of darunavir-resistant HIV-1 protease

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BACKGROUND: Darunavir (DRV) is the latest HIV-1 protease inhibitor (PI) with potent antiviral activity and a high genetic barrier to acquiring drug resistance. However, DRV appears to be more vulnerable to resistance acquisition when used for salvage therapy in PI-failure cases. To better understand this phenomenon, we analysed the molecular and structural mechanisms of DRV resistance.

METHODS: PI-failure case was selected from clinical samples sent to our centre for regular drug-resistance testing. The virus was isolated by co-culture method, followed by DRV-resistance selection by increasing the DRV concentration from 2 to 1,000 nM over 154 days. The baseline protease (PR) sequence of the isolated virus showed L10I/L33F/M46I/F53L/I54V/Q58E/I62IV/L63P/H69R/A71V/G73S/V77I/V82F/L90M/I93L mutations in the protease region. The resistance level of the baseline and DRV-selected isolates were examined by an in-house drug susceptibility assay with the R5-MaRBLE cell line. For more detailed analysis of PI resistant mutations, the gag-pro region of the isolates were amplified and inserted into a Δgag-pro NL4-3 clone. We also crystallized an inactive form of the DRV-resistant PR to obtain its detailed structure. The binding mode of the PR with DRV was analysed by molecular dynamics simulation.

RESULTS: The DRV-resistant virus isolate harvested on day 154 showed 55.5 times higher IC₅₀ for DRV (0.42 nM) than the wild-type NL4-3 clone (0.0075 nM). The sequence of the isolate revealed that 5 additional mutations, V11I, V32I, I47V, I50V and L89V, were selected during culture. We obtained two X-ray crystal structures of the inactive D25N mutant of this DRV-resistant PR, each of which were determined at 1.8 and 2.0 Å resolution in the substrate-free state. The structures showed that the DRV-resistant PR had a conformation similar to that of the open form, whereas wild-type PR is known to have a semi-open form. The simulations also suggest that the DRV-resistant PR bound to DRV would have a flap conformation distinct from that of the wild-type PR bound to DRV.

CONCLUSION: The structural characteristics of the flap in the DRV-resistant PR would influence its resistance to DRV.
ABSTRACT 8

*Meeviral Therapy* 2012; 17 Suppl 1:A16

Mechanism of substrate binding revealed by the N-terminal product complexes of HIV-1 protease

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BACKGROUND: The 13 known substrate cleavage sites of HIV-1 protease share little sequence identity and lack an obvious consensus binding motif. The factors governing this substrate recognition and specificity are poorly understood. And how this specificity is preferentially maintained over inhibitors in drug resistant protease is even harder to explain. While predicting accurately the local preference for a specific amino acid at a particular position in the cleavable substrate sequence, the N-terminal region (residues P3-P1) of substrates is suggested to initiate recognition. We investigated, structurally, the relative preference of N- or C-terminal substrate region in determining binding affinity to protease.

METHODS: To determine preferential affinity of HIV protease for N-terminal substrate sequences, we crystallized active HIV protease with decameric peptides representing viral substrate cleavage sites. Structural analysis was used to understand the structural implications of product versus substrate binding in protease dimer and Isothermal Titration Calorimetry (ITC) and gel filtration chromatography techniques were used to examine the biochemical basis of preferential N-terminal substrate affinity displayed by protease.

RESULTS: We determined seven crystal structures of active protease in complex with decameric peptide sequences corresponding to MA-CA, RH-IN, CA-p2, p1-p6, RT-RH, p2-NC and NC-p1 cleavage sites, respectively. Structural analysis revealed that these decameric peptides were cleaved by the active protease within the crystallization drop and protease crystallized with N-terminal product bound in the active site. The C-terminal product was either missing or disordered. Structural data demonstrates that protease exhibits binding preference for the N-terminal product sequence compared to the C-terminal sequence. The detailed biochemical analysis of N-terminal product binding to protease is currently underway.

CONCLUSIONS: These findings confirm the preferentially higher affinity of N-terminal substrate region in binding to the protease and this knowledge may assist in a more rational approach to inhibitor design to combat the rapid acquisition of resistance. Additionally, our results present a possibility of product inhibition during polyprotein turnover. Since polyprotein processing by protease is a highly regulated component of viral maturation cycle, our further interest is to biochemically investigate the possibility of product inhibition in this process.
ABSTRACT 9

Antiviral Therapy 2012; 17 Suppl 1:A17

Contribution of the gag gene to variation in susceptibility to protease inhibitors between different strains of HIV-1

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BACKGROUND: Resistance to HIV-1 protease inhibitors (PIs) develops by the accumulation of mutations in protease. Mutations in the cleavage site of Gag, the protease substrate, partially compensate for the loss of fitness caused by protease mutations. There is evidence that Gag may contribute directly to resistance, independently of compensating for loss of fitness. We have explored whether natural variation in Gag may contribute to differences in susceptibility of wild-type viruses to PIs.

METHODS: A single-cycle, phenotypic drug susceptibility assay was used to assess the contribution of Gag and protease to the PI susceptibility of six HIV subtype B molecular clones and four subtype B viruses from treatment-naive patients. Unique restriction sites were used for the subcloning of full-length gag-protease or gag and protease separately.

RESULTS: Significant differences in PI susceptibility between the viruses were observed with some strains displaying up to 17-fold decrease in PI susceptibility compared to a wild type control, with variation being seen with respect to six PIs, namely amprenavir, atazanavir, darunavir, lopinavir, saquinavir and tipranavir. For two molecular clones, JRFL and YU2, the reduced susceptibility of full-length Gag-protease was caused solely by the gag gene. In particular, the gag gene of the molecular clone YU2 was responsible for 14-fold, 8-fold and 7-fold reduced susceptibilities to amprenavir, atazanavir and lopinavir, respectively. In the treatment-naive patient viruses, a 17-fold and 16-fold decrease in atazanavir susceptibility of full-length Gag-protease was observed in two patient samples. In contrast to molecular clones, this variation was shown to be caused by protease, despite the absence of major protease resistance mutations and data showing a minor mutation did not play a significant role.

CONCLUSIONS: Variation in the gag gene of HIV-1 contributes to different susceptibilities to PIs. Full-length patient-derived Gag is not currently included in genotypic or phenotypic drug resistance testing, giving the potential for incomplete results. In addition, significant variation in the susceptibility to atazanavir of full-length Gag-protease derived from treatment-naive patient viruses merits further investigation, given the potential role of atazanavir as a first-line PI.
ABSTRACT 10

Antiviral Therapy 2012; 17 Suppl 1:A18

Structural modifications in HIV-1 RT induced by specific RT polymorphisms tightly modulate the first phase of viraemia decay to first line FTC+TDF-containing HAART

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BACKGROUND: We recently identified specific RT polymorphisms modulating virological response to FTC+TDF-containing HAART at week 24. Here, we specifically aim at defining their impact on kinetics of viraemia decay, and on molecular recognition between RT and these drugs.

METHODS: This study includes 373 HIV-1 B subtype-infected patients starting first-line FTC+TDF-based HAART (EFV: 221; LPV/r: 152) with genotypic test within the prior 6 months. No patients have transmitted resistance. Impact of RT mutations on viraemia decay and VS achievement (2 consecutive HIV-1 RNA <50 copies/ml) was assessed by logistic regression adjusted by patients’ demographics, baseline viraemia and CD4 count, EFV/LPV. Structural analysis was based on docking simulations.

RESULTS: The baseline presence of R211G (prevalence: 5.7%) and A98S (prevalence: 10.5%) significantly (P<0.05) correlates with a lower and higher proportion of patients achieving VS by week 24 than patients without these mutations (controls; R211G: 61.9%; A98S: 89.7%; controls: 75.1%), respectively. Multivariate model confirms such correlations (OR R211G: 0.17 [95% CI: 0.34–0.01]; OR A98S: 3.2 [95% CI: 1.0–11.6]; P<0.05).

Moreover, R211G and A98S correlate with a prolonged and faster viraemia decay from baseline to week 4 compared to controls, respectively (median [IQR] R211G: 2.5 [2.2–3.1] log₁₀ copies/ml; median [IQR] A98S: 1.9 [1.0–2.6] log₁₀ copies/ml; median [IQR] Controls: 2.3 [1.9–2.6] log₁₀ copies/ml, P=0.007), and with longer and shorter time to achieve VS (median [IQR] R211G: 16 [11–25] weeks; median [IQR] A98S: 13 [8–16] weeks; median [IQR] Controls: 15 [10–22] weeks, P=0.03), respectively. R211G also correlates with increased proportion of patients with persistent HIV-1 RNA>50 copies/ml at week 48 compared to controls (19.4% versus 6.1%, P=0.02). Results confirmed after adjusting for baseline viraemia.

Consistent with these results, the introduction of R211G in an RT-structural model determines a decreased affinity for TDF comparable or stronger than that observed in presence of TDF-resistance mutations L74V and K65R (WT: -0.88 kcal/mol; R211G: +0.31 kcal/mol; L74V: +0.02 kcal/mol; K65R: -0.37 kcal/mol).

Interestingly, R211G determines the loss of 2 hydrogen bonds between TDF and RT residue 65, reduces the number of crucial interactions with residue D185 involved in RT active site (11 for wild-type versus 1), Y115 (20 versus 0), and M184 (8 versus 0). Furthermore, R211G remarkably decreases the electrostatic energetic contribution, thus further destabilizing RT/TDF-interaction (WT elec: +0.07 kcal/mol; R211G elec: +0.28 kcal/mol).

Conversely, A98S significantly increases RT binding affinity for FTC (WT: -2.74 kcal/mol; A98S: -3.17 kcal/mol), by specifically increasing the number of FTC-interactions with D186 involved in RT active site (5 for wild-type versus 14), F160 (0 versus 4) and M184 (0 versus 2).

CONCLUSIONS: Structural modifications induced by two selected RT polymorphisms can modulate, positively or negatively, HIV-1 decay kinetics during FTC+TDF-based HAART. The knowledge of these mechanisms may be critical to set-up an optimal therapeutic approach aimed at achieving the maximal virological inhibition.
ABSTRACT 11

Antiviral Therapy 2012; 17 Suppl 1:A19

Transient secondary structure alterations in HIV-1 integrase as a mechanism for raltegravir resistance

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BACKGROUND: HIV-1 integrase is an essential enzyme necessary for the replication of HIV as it catalyzes the insertion of the viral genome into the host chromosome. Raltegravir (RAL) was the first integrase inhibitor approved by the FDA for antiretroviral treatment. Drug resistance mutations at residues 140 and 148 in the catalytic loop have been observed in patients who fail RAL. Mutations at 148 confer a 5–10-fold decrease in susceptibility to RAL.

METHODS: Obtaining crystallographic structure information on the Q148H/R, G140S/A primary and secondary mutations has been elusive. Using 10 ns molecular dynamics simulations, we present a detailed analysis of the structural changes induced by these mutations. The catalytic core domain crystal structure PDB entry 1BL3 was used to manually dock RAL into the active site of the integrase core. The initial temperature of the system was set to 70 K, and then increased to 310 K in 5 K increments. After energy minimization using the conjugate gradient method, MD was carried out for 10 nanoseconds using the CHARMM27 force field in a statistical ensemble accounting for a constant number of atoms, temperature and pressure (NPT). The RMSD trajectory, interaction energies and Ramachandran plots were calculated in VMD.

RESULTS: The formation frequency of a transient helix in the catalytic 140’s loop is increased and the length of this helix is extended from 3-residues to 4 in the mutants relative to the wild type. This helix causes reduced flexibility in the protein active site and serves as a gating mechanism restricting the access of RAL to the integrase binding pocket.

CONCLUSIONS: These results suggest that resistance to RAL occurs through a common mechanism of altering the formation frequency of transient secondary structures such as α2 and β5, in addition to the conformational changes, in the 140’s loop, therefore, decreasing the flexibility of the HIV-1 integrase protein. Changes in secondary structure elements are not only required for function but play an important role in the development of HIV integrase resistance to RAL and other INSTIs.

ACKNOWLEDGEMENTS: This work was generously supported by a grant (IISP 37125) from Merck Research Laboratories.
Combinations of primary and secondary integrase mutations in the VIKING Pilot Study: effects on and rationale for dolutegravir dissociation

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BACKGROUND: VIKING is a Phase Ib clinical trial of the integrase inhibitor (INI) dolutegravir (DTG, S/GSK1349572) for INI-experienced subjects harbouring raltegravir (RAL)-resistant HIV-1. To gain a better understanding of clinical responses based on integrase mutation accumulation and combinations, INI binding and molecular modelling studies were conducted with integrase proteins identified from VIKING; these included multiple secondary and RAL-associated signature resistance mutations.

METHODS: Multiple substitutions were introduced into wild-type HIV-1 integrase based on population genotypes observed during VIKING. Dissociation of [3H]-labelled DTG, RAL or elvitegravir (EVG) from integrase/DNA/bead complexes was monitored using unlabelled compound. Structural models of HIV-1 integrase/DNA, both wild-type and with select amino acid substitutions, were constructed from available crystallographic data on HIV-1 integrase and wild-type and mutant prototype foamy virus intasomes.

RESULTS: The addition of Y143H to G140S+Q148H integrase was not detrimental for DTG binding (dissociative $t_{1/2}$ of 3.3 h for G140S+Q148H versus 3.9 h +Y143H). DTG had a half-life of 2.3 h with integrase having substitutions at Y143R+N155H in conjunction with T97A; no impact on DTG dissociation was observed with additional substitutions at L74M or L74M+E138A. For G140S+Q148H, addition of a L74M or E138K substitution did not impact DTG dissociation relative to G140S+Q148H ($t_{1/2}$ of 3–3.3 h versus 3.3 h) but the accumulation of further substitutions (Q148H+3) at L74M, T97A or E138A did significantly impact DTG dissociation. Modelling suggests that E138A/K substitutions may play a role in disrupting the active-site loop, while the L74M and T97A substitutions may impact the configuration of the active site and the placement of the metal coordination complex. With all of the multi-substituted integrases, dissociation of RAL and EVG was fast ($t_{1/2}<1$ h) or binding was insufficient for analysis.

CONCLUSIONS: The presence of multiple integrase substitutions could cause significant disruption in the INI binding pocket thereby impacting development of INI resistance. The protracted DTG binding ($t_{1/2}>1$ h) observed with integrase/DNA complexes containing several RAL-associated signature and secondary integrase substitutions suggests that DTG can tolerate greater disruption in the integrase active site than RAL and EVG and that the accumulation of multiple RAL-associated mutations is required to break through DTG’s barrier to resistance.
**ABSTRACT 13**

*Antiviral Therapy* 2012; 17 Suppl 1:A21

Key patterns of mutations in HBV S open reading frame are involved in mechanisms underlying HBV-induced hepatocellular carcinoma *in vivo*

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**BACKGROUND:** HBV S-gene, encoding the large (LHBs), middle (MHBs) and small (HBsAg) surface glycoproteins, plays a critical role in mediating HBV-induced tumourigenesis. Nevertheless, little information is available regarding genetic determinants along the full-length S-gene correlated with HBV-induced hepatocellular carcinoma (*HCC*) *in vivo*.

**METHODS:** This study includes 67 HBV chronically infected patients (50 [74.6%] genotype D; 14 [20.9%] genotype A; and 3 [4.5%] others): 19 with HCC and 48 asymptomatic chronically infected patients as control. Mutations were defined according to the reference sequence of each specific HBV sub-genotype. Association of Pre-S1/S2 and S mutations with HCC was assessed by Fisher test with Benjamini–Hochberg for multiple comparison correction. Interactions among mutations were analysed by cluster analyses.

**RESULTS:** Patients with or without HCC had median (IQR) log serum HBV DNA of 3.8 (2.5–5.4) and 4.1 (3.2–6) UI/ml respectively. Pre-S1/S2 and S regions were successfully amplified for 17/19 (89.5%) and 16/19 (84.2%) HCC patients, and for 43/48 (89.6%) and 46/48 (95.8%) non-HCC patients, respectively.

Beyond the already known deletions, specific novel mutations in pre-S1 (A49Vsi-L97Iis1-S98Ts1), pre-S2 (F130Ls2) and HBsAg region (N40I, K141N, V177A, P203Q, S210R) significantly correlate with HCC *in vivo* (*P*=10^{-2} to 10^{-4}, after correction). 19/19 patients carry at least 1 of them (range prevalence: 18.8% to 52.9%), while they occur with a prevalence <2.5% (N40I,K141N,V177A,P203Q) or <15% (A49Vsi-L97Iis1-S98Ts1,F130Ls2,S210R) in non-HCC patients. Strong correlations (*P*=10^{-2} to 10^{-3}), observed only in HCC-patients, are found for L97Iis1,A49Vsi (phi=0.97) both localized in pre-S1 region, and for the HBsAg-mutations N40I,K141N, (phi=0.56), and P203Q,S210R (phi=0.83).

The correlated pair A49Vsi and L97Iis1 localize in the LHBs transactivation domain. In addition, L97Iis1 corresponds to the nucleotides 3172–3175 of HBV-genome, localized in S-gene promoter. Conversely, both N40I, and K141N, localize in specific HBsAg HLA-epitopes. The presence of N40I, in the HLA-epitope TSLNFLG-GTTCVLGQ (aa: 37–51) correlates with an increased dissociation score (N40I: 1.762; wild-type: 0.205), suggesting a decreased affinity in the epitope/HLA molecule complex.

Finally, both P203Q, and S210R, reside in a region of the membrane-embedded C-terminal domain known to be critical for HBsAg secretion.

**CONCLUSIONS:** Key patterns of mutations in the full-length S gene significantly correlate with HBV-induced HCC *in vivo* (reduced immune-control?, induction of intracellular anti-apoptotic signalling?, induction of oxidative stress?). Their role as markers in predicting HCC development deserves further investigation.
ABSTRACT 14
Antiviral Therapy 2012; 17 Suppl 1:A22

Antivirals can select resistant HBV variants that are directly cytopathic to the host cell

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BACKGROUND: Drug treatments for chronic hepatitis B include antiviral nucleoside/nucleotide analogues (NAs) which target the viral lifecycle by inhibiting the reverse transcriptase. NA resistance is common and widespread, characterized by point mutations in the overlapping polymerase/envelope genes, which encode amino acid changes in the reverse transcriptase domains of the HBV polymerase. In some cases, concomitant stop codons at the C-terminal end of the surface proteins are also selected. Common surface stop codon mutants include rtM204I/sW196*, which confers resistance to LMV, and rtA181T/sW172* which confers resistance to multiple NAs including LMV and ADV. We hypothesise that some of these NA-selected HBV variants may be more pathogenic due to their altered surface proteins. The aims of this study were to examine the replication and pathogenicity of these variants.

METHODS: Huh7 cells were transfected with infectious HBV encoding surface stop codons rtM204I/sW196*, rtA181T/sW172*, rtV191I/sW182*, or full-length surface proteins rtA181T/sW172L, rtA181V/sL173F, rtM204V/s195M, rtM204I/sW196S. Secretion and expression of altered HBsAg were measured by western blotting and quantitative serology (Abbott Architect). Proliferation, apoptosis, and intracellular HBsAg levels of transfected Huh7 cells were measured using flow cytometry.

RESULTS: The three stop codon variants were completely defective in HBsAg secretion, which could be partially rescued by co-expression with wt HBV. Flow cytometry was used to show that the truncated surface proteins also accumulated to higher intracellular levels than full-length controls. Cells transfected with these variants were less proliferative and had higher levels of apoptosis than HBV encoding full-length surface proteins. The most cytopathic variant was rtM204I/sW196*.

CONCLUSIONS: Some drug-resistant HBV variants selected during NA therapy are directly cytopathic to the host cell, promoting apoptosis. Apoptosis is strongly associated with disease progression and the development of HCC. Hence, although low genetic-barrier drugs may decrease viral load and increase survival in the short term, we predict that there may be long-term detrimental effect in patients who have selected these variants.
ABSTRACT 15

Antiviral Therapy 2012; 17 Suppl 1:A23

The evolution of HIV and HBV drug resistance in a resource-limited setting: the HEPIK study

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BACKGROUND AND AIMS: The prevalence of hepatitis B virus (HBV) coinfection is 17–20% among HIV-infected adults in Ghana; HEPIK is a prospective study assessing the burden and evolution of HIV and HBV drug resistance in a HIV-positive cohort in Kumasi. As HBV screening is not part of routine practice, patients receive HBV-blind first-line antiretroviral therapy (ART) typically comprising lamivudine with zidovudine or stavudine and nevirapine or efavirenz.

METHODS: Consecutive patients testing hepatitis B surface antigen (HBsAg)-positive by Murex EIA were enrolled. Laboratory and clinical evaluations were performed at study entry, including WHO disease stage, CD4 count and hepatic transaminase levels. HIV-1 RNA and HBV-DNA levels were determined by real-time PCR (limit of quantification 50 copies/ml and 14 IU/ml, respectively). Patients with detectable viraemia underwent Sanger sequencing of partial HIV-1 and HBV polymerase genes.

RESULTS: In 2010–2011 HEPIK enrolled 225 HBsAg-positive patients. 78.7% started lamivudine-based ART in 2003–2011; 3.6% received tenofovir. In univariate analyses, patients with a longer experience of lamivudine were more likely to have long duration of HIV diagnosis, more advanced HIV, higher HBV DNA, lower platelet count, higher ALT, higher Fib-4 scores and elastography KpA scores >12.5 (all \( P < 0.05 \)). Among ART-experienced patients, 41.7% and 63.3%, respectively, had detectable HIV-1 RNA or HBV DNA, of which 70.6% and 67.3% showed major drug-resistance mutations. Resistance mutations associated with lamivudine (M204I/V, 97.0%), entecavir (T184S, 3.0%), and telbivudine (M204I/V, 97.0%). No A181T/V was found, indicating that tenofovir remains a viable treatment option in this population. No HIV or HBV drug resistance was detected in ART-naive patients.

CONCLUSIONS: A high proportion of patients receiving lamivudine-based ART showed detectable HIV-1 RNA and HBV DNA and evidence of drug resistance. No evidence of transmitted drug resistance was found among ART-naive patients. The implementation of tenofovir into first-line HAART for HIV/HBV-coinfected patients is urgently required.
ABSTRACT 16

Antiviral Therapy 2012; 17 Suppl 1:A24

Structural dynamic of the NS5B RNA-dependent RNA polymerase as a new target to block HCV replication

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BACKGROUND: The non-structural protein RNA-dependent RNA polymerase (RdRp) NS5B plays a key role in HCV replication and is currently pursued as the most relevant target to develop safe anti-HCV agents. HCV NS5B is a 66 KDa protein. However, the majority of NS5B inhibitors target the catalytic activity of the enzyme allowing the rapid selection of mutations associated with the resistance of the virus. Therefore, there is an urgent need for more effective and selective therapies for HCV. The structure of NS5B has been widely characterized and as other polymerases, NS5B adopted a typical ‘right-hand’ conformation containing the characteristic fingers, palm and thumb subdomains. The activation of NS5B requires conformational changes involving intramolecular contacts as well interactions with viral and host factors in the replication complex.

METHODS: We have elaborated a new strategy based on short interfacial peptides that target protein–protein interfaces or essential motifs involved in NS5B-activation. In a screen of peptides derived from surface accessible motifs of NS5B selected from the X-ray structure and molecular modelling, we have identified a short 15-residues peptide P8 which tightly binds NS5B and inhibits its polymerase activity in vitro in the nanomolar range.

RESULTS: We showed that P8 alters RNA-binding and stabilizes NS5B in an inactive/non-processive conformation. P8 prevents the proper binding of primer/template and of NTPs. We demonstrated that P8 induces NS5B conformational changes that limited primer/template displacement and nucleotides incorporation. Moreover, P8 inhibits HCV-1b replication in replicon HCV-1b with EC50 in the low nanomolar range. Finally, we have identified the major residues in P8 required for NS5B inhibition and proposed derived peptides exhibiting a more potent activity in vitro and in cellulo.

CONCLUSION: Taken together, these results demonstrate that NS5B structural dynamic constitutes an attractive target for HCV chemotherapeutics and for the design of more specific new antiviral drugs.
ABSTRACT 17

Antiviral Therapy 2012; 17 Suppl 1:A25

Hepatitis C virus (HCV) resistance to silibinin and other flavonoid non-nucleoside inhibitors of the HCV RNA-dependent RNA polymerase

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BACKGROUND: Hepatitis C virus (HCV) drug discovery efforts principally focus on the development of compounds that inhibit HCV enzymes, such as the RNA-dependent RNA polymerase (RdRp) or the NS3/4A protease. Flavonoids are a broad class of molecules naturally present in plants. Among them, silymarin is a mixture of flavonolignans extracted from milk thistle, with silibinin as main component. Intravenous infusion of silibinin-hemisucinate (SIL-hem, Madaus-Rottapharm) has been reported to induce dose-dependent reductions of HCV RNA levels in HCV-infected patients. We previously demonstrated that silibinin inhibits HCV RdRp

METHODS: A partial crystallographic structure of HCV RdRp complexed with quercetagentin was used to identify the flavonoid binding site in the HCV RdRp. Site-directed mutagenesis was used to study the effect of amino acid substitutions conferring in vitro resistance to quercetagentin on RdRp function, HCV replication and silibinin inhibitory effect by means of an HCV RdRp enzyme assay and an HCV genotype 1b subgenomic replicon. Finally, 29 non-responders to prior peginterferon-ribavirin therapy infected with HCV genotype 1 were treated with intravenous silibinin, 5 to 20 mg/kg for 14 or 21 days; peginterferon and ribavirin was added at day 8. The full-length RdRp was sequenced at baseline and on treatment in all HCV-RNA-positive samples.

RESULTS: The partial crystallographic structure of HCV RdRp complexed with quercetagentin identified positions F162, G283, R168 and D559 in close contact with the C ring of the flavonoids. In an enzyme assay, quercetagentin inhibitory activity was affected by the D559A substitution, a known NNI-3 (Palm I) resistance-associated substitution, with a 10-fold increase in the IC50. D559A/G/N substitutions decreased RdRp enzyme activity and reduced its susceptibility to SIL-hem (≥ fivefold increase in the IC50). F162A, R168A and G283A completely abolished RdRp activity, whereas F162Y conferred resistance to SIL-hem (≥ fivefold increase in the IC50). In the replicon model, D559A/G/N reduced HCV replication capacity by 97%, and conferred reduced susceptibility to SIL-hem and quercetagentin. In the 29 patients, HCV RNA levels decreased on average by -2.2 ±1 log IU/ml, but none of them was HCV-RNA-undetectable at day 8. Eight amino acid changes were observed during silibinin administration (S19A, V144I, S231R, V235I, A238E, A327T, P461L, R465G and K535Q). None of them was associated with a virological breakthrough and none conferred reduced susceptibility to SIL-hem in vitro.

CONCLUSIONS: We have identified amino acid substitutions in the HCV RdRp associated with resistance to SIL-hem and other flavonoid inhibitors in vitro. However, these substitutions substantially impair HCV replication capacity and no selection of resistant HCV variants has been observed in patients treated with intravenous SIL-hem, at least in the short-term. SIL-hem and quercetagentin thus appear to have a high barrier to resistance, probably due to the poor fitness of resistant HCV variants.
ABSTRACT 18

Antiviral Therapy 2012; 17 Suppl 1:A26

Detection of NS5B S282T by allele-specific PCR in treatment-naive HCV genotype 1/HIV-1 coinfected patients

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BACKGROUND: The HCV NS5B RNA-dependent RNA polymerase (RdRp) is essential for viral RNA replication and is an attractive therapeutical target. Several nucleoside and non-nucleoside polymerase inhibitors (NNI) are in Phase II and III clinical trials. The S282T mutation, associated to nucleoside analogues resistance, is not detected by conventional methods or by ultra-deep sequencing in HCV treatment-naive patients. The aim of this study was to investigate the presence of this minor variant by allele-specific real-time PCR (AS-PCR) in HCV/HIV-1 coinfected patients naive for HCV treatment.

METHODS: We analysed 45 plasma samples of HCV genotype (GT) 1/HIV-1 coinfected patients (20 of GT1a and 25 of GT1b) and naive for PEG-IFN plus RBV treatment. Three independent RT-PCR amplifications of the partial NS5B polymerase gene (875 bp) were performed for each sample. The resulting nested PCRs were pooled and purified; 10^6 copies per sample were added to the real-time AS-PCR developed for the detection of the S282T mutation.

RESULTS: The sensitivity of the AS-PCR assay was 0.1% and the accuracy was down to 0.1%. Allelic discriminatory ability of the specific amplification between equal amounts of mutant and wild-type was more than 15 cycles. The S282T nucleoside analogue resistant mutation was detected in 44% of patients with a range of frequencies of 0.12% to 2.1%. The S282T mutation was not detected by direct sequencing in any of the samples.

CONCLUSION: These results demonstrate the high prevalence of minority drug-resistant S282T resistance substitution. Our results also demonstrate that allele-specific PCR can be used to detect minor HCV NS5B resistant variants in pretreatment samples and to study, in detail, the evolution of mutant viruses during targeted antiviral therapy.
ABSTRACT 19
Antiviral Therapy 2012; 17 Suppl 1:A27

Characterization of NS3 amino acid variants observed in a Phase 1b study of genotype 1 (GT1) and GT3-infected patients with the HCV protease inhibitor, MK-5172

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BACKGROUND: MK-5172, a next-generation HCV protease inhibitor, demonstrates robust in vitro activity against HCV genotype (GT) 1–6 proteases and against GT1 proteases harbouring first-generation protease inhibitor resistance-associated variants. MK-5172 demonstrated rapid and robust antiviral activity in GT1 patients at all doses tested (50–800 mg QD) and at 400–800 mg QD in GT3 patients. In this study, no patient experienced virological rebound during the MK-5172 dosing period. Here, we report the analysis of NS3 variants detected in GT1- and GT3-infected patients before and after MK-5172 dosing.

METHODS: HCV RNA was isolated from plasma at baseline and selected time points following MK-5172 dosing for up to 2 months. Population sequencing was performed on NS3/4a genes RT-PCR amplified from samples with HCV RNA titres >1,000 IU/ml. Amino acid variants were identified by alignment with GT1 and GT3 reference sequences. Clonal NS3/4a sequencing and cell-based phenotypic susceptibility testing was performed on a subset of patient samples with detectable variants.

RESULTS: Three GT1 patients had variants detected at baseline; two with D168E and one with Q41H. All three patients experienced a VL decline to <25 IU/ml. During follow-up, additional patients had variants observed at positions F43, Y56, R155, A156 and D168. Phenotypic analysis demonstrated that pooled NS3 clones isolated from patients at baseline and post-dosing, were inhibited by MK-5172, with EC50 values ranging from 0.7–5.9 nM and 7.5–1,870 nM, respectively. For GT3a patients, no variants were identified at positions F43, Q80, R155 or A156 at any time point. In one patient (800 mg arm), variants were detected at positions K26R and Q168R. This patient experienced a 3 log decline in HCV RNA on-treatment. In vitro, K26R/Q168R confers decreased susceptibility to MK-5172 in a GT3a background.

CONCLUSIONS: During MK-5172 dosing, no viral rebound was observed in GT1- or GT3a-infected patients. The detection of variants in post-treatment samples from GT1-infected patients suggests that 7-day MK-5172 dosing may not sufficiently decrease levels of all pre-existing variants (that have reduced sensitivity to MK-5172) to below that of the wild-type population. Among GT3a patients, only one harboured viruses with K26R/Q168R variants, which conferred a marked decrease in MK-5172 susceptibility in vitro.
Resistance profile of TMC435 in HCV genotype-1-infected patients: virological analyses of the PILLAR and ASPIRE Phase IIb trials

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BACKGROUND: TMC435 is an investigational, potent, once-daily oral HCV NS3/4A protease inhibitor currently in Phase III clinical development. Efficacy and safety of TMC435 (75, 100 or 150 mg once daily) with PegIFN/RBV was assessed in HCV genotype-1-infected, treatment-naive (PILLAR; NCT00882908; n=309) or -experienced (ASPIRE; NCT00980330; n=396) patients. In TMC435-treated patients who did not achieve sustained virologic response (SVR), emerging viral variants were characterized.

METHODS: Population sequencing of the NS3/4A regions was performed at baseline and in isolates from patients who failed to achieve SVR. In vitro susceptibility was assessed using a transient replicon assay as site-directed mutants in a genotype 1a or 1b replicon, or in chimeric replicons with patient-derived NS3 protease sequences.

RESULTS: In PILLAR and ASPIRE, SVR24 was achieved in 81–86% and 67–80% of TMC435 150 mg treated patients, respectively. The main outcomes in TMC435-treated patients not achieving SVR were viral breakthrough (vbt) and viral relapse. In total, 58 patients experienced vbt (57 with sequence information) and 72 experienced relapse (68 with sequence information). Emerging mutations at one or more of the NS3 positions 80, 122, 155 and/or 168 were observed at the time of failure in 56/57 (98.2%) patients with vbt and in 62/68 (91.2%) with relapse. No clear difference in the type of emerging mutations was observed between patients with vbt or relapse. Different resistance pathways were selected in genotype 1a (mainly R155K alone or combined with emerging Q80K/L/R, S122R or D168E, or with pre-existing Q80K) compared to 1b (mainly D168V or Q80R+D168E). Phenotypic analysis of some isolates at baseline and time of vbt (n=23) and relapse (n=16) demonstrated median 1,189-fold and 134-fold increases in TMC435 EC50 values at time of failure, respectively. In 28/55 patients with emerging mutations at time of vbt, no mutations were detected at last follow-up during the studies (median: 61.9 weeks).

CONCLUSIONS: In Phase IIb studies PILLAR and ASPIRE vbt and viral relapse were relatively uncommon and usually associated with emerging viral variants carrying mutations with reduced in vitro susceptibility. Resistance pathways were different between genotype 1a- and 1b-infected patients. Resistant variants were frequently replaced by wild-type virus during follow-up.
ABSTRACT 21

Antiviral Therapy 2012; 17 Suppl 1:A29

Phenotypic characterization of HCV genotype 1 protease sequences derived from patients treated with telaprevir-based regimens in Phase 3 studies

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BACKGROUND: In Phase 3 clinical trials, telaprevir in combination with peginterferon (P) and ribavirin (R) significantly improved SVR rates in chronic HCV genotype 1 (GT1) patients compared to PR alone. Viral variants carrying mutations in the NS3•4A protease region have been observed in patients who do not achieve SVR after treatment with telaprevir-based regimens. Here, we report the phenotypic characterization of HCV GT1 variants in a subset of treatment-naive and treatment-experienced patients participating in the Phase 3 studies ADVANCE, ILLUMINATE and REALIZE.

METHODS: Using a replicon-based phenotyping assay, telaprevir susceptibility of the NS3•4A protease was obtained for paired baseline and failure plasma samples from 22 patients who did not achieve SVR with telaprevir-based treatment (10 GT1a, 12 GT1b) and baseline samples from an additional 21 patients (11 GT1a, 10 GT1b; 20 telaprevir-based treatment failures, 1 patient achieving SVR).

RESULTS: In the 22 patients with paired samples, telaprevir-resistant variants were not detected at baseline by population sequencing (wild-type [WT] protease sequence) while the post-baseline samples had either WT or contained variants with mutations V36A/M, T54A, R155K/T, A156S/T or V36M+R155K. The 21 additional baseline samples had WT (n=16) or had naturally-occurring V36M (n=2), R155K (n=2) or V36L+R155K (n=1) mutations.

Baseline samples with WT protease sequence had a mean telaprevir EC$_{50}$ value of 0.12 μM (range in EC$_{50}$: 0.024–0.44 μM). Baseline samples harbouring telaprevir-resistant variants (n=5) were less sensitive to telaprevir (mean EC$_{50}$ V36M: 1.5 μM [n=2]; R155K: 1.3 μM [n=2]; V36L+R155K: 12 μM [n=1]) than WT samples. The post-baseline samples carrying telaprevir-resistant mutations had fold changes (FC) in EC$_{50}$ consistent with FC values obtained with site-directed mutants carrying the same mutations. Post-baseline samples from patients who failed telaprevir with WT variants showed similar telaprevir sensitivity (mean EC$_{50}$ 0.15 μM) to their corresponding baseline samples (mean EC$_{50}$ 0.10 μM).

CONCLUSIONS: The presence of previously characterized telaprevir-resistant variants at baseline or at time of failure decreased susceptibility to telaprevir by a factor similar to that observed with site-directed mutants, suggesting that the level of resistance conferred by telaprevir-resistant variants is not substantially influenced by patient-specific protease backbone sequences.
ABSTRACT 22

*Antiviral Therapy* 2012; 17 Suppl 1:A30

**Full-length HCV NS3/4A recognizes protease inhibitors better compared to the isolated protease for wild-type and multidrug-resistant variants**

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**BACKGROUND:** Hepatitis C is a global health problem that plagues about 3% of the world’s population. Despite being curable, the current treatment with pegylated interferon and ribavirin is lengthy, not completely effective and intolerable. Pharmacology industry attempts to eliminate this problem by development of directly-acting antiviral drugs against critical viral machinery, such as NS3/4A protease, the primary and indispensable proteolysis machinery for polyprotein maturation. Drug development efforts on NS3/4A protease currently produced two drugs on the market – boceprevir and telaprevir – with promising new protease inhibitors on clinical trials. However, most of these development attempts disregard the helicase domain. NS3 helicase is also an essential part of the hepatitis C virus machinery and covalently linked to the protease. Furthermore, the protease and the helicase domains are interdependent to achieve optimum activity. However, there are conflicting results about whether the helicase domain aids in the protease drug recognition.

**OBJECTIVE:** This research aims to discern the effect of the helicase domain on protease inhibitor recognition for both the wild-type protein and multidrug-resistant variants.

**METHODS:** Multidrug-resistant variants (V36M, R155K, A156T, D168A, R155K+V36M) were created on both the full-length NS3/4A and the isolated protease. In addition, helicase residues were also mutated (M485A, V524A, Q526A, H528A) according to the full length crystal structure (PDB ID: 1CU1). All of these constructs were expressed, purified and the drug inhibition profiles were analysed using a Forster resonance energy transfer based protease cleavage assay for protease inhibitors telaprevir, danoprevir, vaniprevir, MK-5172, BMS-650072 and our in-lab variants.

**RESULTS AND CONCLUSION:** We have observed a slight increase in potency (up to sixfold) when the full-length protein and the isolated protease were compared and slight decrease (up to threefold) in helicase mutants. These results indicate that the helicase domain influence the drug recognition even if no specificity has been engineered to the helicase. This signifies the need for drug development, taking the helicase domain into consideration, so that the drug resistance paradigm can also be overcome.
ABSTRACT 23

Antiviral Therapy 2012; 17 Suppl 1:A31

A virus-based method for quantifying genetic barriers in HCV

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BACKGROUND: The development of drug resistance against direct-acting antivirals targeting HCV has been demonstrated and represents a major reason for treatment failure. We recently demonstrated that not only the number, but also the nature of the nucleotide changes can contribute to the genetic barrier. Deep sequencing data revealed that transitions occur more frequently than transversions. Here, we hypothesized that the selection of transitions is favoured over that of transversions, provided the virus is given access to both pathways.

METHODS: To test this hypothesis we developed an HCV reversion system based on arginine (Arg) to tryptophan (Trp) substitutions strategically introduced into the viral genome. Trp mutant variants are associated with a wide range of fitness penalties, and HCV can theoretically revert by mutating to either of the six Arg codons. To directly test whether HCV reverts preferentially through the facile transition pathway, we engineered an R251W mutation in NS5B within a high titre version of JFH1. Since the wild-type Arg at this position is coded for by CGC, reversion from the Trp (UGG) would require two nucleotide changes. However, changes to Arg (CGG) and Arg (AGG) represent a single transition or transversion, respectively.

RESULTS: We found that the R251W virus is indeed less fit than the original virus, and culturing the mutant variant resulted in a reversion back to Arg. Rather than reverting back to the wild-type CGC codon, the virus rapidly reverted to Arg by selecting the CGG codon, which represents the most facile change amongst the six possibilities. To quantify the effect of genetic barrier, we performed 10 independent infections with the R251W variant and found that 9/10 revertant viruses were generated through the UGG to CGG transition pathway. Only a single revertant was selected through the UGG to AGG transversion pathway.

CONCLUSIONS: The data demonstrate that the selection of transitions is favoured over that of transversions. The experimental system represents a novel virus-based approach to numerically quantify genetic barriers in HCV and perhaps other viruses. In contrast to classic selection experiments, the effects of viral fitness and level of resistance to a given drug are negligible.
ABSTRACT 24

Antiviral Therapy 2012; 17 Suppl 1:A32

Origin of minority drug-resistant HIV-1 variants in primary HIV-1 infection

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BACKGROUND: Minority drug-resistant HIV-1 variants are part of the virus population in some ART-naive subjects. They may arise due to de novo mutagenesis. Alternatively, transmission of minority variants has been suggested but not been proven so far. Here, we provide several lines of evidence supporting the hypothesis that transmission of minority variants occurs.

METHODS: Minority drug-resistant HIV-1 variants harbouring ≥0.3% of the K103N, Y181C, M184V (RT) and/or N155H (integrase) mutations were detected by AS-PCR in plasma samples collected between 2002–2010 from 204 participants of the Zurich Primary HIV Infection cohort (ZPHI) during acute or recent HIV-1 infection. Transmission clusters were identified by phylogenetic analyses of pol sequences from ZPHI participants and >9,900 participants of the Swiss HIV Cohort Study and confirmed by phylogenetic analyses of C2V3C3 env sequences. Bootstrap values for all transmission clusters were 100% within pol and >99% within env sequences (maximum likelihood and neighbour joining phylogenetic trees).

RESULTS: The first line of evidence relies on different prevalences of these mutations: M184V in 16/194 (8.3%), Y181C in 4/153 (2.6%), K103N in 4/192 (2.1%) and N155H in 1/195 (0.5%). The difference is highly significant (P=0.0003) between the long-standing, very commonly detected mutation M184V and the yet very uncommon integrase mutation N155H. If sporadic appearance alone was the reason for the emergence of minority drug-resistant HIV-1 variants in ART-naive subjects, a more similar distribution of these mutations would be expected. The second line of evidence is based on the identified transmission clusters: potential transmitters have been identified for 12 of the 16 subjects harbouring minority M184V variants (all distance <0.015). Of those, four subjects were ART-experienced, selected the M184V mutation as major virus population prior to transmission, and had detectable viral loads at the estimated time of transmission. Interestingly, the M184V mutation was not detected as major virus population at this time by population sequencing, thus, was presumably transmitted as minority variant.

CONCLUSION: Minority drug-resistant HIV-1 variants can appear sporadically, however, they can also be transmitted. To which extent the origin of minority drug-resistant HIV-1 variants determine their impact on ART needs to be further explored.
ABSTRACT 25

Antiviral Therapy 2012; 17 Suppl 1:A33

Antiretroviral drug-resistant minority variants are significantly associated with first-line treatment failure in antiretroviral drug-naive patients

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BACKGROUND: The overall aim was to determine the clinical relevance of low frequency, or minority, HIV-1 drug resistance mutations on treatment outcome by ultradeep pyrosequencing (UDPS). Participant samples from the CIPRA-SA study, a randomised controlled trial of antiretroviral drug monitoring strategies in a resource-poor setting, were available for this study. Participants were antiretroviral drug-naive, or had prior exposure to single-dose nevirapine and/or zidovudine for prevention of mother-to-child transmission (PMTCT), and were monitored longitudinally for a minimum of 96 weeks to a maximum of 2.2 years from enrolment.

METHODS: Protease and reverse transcriptase were amplified from participant plasma samples available prior to treatment initiation (baseline) and at treatment failure. Three overlapping amplicons using HIV-1 subtype C specific primers tagged with 48 multiplex identifiers were generated per sample, and pyrosequenced using the Roche 454 FLX platform. Samples were evaluated for low level variants to ≥1% levels. Sequence data was analysed manually from the AVA software output files, the SANBI UDPS bioinformatics program, and the DeepChek®-HIV v1.0 program. Genotypes were interpreted according to the 2011 IAS–USA drug resistance mutations list and Stanford HIVdb algorithms.

RESULTS: UDPS data from 626 samples evaluated to 1% levels was obtained, and included 554 baseline samples, 60 first-line regimen failures and 12 second-line regimen failures. Among the 554 baseline participants, 363 were antiretroviral drug-naive and 192 had prior exposure for PMTCT, of which 51 and 23 experienced treatment failure, respectively. UDPS detected minority drug resistance mutations in 81 of 554 baseline samples. Generally, baseline minor drug resistance mutations were present at treatment failure. Analysis of the 363 antiretroviral drug-naive baseline samples confirmed the presence of minority drug-resistant variants was significantly associated with treatment failure (P=0.003), whereas minority drug resistance mutations against nevirapine/zidovudine amongst the 192 participants with prior drug exposure for PMTCT were not (P=0.24).

CONCLUSIONS: The presence of acquired minor NNRTI-resistant viruses does not necessarily result in virological failure if patients are on a drug regimen with at least two other active drugs. However, the presence of drug-resistance minority variants in antiretroviral drug-naive individuals at levels >1% are significantly associated with virological failure with first-line antiretroviral therapy.
ABSTRACT 26
Antiviral Therapy 2012; 17 Suppl 1:A34
Prevalence and significance of HIV-1 drug resistance mutations among patients on antiretroviral therapy with detectable low-level viraemia

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BACKGROUND: There continues to be controversy over the significance of low-level viraemia (LLV) and the associated risks of drug resistance accumulation and virological failure (VF).

METHODS: Plasma samples from participants in the SCOPE cohort near the time of LLV (plasma viral load [pVL] <1,000 copies/ml) were ultracentrifuged and HIV-1 PRO, RT, IN and gp41 were amplified and sequenced. Antiretroviral drugs (ARVs) having a Stanford HIV Resistance Score <10 were considered fully active. Adherence was recorded by 30-day self-report. Repeated measures multivariable logistic regression was used to evaluate predictors of resistance mutation accumulation. VF was defined as a pVL ≥ 1,000 copies/ml following the period of LLV without intervening virological suppression.

RESULTS: HIV-1 drug resistance testing was successful in 47 participants with LLV, including 7 (15%) participants with 2 LLV time points and 11 (23%) participants with ≥3 LLV time points. The median (IQR) number of years since HIV diagnosis was 13 (8–19) and 89% of participants were treatment-experienced at entry into SCOPE. The median pVL was 267 copies/ml. In addition to NRTIs, ARVs included PI (66%), NNRTI (11%) or both (23%). Median 30-day adherence was 98% (93–100%). Compared to a prior genotyping, 46% of samples had a new resistance mutation. There was no significant association between pVL and the number of fully active ARVs in the current regimen (Kruskal–Wallis P=0.69). In participants with ≥2 LLV time points, 8 (44%) were found to have accumulated additional resistance mutations over a median of 11 months. In a multivariable regression analysis, fewer fully active ARVs at the prior time point (β=-1.3, [95% CI -2.2, -0.5]; P=0.003) and longer elapsed time (months, β=0.08, [95% CI 0.01, 0.14]; P=0.02) were associated with an increased risk of resistance accumulation after controlling for adherence and pVL. VF followed 16% of LLV time points.

CONCLUSIONS: In this largely treatment-experienced cohort, new drug resistance mutations were frequently discovered at the time of initial LLV and accumulation of resistance mutations was commonly detected over time, especially for individuals receiving fewer fully active ARVs. Strategies for early intervention during LLV episodes (for example, adherence counselling, resistance genotyping, regimen switch) should be further studied.
ABSTRACT 27

Antiviral Therapy 2012; 17 Suppl 1:A35

Deep sequencing analysis of baseline samples from patients treated with rilpivirine in the Phase III studies ECHO and THRIVE shows no association between the presence of minority resistance-associated variants and virological failure

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BACKGROUND: Baseline minority variants, detected using deep sequencing technologies, may influence HIV-1 antiretroviral (ARV) treatment outcome. The impact of baseline NNRTI resistance-associated minority variants on response to rilpivirine-containing regimens was investigated in ARV treatment-naive patients from the Phase III studies ECHO and THRIVE. Samples obtained at time of virological failure (VF) were also analysed and linkage between frequently observed emerging resistance-associated mutations was determined.

METHODS: Population-based and paired-end deep sequences (Illumina®) from 47 subjects experiencing VF and 49 matching responders were analysed at baseline in addition to 48 samples from VFs at failure. Minority variants were defined as having frequencies between 1% (approximate assay sensitivity) and 25%. Linkage analysis was performed by matching sequence reads through flow cell positional information.

RESULTS: Minority NNRTI resistance-associated variants were detected at baseline in 8/47 VFs (17.0%) and 6/49 responders (12.2%; P=0.57) and were V90I (n=1), V106I (n=2), V108I (n=1), V179I (n=1), and V189I (n=3) in VFs, and V90I (n=3), V106I (n=2), V179I (n=1), V179D (n=1), and F227C (n=1) in responders. V90I, V106I and V179I were also found in similar numbers of VFs and responders by population sequencing (PS). Minority NRTI resistance-associated variants M184V (n=1) or L210W (n=1) were detected in two VFs at baseline while none were observed among responders. Deep sequencing of samples collected at time of failure revealed additional subjects with emerging resistance-associated variants E138K (n=5), K101E (n=6), M184I (n=3) and M184V (n=1) in addition to those found by PS (n=22, 8, 25 and 11, respectively). Minority resistance-associated variants at failure were detected by deep sequencing in 4 VFs without evidence of emerging resistance by PS (n=12). Presence of E138K and K101E was mutually exclusive and both mutations were linked to M184I/V in approximately half of the analysed HIV-1 sequences.

CONCLUSIONS: Baseline minority NNRTI resistance-associated variants were mostly polymorphisms, were uncommon in the Phase III ECHO and THRIVE patient population, and were not associated with rilpivirine virological failure. Deep sequencing analysis improved the characterization of VF samples and confirmed two distinct pathways of rilpivirine resistance, characterized either by E138K or less frequently by K101E, both associated with M184I/V.
ABSTRACT 28

Antiviral Therapy 2012; 17 Suppl 1:A36

Reverse transcriptase connection domain mutations were not associated with virological failure or phenotypic resistance in rilpivirine-treated patients from the ECHO and THRIVE Phase III trials (week 96 analysis)

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BACKGROUND: In the ECHO and THRIVE trials (HIV-1 treatment-naïve patients), both rilpivirine (RPV) 25 mg qd and efavirenz (EFV) 600 mg qd plus background N(t)RTIs resulted in a 78% response rate (viral load <50 copies/ml; intent-to-treat-time-to-loss-of-virologic-response [ITT-TLOVR]) at week 96. Connection-domain mutations (CDMs) in the HIV-1 reverse transcriptase can affect virological response to NNRTI-based treatments. This study analysed the prevalence of CDMs and their association with virological failure (VF) and resistance to RPV.

METHODS: Twenty CDMs (E312Q, Y318F/W, G333D/E, G335C, N348I, G359S, A360I/V, V365I, T369I/V, A371V, I375V, A376S, T377M, T386A, I393L and E399D) were considered. The prevalence of CDMs at baseline was determined in RPV-treated patients grouped by subsequent VF or non-VF. The influence of CDMs on RPV fold-change in EC₅₀ (FC) and distribution of the CDMs/RPV RAMs were analysed among VF-patients from the week-96 analysis of the pooled ECHO and THRIVE RPV groups (N=686). Genotypes (baseline/failure time points) were obtained by standard population sequencing (SPS, vircoType®) and further investigated post hoc in a subset of 48 VF patients by ultra-deep sequencing (UDS, Illumina® Inc).

RESULTS: The most prevalent (≥5%) CDMs at baseline (SPS) were G359S (161/686 [23%]), A371V (122/686 [18%]), I375V (97/686 [14%]), E399D (80/686 [12%]), G333E (68/686 [10%]), T377M (59/686 [9%]) and V365I (41/686 [6%]). By SPS, the proportion of patients with ≥1 CDM at baseline was 59% (407/686) in the overall population and 50% (48/96) among VF patients. The distribution of CDMs was comparable among VF patients and non-VF patients. At failure, the proportion of VF patients with treatment-emergent CDMs was low (3/86 with genotypic data at failure [3.5%]). There was no correlation between the number/presence of any CDM and any RPV RAM. Among VF patients with RPV RAMs, the median RPV FC at failure was 8.6 in the presence and 6.7 in the absence of CDMs. Additional CDMs identified by UDS were rare in the subset of 48 VF patients.

CONCLUSIONS: The prevalence of CDMs in the ECHO and THRIVE population at baseline ranged from 0–23%. The presence of CDMs did not influence virological treatment outcome. Treatment-emergent CDMs in RPV VF patients were rare and were not associated with increased resistance to RPV.
ABSTRACT 29

*Antiviral Therapy* 2012; 17 Suppl 1:A37

Drug resistance among HIV-1 seroconverters in the FEM-PrEP Study

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BACKGROUND: Results from HIV chemoprophylaxis trials show differing efficacies for preventing infection. The FEM-PrEP trial of daily oral administration of the combination pill emtricitabine and tenofovir disoproxyl fumarate (FTC/TDF) versus placebo was unable to determine effectiveness for HIV infection prevention in African women. Selection of drug resistant HIV is a concern, especially in settings of reduced efficacy due to low or intermittent product adherence.

METHODS: Clinical drug resistance to FTC/TDF at study visits at or near first evidence of seroconversion was determined by genotype (TRUGENE, Siemens) and phenotype (Phenosense, Monogram Biosciences). Minor variant drug resistance was measured using an allele-specific PCR assay that maintains specificity with divergent viruses. Plasma tenofovir (TFV) and FTC concentrations and cellular tenofovir diphosphate (TDP) concentrations were used to measure adherence levels among resistant cases.

RESULTS: Specimens from 68 women infected during regular study follow-up (33 FTC/TDF, 35 placebo) and from 2 women with pre-study infection (RNA-positive, antibody-negative) who had access to study drug until documented seroconversion, were tested for drug resistance. Viral loads at seroconversion visits ranged from 117 to >1×10⁷ copies/ml (mean, 1.6×10⁵), with no difference between arms. No infections showed clinical resistance to TFV, while five (one placebo, four FTC/TDF) showed genotypic resistance to FTC (M184V/I). Mutations waned over time in the active arm participants following drug termination.

Phenotypic resistance to FTC was seen in 4/4 tested, accompanied by increased susceptibility to TFV, stavudine and zidovudine. Other mutations not associated with exposure to FTC/TDF (K103N, E138A) were co-existent in two subjects randomized to the active arm.

Three subjects showed low-level (0.50–1%) minor variant drug resistance, each at a single mutation, K70E, M184V or M184I. TFV, FTC and TDF concentrations were undetectable in the one active arm participant with minor variant resistance and in two with clinical FTC resistance. Moderate to high concentrations of TFV, FTC and TDF were detected in the additional two active arm participants with clinical FTC resistance.

CONCLUSIONS: FTC-associated drug resistance was observed in FEM-PrEP, but infrequently in the presence of measurable plasma and cellular drug concentrations, consistent with the overall level of adherence to study drug.
Drug resistance patterns among HIV-infected children following pMTCT in South Africa during 2011

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INTRODUCTION: The South African Department of Health implemented new pMTCT guidelines in April 2010. This includes provision of HAART for pregnant women with CD4 counts <350 cells/mm³ and zidovudine and intrapartum nevirapine with extended infant nevirapine during breastfeeding for women who do not meet criteria for HAART. This study proposed drug resistance screening in infants infected with HIV despite possible access to pMTCT interventions to assess the levels of drug resistance selected by pMTCT.

METHODS: Plasma samples from 126 HIV-1-infected infants less than 2 years of age were collected as part of the Finding Infants with HIV Disease: Evaluation of Resistance, pMTCT Failures and Linking Access to Care (FInHDER) Study. Children were recruited from a variety of settings in Johannesburg to represent those who had full, partial or no access to pMTCT interventions. The reverse transcriptase and protease genes were genotyped using a validated in-house method. Genotypic resistance was defined using the Stanford Genotypic Resistance Interpretation Algorithm and the November 2011 International AIDS Society drug resistance mutation list.

RESULTS: From 123 specimens that were amplifiable for genotyping, major NNRTI mutations were detected in 40 (32%). The Y181I/C mutation was most predominant, being detected in 30 (24%) specimens, followed by K103N in 16 (13%) specimens. Other major mutations detected included H221Y (n=7, 6%), G190A/S (n=5, 4%), Y188C (n=3), V108I (n=3), K101E (n=2) and V106I/M (n=2). Minor NNRTI mutations were detected in an additional 9 specimens, and included E138A (n=7), A98G (n=5), V90I (n=3), V179T/D (n=3), F227L (n=2) and K101H (n=1). Resistance to the other drug classes was minimal, with major NRTI mutations being detected in only 6 specimens (M184V [n=2], L74V [n=2], K70R, A62V and V75I) and only minor PI mutations in 4 specimens.

CONCLUSIONS: NNRTI mutations, especially the Y181I/C mutations, continue to occur in one third of newly diagnosed HIV-infected infants and young children, all of whom are eligible for HAART.
ABSTRACT 31

Antiviral Therapy 2012; 17 Suppl 1:A39

Characterization of persistent HIV-1 in a broad spectrum of CD4+ T-cells isolated from peripheral blood and gut associated lymphoid tissue from patients on long-term suppressive therapy

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BACKGROUND: The role of ongoing virus replication in HIV persistence during long-term antiretroviral therapy is unknown. Since residual replication should result in detectable evolution, we investigated the degree of sequence evolution in blood-derived and rectal tissue-derived CD4+ T-cells.

METHODS: Using single-genome and single-proviral sequencing techniques, we obtained 20–50 single viral genomes from pre-therapy plasma samples from five subjects who initiated therapy during acute infection and three subjects who initiated therapy during chronic infection. Pre-therapy plasma viral sequences were compared to single proviral HIV-1 genomes derived from HIV-1-infected T-cells (naive, memory, central- and effector-memory) from peripheral blood (PB) and gut-associated lymphoid tissue (GALT) samples collected after 4–12 years of suppressive therapy. Maximum likelihood phylogenetic trees were constructed using the general time reversible model incorporating rate variation among sites. Evolutionary divergence was explored using root-to-tip analysis (Path-O-Gen).

RESULTS: The geometric mean infection frequency of memory and naive CD4+ T-cells in the PB was 13- and 24-fold higher, respectively, in subjects treated during chronic compared to acute infection. This was also true for effector memory CD4+ T-cells from the GALT (sixfold higher). Phylogenetic analysis revealed clear evidence against any substantial evolution between the pre-therapy plasma-derived HIV RNA sequences and on-therapy intracellular HIV DNA sequences. Numerous intracellular HIV sequences identified after long-term therapy contained replication-incompetent virus. One patient, who initiated therapy during chronic infection, had a predominant intracellular clone in both memory and effector memory T-cells containing a 380 bp deletion after >9 years of therapy.

DISCUSSION: Early initiation of effective therapy results in substantially lower reservoir size in blood and gut. The lack of HIV-1 genetic evolution in the HIV-1-infected CD4+ T-cell populations after years of therapy argues against virus replication as a major cause of persistence in these cell populations. The finding of multiple T-cells with identical replication incompetent virus after long-term therapy is strong evidence that this persistent virus was due to expansion of cells with integrated proviral DNA rather than active viral replication. The role of replication in other tissues and cell types however remains to be defined.
ABSTRACT 32
Antiviral Therapy 2012; 17 Suppl 1:A40

Decay of total HIV-1 DNA and 2-LTR circles with long-term suppressive antiretroviral therapy (ART)

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BACKGROUND: The decay kinetics of plasma viraemia in patients on ART has been characterized, including a plateau phase after 4–5 years during which no further decay occurs. The decay kinetics of HIV-1 DNA and 2-LTR circles are less well understood. We therefore investigated their decrease in patients on long-term suppressive ART.

METHODS: Nine patients initiating ART (without raltegravir) were sampled for a median of 9.2 years (range 3.3 to 11.8 years) of suppression without blips. Peripheral blood mononuclear cells (PBMCs) were obtained at 0, 4, 8–12, 20–30, 40–45 and 52 weeks after ART-initiation, and then every 6 months. Total HIV-1 DNA and 2-LTR circles in PBMCs were measured by qPCR (95% detection limits of 5 and 7.5 cps/reaction, respectively) and normalized per million PBMC (qPCR for CCR5 DNA).

RESULTS: Total HIV-1 DNA declined after ART-initiation from a pre-therapy median of 5,771 cps/10⁶ PBMCs (range 1,800–7,885) to a median of 134 cps/10⁶ PBMCs (range 24–392) after long-term ART (mean 43-fold decrease compared to 58,000-fold decrease for plasma RNA). Two patterns of decay were observed. In 5/9 patients, HIV-1 DNA decayed rapidly (mean 7-fold; range 4.8–8.7) before plateauing within 10 weeks without subsequent decline. In the other patients, HIV-1 DNA declined more slowly and continuously during follow-up. The ratio of 2-LTR to total HIV-1 DNA increased after ART-initiation, peaking by week 8 (from 3.6% to 15.4%), but then 2-LTRs declined linearly from a median of 155.5 cps/10⁶ PBMCs (range 8–860) to <5 cps/10⁶ PBMCs, although remaining detectable in some patients.

CONCLUSIONS: In most patients, HIV-1 DNA levels fell rapidly after ART-initiation but soon reach a plateau without further decline for years. In other patients, the decay was gradual and did not plateau during follow-up. The average decline in HIV-1 DNA (~40-fold) was far less than that of plasma HIV-1 RNA (~50,000-fold). The initial increase in the ratio of episomal to total HIV-1 DNA after ART-initiation is consistent with 2-LTR circles being present in cells that are longer-lived than productively infected ones. The steady decline of 2-LTR circles after many years of ART suggests they have a longer half-life in vivo than proposed previously.
ABSTRACT 33

Antiviral Therapy 2012; 17 Suppl 1:A41

HIV-1 genome is often defective in PBMCs and rectal tissues after long-term HAART as a result of APOBEC3 editing and correlates with the size of reservoirs

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BACKGROUND: Precise characterization of viruses present in reservoirs after several years of antiretroviral treatment will be one major issue to consider in the context of viral eradication. Here, we propose the systematic detection of in-frame stop codons in HIV proviral quasi-species and its application to clinical samples (PBMCs, rectal tissues) to assess the frequency of defective viruses present in cellular reservoirs.

METHODS: PBMCs and rectal biopsy samples from 5 patients on successful long-term HAART (7–13 years) were compared with 5 untreated patients. The presence and quantification of in-frame stop codons in HIV quasi-species were performed using molecular cloning on the reverse transcriptase region. The relationship between the size of reservoir and the frequency of defective genomes was assessed.

RESULTS: We systematically detected defective genomes in all patients on long-term HAART (5/5 patients) in both compartments (PBMCs and rectal tissues) with a high level of defective genomes per sample (overall median percentage: 21%; range: 15–100%) contrasting with no stop codons detected in untreated patients. A high level of defective genomes was correlated to a small size of HIV proviral DNA ($P=0.035$; $r^2=0.24$). Regarding the nucleotide context, guanine (G) to adenine (A) at tryptophan positions was responsible for the appearance of 89% of all in-frame stop codons, mostly in the context of APOBEC3-induced hypermutation.

CONCLUSION: We demonstrate that a great amount of the proviral DNA present after many years of antiretroviral treatment is defective even in the major sites of HIV production (gut-associated lymphoid tissue) likely reflecting APOBEC3 footprints on the viral genome. We propose a scenario whereby defective genomes unable to replicate accumulate during HAART treatment, eventually reaching a viral extinction threshold. In the context of viral eradication, the measurement of the relative amounts of defective and non-defective viruses should be used as a criterion for eradicating HIV instead of measuring only proviral HIV DNA.
ABSTRACT 34

*Antiviral Therapy* 2012; 17 Suppl 1:A42

**Increased risk of virological rebound in patients on antiviral therapy with isolated detectable viral loads <48 copies/ml by Taqman RT-PCR assay**

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**BACKGROUND:** European studies investigating the significance of isolated or persistent low-level viral loads (LLVL) over 12–18 months of follow-up by kinetic RT-PCR assays have yielded conflicting results. We investigated the effects of ‘target undetectable’ (TU) measurements versus those that were detectable but below the limit of quantification (<48 copies/ml; BLQ) on virological rebound in patients followed at two academic medical centres in Boston.

**METHODS:** Cox proportional hazard regression modelling was used to investigate the independent effects of the first VL after introduction of the Taqman RT-PCR assay (time-point T0), T0 CD4 count, race/ethnicity, gender, age and NNRTI use on risk of a confirmed or last VL >50, >200, >400 and >1,000 copies/ml censored at 22 months follow-up. Two separate analyses were performed: one using all patients with VL that was TU or BLQ at T0, and a second using a subset of these patients known to be virologically suppressed during the year prior to T0.

**RESULTS:** 778 patients had VL that was TU (N=596) or BLQ (N=182) at T0 (354 versus 98 patients, respectively, had documentation of suppressed viraemia during the year prior to T0). There were no significant differences between T0 covariates for both studies, with the exception of mean baseline CD4 counts which were significantly lower in the BLQ group (579 versus 657 for patients with undetectable VL for the year before T0; *P*<0.014). BLQ at T0, lower CD4 count and older age were independently associated with a subsequent confirmed VL >50, >200 and >400 copies/ml for all patients (*P*<0.05); a VL BLQ at T0 was independently associated with VL >50 and >200 (*P*<0.05), and marginally associated with VL >400 (*P*=0.058) in patients suppressed during the year prior to T0. Overall failure rates were very low: <5.5% of patients from all cohorts had confirmed VL >1,000 during follow-up. Only 26.4% of patients who failed with >200 copies/ml were on NNRTI-based therapy; 53% of patients with viral rebound re-suppressed to <50 copies.

**CONCLUSIONS:** A detectable VL <48 copies/ml was independently associated with subsequent virological rebound, suggesting that LLVL may be clinically significant and should be followed closely.
ABSTRACT 35
Antiviral Therapy 2012; 17 Suppl 1:A43

Naive T-cells remain a very stable reservoir of HIV after 5 years of suppressive combination antiretroviral therapy

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BACKGROUND: We previously demonstrated that HIV can stably persist in naive T-cells in patients receiving suppressive antiretroviral therapy (ART) for 24 months. The aim of this study was to describe the changes in the naive T-cell reservoir in a longitudinal prospective cohort after prolonged suppressive ART.

METHOD: Peripheral blood mononuclear cells (PBMC) were isolated annually from HIV-infected patients receiving ART over 5 years. Using flow cytometry PBMC were sorted into total memory T-cells (CD3+CD4+CD45RO+), CD31+ and CD31- naive T-cell subsets (CD3+CD4+CD28+CD45RO-). Total HIV DNA was quantified using real-time PCR and the change in HIV-DNA content within subsets over time was modelled using Generalised Estimating Equations (GEE). Single genome amplification using limiting dilution PCR that detects C2-V3-C3 region of envelope gp120 was performed. Phylogenetic analysis of C2-V3-C3 at different times on ART was conducted using ClustalW, Neighbour-Joining trees and estimate evolutionary divergence.

RESULTS: We present an extension of a longitudinal study of patients initiating ART (n=10). At initiation of ART, median CD4+ T-cell count was 157 cells/μl and viral load was 71,700 copies/ml. All patients achieved HIV RNA<50 copies/ml within 6 months of commencing ART and maintained this throughout follow up (2–5 years). Memory T-cells had a significantly higher HIV DNA content compared to both naive T-cells subsets (P<0.001). Using GEE to model changes in HIV-DNA content within the three T-cell subsets over 5 years, a significant decay was observed in both memory (coeff [95% CI] = -0.06 [-0.07 to -0.05]; P<0.0001) and CD31- naive T-cells (-0.02 [-0.03 to -0.01]; P=0.006). The decay was more rapid in the first 12 months compared to subsequent months. This significant biphasic decay was more pronounced in memory T-cells. HIV DNA in CD31+ naive T-cells remained stable with no significant change. Sequencing of the V3 loop (n=3) in T-cell subsets before and after 18 months and 5 years on ART, showed no compartmentalisation between subsets but sequences in T-cells on ART clearly clustered away from T-cells pre-ART.

CONCLUSIONS: HIV-infected CD31+ naive T-cells, largely thought to be recent thymic emigrants, are a very stable reservoir in HIV-infected patients on prolonged suppressive ART.
ABSTRACT 36

Antiretroviral Therapy 2012; 17 Suppl 1:A44

No evidence for evolution of plasma HIV-1 RNA or PBMC HIV-1 DNA during long-term suppressive antiretroviral therapy

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BACKGROUND: Our previous studies of HIV-1 population genetics in plasma during suppressive ART showed a lack of divergence from pre-therapy virus and the emergence of groups of identical sequences after long-term suppression. We have now obtained HIV-1 DNA sequences in PBMCs from the same patients to examine their relatedness to plasma virus.

METHODS: Single-genome pro-pol sequences from PBMCs were obtained from 8 subtype B-infected patients at the time of initiating ART and during suppression for 4–12 years and were compared to plasma RNA sequences from similar time points obtained previously from five of the eight patients. RNA sequences from rebound virus after ART interruption had also been obtained from three of the patients. DNA and RNA sequences were compared and analysed using phylogenetic methods to look for evidence of replication during ART (that is, diversification and branch lengthening compared to pre-ART sequences).

RESULTS: Sequences from cellular HIV-1 DNA and plasma RNA showed no clustering by source in any of the eight patients. There was also no evidence for evolution of HIV-1 DNA or RNA sequences obtained during suppression with ART. In two patients with low viral diversity at the start of ART, no further diversification or divergence was observed after 8 years on ART, but diversification resumed in one of these patients after interrupting ART. Clusters of identical HIV-1 DNA sequences were detected in six of nine patients during long-term suppression but none were divergent from sequences present at the time of initiating ART. In one patient, clusters of identical DNA sequences were detected 3 years after starting ART and the same sequences were still present 4 years later, further showing lack of evolution during ART.

CONCLUSIONS: Detailed analysis of single HIV-1 DNA and RNA sequences obtained longitudinally over 4–12 years on suppressive ART showed no evidence of viral evolution or replication, suggesting that the cells containing these sequences were infected before initiation of ART. The similarity of HIV-1 DNA sequences in blood mononuclear cells and from plasma RNA supports the idea that these cells may be a source of persistent viraemia during ART and of rebound virus following ART interruption.
ABSTRACT 37

Antiviral Therapy 2012; 17 Suppl 1:A45

Jak inhibitors exhibit a novel mechanism of HIV inhibition in primary human and rhesus macaque macrophages and lymphocytes

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BACKGROUND: Current therapy cannot eliminate HIV from all reservoirs, necessitating design of novel therapeutics with targets distinct from the HIV-1 replication cycle. The Jak-STAT pathway is activated in macrophages and lymphocytes upon HIV-1 infection, representing an attractive cellular target.

METHODS: Isolated primary macrophages were activated in m-CSF containing media for 18 h and differentiated for 7 days. Lymphocytes were activated in phytohaemagglutinin-containing medium for 72 h. Cells were treated with various concentrations of Jak inhibitors Tofacitinib, Jakafi, AZD-1480, LY2784544, Cyt387 or AZT (control) for 4 h prior to infection with HIV-1BaL (human macrophages), HIV-1 LAI, HIV-1ΔK65R, HIV-1ΔL74V (human lymphocytes) or RT-SHIV (macaque lymphocytes and macrophages). Cells were then maintained for 6 days before supernatant harvest and p24 or p27 ELISA determination. Cytotoxicity was assessed by MTT and Viacell Trypan blue methods. For combination studies, cells were infected and maintained in various concentrations of Jakafi and Tofacitinib at a 1:4 ratio for 6 days prior to supernatant harvest. CalcuSyn (Biosoft) was used for drug interaction analyses. Latently infected human lymphocytes were incubated with drugs for 2 h prior to reactivation with anti-CD3/anti-CD28. Reactivation was monitored by intracellular p24 (flow cytometry).

RESULTS: Tofacitinib and Jakafi demonstrated EC50 ranging from 0.02–0.08 μM and 0.02–0.3 μM in human and rhesus macaque lymphocytes and macrophages, respectively. No significant cytotoxicity was apparent 2–3 logs >EC50, resulting in a therapeutic window of ≥69. Combination of Tofacitinib plus Jakafi significantly decreased the EC50 and EC90 up to 117-fold, and displayed similar potency against both K65R and L74V mutant HIV-1. These drugs also inhibited reactivation of latent HIV-1 in human lymphocytes at low micromolar EC50 values.

CONCLUSIONS: These data demonstrate for the first time that targeted inhibition of Jak provides a safe, potent, and novel mechanism to inhibit 1) HIV-1 replication in lymphocytes and macrophages; 2) replication of drug-resistant HIV-1; and 3) reactivation of latent HIV-1. These findings merit further work to determine the potential clinical relevance of these FDA approved drugs in a macaque model and eventually in humans.
ABSTRACT 38

Antiviral Therapy 2012; 17 Suppl 1:A46

Direct evidence for the importance and potency of CD8-mediated immunity in control of HIV viraemia

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BACKGROUND: There are several lines of indirect evidence, from both in vitro and non-human primate models, for the importance of CD8+ T-cell mediated anti-HIV activity in regulating infection. However, the antiviral potency of this response remains unclear.

METHODS: We describe a case of a subtype-C-infected elite controller diagnosed with multiple myeloma and treated by autologous stem cell transplantation. He experienced viral rebound co-incident with melphalan-induced leucopenia, with viral control re-established as cell counts recovered. We undertook detailed analysis of plasma HIV RNA, whole blood proviral DNA, and performed CD8 killing assays and delineated CD8 IFN gamma responses by ELISPOT. Single-genome sequencing of env V1-V4 from plasma and PBMC was also undertaken.

RESULTS: The first detectable plasma viral load (6 days post melphalan administration) was 17,000 copies/ml, suggesting a minimal initial HIV doubling time of 0.48 days. After the next measurement at 13 days of 28,000 copies/ml, plasma viraemia decreased, commensurate with recovery of bone marrow, demonstrating two phase decay with half lives of 0.71 and 4.1 days, respectively. At day 13, his CD4:CD8 count was 310:920 cells/mm³, and similar ratios were observed both before transplant and one year later, in the context of consistently elevated total lymphocyte count. Total PBMC HIV DNA levels rose from <10 c to a peak of 127 c/10⁶ cells at day 12, followed by decay with t₁/₂ of 3.8 d (upper limit). Isolated CD8 lymphocytes after immune recovery reduced viral replication by 3 log₁₀ in autologous CD4 lymphocytes, and exhibited strong IFN gamma responses to the Gag epitope TPQDLNTRLML, consistent with his protective HLA B*8101 allele. Neutralising antibody titres were low and directly correlated with plasma viraemia levels. Emerging plasma virus was not clonal.

CONCLUSIONS: We present the first direct evidence for CD8-mediated anti-HIV activity in control of plasma viraemia in a patient. The response can be as potent as HAART in its impact on viraemia. These data provide a standard against which therapeutic vaccines could be compared in their efficacy.
ABSTRACT 39

**Antiviral Therapy** 2012; 17 Suppl 1:A47

Challenges inherent in detecting HIV persistence during potentially curative interventions

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BACKGROUND: The size of the HIV reservoir during long-term effective antiretroviral therapy and in 'elite' controllers is close to the limit of detecting using standard assays. This imposes challenges for the design and assessment of potentially curative interventions. We applied a series of measurements of HIV persistence to the study of the ‘Berlin Patient’, who underwent a haematopoietic stem cell transplant from a donor who was homozygous for the CCR5 delta-32 deletion and who had exhibited clinical evidence of being cured. Our objectives were to (1) determine if HIV had been fully eradicated as a consequence of the transplant and (2) define the potential role of various reservoir measurements in cure research.

METHODS: The subject underwent a series of intensive virological and immunological studies beginning approximately five years after this transplantation. Replication-competent virus was measured in two laboratories, and HIV DNA and RNA levels (from blood and rectal mucosa) were measured in several laboratories using different approaches.

RESULTS: A large volume apheresis was performed and 9 billion PBMCs evaluated for the presence of replication-competent virus. All wells were negative for HIV p24, indicating that the frequency of replication-competent HIV was, therefore, estimated to less than one infected cell per 1.4 billion CD4+ T-cells. A repeat experiment in a second laboratory confirmed these findings. Using a variety of assays and approaches, very low levels of HIV RNA were intermittently detected in plasma, although sequence analysis of these variants were different from each other and different from those present before the transplant. Digital PCR for HIV DNA was negative for 1 copy per 2 million cells with a 95% confidence limit of less than 1.9 copies per million cells. Collagenase-digested rectal biopsy-derived cells were positive for very low levels of HIV DNA but not RNA; no sequence for confirmatory studies could be obtained. HIV antibodies levels were low and declined over the course of approximately 18 months.

CONCLUSION: Although the subject has had intermittent evidence for HIV persistence in some assays in some laboratories, the extremely low levels of virus which were detected, while pushing the limits of sensitivity and specificity, and the inability to match sequence with the subject’s pre-therapy virus, make it impossible to conclude that the subject remains HIV infected.
SESSION 1
Resistance to new antiretroviral agents
ABSTRACT 40

*Antiviral Therapy* 2012; 17 Suppl 1:A51

**Evolution of HIV-1 partial resistance to the CCR5 inhibitor 5P12-RANTES**

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BACKGROUND: Resistance to CCR5 entry inhibitors occurs by selection of HIV-1 viruses capable of CXCR4 use or by evolution of virus to use inhibitor-bound CCR5. We have shown that the CCR5 antagonist 5P12-RANTES (QgPPLMATQS-RANTES10-68) could select for resistant CXCR4-using variants, but evolution of resistance with continued use of CCR5 for entry has not been described previously for this class of macromolecular CCR5 inhibitors.

METHODS: Resistance was selected by multiple rounds of R5 HIV-1 CC1/85 virus propagation with increasing 5P12-RANTES concentrations. In the cited work, the fourth round of selection led to resistance due to CXCR4 use between weeks 30 and 36 of virus passage. To repeat this experiment, virus from week 30 was cultured with 5P12-RANTES concentrations that increased from one IC50 dose (0.12 nM) to 78 IC50 doses (9.36 nM) over a period of an additional 70 weeks. The maximum percentage inhibition (MPI) was determined after each weekly passage. Envelope clones were isolated and sequenced when partial resistance developed. Use of the N-terminal domain of CCR5 was measured by sensitivity to the PA12 antibody, and alternative coreceptor use was determined.

RESULTS: No resistance due to evolution to CXCR4 use was observed. Instead, partial resistance to 5P12-RANTES emerged after 60 weeks of selection and was manifest by MPIs between 50–85%, but no change in IC50. The MPIs stabilized in this range after 90 weeks of selection at 5P12 concentrations between 5 and 9 nM, and uniform envelope sequences were observed after 70 weeks. Partial resistance was associated with five mutations in V3, seven mutations elsewhere in gp120, and three mutations in gp41. Virus isolates with partial resistance to 5P12-RANTES were highly resistant to inhibition by the PA12 antibody. Envelope clones from week 70 showed significantly diminished entry via alternative coreceptors and preserved use of CCR5 compared to baseline virus.

CONCLUSIONS: Development of partial resistance to 5P12-RANTES with maintenance of entry via inhibitor-bound or altered CCR5 can occur after prolonged virus selection, although it takes more envelope mutations and more time than resistance caused by switching to CXCR4 use.
ABSTRACT 41

Antiviral Therapy 2012; 17 Suppl 1:A52

Switching the NNRTI or the PI/r to maraviroc in aviraemic subjects infected with R5 HIV by V3-loop population sequencing: 48-week, prospective, randomized, controlled, pilot study

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BACKGROUND: The safety and efficacy of switching the third drug of ART to MRV in aviraemic subjects infected with R5 HIV according to proviral DNA V3 population sequencing (V3-PS) have not been evaluated in prospective randomized controlled trials.

METHODS: This was a pilot prospective open-label randomized controlled trial including HIV-1-infected aviraemic adults on stable ART for >1 year, self-reported adherence >90% and no ARV resistance or previous virological failure. Candidates were screened for presence of non-R5 HIV by triplicate V3-PS using a 20% Geno2Pheno (g2P) FPR cutoff. Those with R5 HIV were randomized 1:1 to switch the NNRTI or PI/r to MRV (300 mg twice daily) or to continue the same ART. The primary end point was the proportion of subjects with VL<50 copies/ml at week 48. HIV tropism was evaluated in all subjects at weeks 0, 4, 12, 24, 36 and 48 using 454 sequencing of proviral DNA (non-R5 defined as ≥2% HIV with a g2P FPR≤3.75). Non-parametric tests were used to test for differences between and within groups (ClinicalTrials ID NCT00966329).

RESULTS: A total of 30 subjects entered the study, including 15 per arm. They had been on ART for a median (IQR) of 7.4 years (3.0–12.0), and had VL<50 copies/ml for a median of 5.1 years (2.7–8.8). Median baseline and nadir CD4+ T-cell counts were 737 cells/mm³ (515–850) and 336 cells/mm³ (250–409), respectively. One subject in the control arm was lost to follow-up at week 14; one in the MRV arm interrupted therapy due to diarrhoea at week 1. One subject in the MRV arm and one control had non-R5 viruses in PBMcs by 454 sequencing: the subject receiving MRV was the only individual developing VL rebound during the study (VL=180 and 170 copies/ml at weeks 36 and 48, respectively). The remaining subjects, including the non-R5 control, had VL<50 copies/ml at week 48. CD4+ T-cell counts increased 75 cells/mm³ (130–190) and -103 cells/mm³ (-139–23) in the MRV and control arms, respectively (P=0.085). Median lipids and glucose levels improved in the MRV arm (P<0.01) but remained below the upper normality level.

CONCLUSIONS: In this small, prospective, randomized, trial switching the third drug to MRV was safe and overall effective. The only subject developing VL rebound under MRV had non-R5 HIV missed by V3-PS.
ABSTRACT 42

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Determination of HIV-1 tropism from proviral HIV-1 DNA in patients with suppressed plasma HIV-1 RNA using population based- and deep-sequencing: impact of X4-HIV variants on virologic responses to maraviroc?


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BACKGROUND: The clinical utility of tropism determination from HIV-1 DNA in patients with low or undetectable plasma HIV-1 RNA before maraviroc (MVC) prescription as well as the impact of integrated X4 minor population on virological response remains to be demonstrated. We aimed to describe the prevalence of R5 and X4 coreceptor usage as determined from bulk- and deep-sequencing of HIV-1 V3 DNA in treated patients with suppressed plasma HIV-1 RNA, and to evaluate the effect of X4 minority variants on MVC response.

METHODS: V3 bulk sequences generated from HIV-1 DNA were collected from 181 treated patients with suppressed (<50 copies/ml) plasma HIV-1 RNA attending nine European centres. Coreceptor usage was predicted from bulk sequences using the Geno2Pheno_coreceptor algorithm (FPR 10%). Proviral V3 deep sequencing was performed for 84/181 patients and the fraction of viruses predicted as X4 (>1%) was quantified using Geno2pheno[454] tool. The prevalence of low frequency (1–20%) X4 viruses in a subgroup of 41 R5-screened patients who changed therapy to a MVC-based regimen was estimated, with special interest in four patients who had virological failure (VF), defined as two consecutive plasma HIV-1 RNA>50 copies/ml.

RESULTS: V3 bulk sequence analysis in 181 patients showed that the prevalence of R5 and X4 coreceptor usage was 69.6% and 30.4%, respectively. Additional V3 deep sequencing analysis for 84 patients showed that only patients with X4 variants >20% (n=20) were identified as X4 by bulk sequencing; 22 patients harboured 1–20% X4 viruses and the remaining 42 patients had X4 variants <1%. Among the 84 patients, 41 had a switch to MVC-containing regimens. We found that 34.1% of these patients had 1–20% X4 viruses, in comparison with 41.7% of patients not treated with MVC. Three of the four patients who failed on MVC had 1–20% X4 viruses, whereas the prevalence of patients with plasma HIV-1 RNA remaining suppressed under therapy having 1–20% X4 viruses was 25.7%.

CONCLUSIONS: The quantification of X4 minority variants in proviral DNA does not seem to be a predictable tool for MVC response for patients with suppressed plasma HIV-1 RNA who need to change their current regimen.
ABSTRACT 43

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Plasticity of the ATP binding site of HIV-1 RT promotes binding of both INDOPY-1 and its ATP enhancer to the post-translocated complex

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BACKGROUND: Previous studies suggested that nucleotide-competing RT inhibitors (ncRTIs) can bind reversibly to the nucleotide-binding site of the enzyme. Mutations M184V and Y115F in HIV-1 RT were shown to confer resistance to these inhibitors, while K65R confers hyper-susceptibility. An intriguing observation is that the presence of aTP can enhance the inhibitory effects of the prototype compound IndoPY-1. However, the underlying mechanism remains elusive. We hypothesize that aTP traps the inhibitor in the binding site and prevents its dissociation. Residues in HIV-1 RT that facilitate the aTP-dependent excision of incorporated nucleotides may provide a potential binding pocket.

METHODS: We employed biochemical and cell-based assays to decipher the mode of IndoPY-1 and ATP enhancer binding, respectively. Drug susceptibilities were measured in MT-2 cells and PBMCs against mutant viruses with amino acid substitutions in the putative ATP binding pocket. The mutational patterns included TAM1, TAM2 and a cluster of mutations referred to as remodelled TAM: K70G, V75T, K219R and L228R. These mutations were shown to confer resistance to foscarnet, while resistance to thymidine analogues is diminished when compared with TAM.

RESULTS: In agreement with previous studies, TAM1 show insignificant changes in susceptibility to INDOPY-1. TAM2 show 2- to 5-fold increases in IC50 values. By contrast, remodelled TAM show 5- to 10-fold decreases in IC50 values. Opposite trends are seen when IC50 values are measured in the presence of foscarnet. Biochemical structure–activity relationship (SAR) studies with ATP derivatives suggest that the base moiety of the enhancer is an important structural motif that can influence binding.

CONCLUSIONS: The combined data suggest that ATP can stabilize the RT/DNA/INDOPY-1 complex. The specific mode of ATP binding is likely to be distinct from previously described complexes. The nucleotide-binding site is here occupied by INDOPY-1, which excludes a dNTP-like binding mode for the ATP enhancer. Moreover, in excision-competent complexes, ATP binds to the pre-translocated complex, while INDOPY-1 traps the post-translocated complex. Thus, the combined data suggest that ATP can also bind to the post-translocated complex. However, the in vitro susceptibility studies provide genetic evidence to demonstrate that the ATP binding pocket is likely to be modulated in this context.
ABSTRACT 44

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Detailed characterization of HIV-1 mutants selected by 3'-azido-2',3'-dideoxypurine nucleosides in primary human lymphocytes

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BACKGROUND: Nucleoside reverse transcriptase inhibitors (NRTIs) are key components of currently recommended ART, but long-term toxicity and the emergence of NRTI-resistant HIV-1 necessitates discovery of novel NRTI with better safety, potency and resistance profiles. 3'-Azidopurine nucleosides, for example, 3'-azido-2',3'-dideoxyadenosine (3'-azido-ddA [AZA]) and 3'-azido-2',3'-dideoxyguanosine (3'-azido-ddG [AZG]), are potent inhibitors that maintain activity against AZT-resistant HIV-1, and do not readily select for HIV-1 resistance in cell lines. Therefore, we performed selections with AZA and AZG in HIV-1 infected primary human lymphocytes, with a goal of characterizing mutants that may arise in humans with treatment.

METHODS: Serial passage of xxHIV-1_Lai in primary human lymphocytes was performed over 90 weeks (one passage/week) in the presence of AZA (4.2 μM), AZG (2.1 μM) or AZA+AZG (4.2 μM/2.1 μM). Viral RNA from passage supernatants was extracted, amplified by RT-PCR, and sequenced for resistance mutations in HIV-1 RT. Single-genome sequencing analysis was also performed on supernatants showing resistance mutations. Site-specific recombinant mutants were generated accordingly and phenotyping of passaged virus and recombinant mutants was performed in primary lymphocytes.

RESULTS: Resistance mutations K70R, M41L/L74V and F77L/F116L/Q151M were selected in lymphocytes treated with AZA, AZG and AZA+AZG, respectively, emerging at passage 64, 21 and 54, respectively. Additionally, an R358K connection domain mutant was selected by AZA, AZG and AZA+AZG. Population and single genomic sequencing analysis confirmed the TAMs as the main constituent of the viral resistant pools extracted at passage 84, along with R358K on the same viral genome.

CONCLUSIONS: These data are the first to show that drug resistant HIV-1 can be selected by AZA, AZG and AZA+AZG in primary human lymphocytes, and that the combination of AZA and AZG suppresses the emergence of TAMs observed with AZA or AZG alone. HIV-1 escape from AZA+AZG requires the selection of the Q151M complex. This information provides a better understanding of the resistance profiles of 3'-azidopurines.
ABSTRACT 45

Antiviral Therapy 2012; 17 Suppl 1:A56

E138K and M184I mutations in HIV-1 reverse transcriptase emerge concomitantly as a result of APOBEC editing in the absence of drug exposure

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BACKGROUND: Recent studies have demonstrated that cellular APOBEC3 restriction factors contribute to HIV sequence diversification and drug escape by the means of retroviral g-to-a hypermutation. Clinical trials with rilpivirine combined with emtricitabine and tenofovir revealed that patients failing treatment frequently harboured viruses encoding resistance-associated mutations in the HIV-1 reverse transcriptase at position E138K and M184I. Because E138K and M184I mutations may occur as a result of APOBEC3 editing, we propose here to investigate the potential role of APOBEC3 as a source of viral escape to rilpivirine-emtricitabine drug combination.

METHODS: We used a Vif K22H NL4-3 mutant that has suboptimal activity against APOBEC3G to infect MT-2 (APOBEC3G+ cells) and Sup-T1 cells (APOBEC3G cells). After two rounds of infection, we assessed the frequency of HIV mutations in quasispecies recovered from MT2 and SupT1 cells using molecular cloning and sequencing on the reverse transcriptase region. Additionally, to assess the degree of in vivo G-to-A viral hypermutation, a large data of HIV-1 RT proviral sequences from PBMcs recovered from infected patients under HAART was analysed.

RESULTS: In vitro infection of Vif K22H in APOBEC3G+ cells resulted in the generation of a large spectrum of G-to-A mutations, with the coemergence of both M184I and E138K in 8% of clones in the absence of drug exposure. By contrast, infection of APOBEC3G cells with either WT or defective Vif K22H mutant results in minor diversity in proviral quasispecies. In regard to PBMcs sequences, analysis of 605 RT sequences revealed that the copresence of mutations E138K and M184I were never detected in non-hypermutated sequences (n=572) contrasting with a high frequency (24%; n=7/29) in the context of APOBEC3 editing and in the absence of exposure to etravirine/rilpivirine (P<0.001). Importantly, some hypermutated sequences harbouring E138K and M184I mutations encoded an intact protein open reading frame.

CONCLUSIONS: We demonstrate using in vitro experiments and analysing patients PBMcs sequences that M184I and E138K mutations may pre-exist in proviral reservoir at a high frequency prior to drug exposure as a result of APOBEC3-driven mutagenesis. Thus, incomplete neutralization of one or more APOBEC3 proteins may favour viral escape to rilpivirine-emtricitabine.

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Antiviral Therapy 2012; 17 Suppl 1:A57

Resistance and potency of GS-7340, a next-generation tenofovir prodrug

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BACKGROUND: The HIV-1 NRTI tenofovir (TFV), which is metabolized intracellularly to its active form TFV-diphosphate (TFV-DP), is commercially available as the prodrug tenofovir disoproxil fumarate (TDF). GS-7340 is a novel TFV prodrug with greater plasma stability and achieves higher TFV-DP levels in peripheral blood mononuclear cells (PBMCs). GS-7340 has shown improved antiviral activity at lower doses than TDF in clinical monotherapy studies. The activity of GS-7340 against clinical isolates and drug-resistant HIV-1, resistance selection experiments, and in vitro potency differences between TDF and GS-7340 are described here.

METHODS: Antiviral activity was evaluated in PBMCs against a panel of wild-type (WT) and drug-resistant HIV-1 clinical isolates. The GS-7340 resistance profile was further assessed using the PhenoSense™ assay (Monogram) against 24 NRTI-resistant viruses. Resistance selections were performed using dose-escalation methods (MT-2/HIV-1IIIB). Potency experiments were performed in the presence of ARV drugs pretreated for 0–30 min in human serum.

RESULTS: GS-7340 displayed potent antiviral activity against all HIV-1 groups/subtypes (mean EC₅₀ 3.6 nM; n=26). GS-7340 was also active against HIV-2 (mean EC₅₀ 1.8 nM; n=3). The resistance profile of GS-7340 was almost identical to TFV, with highly correlated fold-change values (versus wild-type; R²=0.97), but GS-7340 had much lower EC₅₀ values. Both TFV and GS-7340 selected viruses with the K65R mutation within five passages that displayed a 4–6-fold change in their sensitivity to both TFV and GS-7340. The potency of the two TFV prodrugs was directly compared in antiviral assays. Although their potency was similar without serum pretreatment, TDF activity was significantly reduced in the presence of serum. Unlike TDF, GS-7340 maintained its potency after prolonged serum treatment, reflecting the known plasma stability of GS-7340.

CONCLUSIONS: TDF and GS-7340 show similar activity against HIV isolates from all clades and resistant isolates, and both select K65R in vitro. However, GS-7340 demonstrated superior antiviral potency to TDF in vitro in the presence of human serum, consistent with the higher intracellular TFV-DP levels and greater antiviral activity seen clinically at significantly lower doses than TDF. Furthermore, the higher intracellular TFV-DP levels achieved with GS-7340 may enable activity against previously defined TDF-resistant isolates.
ABSTRACT 47

Antiviral Therapy 2012; 17 Suppl 1:A58

Baseline and emerging resistance to etravirine in HIV-1-infected children and adolescents: final results from the Phase II PIANO Study

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BACKGROUND: The Phase II PIANO Study evaluated efficacy, safety, pharmacokinetics and resistance of etravirine (ETR) plus other antiretrovirals in children and adolescents. Week 48 resistance findings are presented.

METHODS: In this open-label single-arm study, multi-class-experienced HIV-1-infected children (≥6 to <12 years; n=41) and adolescents (≥12 to <18 years; n=60) received ETR (5.2 mg/kg twice daily, maximum daily dose 200 mg twice daily) plus an optimized background regimen of a boosted protease inhibitor plus N(t)RTIs and optional enfuvirtide and/or raltegravir. Participants were required to have full activity to ETR (VircoTYPE HIV-1 test) at screening.

RESULTS: Overall, 82% of adolescents versus 66% of children previously used one or two NNRTIs. In total, 41 (41%) patients were virological failures (VFs): 29 non-responders (8 children) and 12 rebounders (3 children). Matched genotypic and phenotypic data at baseline and end point were available for 23/41 VFs. A higher median number of baseline NNRTI resistance-associated mutations (RMs) was observed in VFs (2.0) versus non-VFs (1.0). Baseline Antivirogram results showed 89.2% (33/37 with phenotypic data) of VFs were sensitive to ETR and 10.8% (4/37) had an ETR fold-change (Fc) between 3 and 13. A total of 17 VFs (41.5%) had ≥1 ETR RMs, of which 7 had an ETR weighted-genotypic score (WGS) predicting an intermediate ETR response. The median ETR FC and ETR WGS increased from, respectively, 0.9 and 0 at baseline to 3.6 and 1.5 at end point. NNRTI RMs (extended list) emerging in ≥3 VFs included Y181C (n=8), V90I (n=3), L100I (n=3) and E138A (n=3). Fewer children (42.9%) than adolescents (65.2%) developed NNRTI RMs. Both genotypic and phenotypic ETR resistance development was observed in 39.1% (9/23) of VFs. In 47.8% of VFs (11/23), no ETR resistance development was observed, whereas in 13.0% (3/23) only phenotype indicated resistance.

CONCLUSIONS: In the PIANO Study, most VFs were non-responders. In 39% of VFs both genotype- and phenotype-based ETR resistance was observed. A higher proportion of adolescents than children previously used NNRTIs, experienced VF and developed NNRTI RMs. Emerging NNRTI RMs in this study were previously defined as ETR RMs in the DUET studies and confirm that the ETR resistance profile is consistent in adolescents and children.
Prevalence of pre-existing resistance associated mutations to rilpivirine (TMC278), emtricitabine, and tenofovir in antiretroviral-naive patients

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ABSTRACT

Background: Rilpivirine is a non-nucleoside reverse transcriptase inhibitor (NNRTI), which proved to be effective against wild-type HIV-1 strains and some NNRTI-resistant mutants. A single tablet of emtricitabine, tenofovir disoproxil fumarate and rilpivirine is also available. The objective of this study was to determine in antiretroviral-naive patients the prevalence of rilpivirine, emtricitabine, and tenofovir resistance associated mutations, described in vitro and in vivo.

Methods: Our database consists of 1,729 treatment-naive samples, from Pitié Salpêtrière Hospital and Bichat Hospital, which have been sent for resistance testing by bulk sequencing from 2008 to 2011. The rilpivirine resistance mutations K101E/P, E138A/G/K/Q/R, V179L, Y181C/I/V, H221Y, F227C and M230I/L (according to the US Food and Drug Administration), and additional NNRTI mutations V90I, L100I, K101T, E138S, V179D/I, Y188L, V189I, G190A/E/S and M230V (International AIDS Society, Stanford database and Agence Nationale de Recherche sur le Sida [ANRS]) were included. M184V/I and K65R were studied for emtricitabine and tenofovir, respectively.

Results: We studied 1,729 antiretroviral-naive infected-patients with 50% of B subtype viruses and 50% of non-B subtype viruses (CRF02 in 27% of non-B samples). Among 1,729 sequences, 344 (19.9%) harboured ≥1 rilpivirine mutation: 295 sequences (17%) had one rilpivirine mutation, 44 sequences (2.5%) had two rilpivirine mutations, 2 sequences (0.12%) had three rilpivirine mutations and 3 sequences (0.17%) four rilpivirine mutations. The mutations found to be most prevalent in this analysis were V179I in 145 cases (8%), V90I in 65 cases (3.8%), E138A in 52 cases (3%) and V189I in 40 cases (2.3%). Of these, the common V179I, V189I and V90I polymorphisms have not been associated with virological failure in Phase 3 clinical studies. According to the ANRS algorithm, 4.85% of samples were resistant to rilpivirine, 3.69% of B subtype viruses versus 6.03% of non-B subtype viruses (P=0.02, χ² test). The prevalences of the K65R and M184I/V were 0.06% (1/1,729) and 1% (18/1,729), respectively.

Conclusions: Prevalence of rilpivirine, emtricitabine and tenofovir resistance mutations was very low in antiretroviral-naive patients. Overall, the prevalence of resistance to rilpivirine was 4.85% by ANRS. However resistance testing should be considered before initiation of NNRTI-based treatment in antiretroviral-naive patients.
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Resistance analysis by historical genotypes and at week 24 in subjects switching from 2 NRTIs + boosted PI to the FTC/RPV/TDF single tablet regimen (Study 264-0106: SPIRIT)

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**BACKGROUND:** SPIRIT is a 48-week randomized study to evaluate switching from a stable regimen consisting of two NRTIs and a boosted protease inhibitor (boosted PI group) to the single tablet regimen of emtricitabine/rilpivirine/tenofovir DF (FTC/RPV/TDF) in virologically-suppressed HIV-1-infected subjects. This analysis describes historical resistance mutations in subjects switching to FTC/RPV/TDF and emergent resistance in virological failures through week 24.

**METHODS:** Historical genotypes were analysed to confirm sensitivity to FTC/RPV/TDF at study entry; mutations not listed in the report had the wild-type amino acid imputed. Confirmed virological failures through week 24 or discontinuation samples with >400 copies/ml HIV-1 RNA were analysed for emergent resistance by PhenoSense gT (Monogram Biosciences).

**RESULTS:** In this study, 476 subjects were randomized and treated: 317 to FTC/RPV/TDF and 159 to continue their boosted PI regimen. Historical genotypes (n=475) were used to determine sensitivity to FTC, RPV and TDF according to the following list of 13 exclusion mutations in RT: K65R, K101E/P, E138G/K/R/Q, Y181C/I/V, M184V/I or H221Y. One subject with L100I+K103N was also excluded. Of those in the FTC/RPV/TDF group, 40 subjects (13%) had evidence of ≥1 pre-existing primary resistance mutation: 31 had primary NNRTI-R consisting of K103N (n=17), V108I (n=3), E138A (n=6) and G190A/S (n=4), 4 had an exclusion mutation (E138G, E138K, E138Q and H221Y), 4 had primary NRTI-R and 7 had primary PI-R mutations. After switching to FTC/RPV/TDF, 38 of 40 subjects maintained virological suppression at week 24; 2 discontinued while suppressed before week 24. Drug resistance-associated mutations emerged in two subjects with virological failure (2/317; 0.6%) in the FTC/RPV/TDF group and one subject (1/159; 0.6%) in the boosted PI group. In the FTC/RPV/TDF group, one subject had M184I at discontinuation (day 45); the other subject had HIV-1 RNA>50 copies/ml at baseline and V90I, L100I, K103N and M184V at week 4. In the boosted PI group, one subject had M184V at week 12.

**CONCLUSIONS:** The rate of resistance development was low in these virologically suppressed subjects switching from a stable boosted PI-based regimen to FTC/RPV/TDF (<1% of subjects). Subjects with pre-existing mutations, such as K103N, remained suppressed through 24 weeks of therapy with FTC/RPV/TDF.
ABSTRACT 50
Antiviral Therapy 2012; 17 Suppl 1:A61

Follow-up analysis of integrase sequence alterations in the course of non-failing raltegravir treatment regimens

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BACKGROUND: Raltegravir (Ral), the only integrase inhibitor approved for use in HIV therapy, has been recently licensed in Israel. More than 30 different mutations have been associated with resistance to Ral. However, little is known about the effect of integrase sequence alterations that by themselves do not induce treatment failure, on the development of drug resistance. Our objective was to identify integrase-related sequence alterations in the different HIV-1 subtypes present in Israel in the course of non-failing Ral-including antiretroviral treatment (ART) regimens. We have also followed-up a cohort of our patients aiming to evaluate the integrase sequence at different time points under therapy.

METHODS: A total of 176 samples from 153 Ral-treated (50 samples) or Ral-naive (40 samples from drug-treated, 86 samples from drug-naive) patients were analysed. From 15 individuals, we also analysed two consecutive samples, ≥6 months apart. RNA (when plasma viral load was >1,000 copies/ml) or DNA were isolated and used for subtype classification and integrase sequence analysis.

RESULTS: Subtype classification revealed 43% of the samples to be subtype B, 39% subtype C and 18% subtypes A/AE or D (one individual). As expected, no major resistance mutations were identified in this studied population; however, several minor mutations and other sequence alterations, significantly related to Ral treatment were observed, including L68V/I and G163R/K which were observed in Ral-treated samples only. V72I, observed in Ral-naive individuals in all subtypes, was diminished upon Ral treatment. In the group of patients for which two consecutive samples were analysed, viral evolution was evident even under undetectable plasma HIV viral load, as expressed by differences in the integrase sequences between samples from the same individual, suggesting transcriptional activity of the virus.

CONCLUSIONS: In the course of non-failing Ral treatment, only mutations currently unknown to result in Ral resistance, were identified. The clinical relevance of these sequence changes is yet unclear. Our study also demonstrates the ongoing evolution of the viral reservoir even upon effective treatment regimens. We will continuously follow-up the integrase sequence in these patients.
ABSTRACT 51

Antiviral Therapy 2012; 17 Suppl 1:A62

High barrier to resistance for dolutegravir (DTG, S/GSK1349572) against raltegravir resistant Y143 mutants: an in vitro passage study

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BACKGROUND: Dolutegravir (DTG; S/GSK1349572) is a once daily unboosted INI with limited cross resistance to other INIs in vitro, and currently under Phase 3 clinical development which includes investigation of subjects with raltegravir (RAL) resistance (VIKING study). Three main pathways (Y143, Q148 and N155) of RAL resistance have been described; we have previously reported a high barrier to resistance in an in vitro passage study starting with the RAL resistant associated and main pathway mutations E92Q, Q148H/K/R and N155H. Here we report an in vitro passage study starting from the remained main pathway mutant, Y143 c/r.

METHODS: Wild-type HIV-1 infectious molecular clone NL4.32 and RAL-resistant site-directed mutants (Y143C or R) were passaged for 56 days with INIs in MT-2 cells. We investigated the anti-HIV activity (EC50) against viral pools using HeLa-CD4 cells carrying a reporter β-galactosidase gene driven by HIV-1 LTR. Fold change (FC) was calculated as a ratio of EC50 to that of wild-type.

RESULTS: An initial concentration of 32 nM of DTG completely prevented replication of Y143C/R. With an initial concentration of 6.4 nM DTG, Y143R could replicate, but no additional amino acid substitutions were detected during the passage. Y143C also could replicate, but only for a maximum of 3 weeks; these viruses did not accumulate additional substitutions. In case of RAL, viral replication of Y143C/R occurred in initial concentration of 160 nM – starting from Y143R, L74M/Y143R (FC=78) and Y143R/N155H (FC≥1,400) were isolated at day 56, whilst when starting from Y143C, Y143C was replaced by Y143R (FC=11–51) in some wells at day 28. E92Q/Y143R (FC=45), Y143R/G163R (FC=21) and Y143C/G163R/E170A were isolated at day 56.

CONCLUSIONS: DTG (at 32 nM) prevented viral replication of Y143C/R completely; no additional substitutions were observed during passage. Our results are consistent with the outcome of VIKING study, in which all subjects who had Y143, N155 or Q148 single mutant achieved the primary end-point at day 11 (18/18 [100%] in cohort I and 12/13 [92%] in cohort II). DTG is thus expected to be a potent and durable therapy for subjects with mutations at position 143 in HIV integrase.
SESSION 2
Mechanisms of HIV drug resistance
ABSTRACT 52

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Higher CD4 affinity can confer resistance to maraviroc and could also lead to enhanced entry efficiency, replicative fitness and sensitivity to CD4i antibodies

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BACKGROUND: Maraviroc (MVC) binds a hydrophobic cavity within CCR5, alters conformation and prevents interaction with the HIV-1 gp120 V3 loop. Aside from outgrowth of CXCR4 using virus, different evolutionary pathways in HIV-1 env appear to confer MVC resistance, but always linked to V3 loop changes. The primary mechanism of MVC resistance appears related to use of MVC-bound conformations of CCR5.

METHODS: MVC resistance was selected in a panel of R5 using primary HIV-1 isolates using drug escalation in culture. Gp120 sequences from passage control and MVC-treated cultures were cloned into NL4-3 via yeast-based recombination followed by testing clonal virus for drug susceptibility, replicative fitness, CD4/CCR5 affinity via Affinofile and binding assays. Molecular models were built from the 1G9M and 2QAD gp120-CD4 structures.

RESULTS: After 21 weeks of passage, a primary isolate HIV-1 developed >1,000 fold resistance to MVC. Nine mutations were identified in >100 gp120 sequences, which were then analysed for MVC sensitivity. Instead of V3 loop mutations, MVC-resistant viruses harboured N425K capable of replicating in presence of high MVC concentrations (MPI effect) and with a 70-fold shift in MVC IC50 values. N425K was associated with higher replicative fitness than the parental virus, had higher binding affinity to soluble CD4, and was efficient at infecting 293T cells bearing very low levels of CD4 but across a wide range of CCR5 on the cell surface. Using the COOT programme and energy minimization conditions, enhance CD4-gp120 interactions appeared related to the K425 side chain in gp120 forming a new hydrogen bond with the oxygen from S42 of CD4 as well as cation-pi interaction with F43 aromatic ring.

CONCLUSIONS: Emergence of N425K is responsible for enhanced CD4 binding and represents a novel mechanism for MVC resistance, which could be overlooked in MVC treatment failures. K425 is found in less than 1% of all circulating HIV-1 isolates. We suspect that the MVC resistance N425K mutation may prolong the envelope glycoproteins binding to CD4, eliciting broadly neutralizing antibodies, and lead to hypersusceptibility to CD4i antibodies.
ABSTRACT 53
Antiviral Therapy 2012; 17 Suppl 1:A66

Higher desolvation energy reduces molecular recognition in multidrug-resistant HIV-1 protease

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BACKGROUND: Peptidomimetic design based on HIV-1 protease substrates is a popular strategy for the design of HIV-1 protease inhibitors. Four clinical isolates of multidrug-resistant HIV-1 protease have been studied in our laboratory. A multidrug-resistant HIV-1 protease isolate, MDR 769, was previously crystallized, and the protease adopts a wide-open flap conformation in the absence of ligand.

METHODS: Four multidrug-resistant HIV-1 protease isolates were expressed in E. coli and tested for resistance to nine US FDA-approved HIV-1 protease inhibitors. In addition, the four MDR proteases were assayed for binding preference to the cleavage substrates MA/CA, CA/p2, p2/NC, NC/p1, p1/p6, TF/PR, PR/RT, RT/IN and RT (internal). The inactive (D25N) MDR 769-p2/NC complex was crystallized with 0.1 M citric acid and 2.4 M (NH4)2SO4 at pH 5.2, while the inactive MDR 769-CA/p2 complex was crystallized with 0.1 M MES and 2.4 M (NH4)2SO4 at pH 6.0. Based on the inactive MDR 769 complex structures, four multidrug-resistant HIV-1 protease isolates and a wild-type HIV-1 protease were modelled and simulated for 10 ns using NAMD V2.7b.

RESULTS: A multidrug-resistant HIV-1 protease isolate, MDR 769, was co-crystallized with two heptapeptides that display different levels of recognition by the wild-type and multidrug-resistant HIV-1 protease. The two heptapeptides represent the p2/NC cleavage site and a mutated CA/p2 cleavage site. A wild-type HIV-1 protease and four multidrug-resistant HIV-1 proteases in complex with the p2/NC and CA/p2 P1′F peptides were modelled based on the MDR 769 co-crystal structures and examined during a 10 ns molecular dynamics simulation. The molecular dynamics study reveals that the multidrug-resistant HIV-1 proteases require higher desolvation energy to form complexes with the heptapeptides.

CONCLUSIONS: The results suggest that desolvation of the HIV-1 protease active site is an important step in the protease–ligand complex formation as well as drug resistance. Therefore, the desolvation energy could be considered as a parameter in the evaluation of future protease inhibitor candidates.
ABSTRACT 54

Antiviral Therapy 2012; 17 Suppl 1:A67

Occurrence of HIV-1 Gag and protease mutations after failure of darunavir-containing therapies

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BACKGROUND: Treatment of HIV-infected patients can result in failure because of drug resistance. Resistance interpretation tools focus on drug target regions (for example, protease [PR]). Resistance development in HIV is much more complex and mutations in regions beside the drug targets occur, conferring by themselves and/or increasing resistance. The cooperation of Gag and PR mutations is crucial in the evolution of resistance to protease inhibitors (PIs). Here, we focused on Gag, the substrate of the protease, in darunavir (DRV) failing therapies.

MATERIALS AND METHODS: 43 HIV-1 isolates were analysed in the complete gag and pol gene (17 after more than 3 months after the start of DRV-containing treatment [failure group] and 26 from patients whose HIV replication had been successfully suppressed for at least 6 months [success group]). Resistance mutation penalty scores for PR and RT mutations were received from the Stanford HIVdb. Penalty scores for Gag mutations were set appropriate to those for PR mutations.

RESULTS: The comparison of both groups showed a higher number of any therapy-associated (TA) Gag mutations in the failure group. PR mutations associated with PI resistance were found in 14 of 17 isolates in the failure group (success: 12/26). Each isolate in this group harboured at least one TA cleavage site (CS) mutation. The most prominent TA-CS mutations V128I, A431V and I437V were found in 4 or 5 out of 17 isolates. The genotypic DRV resistance interpretation including Gag mutation penalty scores would lead to an increase in DRV resistance in 5 of 17 isolates. In particular, the isolate with intermediate DRV resistance showed, beside the DRV-scored mutations in the PR (V32I/I84V), TA-CS and non-CS mutations in Gag (V128I/V370M/A431V/P453L). Considering the Gag mutation scores, this isolate would be predicted as high-level resistant to DRV (PR score: 30 + Gag score: 30=60). The score without Gag seemed to underestimate the DRV resistance in at least 1 of 17 isolates.

CONCLUSIONS: The consideration of treatment-associated Gag mutations in the genotypic resistance interpretation algorithms can increase the accuracy of the resistance scoring for PIs. Further improvement can be achieved by scoring of combination rules of Gag and protease mutations.
ABSTRACT 55

Antiviral Therapy 2012; 17 Suppl 1:A68

Resistant minority species are rarely observed in patients on darunavir/ritonavir monotherapy

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OBJECTIVES: To analyse emergence of resistant viruses in patients failing darunavir monotherapy including minority species, and to investigate the impact of baseline reverse transcriptase (RT), protease (PR) and gag mutations on virological failure (VF) occurrence.

METHODS: Nine of the 225 HIV-1-infected patients enrolled in the MONOI Trial (darunavir/ritonavir monotherapy or darunavir/ritonavir + 2 nucleosides reverse transcriptase inhibitors [NRTIs] in a switch strategy) experienced VF defined as 2 plasma HIV-1 viral load >400 copies/ml at least 2 weeks apart. Among these nine patients on VF, five were in the darunavir/ritonavir monotherapy arm and four in the triple darunavir/ritonavir therapy arm. Bulk sequences of the PR, RT and gag genes at baseline (on DNA) and at the time of VF (on RNA) were determined on all patients with 2 VL >50 copies/ml at least 2 weeks apart (n=47). PR and gag gene clonal analysis was performed on plasma samples of the nine patients on VF.

RESULTS: There was no association between mutations in RT, PR and gag genes in DNA and VF occurrence. None of patients demonstrated selection of darunavir resistance mutation among the 47 patients with a viral load >50 copies/ml at least 2 weeks apart. Virus of one patient on nine with VF selected minority variants with darunavir resistance mutations at position 32, 47 and 50. Clonal analysis of the gag region in the nine VF did not show any selection of minority variants.

CONCLUSIONS: In patients in case of failure to darunavir/ritonavir monotherapy, we did not show any selection of darunavir resistance mutation using standard genotype testing. However, virus of one patient among nine failures presented minority variants with darunavir resistance mutations.
ABSTRACT 56

Antiviral Therapy 2012; 17 Suppl 1:A69

Description of the L76V multidrug resistance protease mutation in HIV-1 B and ‘non-B’ subtypes

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BACKGROUND: To describe the prevalence of the L76V multidrug protease inhibitors resistance-associated mutation (PI-RAM) in relation with patient characteristics and protease genotypic background in HIV-1 B- and ‘non-B’-infected patients.

METHODS: Frequency of the L76V mutation between 1998 and 2010 was surveyed in the laboratory database of three clinical centres: two in Paris, France and one in Roma, Italy. Major PI-RAMs were identified according to the IAS-USA list. Fisher’s and Wilcoxon tests were used to compare variables.

RESULTS: Among the overall 29,643 sequences obtained from both drug-naive and drug-treated patients between 1998 and 2010, the prevalence of the L76V mutation was 1.50%, while was 3.94% in PI-resistant virus (n=5,209), derived from PI-experienced patients. A similar prevalence was observed among B- and ‘non-B’-infected patients (1.32% versus 1.63%, P=0.083). 179 patients harbouring L76V-mutated virus with known clinical and therapeutic history were further analysed, including 66% and 34% of B- and ‘non-B’-infected patients, respectively. CRF02_AG was the most prevalent ‘non-B’ subtype (47%). Median time since diagnosis of HIV-1 infection and median time under antiretroviral-based regimen were both shorter in ‘non-B’- compared to B-infected patients (8 versus 11 years, P<0.0001; and 7 versus 8 years, P=0.004). Although the duration of PI-based regimen was similar (60 months), ‘non-B’-infected patients had been pre-exposed to a lower number of PI (2 versus 3, P=0.016). Similar proportion of B- (76%) and ‘non-B’-infected (75%) patients were pre-exposed to lopinavir. Among the 41 patients without lopinavir pre-exposure, half had previously received indinavir. The L76V was associated with a lower number of major PI-RAM in ‘non-B’ versus B samples (3 versus 4, P=0.001). The M46I/L was the most prevalent associated PI-RAM both in B and ‘non-B’ samples (92 and 82%, respectively). The L76V as single major PI-RAM was significantly more frequent in ‘non-B’ versus B subtypes (10% versus 2%, P=0.014). Four out of the six ‘non-B’-infected patients failing a PI-based regimen with the single L76V major mutation were on first-line lopinavir-based regimen.

CONCLUSIONS: The frequency of selection of the L76V PI-RAM was similar between B- and ‘non-B’-infected patients. However, in ‘non-B’-infected patients this mutation appeared more rapidly and was associated with less PI-RAM.
ABSTRACT 57

Antiviral Therapy 2012; 17 Suppl 1:A70

Hypermuation in PRO and RT gene of HIV-1 and the correlation with Vif

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BACKGROUND: The inhibition of viral replication by APOBEC3 proteins is due to the hypermutation of minus strand virus DNA (G to A mutations). This antiviral activity is neutralized by the virion infectivity factor (VIF) that prevents the incorporation of APOBEC3G into progeny HIV-1 virions and promote its degradation. Some mutations in target genes of treatment (PRO/RT) are associated with mutations G to A and may be a consequence of the effect of APOBEC. In this work, we evaluate the levels of G to A hypermutation in RT and PRO genes and the correlation with resistance mutations from samples of patients with treatment failure.

METHODS: The RT and Pro gene were sequenced from 640 samples of individuals with treatment failure, using RT and protease inhibitor from clinical follow-up in the Brazilian programme National Network of HIV-1 genotyping. The levels of hypermutations of each sequence were evaluated. The samples with statistical significance for hypermutation levels were selected and the vif gene sequenced.

RESULTS: Using a subtype B standard, 11 samples profile hypermutation P<0.09, 7 samples showed hypermutation profile of P<0.05. Using a default subtype C, two samples that are of subtype C showed hypermutation profile of P<0.05, using a subtype F sample showed no profile hypermutation. In 18 samples with hypermutation profile of P<0.09 the mutation M36I, L63P, L89M, I93L at the protease, K103N, M184V at the RT more frequently. One sample showed a stop codon in the areas that are relevant to the interaction of cellular proteins with VIF. Could not find any mutation in the active domains of Vif, since these viruses are replicating competent. We found other Vif polymorphism with high frequency between amino acids 33–39 and 122–128. These polymorphisms maybe modulating the activity of Vif, allowing a residual activity of Apobec causing resistance mutations.

CONCLUSIONS: Mutations in RT and protease maybe due to hypermutation process APOBEC of the genome, since a functional VIF less active but not discriminate all activity hypermutation. Hypermutation contributes to the variability of the virus and may facilitate the escape immune or drug selection pressure. New tests will be made to examine whether hypermutation decreases the genetic barrier and impacts on ARV treatment.
ABSTRACT 58

Antiviral Therapy 2012; 17 Suppl 1:A71

Mutations in the connection domain of HIV-1 RT contribute to drug resistance

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BACKGROUND: Recent studies have shown that mutations outside of the polymerase domain of RT contribute to drug resistance. In this study, we investigate role and mechanism of several substitutions in the connection and RNase H domain of RT that are found with increased frequency in treated patients.

METHODS: Mutations R356K, G359S, A400T, I506L and K527N were introduced by site-directed mutagenesis into the wild-type HIV-1 molecular clone, and in a background containing M184V or (M41L+T215Y). In vitro drug susceptibility of these viruses was assayed on TZMbl cells. DNA polymerization and RNase H activity were studied using virion-derived RT enzymes, an RNA template mimicking the HIV-1 PBS region and a DNA primer complementary to the PBS. The products were analysed on denaturing sequencing gels.

RESULTS: Mutations R356K, G359S, I506L and K527N did not affect the susceptibility to NRTIs and NNRTIs. Substitution A400T is a polymorphism that increases in frequency in the treated population. We demonstrate that mutation A400T contributes to resistance to non-nucleoside RT inhibitors. A five-fold increase in nevirapine resistance was measured. Biochemical assays were performed to investigate the mechanism by which this mutation influences NNRTI susceptibility. Mutation A400T does not affect DNA polymerization in the wt and (M41L+T215Y) backgrounds. However, this mutation was found to partially restore the reduced DNA polymerization of the M184V variant. RNase H cleavage was reduced by mutation A400T in all backgrounds.

CONCLUSIONS: These results support a model in which mutation A400T increases NNRTI resistance by reducing template RNA degradation, thereby providing more time for dissociation of the NNRTI and re-initiation of DNA synthesis.
ABSTRACT 59

Antiviral Therapy 2012; 17 Suppl 1:A72

Structural dynamics of HIV-1 reverse transcriptase as a new target to block HIV replication

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BACKGROUND: Reverse transcriptase (RT) plays an essential role in the replication of human immunodeficiency virus type-1 (HIV-1) and remains a primary target of anti-HIV-1 drugs. The biologically active form of HIV-1 RT is a heterodimer of two subunits, p51 and p66, each consisting of distinct sub-domains: the fingers, the palm, the connection, the thumb and the RNase H sub-domains, the latter only present in p66. The formation of RT is a two-step mechanism, involving first the rapid association of the two subunits: dimerization, followed by conformational changes: maturation yielding the biologically active form of the enzyme.

METHODS: We have elaborated a new strategy based on short interfacial peptides that target protein–protein interfaces involved in RT activation. RT maturation mainly involves interactions between the thumb domain of p51 and RNase-H domain of p66. In a screen of peptides derived from the thumb domain of this enzyme, we have identified a short peptide of Paw, which inhibits the maturation step and abolishes replication of HIV-1 LAI with an IC50 of 1 nM. We have identified the sequence and structural features required for Paw activity and derived a shorter 12-mer peptide P27.

RESULTS: We demonstrated that P27 is 10-fold more potent in vitro (Ki: 45 nM) and exhibiting a potent antiviral activity with an IC50 of 0.12 nM and a selectivity index of about 5,400. P27 only binds dimeric RT and stabilizes it in an inactive/non-processive dimeric conformation. From a mechanistic point of view, P27 prevents proper binding of primer/template and of dNTP. We have showed that P27 does not act as classical non-nucleoside inhibitors, such as efavirenz, and that sub-nanomolar concentrations of P27 block the replication of multidrug-resistant strains. Moreover, a stable cyclic-D form of P27 can be used in synergy with other NRTI and NNRTI and we were unable to isolate P27 resistance strain after 6 months.

CONCLUSIONS: Taken together, these results confirmed that HIV-1 RT maturation constitutes an attractive target for AIDS chemotherapeutics and for the design of more specific new antiviral drugs that can bypass resistance limitation.
ABSTRACT 60

*Antiviral Therapy* 2012; 17 Suppl 1:A73

Risk factors for raltegravir resistance development in clinical practice

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BACKGROUND: Raltegravir resistance is conferred by integrase mutations through three main genetic pathways: Q148R/H/K, N155H and Y143H/R/C. The aim of this study was to define the best conditions of raltegravir use to avoid the selection of resistance mutations in case of virological failure.

METHODS: One hundred sixty-one (161) patients failing a raltegravir-containing regimen with two consecutive HIV-1 viral loads quantified above 20 copies/ml were studied. Ten parameters (HIV-1 RNA at baseline and failure, CD4 at baseline and failure, genotypic sensitive score [GSS] of the treatment associated with raltegravir, protease inhibitors used in the regimen or not, time spent on raltegravir, subtype, sex and age) were investigated, using a logistic regression, as predictive factors of the emergence of at least one of the main resistance mutations to raltegravir. Phenotypic susceptibility to raltegravir was studied in 16 patients without main resistance mutations to raltegravir at failure.

RESULTS: At failure, 46 (28.6%) patients had occurrence of at least one integrase resistance mutations at one of the three main residues, whereas 115 (71.4%) had no mutation. Nineteen patients (11.8%) and 21 (13%) showed mutations linked to the 148 and 155 pathways, respectively. Two (1.2%) patients showed mutations linked to the 143 pathway and 4 (2.5%) others showed a mixture of mutations linked to two different pathways. High HIV-1 viral load level at failure (OR=2.8, *P*<0.001) and low GSS of the drugs combination associated to raltegravir (OR=11.6, *P*<0.001), were independently associated with the selection of integrase mutations to raltegravir. The percentages of patients with integrase resistance mutations were 7.7% versus 48% when HIV-1 viral load is ≤200 or >200 copies/ml and 48% versus 9% when GSS <2 or ≥2. Among patients without main resistance mutations, two patients showed raltegravir phenotypic resistance, one naturally with the F121Y at baseline, the other acquiring the G118R at failure.

CONCLUSIONS: Our results show that to avoid the selection of raltegravir resistance mutations, patients have to be treated with at least two active drugs in combination with raltegravir and to maintain an HIV-1 viral load <200 copies/ml.
ABSTRACT 61

Antiviral Therapy 2012; 17 Suppl 1:A74

Quantitative analysis of the role of APOBEC3G in the emergence of M184I in vivo

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BACKGROUND: Drug resistance mutations emerge in genetically diverse populations in response to selection by antiretroviral therapy. Error-prone reverse transcription and human APOBEC3G (A3G) restriction represent two mechanisms generating genetic diversity in vivo. The specific role of A3G in generating viral diversity and the development of resistance in vivo has not been extensively investigated. M184I occurs at a canonical A3G sequence. To investigate whether A3G contributes to the emergence of M184I in vivo, we examined viral populations from longitudinal plasma samples from patients with M184I.

METHODS: Genotypes (TRUGENE) from patients in the NIAID/CCMD clinic were screened for M184I mutations. Single genome sequencing (SGS) was performed using plasma samples stored at the time of the genotype and preceding and subsequent time points. Sequences were aligned and phylogenetic analyses performed with MEGA 5.05. To quantitatively investigate the role of A3G, sequences were analysed using a standard position-specific scoring matrix, as well as a newly developed algorithm to identify all potential A3G target sites.

RESULTS: We screened 1,780 genotypes and identified 11 (0.6%) with M184I; longitudinal sampling was available from 9, and 355 sequences (range 16–71/patient) were obtained. Patients were treatment-experienced (median drug exposure 2,294 d, range 106–4,107 d), often with multiple drug resistance mutations. Quantitative A3G analyses using two approaches revealed that all sequences had A3G changes (mean 13.49/sequence, range 1–22), but there was no correlation between the frequency of A3G changes and overall genetic diversity. In individual samples from 6/9 patients, the number of A3G changes was not significantly different between M184I-containing sequences and other sequences obtained at the same time with other amino acids at this position (P>0.05). Samples from two patients had significantly more A3G changes in M184I sequences than in M184V (P=0.009 and 0.019) and one had more in M184V than M184I (P=0.0002). Sliding window analysis did not reveal islands of A3G changes around position 184.

CONCLUSIONS: Our results do not support the conclusion that A3G is important for resistance mutations arising in vivo. The wide range of A3G changes among patients is likely the result of other patient-specific variables, including number, type and A3G content of infected cells.
SESSION 3
HIV pathogenesis, fitness and resistance
ABSTRACT 62

*Antiviral Therapy* 2012; 17 Suppl 1:A77

Dynamics of recombination in HIV-1 following superinfection described using single genome sequencing

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BACKGROUND: There are few detailed studies describing the genesis of recombinant HIV-1 strains or on the potential impact of recombination on resistance. We describe here an analysis of recombination events occurring in plasma and semen in a patient infected with multidrug-resistant subtype D virus and then superinfected with wild-type subtype B virus.

METHODS: Population and single genome sequencing (SGS) were performed on sequential plasma samples and one semen sample over an 87 week period from diagnosis. The patient was treatment-naive during this period.

RESULTS: Initial population sequencing on diagnosis showed a subtype D virus with reverse transcriptase drug resistance mutations D67N, K70R, A98G, K101E, Y181C, G190A, T215L and K219E, which was maintained at week 34 post-diagnosis, and the homogeneity of this sample was confirmed by SGS. At 54 weeks, population sequencing showed many mixed peaks, and by SGS 18/25 genomes were similar to the virus seen at diagnosis, 1/25 was subtype B with no resistance, and 3/25 were recombinant DB virus with variable resistance. At 85 and 87 weeks, SGS of blood plasma showed just 2/43 genomes were the same as the initial resistant virus, 3/43 were drug sensitive presumed superinfecting subtype B virus, while 39/43 (91%) genomes were DB recombinants, all but one of which had lost the original resistance mutations. Many different breakpoints were observed in the recombinants but at the final time point most showed breakpoints close to the end of protease with the original subtype D virus genome in the region leading up to the breakpoint and then superinfecting subtype B virus thereafter. In contrast, SGS of a semen sample taken at 87 weeks, showed 18/21 (86%) of genomes were subtype B and only 3/21 (14%) were DB recombinants and all were drug sensitive.

CONCLUSIONS: Multiple recombinants with different breakpoints were seen following superinfection in this patient, followed by outgrowth in blood plasma of a few predominant species which were no longer drug resistant. The proportion of virus variants in semen was not an accurate reflection of the blood plasma, possibly indicating that that the recombinants were being selected independently in different body compartments.
ABSTRACT 63

Antiviral Therapy 2012; 17 Suppl 1:A78

Colinearity of protease mutations in HIV-1 samples with high-level protease inhibitor class resistance

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BACKGROUND: Considering the decreased fitness of PI resistance mutation and the disparate and often antagonistic effects of PI resistance mutations on different PIs, we sought to determine whether pan-PI-resistant virus populations are composed predominantly of viruses with resistance to all PIs or contain subpopulations with different patterns of PI resistance mutations.

METHODS: We performed 454 deep sequencing of plasma virus samples from nine patients who received a median of five PIs over a median 8 years, had high-level genotypic and/or phenotypic resistance to all licensed PIs, and a plasma HIV-1 RNA level ≥4.5 log copies/ml. A median of 2,000 high quality reads were obtained per sample. The sensitivity was estimated to be ~1% based on limiting dilution analysis of viral cDNA and a post-hoc analysis that included the technical error rate and the distribution of synonymous and non-synonymous nucleotide variants.

RESULTS: The 9 virus samples had a median of 12 PI resistance mutations by direct PCR sequencing. With three exceptions, each sample had high-level resistance to each PI (PhenoSense assay; Monogram, South SF). The median fold decrease darunavir susceptibility was 142 and the median RC (PhenoSense; Monogram) was 11%. For each of the 9 samples, deep sequencing showed that each virus in the sample contained nearly all of the mutations detected by direct PCR sequencing. A median 94.9% of deep sequence reads had each PI resistance mutations detected by direct PCR sequencing that was not part of an electrophoretic mixture (median one mutation per sample). A median of 5.0% of reads had all but one of the mutations detected by direct PCR sequencing that was not part of an electrophoretic mixture.

CONCLUSIONS: The colinearity of PI resistance mutations in the nine virus samples shows that pan-PI-resistant viruses can replicate in vivo despite their highly mutated protease enzymes. It also negates the rationale for using combinations of ritonavir-boosted PIs for treating such viruses. The marked colinearity of PI resistance mutations in pan-PI-resistant viruses may result from the unique requirements for darunavir resistance, the extensive cross-resistance conferred by many accessory PI resistance mutations, and by limits to wild-type reversion imposed by interlocking of primary and accessory PI resistance mutations.
ABSTRACT 64
Antiviral Therapy 2012; 17 Suppl 1:A79

Increasing frequencies of silent mutations in the protease and the reverse transcriptase of treatment-naive HIV-1 isolates

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BACKGROUND: The evolution of HIV-1 is driven by the immune system and antiretroviral treatment targeting viral proteins. Recently the RNA structure of the viral RNA genome was also considered to be an important part of genetic code. Due to the error-prone nature of HIV-1 replication mutations throughout the whole genome frequently occur. Even though the viral proteins have extensively been analysed in treatment-naive and treatment-experienced HIV-1 isolates, silent mutations possibly influencing the RNA secondary structure of the viral genome have not been analysed in detail.

METHODS: HIV-1 subtype B genotypes obtained from 1,507 treatment-naive patients and from 682 treatment-experienced patients were analysed (years: 2001–2011). All sequences were compared with HxB2 and differences were scored as either silent mutations or mutations changing the amino acid sequence of protease (PR) and reverse transcriptase (RT).

RESULTS: In treatment-naive HIV-1 isolates the proportion of codons carrying silent mutations in the PR and RT continuously increased over time. In 2001 the frequencies of codons with silent mutations were 5.5% in the PR and 8.7% in RT, whereas in 2011 silent mutations were detected in 7.5% and 10.8%, respectively. By contrast the proportion of codons carrying silent mutations remained stable over time and was not specifically accumulating in the group of treatment-experienced HIV-1 isolates carrying PR and/or RT resistance mutations. In general, the number of silent mutations did not seem to influence the viral load of treatment-naive or treatment-experienced patients. The silent mutations were located throughout both viral genes and were hardly observed at positions selected by antiretroviral treatment. In one case a silent mutation could hamper the emergence of a drug resistance mutation. At position RT151 the wild-type codon (CAG) was more and more often replaced by the codon (CAA), which would increase the genetic barrier for the occurrence of the drug-selected RT mutation Q151M (ATG).

CONCLUSION: Silent mutations in PR and RT accumulated in treatment-naive HIV-1 subtype B isolates over time indicating the ongoing evolution of HIV-1 in humans. Silent mutations were not selected by antiretroviral treatment but can influence the genetic barrier of drug resistance mutations.
The combinations of NNRTI mutations and IN inhibitor (INI) mutations in HIV-1 affect viral fitness and drug susceptibility

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BACKGROUND: Non-nucleoside reverse transcriptase inhibitors (NNRTI) and integrase (IN) strand-transfer inhibitors (INSTI) are key components of antiretroviral regimens. Preliminary data suggest that the combination of NNRTI and INSTI resistance mutations impair HIV replication capacity. We conducted a more extensive investigation of the combined effects of NNRTI and INSTI resistance mutations on drug susceptibility and viral fitness.

METHODS: We constructed vectors deleted in pol, with wild-type nef or with a sequence tag or reporter gene inserted into nef in an HIV-1NL4-3 backbone. These vectors allow us to cassette in pol carrying mutations of interest in PR, RT and/or IN. INSTI resistance mutations Y143C, N155H and G140S/Q148H were introduced by site-directed mutagenesis alone or in combination with NNRTI resistance mutations K103N, E138K or Y181C. Susceptibility to efavirenz (EFV), rilpivirine (RPV) or raltegravir (RAL) and fitness of the dual INSTI- and NNRTI-resistant viruses were compared with the singly resistant viruses.

RESULTS: As expected, replication capacity (RC) and infectivity profiles in the absence of drug showed that viruses carrying NNRTI or INSTI resistance mutations mutants were less fit than WT, and viruses carrying NNRTI+INSTI resistance mutations were less fit than singly resistant viruses. In the presence of EFV K103N mutants with N155H or G140S/Q148H were fitter than K103N alone. Similarly, in the presence of EFV the E138K/G140S/Q148H mutant was fitter than E138K alone. No effect of INSTI resistance mutations on the fitness of Y181C mutants was observed. Conversely, E138K and Y181C improved the fitness of the G140S/Q148H mutant in the presence of RAL; K103N had no effect. The NNRTI resistance mutations had no effect on RAL susceptibility; likewise, G140S/Q148H had no effect on EFV or RPV susceptibility. However, the K103N/G140S/Q148H and E138K/G140S/Q148H had significantly greater fold increase in EFV IC₅₀ compared with the single NNRTI mutants, respectively, and the E138K/G140S/Q148H mutant had significantly greater fold increase in RAL IC₅₀ compared with G140S/Q148H alone.

CONCLUSIONS: Certain combinations of NNRTI and INSTI resistance mutations resulted in enhanced resistance to RAL and NNRTIs, respectively. Biochemical characterization of these mutants is needed to understand the mechanism of these interactions.
ABSTRACT 66
Antiviral Therapy 2012; 17 Suppl 1:A81

The emergence of HIV-1 integrase mutation Y143G upon re-initiation of raltegravir-containing therapy confers high-level resistance to raltegravir and improved viral fitness

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BACKGROUND: Raltegravir (RAL), the first approved HIV-1 integrase (IN) inhibitor, may be used in patients who have failed on therapy with protease (PR) and reverse transcriptase (RT) inhibitors. We examined the dynamics of the development of resistance mutations to RAL and their interactions with drug resistance mutations in PR and RT genes.

METHODS: Three to five sequential samples from two patients who had failed RAL-containing salvage therapy were analysed using single-genome sequencing of the whole pol gene followed by phylogenetic analyses. The effect of IN mutations on drug susceptibility and replicative capacity was determined using recombinant viruses expressing patient-derived pol genome segments in a single-cycle replication assay.

RESULTS: We generated 62 and 117 single genomes from each patient, respectively. The genomes contained all three major IN drug resistance mutations at Y143, Q148 and N155 albeit on separate genomes, associated with various accessory mutations. The data showed linkage of IN drug resistance mutations with PR and RT drug resistance mutations in 116 single genomes from both patient samples. Of interest, one of the patients had all three major IN drug mutations Y143R/C (n=14), Q148R (n=10) and N155H (n=1) present as eight unique drug-resistant variants at 4 months after initiation of RAL-containing therapy. However, 4 months after cessation of RAL-containing therapy none of the single genomes contained IN resistance mutations except for one (1/39) which had the rare Y143G plus G163R mutation. Phylogenetic analyses showed that this was due to re-emergence of viruses from before initiation of RAL therapy rather than by reversion. Upon re-initiation of RAL-containing therapy the Y143G+G163R variants were present in all the single genomes (n=13). Recombinant viruses expressing the patient-derived Y143G+G163R exhibited a significant decrease in RAL susceptibility (98-fold) which was higher than that of two variants (N155H+V151I and Y143C+G163R). However, the Y143G+G163R variant demonstrated the highest replicative fitness (83% of wild-type virus) compared to the other variants (34–49%).

CONCLUSIONS: We demonstrate the presence of all three major IN resistance mutations in the same patient at the same time. The data confirm previous findings that the interplay between drug susceptibility and replication fitness dictates the emergence of IN resistance mutations.
ABSTRACT 67
Antiviral Therapy 2012; 17 Suppl 1:A82
Antiviral activity of dolutegravir and replication capacity of raltegravir-resistant strains in human primary macrophages and lymphocytes

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INTRODUCTION: The goal of the study was to compare the activity of dolutegravir, a next generation integrase inhibitor, in primary human macrophages (MDM), peripheral blood mononuclear cells (PBMCs) and lymphocytes, using both wt- and raltegravir-resistant-strains.

METHODS: MDM and PBMCs were infected with p81.A-HIV-1-strain, while C8166-T-lymphocytic cells were infected with HXB2. Viral production was analysed by HIV-p24-gag production. In C8166 T-cells, cytopathic effect was also analysed by optical microscopy. Quantification of integrated, unintegrated and 2-LTR-circles-HIV DNA was performed by real-time PCR.

RESULTS: Analysing the p24-production, a similar range of dolutegravir activity was found in MDM and PBMCs (EC50 and EC90: in MDM =2.5 ±2.1 nM and 18.4 ±11.3 nM; in PBMCs =0.1 nM and 19.3 nM, respectively). Based on syncytium inhibition, Dolutegravir EC50 and EC90 in C8166 were 4.3 ±1.6 nM and 52.9 ±20.3 nM, respectively.

Overall, a similar antiviral activity against HIV-1 wt-strains was observed with raltegravir (EC50 and EC90: in MDM =3.7 ±1.1 nM and 9.4 ±0.2 nM; in PBMCs= 0.5 ±0.6 nM and 19.2 ±8.6 nM; in C8166 =6.4 ±1.3nM and 65.8 ±8.1nM, respectively).

As expected, the activity of dolutegravir was superior to raltegravir in raltegravir-resistant strains. In C8166 T-cells, N155H- or Y143C-resistant virus had fold change resistance of 1.2 and 0.9 in presence of dolutegravir, while of 17.6 and 2.1 in presence of raltegravir, respectively.

The replication capacity (in absence of drugs) of N155H-resistant virus was strongly reduced compared with 100% of each wt virus in MDM, PBMCs and C8166 T-cells (36.5 ±8%, 29.1 ±12% and 29.5 ±26%, respectively).

Interestingly, in MDM at 30 days post-infection, no difference was observed in integrated HIV DNA between N155H-81A and wt-81A (368 versus 386 copies/1,000 cells), while there was a high reduction of unintegrated forms with N155H virus compared with wt-81A (57 versus 229 copies/1,000 cells). In presence of drugs (dolutegravir 100 nM, raltegravir 100 nM), in agreement with total p24-production inhibition, there was a strong reduction of all HIV DNA forms in MDM infected with both wt-81A and N155H viruses.

CONCLUSIONS: Dolutegravir efficiently reduces HIV-1 replication in MDM, PBMCs and C8166 T-cells, with the potential to be effective in different HIV cellular targets. Raltegravir-resistant viruses show reduced replication capacity in all tested cells, suggesting that MDM and lymphocytes might act as reservoir more for wt virus than for resistant/low-fitness virus.
ABSTRACT 68

Antiviral Therapy 2012; 17 Suppl 1:A83

Comprehensive characterization of LEDGF/p75 in a HIV-1-infected patient cohort

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BACKGROUND: Lens epithelium derived growth factor interaction inhibitors (LEDGINs) are an emerging new class of allosteric integrase inhibitors. Few data are available about the genetic variability, mRNA and protein expression levels of LEDGF/p75 in HIV-1-infected patients. The present study evaluated the relation between LEDGF/p75 genetic variation, mRNA/protein expression levels and HIV-1 disease progression in order to further validate LEDGINs in clinical practice.

METHODS: Samples were derived from a therapy-naive cohort at the Ghent University Hospital and from the Spanish long-term non-progressor cohort, kindly provided by the HIV BioBank integrated in the Spanish AIDS Research Network (RIS). High resolution melting curve analysis and Sanger sequencing were used to identify all single nucleotide polymorphisms (SNPs) in the coding region and 3′ UTR of LEDGF/p75. We quantified LEDGF/p75 mRNA derived from patient PBMCs using RT-qPCR with validated reference genes for normalization. Western blotting was performed on selected samples.

RESULTS: A total of 325 samples were investigated, 291 (90%) of Caucasian and 34 (10%) of African origin and comprising Elite controllers (n=49) and Viraemic controllers (n=62). We identified 24 SNPs, including 5 in the coding region (2 synonymous and 3 non-synonymous), 17 in the flanking non-coding regions and the 3′ UTR, and 2 additional tagSNPs. The minor allele frequency of the SNPs in the coding region was very low and none of the variants had a major impact on protein structure according to SIFT and PolyPhen score. One intronic SNP (rs2737828, n=13) was significantly under-represented in Caucasian HIV patients (P<0.0001) compared with healthy controls (HapMap). No splice variants were detected. There was no correlation between mRNA expression levels of LEDGF/p75 and disease progression. Of note, low levels of protein expression, as determined by western blot, did not exclude a high viral load.

CONCLUSIONS: The data of this investigation support the idea that LEDGF/p75 might influence HIV susceptibility in Caucasians. Very low levels of LEDGF/p75, previously linked with decreased integration in transcriptional active regions in vitro, did however not result in lower viral load. As LEDGF/p75 expression levels differ substantially among patients, LEDGINs will have to compete with their cellular competitor for IN binding in case of high expression.
ABSTRACT 69

Antiviral Therapy 2012; 17 Suppl 1:A84

Significant HIV-1 subtype differences observed in the development of accessory mutations associated with high-level resistance to reverse transcriptase inhibitors

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BACKGROUND: Accessory mutations which either increase resistance levels or restore viral fitness have been described for HIV-1 subtype B. However, there are few data on such mutations in other subtypes. Here we compare accessory mutations associated with high-level resistance to reverse transcriptase (RT) inhibitors in HIV-1 subtypes B and C.

METHODS: Codon changes associated with the accumulation of mutations conferring resistance to thymidine analogues (TAMs) and to non-nucleoside reverse transcriptase inhibitors (NNRTI) in HIV-1 RT codons 1–400 of subtypes B and C within the UK HIV Drug Resistance Database were analysed. Sequences of 5,463 subtype B samples from treated patients were compared with those of 1,918 subtype C samples. To determine if changes associated with TAMs affected drug sensitivity, some of these mutations were tested in gag–pol subtype B and subtype C backgrounds with or without TAMs in a single replication cycle drug susceptibility assay.

RESULTS: Significant differences between subtype B and C accessory mutations associated with TAMs were observed at codons 43 (27.0% change with 4+ TAMs from patients with no TAMs in B versus 2.5% in C; \( P < 0.001 \)); 118 (36.3% change in B versus 16.2% in C; \( P < 0.001 \)); 135 (12.5% change in B versus 28.0% in C; \( P < 0.001 \)); 203 (16.9% change in B versus 22.0% in C; \( P = 0.01 \)) and 326 (2.6% towards consensus in B versus 7.6% away in C; \( P = 0.001 \)). For accessory mutations associated with accumulation of NNRTI resistance, significant differences were observed only at codons 43 (10.2% change in B versus 0.5% in C; \( P = 0.002 \)) and 68 (5.2% change in B versus 10.3% in C; \( P = 0.003 \)). However, codon changes K43E, E44D and V118I were found to have no effect on resistance to zidovudine, stavudine, lamivudine and abacavir with or without other TAMs in both subtypes B and C background in a phenotypic assay.

CONCLUSIONS: Significant differences between subtypes B and C were observed in the development of accessory mutations associated with high-level resistance to reverse transcriptase inhibitors.
ABSTRACT 70

Antiviral Therapy 2012; 17 Suppl 1:A85

Concordance between HIV-2 genotypic coreceptor tropism predictions based on plasma RNA and proviral DNA

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BACKGROUND: Most of HIV-2-infected patients naturally displayed undetectable plasma viral load in absence of antiretroviral treatment. Thus, proviral DNA V3 loop study may be a promising approach to determine if a patient is eligible for maraviroc use. In this study, we assessed HIV-2 tropism in paired plasma and peripheral blood mononuclear cells (PBMC) samples.

METHODS: HIV-2 proviral DNA and viral RNA V3 loop sequences were obtained from 43 clinical isolates issued from 43 patients (Group A, n=27; Group B, n=15; Group H, n=1) with detectable plasma RNA (>100 copies/ml). HIV-2 R5 or X4 tropism was interpreted using the four gp105 V3 loop major determinants of CXCR4 coreceptor use we previously described (L18X, V19K/R, V3 global net charge, insertions at position 24). Chi-square and Wilcoxon tests were performed to analyse categorical and quantitative variables, respectively.

RESULTS: Median HIV-2 RNA level and CD4 cell count were 2,350 copies/ml (IQR=921–10,980 copies/ml) and 289/mm³ (IQR=183–414/mm³), respectively. Genotypic prediction of HIV-2 RNA tropism was as follows: 31 were classified as R5 (72%) and 12 as X4/dual-mixed (28%). Genotypic prediction of HIV-2 DNA tropism was as follows: 20 were classified as R5 (47%) and 23 as X4/dual-mixed (53%). Thus, a higher proportion of X4/dual-mixed tropic virus was observed in proviral DNA than in plasma RNA (P=0.028). Among the 43 tested clinical isolates, viral tropism was concordant between plasma RNA and proviral DNA in 32 cases (74%), including 20 R5 and 12 X4/dual-mixed. All the discordances (n=11) were attributable to the prediction of R5 in plasma RNA and of X4/dual-mixed in proviral DNA. Median CD4 cell counts of patients with R5 and X4/dual-mixed viruses in plasma were 307 and 262/mm³, respectively (P=0.86). No statistical association was found between HIV-2 RNA plasma level and HIV-2 viral tropism predicted either from RNA or from DNA.

CONCLUSIONS: In our study a higher proportion of X4/dual-mixed virus was observed in proviral DNA than in plasma RNA, as described in HIV-1 infection. In addition, we showed that tropism prediction based on proviral DNA might be a useful tool in HIV-2-infected patients with undetectable viral load.
SESSION 4
HCV & HBV drug resistance
ABSTRACT 71
Antiviral Therapy 2012; 17 Suppl 1:A89

Heterodimeric second-generation NS5A inhibitors with enhanced potency against genotype 1a replicons and clinically relevant resistant mutations

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BACKGROUND: The demonstrated properties of first-generation NS5A inhibitors (high potency, low dose, convenient QD dosing schedule and low potential for drug–drug interactions) have made these molecules attractive candidates for inclusion in all-oral regimens for the treatment of hepatitis C viral infection. However, first-generation NS5A inhibitors also have exhibited sub-optimal clinical efficacy in patients with genotype 1a, the most common subtype in the United States and northern Europe, due to their low genetic barrier to highly fit mutants. To address this important medical need, we have discovered a novel class of heterodimeric NS5A inhibitors with significantly improved potency against genotype 1a as well as the resistant mutants commonly observed in genotype 1a patients.

METHODS: Using a multivalent approach to lead discovery, structurally novel heterodimeric NS5A inhibitors with picomolar genotype 1b and genotype 1a potency were identified. Optimization was guided by in vitro replicon assays against wild-type and mutant constructs, as well as colony formation studies. Pharmacokinetic properties were measured in mice and dogs. Metabolic stability was assessed in human hepatocytes. Off-target activity was measured using standard procedures.

RESULTS: A focused lead optimization programme yielded multiple heterodimeric NS5A inhibitors with low picomolar activity in genotype 1a and genotype 1b replicons. Against a battery of resistant mutants of genotype 1a previously encountered in HCV patients with first-generation NS5A inhibitors, these compounds displayed EC50s in the pM to low nM range. This improved resistance profile was confirmed by colony formation assays at both genotype 1a and 1b. These compounds possessed pM to low nM potencies against a panel of intergenotypic replicons, including those with the common L31M sequence in genotype 2. The absorption and metabolic stability of these compounds in preclinical studies supports their potential for QD dosing in humans.

CONCLUSIONS: We have discovered novel pan-genotypic heterodimeric NS5A inhibitors that maintain the attractive properties of first-generation compounds while offering significantly enhanced efficacy against genotype 1a resistant mutants. These data support progression into clinical trials and potential inclusion in an all-oral regimen suitable for a broad range of HCV patients, including those with genotype 1a.
ABSTRACT 72

Antiviral Therapy 2012; 17 Suppl 1:A90

Dynamics of HCV mutations during telaprevir treatment dissected using ultra-deep pyrosequencing (UDPS)

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BACKGROUND AND AIMS: Telaprevir (TVR) is a newly protease inhibitor (PI) used in chronic hepatitis C treatment in association with PEG-interferon and ribavirin. During therapy, the dynamics of emerging PI resistance are not well-understood. We used UDPS to characterize the spectrum of low prevalence TVR resistance mutations in HCV and we compared with Sanger sequencing.

METHODS: 25 cirrhotic patients (genotype 1b, n=18; genotype 1a, n=7) previously treated with PEG-interferon and ribavirin (7 null-responders, 11 partial non-responders [NR], 6 relapers and 1 breakthrough) were retreated with PEG-interferon, ribavirin and TVR. The distribution of PI-resistant variants was assessed at every HCV-RNA-positive time point by UDPS and Sanger method. Methodological error was determined by pyrosequencing of plasmid and replicon.

RESULTS: A median of 4,654 clones in each sample was studied by UDPS. No described resistant mutations were detected in the plasmid or replicon controls. Resistant variants to HCV-PI were found at baseline in 16/25 patients, responders or not to TVR: V36A/M (n=2, 0.44–35%), T54A/S (n=3, 0.25–2.8%), V55A (n=8, 0.2–0.48%), Q80R/K (n=7, 0.32–7.8%), R155K/T/Q (n=5, 0.2–2.3%), A156S/T/V (n=3, 0.1–2%), D168A/V/T/H (n=2, 0.1–13%), V170A/T (n=3, 0.17–0.4%) detected by UDPS only and Q80K (n=1, 64%) and T54A/S (n=1, 49.5%) detected by UDPS and direct sequencing. 4/25 patients (all genotype 1a) failed to eradicate HCV at W4–12 and stopped treatment. 3/4 NR to TVR harboured variants resistant to PI at baseline (mutation R155K/T/Q in all cases) versus 13/20 responders to TVR. During treatment failure, a progressive increase in proportion of TVR-resistant variants was observed, in particular variants at positions 36 and 155. These variants were found as soon as W2 by UDPS and direct sequencing. For the four patients that failed to eradicate HCV, a progressive reversion of PI resistance mutations was observed as soon as treatment interruption.

CONCLUSIONS: PI-resistant variants pre-exist in the majority of HCV patients before treatment, both in responders and NR to TVR. During treatment failure, which occurred in some genotype 1a patients, a significant enrichment in resistance mutations V36A/M and R155K/T/Q was observed. Follow-up of patients at failure of TVR exhibited progressive reversion of resistant mutants to wild-type virus.
ABSTRACT 73

Antiviral Therapy 2012; 17 Suppl 1:A91

Deep sequencing at baseline fails to identify prior null-responder patients at risk of failing telaprevir plus peginterferon/ribavirin therapy

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BACKGROUND: Naturally occurring telaprevir-resistant viral variants exist in all HCV patients, most often at low frequencies. The utility of population sequencing-based resistance testing to identify patients at risk of treatment failure is low, since only a few patients (<5%) have resistant variants as the dominant species before treatment. The impact of baseline resistance could be higher with the use of more sensitive sequencing techniques, especially in patients with a poor interferon response.

METHODS: Prior null-responders (10 genotype 1a and 5 genotype 1b) with diverse treatment outcomes on telaprevir-based treatment (5 with sustained virological response [SVR], 9 on-treatment virological failures and 1 relaper) in the Phase III REALIZE study were analysed at baseline, during treatment and follow-up. Paired-end deep sequencing of a fragment spanning the HCV NS3•4A regions was performed using Illumina® technology. Telaprevir-resistant variants (V36A/M, T54A/S, R155K/T and A156S/T/V) were reported when present in ≥1% of sequence reads.

RESULTS: Telaprevir-resistant variants were detected by deep sequencing at baseline in only 3 of the 15 patients: in none of the 5 patients that achieved SVR and in 3 of the 10 patients that did not achieve SVR. These 3 patients (all on-treatment virological failures) had a baseline variation R155K (67%), T54S (2%) or T54A (1%). 8 of the 10 patients who did not achieve SVR had the V36M and R155K variants present at time of failure. The frequency of these mutations decreased gradually after treatment in patients with follow-up data available. At week 72, V36M remained detectable in 3/5 patients (at 3–8% frequency), whereas R155K was no longer detectable by deep sequencing (<1%) in any of these 5 patients.

CONCLUSIONS: In this limited dataset of null-responders, deep sequencing only identified a small fraction of patients at risk for treatment failure. The frequency of telaprevir resistance-associated mutations decreased over time after treatment in patients without SVR, confirming and extending previous findings obtained with population and clonal sequencing.
ABSTRACT 74
Antiviral Therapy 2012; 17 Suppl 1:A92

Evolution and mutation linkage of naturally occurring variants resistant to HCV protease inhibitors in cirrhotic liver transplant patients analysed by deep sequencing

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BACKGROUND: Increasing evidence suggests that variants with decreased susceptibility to HCV protease inhibitors (PIs) occur naturally and pre-exist at low levels within HCV populations. However, in some individuals, resistance-associated variants (RAVs) may circulate as dominant quasispecies that persist long-term in vivo.

The relative abundance, temporal dynamics and mutation linkage of naturally occurring RAVs within viral quasispecies have not been examined in detail, especially in cirrhotic patients undergoing liver transplantation.

METHODS: Partial NS3 gene sequences from 61 serum samples in 20 HCV-infected subjects were analysed, including 53 longitudinal samples from 12 liver transplant recipients. The prevalence and abundance of RAVs to PIs were determined using 454/Roche pyrosequencing, and their mutation linkage was identified using Illumina paired-end sequencing.

RESULTS: The natural prevalence of Q80K and V55A variants was high (77% and 82% of subjects, respectively). Q80K confers reduced susceptibility to macrocyclic PIs and V55A is associated with resistance to linear ketoamides. Q80K was the dominant quasispecies (>50% of pyrosequence reads) in 7 (35%) subjects, whereas V55A was a minority quasispecies (<30% of reads) in nearly all subjects. Substitutions at V36, T54, and V170 were prevalent (20–50% of subjects), but at low frequencies within quasispecies populations (<30% of reads). Variants associated with high-level resistance to PIs (substitutions at positions 155, 156 and 168) were uncommon. Standard population sequencing failed to detect minority variants in most subjects. Mutation linkage of low frequency RAVs was identified using sensitive Illumina paired-end sequencing. Longitudinal analysis revealed fluctuating frequencies of RAVs in transplant recipients over time. Although significant genetic drift occurred during liver transplantation in some subjects, no genetic bottleneck was observed.

CONCLUSIONS: Naturally occurring RAVs are more common than previously reported. Most RAVs circulate at low frequencies not readily detectable by standard population sequencing, but variants conferring high-level resistance to PIs are uncommon. RAVs are highly dynamic in vivo, which may be related to immune suppression associated with liver transplantation. The methods described here should provide a framework for longitudinal analyses of both the dynamics and mutation linkage of RAVs in patients undergoing direct-acting antiviral therapies.
ABSTRACT 75

Antiviral Therapy 2012; 17 Suppl 1:A93

454 Deep-sequencing analysis of newly described rare HCV NS3 protease resistance mutations in patients treated with telaprevir or boceprevir

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BACKGROUND: Resistance mutations emerging during direct antiviral therapies, including HCV NS3 protease inhibitors (PI), telaprevir (TVR) and boceprevir (BOC), were associated with treatment failure. PI resistance mutations selected at >5% frequency after TVR/BOC treatment are located at positions 36, 54, 55, 155, 156 and 170 and were already well-described. Analysis of a large number of minor variants revealed resistance relevant mutations also at positions 87 (BOC-IC50 2.8× wt), 117 (TVR-IC50 2.6×; BOC-IC50 4.0×) and 174 (TVR-IC50 3.3×; BOC-IC50 5.9×). Pre-existence and long-term follow-up stability should be described in detail by deep-sequencing analysis.

METHODS: Deep-sequencing analysis of the NS3-protease gene was performed in HCV genotype (gt)-1-infected patients, who received TVR or BOC in Phase Ib studies, at baseline (BL) and long-term follow-up (LT-FU; medium 4.2 years). The viral quasispecies were analysed by using the 454 FLX genome sequencer. High-quality reads were mapped to the NS3-gene of the HCV-J/HCV-H77 reference. Nucleotide variant distributions were called for each variant position using the SAMtools suite.

RESULTS: In both subtype 1a and 1b, exchange of one nucleotide is sufficient to generate each of the three analysed variants (A87T, R117H and S174F). A87T was present in two BOC patients at BL and LT-FU and in one BOC patient only at LT-FU (all gt1b). Three of the TVR-patients exhibit A87T at BL, two of them also at LT-FU and one additional only at LT-FU (all gt1b). R117H was found to be pre-existing between 58–99% at BL in one BOC- (gt1b) and in two TVR-treated patients (1× gt1a and 1× gt1b). R117H is still present at LT-FU with 68% and 98% frequency, respectively. At position 174, we found phenylalanine as a dominating pre-existing and persisting variant in two BOC-treated patients (1× gt1a 44% BL, 43% LT-FU; 1× gt1b 99% BL, 99% LT-FU).

CONCLUSIONS: Two of three newly described minor variants, 117H and 174F show potential for pre-existence and long-term persistence in patients treated with TVR/BOC. No differences between gt1a and gt1b were observed. Determination of the impact of these three variants on re-treatment and influence on triple-therapy will be of great importance.
ABSTRACT 76

Antiviral Therapy 2012; 17 Suppl 1:A94

Prevalence of pre-existing NS5A and NS3 drug resistance mutations in direct-acting antiviral (DAA)-naive HCV genotype-1a-infected patients using semiconductor deep sequencing technology (Ion Torrent)

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BACKGROUND: The Ion Torrent platform uses ‘semiconductor sequencing’ based on the detection of hydrogen ions released from the incorporation of dNTPs during DNA polymerization. Ion sensors similar to a pH meter are embedded within a high-density chip with 1.2 M to 11 M wells. No modified dNTPs or laser/optics are required. Each well holds a different DNA template with a sequencing run yielding 500 K to 5 M unique reads. NS5A inhibitors are an important new class of HCV DAAs that when paired with NS3 inhibitors have led to HCV cures in some patients without the use of PEG-IFN/RBV. However, aa changes in NS5A (M28A/T/V, Q30E/H/R, L31M/V, H58D/P and Y93H/C/N) and NS3(R155K/T/Q, A156S/V/T, D168A/V/T/H/E and V170A/T) can lead to high-level drug resistance and are strongly associated with virological failure.

OBJECTIVES: To determine the prevalence of HCV genotype 1a variants possessing NS5A and NS3 drug-resistant variants in DAA-naive patients using deep sequencing (Ion Torrent) and Sanger sequencing (SS).

METHODS: NS5A and NS3 genes from DAA-naive HCV-1a-infected patients were sequenced using deep sequencing (Ion Torrent) with an average read coverage depth of 7,862 for NS5A and 6,239 for NS3 mutations and SS.

RESULTS: Samples from 75 HCV-1a-infected DAA-naive subjects were evaluated by Ion Torrent sequencing. Variants possessing any mutation associated with NS5A drug resistance were identified in 20% (15/75) by SS versus 34.7% (26/75) by deep sequencing (P=0.04); resistant variant level range: 0.33–99.6%. 21% (16/75) had mutations known to confer >340 FC in resistance (M28T/A, Q30E/H/R, L31M/V, H58D/P and Y93H/C/N) and NS3(R155K/T/Q, A156S/V/T, D168A/V/T/H/E and V170A/T) can lead to high-level drug resistance and are strongly associated with virological failure.

No sample had variants with both NS5A and NS3 resistance by deep sequencing. There was good concordance between SS and Ion Torrent for HCV variants identified at levels >20% with 98.7% of NS5A and 100% of NS3 samples being concordant.

CONCLUSIONS: Deep sequencing identified more DAA-naive HCV-1a-infected patients harbouring pre-existing DAA-resistant variants. There was good concordance between Ion Torrent sequencing and SS for mutations at high variant levels. Baseline genotyping for NS5A drug resistance mutations may be necessary when using NS5A DAA strategies without PEG-IFN.
ABSTRACT 77

Antiviral Therapy 2012; 17 Suppl 1:A95

Re-emergence of vaniprevir (MK-7009) resistance-associated amino acid variants (RAVs) following retreatment of patients previously exposed to MK-7009

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INTRODUCTION: Vaniprevir (MK-7009) is an oral HCV-NS3/4A protease inhibitor being assessed in combination with peginterferon alfa-2a (P) and ribavirin (R) in patients with chronic hepatitis C genotype (G)1 infection. Patients enrolled in MK-7009 Phase Ib or II clinical studies were offered enrolment into an MK-7009 rollover study, where patients received either 300 or 600 mg BID of MK-7009 plus P/R for 48 weeks (PN028). In this analysis, we describe clonal sequence data from five patients that experienced virological failure in the PN028 study that had been previously exposed to MK-7009; two patients received 7 days MK-7009 monotherapy (125 mg QD), three patients received MK-7009/P/R.

METHODS: HCV RNA measurements were made using the Roche Cobas TaqMan assay with a LOD of 10 IU/ml. HCV viral RNA was isolated from patient plasma and the HCV NS3/4a gene amplified using standard nested RT/PCR techniques and clonal sequencing (40–90 clones) performed.

RESULTS: In one patient that received 7 days of MK-7009 monotherapy, RAV R155K was detected in the majority (96.8%) of viruses 14 days after MK-7009 dosing. Prior to re-treatment in PN028, (908 days later) R155K was no longer detected; however, at failure, following MK-7009/P/R therapy, RAV D168T was detected in >95% of viral clones. Another patient that received 7 days of MK-7009 had RAV R155K (44%) detected after monotherapy dosing. This RAV was not detected prior to subsequent MK-7009/P/R therapy in PN028 (after 832 days), but R155K was again detected in over 95% of virions at virological failure after MK-7009/P/R therapy.

Three patients received a second MK-7009/P/R treatment after failing a first MK-7009/P/R treatment with detectable RAVs. 2/3 patients had RAVs detected at baseline prior to the initiation of the second MK-7009/P/R treatment (316–367 days later), while 1/3 patients had wild-type viruses. All three patients experienced lower HCV RNA declines during the second MK-7009/P/R treatment and had the same MK-7009 RAVs detected at both virological failure time points.

CONCLUSIONS: MK-7009 RAVs detected at virological failure in MK-7009 P/R-treated patients were also detected following a second MK-7009/P/R treatment, despite their inability to be detected by clonal analysis prior to the second treatment. This indicates that RAVs can persist and potentially impact subsequent therapy with the same PI.
ABSTRACT 78

Antiviral Therapy 2012; 17 Suppl 1:A96

Early viral kinetics and emergence of resistance in cirrhotic patients with HCV genotype 1 and prior peginterferon/ribavirin treatment failure: subanalysis of the REALIZE Phase III Study

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BACKGROUND: In the Phase III REALIZE trial in patients with chronic HCV genotype 1 infection who had failed prior peginterferon/ribavirin (PR) treatment including non-responders and relapsers, telaprevir plus PR resulted in higher SVR rates in cirrhotic patients compared with PR alone. In prior non-responders, however, SVR rates were higher in non-cirrhotics than in cirrhotics, while in prior relapsers SVR rates were similar irrespective of cirrhosis status. The effects of liver fibrosis stage on early viral kinetics and emergence of resistance were evaluated.

METHODS: Patients with prior PR treatment failure received placebo or telaprevir (T) 750 mg q8h for 12 weeks, with or without lead-in (that is, 4 weeks PR prior to starting telaprevir), in combination with 48 weeks of PR. HCV NS3•4A population sequencing was performed at baseline and time of failure, and telaprevir-resistant variants (V36A/M, T54A/S, R155I/K/M/T, A156S/T/V) were identified in non-SVR patients. Changes in HCV RNA from baseline and emergence of telaprevir-resistant variants were evaluated in patients with no/minimal or portal fibrosis (F0–F2, n=273), bridging fibrosis (F3, n=118) and cirrhosis (F4, n=139).

RESULTS: The change in HCV RNA from baseline at early time points (≤week 4) in telaprevir-treated patients (T12/PR48) was similar regardless of baseline fibrosis stage for all prior response categories. 75% (F0–F2), 67% (F3) and 47% (F4) of telaprevir-treated patients achieved SVR. In non-SVR patients, 74% (F0–F2), 59% (F3) and 75% (F4) had detectable telaprevir-resistant variants at failure. SVR rates were 87%, 85% and 84% in prior relapsers and 57%, 46% and 22% in prior non-responders with F0–F2, F3 and F4 fibrosis, respectively. Emergence of resistance in non-SVR patients occurred in 65%, 50% and 50% of prior relapsers, and 77%, 61% and 78% of prior non-responders with F0–F2, F3 and F4 fibrosis, respectively. In addition, no difference in type of emerging mutations was observed across fibrosis stages.

CONCLUSIONS: Although prior non-responder cirrhotic patients in REALIZE had lower SVR rates than non-cirrhotics, fibrosis stage had no effect on early viral kinetics. Further, the frequency and type of telaprevir-resistant variants emerging in non-SVR patients in REALIZE was similar irrespective of fibrosis stage, in line with observations in treatment-naive patients.
ABSTRACT 79

**Investigation of viral and host factors associated with outcome of acute hepatitis C in a cohort of patients with HIV coinfection**


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**BACKGROUND:** An increasing number of acute hepatitis C outbreaks is reported in Europe among HIV-infected men who have sex with men (MSM). To investigate the reasons for spontaneous clearance (SC) versus persistent HCV infection, we started a prospective study of MSM for the analysis of viral, clinical and host factors affecting the outcome of acute hepatitis C.

**METHODS:** At diagnosis and every 4 weeks thereafter, blood was collected as yet from 50 patients with acute hepatitis C and HIV coinfection for the investigation of virological/biochemical parameters. We performed clonal sequencing of the NS3 protease to characterize mutational patterns possibly associated with the outcome of HCV infection as well as interleukin-28B (IL28B) genotyping (rs129797860).

**RESULTS:** HCV persisted in 43 (86%) patients, 4 (8%) displayed SC and the outcome is unclear for 3 (6%) individuals. IL28B genotypes CC, CT and TT were displayed by 22 (44%), 25 (50%) and 3 (6%) of patients. Due to the small numbers of SC, no association with IL28B genotype was detected. Highly fluctuating viral loads were observed before therapy initiation in patients with CC genotype compared with CT/TT genotypes (median delta HCV RNA viral load 2.8 log$_{10}$ versus 1.4 log$_{10}$ IU/ml). Within 100 days after diagnosis, the maximum viral load drop was higher in patients with fluctuating viraemia (FV; median 2.8 log$_{10}$ IU/ml) and in individuals with SC (median 3.3 log$_{10}$ IU/ml) compared with patients with plateau viraemia (median 0.5 log$_{10}$ IU/ml). Individuals with FV and SC taken together showed higher median peak bilirubin (2.0 versus 0.8 mg/dl) and ALT levels (1,032 versus 265 U/l) in relation to patients with plateau viraemia. We observed no mutational NS3 protease patterns in correlation with the acute hepatitis C outcome. Currently, cell culture assays are established for the functional analysis of patient NS3 proteases.

**CONCLUSIONS:** In SC patients and individuals with FV and persistent outcome of acute HCV infection, a maximal viral load drop ≥2.8 log$_{10}$ IU/ml is observable, which is associated with higher peak bilirubin and ALT levels. In comparison to IL28B CT/TT genotypes, individuals with CC genotype display highly fluctuating HCV RNA concentrations.
ABSTRACT 80

Antiviral Therapy 2012; 17 Suppl 1:A98

Characterization of baseline NS3 protease polymorphisms and drug resistance development in HCV-1-infected patients starting a protease-inhibitor-containing regimen

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BACKGROUND: The rate of virological response to PI treatment, especially in presence of baseline NS3 polymorphisms and of drug-resistant variants, is not yet defined. Therefore, aim of this study was to assess the impact of baseline NS3 protease polymorphisms and drug resistance mutations on the virological response to boceprevir/telaprevir treatment in clinical practice.

METHODS: We analysed 21 HCV-infected patients (HCV-1b=17; HCV-1a=4) starting an anti-HCV treatment with boceprevir (n=17) or telaprevir (n=4) plus PEG-interferon/ribavirin. HCV RNA (assay detection-limit =12 IU/ml) was evaluated at baseline and during follow-up (48 h-4-8-12-16-24-weeks of PI treatment). NS3-protease sequences at baseline and at different time points after therapy were available for 11/21 patients.

RESULTS: The 17 boceprevir-treated patients received PI treatment for a median (IQR) time of 16.6 (13.0–22.1) weeks, with a median (IQR) HCV viraemia at baseline of 5.9 (5.2–6.4) log IU/ml. Of them, 12 (70.6%) achieved undetectable HCV RNA: 9 patients at week 4, 2 at week 8 and 1 at week 12 of PI treatment. One HCV-1a patient with the putative resistant M175L variant at baseline reached undetectability at week 4. Five patients (1=HCV-1a, 4=HCV-1b) had still detectable HCV RNA (median [IQR]=1.9 [1.8–2.0] log IU/ml) after a median (IQR) time of 12.1 (9.0–13.0) weeks. They were all non-responders to previous standard-of-care. One HCV-1b cirrhotic patient presented the resistant variant T54S at baseline and during follow-up, and even if with favourable CC IL-28b-genotype, was still positive (83UI/ml) after 8 weeks of PI treatment. One non-responder HCV-1b patient developed the major resistance mutation T54A at week 8 (HCV viraemia: 5.2 log UI/ml). No other resistance mutations were observed either at baseline or during follow-up.

Four patients received telaprevir-treatment for a median (IQR) time of 2.1(2.0–3.1) weeks. All had C/T IL-28b-genotype and were non-responder to previous standard-of-care, with a median (IQR) HCV viraemia of 6.8 (5.8–7.0) log IU/ml. Two HCV-1b patients reached undetectable HCV RNA before week 4 of PI treatment. Differently, 2 HCV-1a patients had still detectable HCV viraemia, even if with a 2.2–3.6 log IU/ml decrease at 48 h and 4.9–5.1 log IU/ml decrease at week 2. Both patients presented the resistance-associated variant M175L at baseline, together with an isoleucine at the resistance-associated position V170.

CONCLUSIONS: At baseline, major PI resistance mutations were undetectable (at least by population sequencing), and the emergence of T54A was associated with one boceprevir virological failure. The baseline presence of NS3 protease polymorphisms in HCV-1-infected patients may have a role in slowing HCV RNA decay under PI treatment. Confirmation by long-term studies is now warranted.
ABSTRACT 81

Antiviral Therapy 2012; 17 Suppl 1:A99

HCV screening project of patients on dual interferon-α and ribavirin therapy

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AIM: Many patients suffering from chronic HCV infection do not respond to the dual treatment combination of the unspecific antiviral agents pegylated interferon-α (IFN-α) and ribavirin (RBV). Therapy outcome depends on both host and viral factors, such as host IL28B cytokine polymorphisms, viral genotype or presence of viral resistance mutations. The aim of this prospective, non-interventional study is to collect data about HCV baseline polymorphisms and resistance mutations in the NS3 protease gene (targeted by most upcoming HCV drugs), viral quasispecies distribution and clinical outcome from patients during dual therapy.

METHODS: Viral RNA was gained from blood samples. The NS5B region was amplified, sequenced and subtyped by BLAST search using the Los Alamos online database and geno2pheno[HCV]. Subsequently, the NS3 region was amplified using subtype-specific primers, sequenced and also analysed with geno2pheno[HCV] with respect to resistance mutations to boceprevir and telaprevir.

RESULTS: 115 blood samples from 90 patients were used in this study. 104/115 viruses could be subtyped. The most prevalent subtypes were 1a (33%), 1b (34%) and 3a (28.0%). The NS3 region from 91/104 (88%) viruses could be amplified and sequenced (1a=27/34 [79%]; 1b=35/36 [97%]; 3a=27/29 [93%]). In four samples, resistance mutations against protease inhibitors were scored (three samples with 117H and one sample with 168G).

CONCLUSIONS: The current strategy of amplification and sequencing of the NS3B region and interpretation with geno2pheno[HCV] currently allows genotyping/subtyping and may, in the future, allow the analysis of resistance mutations to drugs developed to target this protein. Subsequent analysis of NS3 and prediction with geno2pheno[HCV] allows the prediction of susceptibility to the protease inhibitors boceprevir and telaprevir. By collecting additional viral and clinical data from patients on protease inhibitor treatment we will continuously improve the geno2pheno[HCV] interpretation system.
ABSTRACT 82

*Antiviral Therapy* 2012; 17 Suppl 1:A100

Comparison of *in vitro* susceptibility to TMC435 of clinical isolates with site-directed mutants in a genotype 1 replicon system

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**BACKGROUND:** Phenotypic analysis to assess *in vitro* susceptibility to HCV NS3/4A protease inhibitors can be performed by engineering site-directed mutations (SDMs) or introducing patient-derived NS3 protease sequences into a replicon backbone. Here, we compare the phenotypes from patient-derived viral sequences carrying TMC435 (an investigational, once-daily, oral protease inhibitor in Phase III development) resistance-associated single or double mutations with the corresponding SDMs.

**METHODS:** *In vitro* susceptibility of HCV genotype 1 clinical isolates to TMC435 was determined in a transient replicon assay. The assay comprised chimeric genotype 1b replicons containing a patient-derived NS3 protease domain (amino acids 6–191) or replicons containing single (Q80K, Q80R, R155K, D168E or D168V) or double (Q80K+R155K or Q80R+D168E) mutations. Fold change (FC) in EC₅₀ was calculated compared to wild-type replicon (ET).

**RESULTS:** For clinical isolates without mutations at NS3 positions 43, 80, 155, 156 and 168 (known to be associated with reduced susceptibility to TMC435), median FC was 0.4 (interquartile range [IQR] 0.3–1). Activity of TMC435 against clinical isolates was comparable to activity against SDMs carrying the respective mutations. For clinical isolates with Q80K and Q80R+D168E mutations, median FCs (IQR) were 10 (6.4–13) and 726 (300–876), respectively, compared with 7.7 (6.7–9) and 408 (339–605), respectively, for the SDMs. For clinical isolates with an R155K mutation (all genotype 1a), the median FC (IQR) of 83 (56–102) was higher than that observed for the R155K SDM (32 [24–42]) in a genotype 1b replicon, but comparable to the R155K SDM in a genotype 1a replicon (90 [85–116]). A higher FC was observed in two clinical isolates with an R155K mutation than for the rest of this group. Further analyses identified the presence of an S122R variant, which as an SDM alone reduced TMC435 activity by 22-fold and as S122R+R155K by 207-fold.

**CONCLUSIONS:** In general, a good concordance between the phenotypes of clinical isolates and matching SDMs was observed, suggesting that previously identified TMC435 resistance-associated mutations are the most common drivers of resistance observed in clinical isolates. In one R155K isolate with a higher than expected FC, an S122R mutation was identified that could have contributed to the additional resistance observed.
ABSTRACT 83

*Antiviral Therapy* 2012; 17 Suppl 1:A101

Cross-sectional characterization of the susceptibility of HCV genotype 1 patient isolates to direct acting antiviral (DAA) agents

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BACKGROUND AND AIMS: DAAs that target HCV replication offer significant promise for the curative treatment of HCV-infected individuals. In an effort to standardize the preclinical and clinical evaluation of DAAs, we generated genotypic and phenotypic assays to assess resistance to NS3 protease, NS5A and NS5B polymerase inhibitors and surveyed a large panel of patient isolates that were submitted for routine viral load testing.

METHODS: Genotype 1(a/b) NS3/4A, NS5A and NS5B sequences were amplified from patient plasma and subjected to conventional sequence analysis. A Con1 luciferase-reporter replicon was engineered to generate replicons that incorporate patient-derived NS3 protease, NS5A or NS5B sequences. One or more DAA resistance mutations were also introduced into reference replicons by site-directed mutagenesis. Replicons containing SDM or patient virus sequences were used to evaluate DAA susceptibility (IC50 fold change) and replication capacity (RC) relative to reference replicons.

RESULTS: Susceptibility to multiple protease, NS5A and polymerase inhibitors was characterized using a panel of 19 NS3, 18 NS5A and 17 NS5B site-directed mutants (SDMs), which enabled assessments of cross-resistance among inhibitors. The assessment of protease, NS5A and polymerase inhibitor susceptibility for replicons containing sequences derived from a broad collection (n>100) of patient isolates was used to define the biological variation in DAA susceptibility. Based on this initial survey, genotype 1 variants display susceptibilities to protease, NS5A and polymerase inhibitors that vary within an approximate 1, 2 and 3 log10 range, respectively. Amino acid substitutions at acquired DAA resistance association positions in a minority of isolates largely accounted for this broad variation in susceptibility.

CONCLUSIONS: HCV replicons containing SDMs and patient sequences derived from a broad panel of patient isolates were used to document the natural variation in susceptibility of GT1 isolates to inhibitors of HCV protease, polymerase and NS5A. Amino acid substitutions at acquired DAA resistance association positions in a minority of isolates largely accounted for this broad variation in susceptibility.
ABSTRACT 84

Antiviral Therapy 2012; 17 Suppl 1:A102

In vitro characterization of the pan-genotype activity of the HCV NS3/4A protease inhibitors boceprevir and telaprevir

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BACKGROUND: The HCV-NS3 protease inhibitors, boceprevir (BOC) and telaprevir (TVR), combined with peginterferon alfa-2a/b plus ribavirin have recently been approved for treatment of HCV genotype 1 (G1) infection. In a previous Phase Ib study, 14 days of BOC (400 mg TID) monotherapy was associated with a decrease in HCV RNA in G2/3-infected patients, comparable to the viral load decline observed in G1-infected patients receiving the same dose. Here, the in vitro pan genotypic activity of BOC across HCV genotypes 1–6 (G1–6) was further assessed, and compared to TVR using a panel of NS3/4A sequences cloned from protease inhibitor treatment-naive patients.

METHODS: NS3/4A sequences were generated by RT/PCR amplification of HCV RNA from plasma samples, purchased from Teragenix Inc. (Ft. Lauderdale) and BioCollections Worldwide Inc. (Miami, FL), from patients infected with HCV genotypes 1a, 1b, 2a, 2b, 3a, 4a, 5a and 6a. BOC and TVR activities were assessed using a cell-based phenotypic assay, kinetic enzyme assays and a chimeric JFH1-G2a/G3aNS3 replicon cell line.

RESULTS: Average BOC activity against NS3 proteases from patients infected with G1b, G2a/b, G3a, G4a, G5a and G6a remained within a 1.1–3.2-fold range of G1a activity. TVR inhibition fell within a 1.6–3-fold range versus G1b, G2a/b, G5a and G6a isolates, whereas average fold-shifts against G3a and G4a NS3/4A proteases were 8.0-fold and 5.9-fold, respectively. The pattern of BOC and TVR activity assessed with the kinetic enzyme assay, using a panel of purified G1–G6 NS3/4a proteases, was similar to that found in the cell-based assay for each inhibitor. BOC and TVR were found to have equipotent activities against the JFH1G2a replicon (BOC EC50=283 ±36 and TVR EC50=252 ±54). The inhibitory activities against the chimeric JFH1-G2a/G3aNS3 replicon were BOC (EC50=159 ±5) and TVR (EC50=953 ±103).

CONCLUSIONS: The in vitro activity of BOC and TVR across clinical isolates encompassing G1, 2, 3, 4, 5 and 6 supports further clinical evaluation of both BOC and TVR in G2, 5 and 6 and BOC in G3 and 4.
ABSTRACT 85

Antiviral Therapy 2012; 17 Suppl 1:A103

Genetic characterization of non-structural HCV regions and response to standard HCV treatment

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BACKGROUND: Standard HCV treatment, interferon (IFN) combined with ribavirin, induces a sustained virological response (SVR) in only 50% of patients infected with HCV genotype 1a. However, the correlation between treatment outcome and the genetic variability of HCV genotype 1a is unclear. To address this issue, we used single-genome sequencing to investigate whether the genetic diversity of non-structural HCV proteins impacts treatment response.

METHODS: Pre-therapy plasma samples were obtained from 10 genotype 1a HCV-infected patients (viral load: 70,000 to 8,000,000) with different responses to standard HCV therapy: 5 SVR, 3 non-sustained responders (NSR) and 2 non-responders (NR). For single-genome sequencing, a 3 kb region encompassing the NS5A and first 1,760 nucleotides of NS5B was amplified and sequenced. Sequences from 11–23 single viral genomes from each pre-therapy plasma sample were obtained. Using phylogenetic analysis, the genetic variation and average pairwise distance was determined for each sample.

RESULTS: Patients with higher HCV RNA levels presented a lower percentage of identical nucleotide sites. Although the genetic diversity of the NS5A-NS5B region ranged from 1 to 3.4%, there was no correlation between sequence diversity, baseline RNA levels and treatment outcome. However, the average sequence diversity was greatest for patients with a non-sustained response to therapy (NSR [2.9%] > SVR [1.7%] > NR [1.2%]). Three patient samples, two NSR and one SVR, were found to contain recombinant viral strains. Separate analysis of the NS5A and NS5B regions, revealed a higher average sequence diversity in the NS5A (avg. 2.4%) region compared to the NS5B (avg. 1.6%). The heterogeneity of the IFN sensitivity-determining region located in the carboxy-terminal region of the NS5A was not associated with treatment response in these patients.

CONCLUSIONS: The non-structural proteins of HCV, NS5A and NS5B, are genetically variable: 30% of patient samples in this study revealed a 3% sequence diversity or greater; recombinant viral strains were also found in 30% of the patients. This genetic diversity was not found to be predictive of treatment response; however, non-sustained responders had the most genetically diverse viral strains. This indicates an initial treatment response by some viral strains with subsequent rebound of other, less treatment-susceptible, strains.
ABSTRACT 86

Antiviral Therapy 2012; 17 Suppl 1:A104

Measurement of HCV viral load, viral genotype and IL28B genotype from dried blood spots (DBS) and a dried ambient transport matrix (ViveST) using the Abbott m2000 system

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BACKGROUND: The use of specimen types other than plasma for HCV diagnostic assays may eliminate logistical limitations and improve clinical management of patients on antiviral therapy in resource-limited settings. This study evaluates the performance of the RealTime HCV viral load (RTVL), Abbott RealTime HCV Genotype II (RTGTII) and prototype IL28B genotype (IL28B) assays with DBS using alternate extraction protocols available with the Abbott m2000 system. This study also compares the performance of ViveST, a dried ambient transport matrix, to plasma for viral load determination with the RTVL assay.

METHODS: Citrated whole blood from 10 HCV-positive patients was used to prepare DBS on Whatman 903 paper. Parameters evaluated during protocol optimization included sample type, volume, number of DBS per test, extraction chemistry and extraction protocols. The optimal extraction protocol was selected and the performance of the RTVL assay was assessed. Suitability of DBS for the RTGTII and IL28B assays was assessed using the standard m2000 protocols. The performance of the ViveST sample collection device for RTVL and RTGTII was evaluated separately using plasma samples from 19 subjects and compared to frozen plasma.

RESULTS: HCV viral genotype and IL28B genotype from DBS was demonstrated using the standard protocols and gave expected results. For RTVL, a two 75 μl spot, 1 ml total nucleic acid (TNA) extraction procedure gave the closest correlation to results obtained with plasma with a mean bias (plasma–DBS) of 0.41 log IU/ml. All DBS replicates of the lowest viral load tested (250 IU/ml, n=10) were positive. Good correlation was observed between the plasma RTVL and ViveST using the standard protocol with a mean bias (plasma–ViveST) of 0.32 log IU/ml. RTGTII results demonstrated 100% concordance between ViveST and frozen plasma with tested genotypes (1, 1a, 1b, 2 and 3).

CONCLUSIONS: DBS is a suitable specimen type for RTVL, RTGTII and IL28B testing on the m2000 platform. The ViveST device performed well for RTVL and RTGTII assays. Future studies of the clinical utility of these protocols are warranted.
ABSTRACT 87

Antiviral Therapy 2012; 17 Suppl 1:A105

Development and evaluation of an HCV NS5B polymerase replicon-based phenotyping assay

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BACKGROUND: Numerous direct-acting antiviral (DAA) agents targeting the HCV polymerase (NS5B) are currently in clinical development. To evaluate drug susceptibility against polymerase inhibitors, we developed a cell-based phenotypic assay based on analysis of con1b replicons containing NS5B regions derived from HCV-1a/b-infected patient samples. We describe the phenotyping assay and demonstrate feasibility on a panel of clinical isolates, which were tested against polymerase inhibitors representing different published chemical entities.

METHODS: The HCV NS5B sequence was amplified from 30 HCV-1a and 30 HCV-1b plasma samples from treatment-naive patients by RT-PCR using subtype-specific primers. The ET replicon (pFK_i341_PILuc_NS3-3′_ET) was genetically modified to allow efficient insertion of NS5B domains derived from clinical isolates using the In-Fusion™ cloning method. Replicon RNA was transferred into Huh7 cells by electroporation. Replication was determined in the presence and absence of polymerase inhibitors by measuring luminescence (384-well format) after 48 h. Experiments were performed at least in triplicates. Relative replication capacity (RRC), EC50 and EC 50 fold change (FC) values were calculated.

RESULTS: NS5B amplicons were generated from 28/30 (93%) HCV-1a and 28/30 (93%) HCV-1b isolates and were subsequently cloned into the shuttle backbone. Analysis of the NS5B sequences confirmed the original subtype annotation and demonstrated that the isolates were phylogenetically distinct. All 56 NS5B shuttle replicons showed replication signals >10 signal/noise ratio with RRCs in the range from 1% to 32% (Q1=4%; Q3=17%) with 46/56 (82%) samples showing standard deviations (sd) of <0.3 log10. Drug susceptibility of the 56 samples was evaluated and EC50 and FC fold change (FC) values were calculated.

CONCLUSIONS: An NS5B replicon-based phenotyping assay using the InFusion™ method was developed to support research and clinical trials on NS5B inhibitors in patients infected with HCV subtype 1a/b. Optimized assay parameters were identified, which resulted in an overall success rate of 93% for the complete NS5B phenotyping assay procedure and excellent sds.
ABSTRACT 88

Antiviral Therapy 2012; 17 Suppl 1:A106

Role of serum HBsAg quantitation in HIV+ patients with chronic hepatitis B during long-term exposure to tenofovir

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BACKGROUND: Achievement of HBsAg seroconversion is the closest outcome to clinical cure in chronic HBV infection. Interferon-based therapy increases the likelihood of achieving HBsAg clearance in HBV-monoinfected patients. The aim of our study was to examine the rate of HBsAg seroconversion and role of periodic HBsAg quantitative monitoring in a large group of HIV–HBV-coinfected patients treated with tenofovir (TDF).

METHODS: All HBsAg+ patients seen at three clinics in Spain were identified. Demographics, coinfections, laboratory parameters (HIV RNA, HBV DNA, HBeAg, HBsAg and HBV genotype) were recorded. Serum HBsAg levels were quantified using the Abbott ARCHITECT assay which depicts a dynamic range from 0.05 to 250 IU/ml.

RESULTS: A total of 147 chronic HIV–HBV-coinfected patients were identified. HBeAg+ 45%; HCV RNA+ 20.2%; HDV Ab+ 26.7%. HBV genotypes A and D were the most common (36% and 28%, respectively). The rate of HBeAg loss was 7.1%. HBsAg loss was 6.8%. Overall 93% of patients were on TDF (median length 47 months). Nearly 30% had initiated TDF having undetectable HBV DNA (<200 IU/ml). Median HBV DNA in patients with baseline detectable HBV DNA was 6.4 [IQR: 4.8–8]. Overall 60% of patients had received lamivudine before beginning TDF.

Longitudinal follow-up (median, 5 years) could be performed in a subset of 51 patients with stored serum specimens. Patients who experienced HBsAg clearance had lower baseline HBsAg levels than those who remained HBsAg+ (1.4 [0.3–2.1] versus 4 [3.2–4.6] log IU/ml; P=0.001). The former experienced more pronounced HBsAg decreases than those with persistent HBsAg during follow-up (0.5 versus 0.3 log IU/ml; P=0.027). Median baseline HBsAg levels were lower in HBV genotype D than A (3.5 [2.2–4.3] versus 4.2 [2.3–5] log IU/ml; P=0.03). HBeAg+ patients had baseline greater HBsAg levels than HBeAg-negative patients (4.4 [4–5] versus 3.4 [2.4–4.3] log IU/ml; P=0.001). In multivariate analysis, baseline HBsAg levels and HBsAg decreases >0.5 log/ml/year were independent predictors of HBsAg clearance.

CONCLUSIONS: Baseline quantitation and periodic monitoring of serum HBsAg predicts HBsAg clearance at 5 years in HIV–HBV-coinfected patients treated with TDF.
ABSTRACT 89

Antiviral Therapy 2012; 17 Suppl 1:A107

Evolution of a multidrug-resistant mutation during tenofovir treatment in a hepatitis B patient with sequential antiviral therapy

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BACKGROUND: The rtA194T HBV polymerase mutation recently did not confer in vitro resistance to TDF as a single mutation or in lamivudine (LAM)-resistant viral background. We experienced one case of virological and biochemical breakthrough during TDF treatment of HBV patient who received sequential antiviral therapy.

CASE: A 50-year-old man diagnosed with HBeAg-positive chronic hepatitis B (CHB) had received entecavir (ETV) 1 mg/day treatment for 120 weeks following an initial unsuccessful LAM treatment. The therapy had been switched to LAM and adefovir (ADV) for 24 weeks. Because of the medical insurance system, he did not receive ETV 1 mg/day and ADV combination therapy. This led only to a temporary HBV DNA decrease which soon was followed by virological and biochemical breakthrough despite the lack of known ADV resistance mutations. The therapy had been switched to TDF monotherapy for 1 year. However, the levels of HBV DNA and ALT rebounded from 3.8 log10 copies/ml and 23 IU/ml at week 24 to 7.0 log10 copies/ml and 357 IU/ml at week 48. The virological and biochemical breakthrough were developed without known rtA194T mutation. Analysis from 13 serial samples with multidrug-resistant mutations on direct sequencing showed rtL180M, rtM204V/I mutations and novel mutations (rtA200V, rtF221Y, rtS223A) continuously, and rtT184T/L mixed mutations intermittently. Among serial samples, a total of 40 clones from four different serum samples were analysed retrospectively. Dominance of a clone carrying rtL180M, rtM204V/I, rtF221Y, rtS223A, rtL84M, rtI91F, rtR153Q, rtT184A/S, rtV191I combined mutations without previous reported rtA194T mutation. This viral strain might induce decreased susceptibility to nucleosides and nucleotides. TDF resistance may emerge due to multisite polymerase mutations rather than single-site polymerase mutation.

CONCLUSIONS: A case is described who developed a high degree of resistance to TDF caused by a HBV virus strain bearing rtL180M, rtM204V/I, rtF221Y, rtS223A,
ABSTRACT 90

Antiviral Therapy 2012; 17 Suppl 1:A108

Simultaneous emergence of entecavir resistance mutations in a nucleoside-naive chronic hepatitis B patient

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BACKGROUND: Entecavir (ETV) has potent antiviral activity against hepatitis B virus (HBV), and the emergence of drug resistance is rare in nucleoside-naive patients. Resistance requires the simultaneous appearance of three mutations which account for the very low resistance profile of ETV. We experienced one case of genotypical ETV resistance with viral rebound during ETV treatment of nucleoside-naive patients with chronic hepatitis B (CHB).

CASE: A 50-year-old HBeAg-positive man received ETV 0.5 mg/day for 120 weeks. The level of HBV DNA was 9.0 log10 copies/ml at baseline, decreased to a nadir of 2.7 at week 60, and then rebounded to 6.0 at week 108 and 7.5 at week 120. The serum alanine aminotransferase (ALT) level did not increase during ETV treatment. The ETV-resistance-related substitution (T184A) and lamivudine (LAM)-resistance-related substitutions (L180M and M204V) were detected by sequence analysis at week 96.

CONCLUSIONS: The three substitutions associated with ETV and LAM resistance developed simultaneously without complete suppression in a nucleoside-naive CHB patient after extended therapy. In spite of the extremely rare chance of viral mutation during ETV treatment, treatment-naive patients with high pre-treatment viral loads and detectable HBV DNA during treatment should be carefully monitored or undergo targeted surveillance for resistance.
SESSION 5
Clinical implications of resistance
ABSTRACT 91

Antiviral Therapy 2012; 17 Suppl 1:A111

Impact of NNRTI-resistant minority variants and NNRTI regimen on resistance genotype after virological failure

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BACKGROUND: In a recent pooled analysis, drug-resistant HIV-1 minority variants (MVs) increased the risk of virological failure (VF) for first-line NNRTI-based regimens twofold. Using that pooled dataset, we evaluated the relationship between baseline NNRTI-resistant MVs, NNRTI regimen, and other factors on the likelihood and types of resistance mutations detected after VF.

METHODS: Resistance data at VF were available from seven studies included in the MV pooled analysis. Multivariable logistic regression with backward elimination and stratified by study was used to evaluate predictors of NNRTI resistance at VF. Individuals without baseline NNRTI-resistant MVs detected were considered to have 0% MVs; a sensitivity analysis was also performed imputing 10% of the assay limit of detection for these individuals.

RESULTS: Genotypic resistance data were available from 240 of the 319 participants with VF. In the 80 participants with NNRTI-resistant MVs detected at baseline, increasing MV copy number was associated with a higher probability of NNRTI resistance at VF (25% with 1–9 copies/ml, 39% with 10–99 copies/ml, 58% with 100–999 copies/ml and 88% with ≥1,000 copies/ml; Cochran–Mantel–Haenszel P=0.04). This association was largely unchanged in a sensitivity analysis including imputed baseline NNRTI-resistant MV copy numbers for the 118 participants without detectable MVs. In multivariable logistic regression analysis, higher NNRTI MV copy numbers, non-white race and nevirapine use were all associated with a higher risk of NNRTI resistance at VF. Among participants on an efavirenz-based regimen, K103N was the most frequently observed resistance mutation at VF regardless of the MV detected at baseline (77%, 95/124). However, the presence of Y181C MV at baseline was associated with a higher probability of Y181C detection after VF (18% versus 3%; P=0.01). For individuals without detectable pre-existing NNRTI-resistant MVs, Y181C was detected after VF in 75% (9/12) of individuals on a nevirapine-based regimen compared with 4% (3/79) on an efavirenz-based regimen (P<0.001).

CONCLUSIONS: In treatment-naive individuals, NNRTI regimen choice and pre-existing NNRTI-resistant MVs were both associated with the probability and type of resistance mutations detected after virological failure.
ABSTRACT 92
Antiviral Therapy 2012; 17 Suppl 1:A112

Optimized antiretroviral drug selection achieves rapid and sustained suppression of viral replication, despite transmission of HIV-1 exhibiting resistance to five-drug classes

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BACKGROUND: Transmission of HIV-1 exhibiting resistance to protease (PR) and reverse transcriptase (RT) inhibitors is well documented, but is not well established for integrase (IN) or fusion inhibitors. We describe a case of transmitted drug-resistant virus involving PR, RT, IN and fusion inhibitors in a patient who was hospitalized in 2010 with a severe flu-like illness. HIV antibody testing initially resulted negative during hospitalization, but was positive 6 months later and confirmed by western blot.

METHODS: Mutations associated with reductions in susceptibility to inhibitors of HIV-1 PR, RT, IN and fusion were identified by conventional DNA sequencing. Phenotypic susceptibility to PR, RT, IN and fusion inhibitors, and coreceptor tropism were assessed using well-established single-cycle replication assays.

RESULTS: Baseline CD4+ cell count and HIV-1 viral load were 376 cells/mm³ and 211,540 copies/ml, respectively. The baseline virus harboured mutations associated with resistance to inhibitors of PR (L10Y, I13V, K20I, E35D, M36I, K43T, I62I/V, V82A), RT (M41L, D67N, L74V, V118I, K101E, Y181C, V189I, G190S) and IN (G140S, Q148H). Novel substitutions (Q40R, N43S) at amino acid positions in gp41 previously associated with enfuvirtide resistance were identified. Reductions in susceptibility to PI, NRTI, NNRTI, IN and enfuvirtide were confirmed by phenotypic testing. Coreceptor tropism testing identified a predominantly R5 virus population with a significant component of dual-R variants. Based on resistance and tropism profiles, a regimen of ritonavir-boosted darunavir, tenofovir/emtricitabine and maraviroc was implemented resulting in viral suppression which was rapid and maintained at 6 months.

CONCLUSIONS: This case study represents the first confirmed report of the transmission of HIV-1 exhibiting resistance to five antiretroviral drug classes, and includes the description of two novel resistance mutations in gp41 that confer resistance to enfuvirtide. This case also represents the third documented case of transmitted IN inhibitor-resistant HIV-1 and highlights the emerging value of assessing integrase inhibitor resistance before treatment initiation in newly infected patients.
ABSTRACT 93

Antiviral Therapy 2012; 17 Suppl 1:A113

Transmitted HIV-1 drug resistance associated with virological failure of NNRTI-based ART: representative codons and their concentration in plasma

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OBJECTIVES: To identify HIV-1 pol resistance codons in antiretroviral-naive Kenyans (that is, transmitted resistance) and the concentration of mutant virus predictive of virological failure (VF) to first-line non-nucleoside reverse transcriptase inhibitor (NNRTI)-based ART.

METHODS: Pre-ART plasma specimens studied for HIV-1 resistance were from Kenyans participating in a randomized trial to maximize antiretroviral adherence and minimize WHO-defined VF with a plasma HIV RNA >5,000 c/ml at 6, 12 and 18 months of ART. HIV-1 pol mutations associated with NNRTI and NRTI resistance were evaluated by three methods: first, an oligonucleotide ligation assay (OLA) for reverse transcriptase codons 103, 181, 190 and 184 (lower limit of detection 2%) was performed on all specimens. Second, consensus sequencing (CS) was performed on specimens with mutant or indeterminate codons by OLA. Third, pyrosequencing of ≥1,000 amplifiable viral templates was performed on pre-ART specimens from all subjects with virological failure, and/or resistance by OLA or CS; and is ongoing on matched case-control specimens.

RESULTS: OLA detected a total of 16 resistance mutations in 11 of 324 available/amplifiable pre-ART specimens. CS detected seven additional mutations (2 M41L, D67N, L74V, V106A, L210W and T215Y) in 3/11 subjects who had mutants by OLA. The 11 subjects represented 8/42 (19%, 95% CI 9.7, 33.6%) with VF; 1/46 (2%, 95% CI 0.01, 12.4%) with HIV-1 RNA 200, 5,000 c/ml, and 2/243 (<1%, 95% CI 0.03, 3.24%) with viral suppression (<200 c/ml; P<0.0001). Among the 8 with VF, 4 had 2–8 NNRTI/NRTI mutations. The three without VF had K103N only by OLA; one also had M41L by CS. The median mutant concentration detected by OLA (n=16) was ‘high’ (71%, range 4–100), including those without VF (K103N at 81%, 73% and 74%). Pyrosequencing detected an additional 9 mutant codons, at a median concentration of 0.15% (range 0.11–5.9). Two of these mutations were among 6 subjects with VF and mutants by OLA, and 7 in 21 others with VF but without mutations by OLA.

CONCLUSIONS: High concentrations of transmitted NNRTI mutations in pre-ART specimens were highly associated with VF. Whether very low concentrations of mutants (<2%), detected by pyrosequencing are associated with virological failure awaits completion of pyrosequencing of specimens from individuals with virological suppression.
ABSTRACT 94

_Antiviral Therapy_ 2012; 17 Suppl 1:A114

Clinical trial evidence with respect to the use of 3TC to maintain M184IV mutations in patients changing therapy

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**BACKGROUND:** The M184I/V drug mutation, associated with resistance to 3TC and FTC, is also known to adversely impact on reverse transcriptase function and viral replicative fitness. We sought to determine whether maintenance of M184I/V through continued use of 3TC after treatment failure might have virological benefit in the context of large randomized clinical trials that led to the approval of tipranavir (TPV) for treatment-experienced patients.

**METHODS:** We examined viral loads (VL) in patients who participated in the RESIST clinical trials, where TPV plus optimized background therapy (OBT) was compared with control protease inhibitor (CPT) plus OBT. Choice of whether to include 3TC as a part of OBT was left to the discretion of physicians. Our analysis included patients enrolled in both the CPT+OBT and TPV+OBT arms of the studies. Analysis population included those patients using 3TC and with M184I/V mutation at baseline. Analysis compared those who continued 3TC to those who discontinued 3TC with respect to week 8 VL change from baseline and week 48 VL response, with adjustment for the activity score for the rest of the treatment regimen.

**RESULTS:** Those who discontinued 3TC had numerically superior response at both time points when compared with those who continued 3TC: -1.21 log_{10} VL versus -0.96 log_{10} VL at week 8 and Mantel Haenszel odds ratio 1.46 (0.94, 2.27; 95% CI) at week 48.

**CONCLUSIONS:** These findings do not support the notion that maintenance of the M184I/V mutation through continued use of 3TC in secondary treatment regimens provides virological benefit.
ABSTRACT 95
Antiviral Therapy 2012; 17 Suppl 1:A115

Drug resistance prevalence after failing antiretroviral therapy (ART) with tenofovir (TDF) + emtricitabine/lamivudine (3TC/FTC) + efavirenz (EFV) versus the single tablet regimen (STR) Atripla: the ATRES study

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BACKGROUND: STRs for the treatment of HIV prevent partial interruptions of therapy, potentially lowering the likelihood for selection drug resistance mutations associated with virological failure. We tested this hypothesis in a large multicentre retrospective analysis.

METHODS: We analysed the proportions of patients developing drug resistance mutations (DRMs) associated with VF to initial ART in two groups of patients: those receiving the STR Atripla versus the non-STR combinations of TDF+3TC/FTC+EFV. We used data from eight cohorts and four randomized studies. The eight cohorts were from Tor Vergata University (Italy), Stanford University (USA), British Columbia University (Canada), Emory University (USA), RIS (Spain) and Guadalajara University (Mexico), and two clinics in Barcelona. The four randomized studies included GS903, GS934, MSD-004 and STARTMRK trials. VF was defined as having ≥2 plasma HIV-1 RNA levels above the limit of detection. Only patients with a genotypic resistance test within 3 months of VF, follow-up ≥6 months and adherence ≥90% to ARV were included.

RESULTS: A total of 184 patients were included in the final analysis, 122 were from cohort studies (66.3%) and 62 (33.7%) from clinical trials. The overall proportion of patients with ≥1 DRM was 64%. 57% in the STR group versus 67% in the non-STR group (P=0.10, Fisher’s exact test). The proportions of patients with drug-specific DRMs, however, did not differ for the individual drug components comparing the STR-group with the non-STR group: (i) TDF DRMs (65R, 70E): 16% versus 14% (P=0.82); (ii) 3TC/FTC DRMs (184V/I): 31% versus 33.3% (P=0.86); and (iii) NNRTI DRMs (90I, 98G, 100I, 101E/P/H, 103N/S, 106A/M, 108I, 126A/G/K/Q/R, 179D/F/T, 181C/I/V, 189L/I/H, 190A/S, 225H, 227C, 230I/L): 50% versus 57% (P=0.42) The proportions of patients in the cohort studies with ≥1 DRM was significantly lower among the STR (52%) versus non-STR (75%) groups (P=0.009). There was a trend to a lower proportion of patients with M184V/I in those receiving FTC-group (n=127; 12%) versus 3TC-group (n=57; 21%; P=0.117).

CONCLUSIONS: Compared with patients receiving the STR Atripla®, those receiving the same/similar drug components separately could be at an increased risk of developing drug resistance following the development of VF.
ABSTRACT 96

Antiviral Therapy 2012; 17 Suppl 1:A116

Could treatment-emergent drug resistance be used as the primary endpoint in HIV clinical trials?

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BACKGROUND: While most HIV clinical trials use HIV RNA suppression as the primary efficacy endpoint, it is unclear when trials can detect statistically significant differences in treatment-emergent drug resistance between treatment arms. Methods of selecting/sampling patients for resistance testing and statistical analyses have not been standardized.

METHODS: Rates of treatment-emergent drug resistance (defined as primary NRTI, NNRTI or PI IAS-USA mutations) were analysed from 15 trials in treatment-naive patients, and five switch trials. Sample sizes using drug resistance as the primary endpoint were calculated for (i) difference trials (control arm 2 NRTI+EFV), assuming reductions in absolute risk of resistance (ii) non-inferiority trials, with a delta (non-inferiority margin) of 3% in absolute risk of resistance. Sample size calculations were conducted using NQUERY software, assuming 5% significance level, 80% power and 1:1 randomization. Exact methods were used when event rates were close to 0%.

RESULTS: The percentage of patients with treatment-emergent IAS-USA mutations after 96 weeks ranged from 0 to 6% for first-line 2 NRTI/efavirenz treatments, 0 to 1% for first-line 2 NRTI/PI/r treatments and 0 to 1% in switch trials of boosted PIs. The percentage of randomized patients with resistance development was at least 3% in first-line trials of 2 NRTI/efavirenz when all patient samples with HIV RNA >50 copies/ml were genotyped (including after discontinuation of randomized treatment). First-line trials of 2 NRTI/efavirenz showed lower percentages with resistance when genotyping was only performed when HIV RNA was >400–1,000 copies/ml on randomized treatment and/or no testing of discontinued patients. The sample size to detect a reduction in drug resistance from 3% on 2 NRTI/EFV to <0.5% on 2 NRTI/PI/r would be 377 patients per arm. Non-inferiority designs would require 400 patients per arm, assuming a 3% risk of resistance in the control arm and delta of 3%.

CONCLUSIONS: HIV clinical trials of approximately 800 patients (400 per arm) could be designed to detect differences of at least 3% in the absolute risk of treatment-emergent HIV drug resistance between treatments or non-inferiority. However this would require a standardized approach, with Intent to Treat analyses, testing of all samples with rebound in HIV RNA>50 copies/ml and genotyping of all patients after drug discontinuation.
ABSTRACT 97

Antiviral Therapy 2012; 17 Suppl 1:A117

Trends in antiretroviral therapy use and drug resistance profiles by calendar in Spain

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BACKGROUND: The current goal of antiretroviral therapy is to reach undetectable viraemia regardless being antiretroviral-naive or -experienced patients. However, side effects and resistance still preclude reaching this goal in a subset of patients. More potent drugs, safer and easy to take are still needed. Increasing the number of HIV persons with undetectable viraemia is of benefit for both infected individuals and community, as it may halt the likelihood of further transmission events, including drug-resistant HIV strains.

METHODS: All patients attended from January 1999 to December 2011 at one large reference HIV clinic located in Madrid were identified. Antiretroviral therapy, plasma HIV RNA and drug resistance mutations were assessed at baseline and periodically during follow-up.

RESULTS: A total of 4,279 different HIV-infected individuals were identified. There was a significant increase in the proportion of patients on antiretroviral therapy over time. It was 60.1% in 1999 and risen to 79.8% in 2011 (P<0.001). On the other hand, the proportion of treated patients with undetectable viraemia increased from 53.1% in 1999 to 91.9% in 2011 (P<0.001).

A subset of 2,662 patients experienced virological failure at any given time point during the study period and underwent drug resistance testing. Median plasma HIV RNA in this population at the time of analysis was 3.3 log copies/ml and median CD4 counts were 340 cells/mm³. Of them, 51.2% were failing protease inhibitors (PIs) and 30.3% non-nucleoside RT inhibitors (NNRTIs). HIV-1 non-B subtypes were found in 7.8% of cases and X4 tropism was recognized in 46.3%. The overall prevalence of drug resistance mutations in this population was 18.6% for NRTIs, 18.4% for NNRTIs and 15.7% for PIs. Interestingly, the rate of drug resistance mutations was significantly higher among patients with X4 viruses (29.4% for NRTIs, 29.7% for NNRTIs and 32.8% for PIs; P<0.0001). It is noteworthy that the proportion of failures with drug resistance mutations increased by calendar, being <4% for each drug family in 1999 but rising to 11% for NRTIs, 21% for NNRTIs and 23% for PIs (P<0.0001) in 2011.

CONCLUSIONS: The expanded use of antiretroviral therapy in recent years has significantly modified the rate and drug resistance profile of virological failures. Whereas the newest antiretroviral combinations account for a lower rate of virological failures, current escape viruses in these patients harbour multiple drug resistance mutations, generally reflecting past exposure to suboptimal therapies. This information may be relevant when considering treatment as prevention at community level.
ABSTRACT 98

Antiviral Therapy 2012; 17 Suppl 1:A118

Long-lasting protection of genotypic activity of NRTIs and PIs by boosted PI-containing regimens

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BACKGROUND: Drug resistance mutations generally accumulate if patients stay continuously on a failing combination antiretroviral therapy (cART). We aimed to evaluate the dynamics of loss of genotypic activity after virological failure.

METHODS: We studied patients from the Swiss HIV Cohort Study who started first-line cART with ≥2 NRTIs and a ritonavir-boosted PI (PI/r) or an NNRTI. Patients were included if they initially achieved viral suppression (<50 HIV-1 RNA copies/ml) and later on failed cART. We studied the loss of genotypic activity over time by analysing genotypic drug resistance tests (GRTs) after virological failure (midpoint between last undetectable and first detectable viral load). The activity of antiretroviral drugs was defined as ‘lost’ if the Stanford algorithm penalty score was >30. To identify potential risk factors associated with early emergence of NRTI mutations (<6 months after failure), we performed a multivariable logistic regression adjusted for sex, transmission group, age, subtype, CD4 cell count and viral load at time of GRT, NRTI and non-NRTI treatment components.

RESULTS: A total of 78 GRTs from PI/r-treated and 104 from NNRTI-treated patients were analysed. Overall, 31, 33 and 23 and 36, 24 and 35 were performed <3, 3–6, >6 months after failure, respectively. The percentage of patients who have lost PI/r activity was 3.2%, 4.4% and 0% <3, 3–6, >6 months after failure compared with 45.5%, 44.4% and 60.0% of those who have lost NNRTI activity (P≤0.001 for all time points). Most interestingly, the risk of losing the activity of ≥1 NRTIs was also lower in patients who were treated with PI/r compared with NNRTI<3, 3–6 and >6 months after failure: 9.7% versus 39.4% (P=0.009), 8.7% versus 41.7% (P=0.008) and 16.7% versus 57.1% (P=0.003). The risk to accumulate an early NRTI mutation was strongly increased by NNRTI-based compared to PI/r-based cART (multivariable odds ratio 10.8 [95% CI 3.0, 38.3]; P<0.001).

CONCLUSIONS: The loss of activity of PI/r and NRTIs is low among patients treated with PI/r, even after long-lasting exposure to the failing therapy. Thus, more options remain for second-line therapy. This finding could be of high relevance for settings with poor or lacking virological monitoring.
SESSION 6
Drug resistance in resource-poor countries
ABSTRACT 99

Antiviral Therapy 2012; 17 Suppl 1:A121

ZDV resistance mutations detected by pyrosequencing in women treated with single-agent ZDV for prevention of mother-to-child transmission of HIV-1

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OBJECTIVES: In 2010, the WHO revised their recommendations for prophylaxis of mother-to-child transmission (PMTCT) of HIV-1. Single-agent zidovudine (ZDV), option A, will likely be relied upon for women with >350 CD4/µl in low-income nations due to greater cost of triple ARV (ART). We used deep-sequencing to test whether minority variant ZDV mutations will be selected for in women with prolonged ZDV monotherapy for PMTCT.

METHODS: HIV-1 subtype-E-infected, ARV-naive, pregnant Thai women with CD4 counts >250 cells/µl were enrolled and treated with ZDV monotherapy beginning at 28 weeks gestation. We developed HIV-1 subtype-E-specific, pol region primers to detect resistance mutations M41L, D67N, K70R, L210W, T215Y/F and K219Q/E. The concentration of amplifiable viral templates was determined for each specimen, with ~1,000 templates submitted for 454 pyrosequencing (Roche). Concentrations of ZDV-associated mutations were calculated.

RESULTS: Specimens from 27 women on ZDV for a mean of 10.05 weeks (range 7.57–15.85 weeks) were analysed. Plasmid control data revealed an error rate of 0.00% (range 0.00–0.81%) in pyrosequencing; therefore, we used 1.6% as the limit of detection. ZDV-associated resistance mutations were detected at delivery in 3/27 (11.1%) subjects. Two subjects had mutations at two codons each (D67N/K70R, D67N/K219Q), K70R was detected in 7.4% (2/27), D67N in 7.4% (2/27) and K219Q in 3.7% (1/27). All three women had mutant concentrations below the limit of detection: K70R at 2% viral population in one subject, D67N at 2% and 10% in two subjects. M41L, L210W and T215Y/F were not detected.

CONCLUSIONS: In women treated with short-duration of ZDV (mean 10.5 weeks) for PMTCT, resistance-associated mutations were frequent, occurring in 11.1%. Studies are needed to evaluate: (1) a larger number of women; (2) women who take ZDV for a longer duration, as recommended by the WHO beginning at 14 weeks gestation; (3) whether mutations decay; and (4) the clinical consequences of ZDV mutations on later ART.

These data raise the question of whether those women and children exposed to ZDV prophylaxis will experience higher rates of virological failure and poor clinical outcomes when later started on ART, as has been demonstrated with single-dose nevirapine.
ABSTRACT 100

*Antiviral Therapy* 2012; 17 Suppl 1:A122

Virological outcome and frequency of drug resistance mutations in HIV-infected patients receiving first-line ARV regimen and monitored with the public health approach in Southeast Asia and sub-Saharan Africa

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BACKGROUND: In resource-limited countries (RLC), antiretroviral treatment (ART) initiation and switch are essentially based on patient’s clinical assessment. We conducted a large cross-sectional study to estimate the virological outcome and drug resistance mutations (DRM) among patients experiencing 12 and 24 months first-line ART in Burkina-Faso (BF), Cameroon (CM), Senegal (SN), Togo (TG), Thailand (TH) and Vietnam (VN).

METHODS: Between 2009 and 2011, we recruited adult patients attending national ART centres for their 12 ±2 or 24 ±2 months visit. Observance data were collected using questionnaires and patients’ logs. Viral load (VL) was performed for each patient and samples with VL≥1,000 copies/ml were tested for genotypic drug resistance. Mutations were interpreted using the 2011v21 ANRS algorithm.

RESULTS: Overall, 3,100 (1,649 at M12 and 1,451 at M24) patients were enrolled in the six countries. Median age ranged from 31 to 40 years and median CD4 values at ART initiation from 70 cells/µl in TH to 174 in BF. Up to 90% of ARV combinations were 3TC+AZT/d4T+NVP/EFV. ART failure rate at M12 (VL≥1,000) was ≤5% in BF and TH, ≥10% in CM, SN and VN, and 17% in TG. At M24, failure rates were ≤5% in BF and TH, ≥10% in SN and VN, 13% in CM, and almost 26% in TG. Among failing patients, between 57% in CM and 100% in TH carried ≥1 major DRM at M12, and between 72% and 96% at M24, respectively, in CM and TG. DRMs included both NRTI (mostly M184V) and NNRTI mutations. There was an increase accumulation of DRM in patients at M24, leading to resistance to drugs that were not part of the current regimen as ddI, ABC, TDF for NRTIs and ETR for NNRTIs. Patients who declared to have missed at least 1 day of treatment the week before the visit were more likely to present virological failure than those who did not (P<0.001).

CONCLUSIONS: Our study demonstrated that the current ART monitoring recommendations in RLC can lead either to successful programme performance or to critical situations and confirmed the important role of adherence in viral suppression.
ABSTRACT 101

Antiviral Therapy 2012; 17 Suppl 1:A123

An in-depth resistance analysis of HIV-1 subtype-C-infected patients failing a lopinavir/ritonavir (LPV/r) second-line regimen in the South African private sector

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BACKGROUND: Major protease inhibitor (PI) resistance mutations have been reported to be infrequent in patients experiencing virological failure on a second-line regimen. Several factors may contribute to infrequent PI resistance: poor regimen adherence, selection of gag-cleavage site mutations, or selection of complex mixtures of minor variants. This study examined protease and gag-cleavage site mutants, both as major and minor variants, in 32 patients experiencing virological failure on a second-line regimen who were treated by private sector physicians in South Africa.

METHODS: Population-based sequencing of gag-cleavage sites, protease and partial reverse transcriptase (RT) were performed in all patients (n=32), and deep sequencing of protease and RT (HIV-1 RTP collaboration plates) on a GS-Junior Phase-B (Roche-454 Life Sciences) was performed in a subset (n=16). A control group (n=30) of PI-naive patients was included. Previously reported gag-cleavage site mutations and IAS-USA 2011 mutations are presented.

RESULTS: All 32 subjects were subtype-C-infected and were on two nucleoside RT inhibitors plus LPV/r for a median of 37 months. Median HIV-1 RNA and CD4 count were 4.19 log10 copies/ml and 152 cells/μl, respectively. 15 (47%) subjects had major PI resistance mutations, 11 (34%) had RT mutations only, and 6 (19%) had no mutations. The most frequent major PI mutations were: V82A (67%), I54V (67%), M46I (53%), L76V (27%) and L90M (20%). Of 28 patients with gag-cleavage sequences, 16 had gag-cleavage mutations: A431V (26%), K436R (14%), L76V (27%) and L90M (20%). Of 28 patients with gag-cleavage sequences, 16 had gag-cleavage mutations: A431V (26%), K436R (14%), I437T/V (11%), L449P (4%) and P453L (14%). 13 of the 16 (81%) with gag-cleavage site mutations also had PI mutations. Comparison to PI-naive patients revealed significant association of A431V (P<0.0001), K436R (P=0.006) and I437T/V (P<0.0001) with PI exposure. Deep sequencing revealed that only 2 of 16, both with PI mutations detected by population sequencing, had additional PI mutations (I47V-1.5%; I50V-1.2%; I84V-4.7%).

CONCLUSIONS: 47% of patients failing second-line treatment in the private sector had PI resistance mutations, which is higher than previously reported in the South African rollout programme suggesting population-sector influence. Sequencing of gag-cleavage sites identified three additional patients with PI resistance mutations. Deep sequencing did not identify additional patients with PI resistance.
BACKGROUND: There is limited information about activity of second-generation non-nucleoside reverse transcriptase inhibitors (NNRTIs) in HIV-1 non-subtype-B infections, in resource-limited settings. Over five million individuals are accessing first-line treatment, generally consisting of two nucleoside reverse transcriptase inhibitors (NRTIs) combined with nevirapine (NVP) or efavirenz (EFV). This study describes mutations and predicted level of susceptibility to etravirine (ETR) and rilpivirine (RPV) in subjects screening for ACTG A5230 (a study of lopinavir/ritonavir monotherapy after first-line failure in Thailand, South Africa, India, Malawi and Tanzania).

METHODS: HIV-1 RNA (VL), CD4, HIV-1 antiviral resistance and subtype determination were performed at screening. Resistance profiles were interpreted with the Stanford algorithm (11/30/2011). NNRTI mutation associations with subtype, VL, CD4 and site were evaluated by Kruskall–Wallis Test.

RESULTS: 207 individuals were screened; 148 were successfully sequenced. 64% of sequence failures had VL < 1,000 copies/ml. Of the 148, 59% were female; 73% were on a NVP-containing regimen. Subtype C was most prevalent (66%), followed by AE (18%), A1 (8%) and D (7%). Median (interquartile range [IQR]) prior NNRTI duration was 3.25 years (2–4.4); median VL was 4.39 log10 copies/ml. The number of NRTI mutations was associated with site (P<0.001), but not subtype (P=0.092). Several NRTI and NNRTI mutations were observed in >10% of subjects; 50% had ≥3 NNRTI mutations. Median (IQR) resistance scores for EFV, NVP, ETR and RPV were 65 (50–90) and 90 (65–120), 25 (5–35) and 25 (0–35), respectively. Y181C was observed more frequently in NVP-exposed subjects (51% [NVP], 8% [EFV]); K103N in EFV-exposed (31% [NVP], 61% [EFV]). A median of two mutations associated with ETR resistance and one associated with RPV resistance were observed. 60% of subjects were classified as resistant (low-high); 32% as fully susceptible to both ETR and RPV. Compared to EFV, prior NVP exposure was associated with higher ETR and RPV resistance (P<0.001).

CONCLUSIONS: Presumed prolonged virological failure with first-line NNRTI-based treatment resulted in high-level resistance to NVP, EFV and NRTIs. However, 32% of subjects remained fully susceptible to ETV and RPV. Frequent monitoring to identify virological treatment failure would reduce the selection of drug resistance mutations and potentially preserve treatment options.
ABSTRACT 103

Antiviral Therapy 2012; 17 Suppl 1:A125

Virological outcome of adult patients receiving first-line antiretroviral therapy in Maputo, Mozambique

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INTRODUCTION: In Mozambique, 11.5% of the total population is infected with HIV. Antiretroviral treatment (ART) was introduced in 2004 and by 2009, 38% of patients in need of treatment had access to the drugs from the 3% coverage in 2004. To date, no study has evaluated the effectiveness of Mozambican treatment strategy on a virological level in patients attending HIV care services in a public clinic in Maputo city.

METHODS: We recruited 274 adherent patients receiving first-line regimens divided in three groups according to drug exposure: group A, 6–12 mo (n=94); group B, 12–24 mo (n=81) and group C, 24–++ mo (n=99). Venous blood was collected for TCD4+ counting, viral load and drug resistance analysis. Ethics approval: INS IRB Ref:307/CNBS.

RESULTS: The majority received fixed-dose combination of lamivudine/stavudine/nevirapine (87.1%), followed by lamivudine/stavudine/efavirenz (9.7%) and lamivudine/zidovudine/efavirenz (3.2%). Increase in TCD4+ count was observed with increased ARV exposure, with a statistically significant increase (median 104 cells; P<0.05) in group C (GC; median 1,047.5 days). Maximal viral suppression is observed 12–24 mo after treatment initiation (90.1%). Both virological (VF) and immunological failure (IF) tend to increase with increasing exposure time (12–24 mo [9.9%; 22.2%] and 24–++ [23.2%; 11.1%]; P<0.05, 95% CI [4.4–18.0; 13.7–32.8 versus 15.3–32.8; 5.7–19.0]). Lack of correlation between VF and IF was observed with the occurrence of INR in all groups, with significant decrease with increased exposure to ARV. Incomplete VL suppression was observed in GA (85%) and GC (57%). Correlation between VF and DRM in 46% of the cases was seen, and there was an accumulation of DRM with time. The DRM profile reflected ART regimen utilized, the most common NRTI mutation observed was M184V (66%), followed by NNRTI DRM G190A (81.9%) and Y181C (52.9%). TAMs were observed with increasing exposure to NRTIs and TAM 2 was the main mutational pathway (n=5) versus TAM 1 (n=2).

CONCLUSIONS: Treatment strategies are effective in attaining sustainable virological response. The presence of more complex patterns on DRM in patients with longer period of exposure reinforces the need for VL testing. Immunological criteria force patients to remain in failing therapies compromising future treatment options based on NRTI due to elevated number of TAMs and NAMs.
ABSTRACT 104

*Antiviral Therapy* 2012; 17 Suppl 1: A126

HIV-1 drug resistance amongst adults in a routine rural HIV clinic in Kenya

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**INTRODUCTION:** The rapid scale-up in the provision of antiretroviral therapy (ART) in sub-Saharan Africa (sSA) has been implemented in the context of minimal monitoring and support, presenting a potential for emergence and spread of HIV drug resistance.

**OBJECTIVES:** To describe the prevalence of transmitted and acquired HIV-1 drug resistance amongst adults in a routine rural HIV clinic in Kenya.

**METHODS:** Plasma samples were collected from two cross-sectional studies conducted amongst adults (≥15 years old) attending routine HIV care at the Kilifi District Hospital between 2008 and 2010. Samples from recently diagnosed ART-naive clients prior to starting treatment were used to describe transmitted drug resistance (TDR). Those from clients who had been on ART for at least 6 months, with a viral load of >400 copies/ml, were analysed to describe acquired drug resistance (ADR). A portion of the pol subgenomic region of the HIV containing the protease and part of the reverse transcriptase gene was amplified and sequenced. Resistance mutations were identified according to the Stanford HIV drug resistance database. TDR strains were interpreted using the WHO list for surveillance of drug resistance mutations.

**RESULTS:** Of 182 recently diagnosed ART-naive adults, only 2 (1.1% [95% CI 0.1–3.9]) TDR mutations to nucleoside reverse transcriptase inhibitors (NRTIs; n=1 [T215D]) and protease inhibitors (PIs; n=1 [M46L]) were identified. Of the 238 clients on ART over a median duration of 14 (IQR 10–19) months, 62 (26%) had viral loads >400 copies/ml. ADR was detected in 31/62 (50.0% [95% CI 37.0–63.0]) adults. Mutations conferring resistance to NNRTIs were most common (n=29, 94%), followed by NRTI mutations (n=26, 84%). Major PI mutations were not observed. The most prevalent ADR mutations were the M184V mutation (n=23, 74%) and the K103N mutation (n=12, 39%).

**CONCLUSIONS AND RECOMMENDATIONS:** Despite reports of an increase in the prevalence of TDR in some urban African settings, we report low levels of TDR with a considerably high prevalence of ADR amongst adults in a routine rural setting. Continued broader surveillance, improved monitoring and support is needed to limit the emergence and spread of HIV drug resistance in sSA.
ABSTRACT 105
Antiviral Therapy 2012; 17 Suppl 1:A127

Trends of HIV-1 drug resistance during the past 11 years of ARV treatment in Uganda

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BACKGROUND: The HIV global epidemic is still a major challenge with more than 50% of the infected individuals in sub-Saharan Africa. Of concern still is that after close to 30 years of the epidemic, the number of new infections annually is still alarming with 2.7 million new infections in 2010. With the rollout of antiretroviral therapy (ART), only 37% of the estimated number of people that need antiretroviral therapy in sub-Saharan Africa are on treatment. However, major challenges that come with this rollout of therapy include emergence as well as transmission of drug resistance mutations.

METHODS: We performed genotypic resistance testing using an in-house technique. With this technique, we looked at drug resistance profiles from as early as 1999 up to date. Over 1,000 genotypes have been performed on patients a viral load greater than 2,000 copies/ml. We have analysed both the reverse transcriptase (RT) and the protease (PR) regions for drug resistance as well as subtypes.

RESULTS: With the universal rollout of ART in Uganda, the most frequent drug resistance mutation (DRM) to NRTIs was M184V, conferring 3TC and FTC resistance (>60% of all subtype A and D samples tested). The collection of mutations mostly responsible for thymidine analogue resistance (TAMs; sites 41, 67, 70, 75, 210, 215 and 219) were found at a low but similar frequency in both subtype A and D despite the fact that AZT is one of the most prescribed drugs in Uganda. The most frequent DRMs to NNRTIs (NVP and EFV) were K103N and G190A, similar in both subtype A and D samples. There was slightly less Y181C (conferring mostly NVP resistance) in subtype A versus D samples. Analysis by drug showed that patients harbouring subtype D virus were more likely to fail when compared with their subtype A counterparts. We had expected to observe a decrease DRMs/sample/year with the rollout of HAART in Uganda (2005–2009). However, the level of DRMs/sample/year remained remarkably constant.

CONCLUSIONS: Even with the rollout of ART, the burden of drug resistance is still a major challenge that needs to be addressed through regular monitoring of patients.
ABSTRACT 106

Antiviral Therapy 2012; 17 Suppl 1:A128

Comparison of predicted susceptibility between genotype and virtual phenotype HIV drug resistance interpretation systems among treatment-naive HIV-infected patients in Asia: TASER-M Cohort analysis

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OBJECTIVES: Accurate interpretation of HIV drug resistance (HIVDR) testing is challenging, yet important for patient care. We compared genotyping interpretation, based on the Stanford HIV Sequence Database HIVdb, and virtual phenotyping, based on the Janssen Diagnostics BVBA vircoTYPE™ HIV-1, and investigated their level of agreement in antiretroviral (ARV)-naive patients in Asia, where non-B subtypes predominate.

METHODS: Sequence files from 1,301 ARV-naive patients enrolled in the TREAT Asia Studies to Evaluate Resistance – Monitoring Study (TASER-M) were analysed by both interpreting systems. Interpretations were grouped into two levels: susceptible and non-susceptible; and three levels: susceptible, intermediate, and resistant. The level of agreement was analysed using the prevalence adjusted bias adjusted kappa (PABAK).

RESULTS: Overall, the agreement between the two systems was high, with emtricitabine and darunavir/r having 100% agreement and the remaining ARVs being in ‘almost perfect agreement’, using Landis and Koch’s categorization. The ARV with the highest discordance was efavirenz (75/1301, 5.8%), followed by zidovudine (21/1301, 1.6%), and etravirine and abacavir (18/1301, 1.4% each). All 75 efavirenz discordances arose from susceptible Stanford HIVdb versus non-susceptible vircoTYPE™ HIV-1 predictions. The lowest PABAK (0.8847) was also observed for efavirenz. Protease inhibitors had highest level of concordance with PABAKs all above 0.98, followed by nucleoside reverse transcriptase inhibitors with PABAKs above 0.96 and non-NRTIs with the lowest PABAK of 0.8847. 68/75 patients with discordant efavirenz results harboured the V179D/E mutations compared with 7/1,226 with no efavirenz discrepancy (P-value <0.001). No other efavirenz-associated mutations were detected in the 75 discrepant patients. Among the subgroup with non-susceptible efavirenz vircoTYPE™ HIV-1 predictions, the median predicted fold-change for those with discrepancies was nearer to the biological cutoff point (3.3) than those without discrepancies (P<0.001). In the three-level comparison, all but one of the discrepancies was from intermediate versus susceptible or resistant pairs.

CONCLUSIONS: The two systems agreed well with lowest concordance observed for efavirenz. V179D/E was present in high proportion of sequences with efavirenz discrepancy. When interpreting HIVDR, especially in non-B subtypes, clinical correlation is crucial, in particular when efavirenz resistance is interpreted based on V179D/E.
ABSTRACT 107

*Antiviral Therapy* 2012; 17 Suppl 1:A129

Field study of the utility of dried blood spots (DBS) for HIV-1 drug resistance (HIVDR) genotyping in Kampala, Uganda: storage for 2 weeks and shipping at ambient temperature has no effect on genotyping efficiency


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BACKGROUND: DBS are an alternative specimen type for HIVDR genotyping in settings without resources for collection, storage and shipment of frozen plasma. Data relating to DBS storage and shipment conditions in real-world settings are limited. We compared genotyping rate and resistance profiles from DBS stored and shipped at different temperatures to plasma specimens collected from virologically failing patients in Uganda.

METHODS: Plasma and 4 DBS cards from anti-coagulated blood and a fifth card from finger prick blood were prepared from 103 patients receiving ART for >6 months with viral load >1,000 copies/ml. DBS were dried overnight, packaged in plastic bags containing desiccant and a humidity indicator, stored at ambient temperature (AT) for 2 or 4 weeks or frozen at -80°C, and shipped from Uganda to the US at AT or frozen on dry ice for genotyping using a sensitive in-house method. Temperature loggers were used to monitor DBS AT shipments.

RESULTS: Median plasma viral load was 4.8 log10 copies/ml (range 3.0–6.5). Median time on ART was 46 months (range 0.5–120). Peak temperatures during shipment were between 28–40°C. Plasma (97%) and DBS (98%) stored and shipped frozen were genotyped with similar efficiency. DBS stored frozen (97%) or at AT for 2 (93%) weeks and shipped at AT had similar genotyping rates. Genotyping rate was reduced for DBS stored at AT for 4 weeks (89%, \(P=0.03\)) or prepared from finger prick blood (78%, \(P<0.001\)), compared to DBS prepared from anti-coagulated blood and handled similarly. 90% of patients had mutations associated with resistance to NRTI (predominantly M184V, M41L and T215Y), 88% to NNRTI (mostly K103N and G190A) and 4% to PI. HIVDR profiles were similar between plasma and DBS specimens.

CONCLUSIONS: Genotyping rates were unchanged when DBS were stored dry at AT for 2 weeks in an African setting compared to frozen plasma or frozen DBS. AT shipment of previously frozen DBS does not affect genotyping efficiency. DBS prepared directly from finger prick had a lower genotyping rate. This study delineates optimal DBS collection, storage and shipping conditions and demonstrates that DBS are a suitable specimen type for genotyping in resource-limited settings.
ABSTRACT 108

Antiviral Therapy 2012; 17 Suppl 1:A130

Dried blood spots (DBS) for HIV-1 viral load and drug resistance monitoring in HAART-treated patients from Africa and Asia: the ANRS 12235 Study

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BACKGROUND: Dried blood spots (DBS) can be an alternative to plasma (PL) for measurement of HIV viral load (VL) and drug resistance genotyping in resource-limited countries. Here, we evaluated the reliability of DBS to detect virological failure and drug resistance mutations in HIV+ patients treated in national HAART programmes in Africa and Southeast Asia.

METHODS: From August 2009 to April 2011, plasma and DBS specimens were simultaneously obtained from patients receiving first-line treatment for 24 months in Burkina-Faso (BF), Cameroon (CM), Senegal (SN), Togo (TG) in Africa and Thailand (TH) in Southeast Asia. DBS were stored for 2–4 weeks at ambient temperature. HIV-1 RNA extraction and detection were performed with methods available on-site: Nuclisens EasyQ in TG, Nucliens extraction and generic HIV-1 quantification in BF, and Abbott-m2000rt in CM, SN and TH. Both DBS and plasma specimens were genotyped when PL VL ≥ 1,000 copies/ml. Drug resistance mutations (DRM) were interpreted using the ANRS v2011 algorithm.

RESULTS: Overall, we compared 316 (189 with PL VL < 1,000 copies/ml) PL-DBS pairs for VL: 60 in BF, CM and TH, 52 in SN and 84 in TG. In CM, SN and TH, the sensitivity of DBS to detect virological failure was 94% (83–99) and the specificity 93% (86–97). In BF, sensitivity and specificity were 94%, 61% and in and TG 85%, 96% respectively. 90% of DBS with PL VL ≥ 1,000 copies/ml, were successfully PCR-amplified in the RT gene. Among the 83 PL-DBS pairs successfully genotyped, 68/83 presented at least one DRM in both PL and DBS with comparable drug resistance interpretation, despite some sequence differences mainly due to double population. Two samples presented different DRM and interpretation profiles.

CONCLUSIONS: This study showed that DBS stored at ambient temperature can be used for VL and DRM monitoring in HAART-treated patients from remote areas of Africa and Thailand, provided some adaptation of VL interpretation. Thresholds for treatment failure determined from DBS need to be adapted according to the VL assays used because of their differences in sensitivity and specificity.
ABSTRACT 109

*Antiviral Therapy* 2012; 17 Suppl 1:A131

Economic transport of dry plasma between clinical and laboratory sites for RLS to enhance HIV/AIDS patient clinical care – a pathway for scale-up of testing for markers of infectious disease (ID)

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BACKGROUND: Six million HIV/AIDS patients are now receiving ART in RLS, and data indicates resistance is increasing between 10 and 38% per year. Real-time molecular and serological testing is essential for optimum clinical care of these patients. A matrix for transport of dried plasma has been used for economic, stable movement of patients’ samples from RLS and RRS for genotypic and serological analysis to support clinical care. We evaluated the pathways, processes, timelines, costs and outcomes for transport and testing of 757 dried versus paired frozen plasma (DvPFP) for molecular and serological measures to support patient care.

METHODS: HIV/AIDS patient samples (n=757) were sedimented for 2–4 h and 1 ml of plasma was loaded onto the matrix (Vive-ST, VivebioCorp.), air-dried, posted to laboratories and stored at RT, paired plasma frozen at -70°C. Analysis of DvPFP included: genotypic resistance (TRUGENE, SiemensCorp), viral load, serological and biochemical markers using POC (Determine; Alere) and the Mini-Vidas analyser (Biomerieux) measuring Syphilis IgG, HIV1/2, hepatitis A, measles IgG, rubella IgG, EBV and IgE, TSH, PSA and Ferritin. Statistical analyses were performed using SPSS version 17.

RESULTS: HIV-1 genotyping from 92.3% of DvPFP showed mean similarity scores of >98% and >99%, respectively, at nucleotide and amino acid level for resistance-associated mutations (P: NS) and VL was highly correlated (R²=0.97; P<0.001). There was 100% concordance for DvPFP with POC HIV 1/2 and syphilis tests. HIV 1/2 EIA showed equivalence for NOD values in all DvPFP tested (R²=0.993; P<0.001). Significant correlations were also found for hepatitis A (R²=0.94; P<0.01), measles IgG (R²=0.953; P<0.002), rubella IgG (R²=0.95; P<0.001), EBV IgG (R²=0.975; P<0.01) IgE (R²=0.98; P<0.01), TSH (R²=0.973; P<0.01), PSA (R²=0.97; P<0.01) and ferritin (R²=0.982; P<0.001).

CONCLUSIONS: We demonstrated the utility of dry plasma transport between sites for timely, accurate and reliable measures of molecular, serological and biochemical markers to support patients in RLS with HIV/AIDS. The matrix provided a highly flexible means of storing and transporting plasma to monitor IDs, with low cost, no temperature or time constraints.

This process could provide an important key for the delivery of individualised care of adults and children in RLS, and a means of carrying out clinical trials and community audits at much lower costs.
ABSTRACT 110

*Natraly polymorphisms in HIV-1 integrase isolates from Brazil: identification of dolutegravir-associated resistance mutations*

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**BACKGROUND:** Integrase was an attractive target for antiretroviral therapy so integrase inhibitors (INIs) were developed in recent years. Raltegravir was the first INI licensed and elvitegravir is in the late-phase of development showing good results. Dolutegravir, a promising second-generation INI, is in early stages of clinical development presenting strong antiviral activity. Resistance mutations for INIs have been described in non-exposed individuals and some occur as natural polymorphisms. Here we analysed integrase sequences obtained from INI-naive patients from Rio Grande do Sul (Brazil) to investigate the presence of natural polymorphisms and resistance mutations for dolutegravir.

**METHODS:** Proviral DNA was extracted from plasma of 41 drug-naive and 42 antiretroviral-experienced patients from Rio Grande do Sul, all naive for INIs. Complete sequences of integrase were amplified by nested-PCR and further sequenced. Sequences were manually edited and the subtype determination was carried out using Stanford algorithm. Amino acid sequences were analysed for detection of natural polymorphisms and resistance mutations previously described to be associated with the recent INIs (raltegravir, elvitegravir and dolutegravir).

**RESULTS:** The subtyping analysis using Stanford algorithm revealed 48.2% of sequences as subtype B, 48.2% as subtype C, 2.4% as subtype F and 1.2% as CRF02_AG. Consensus sequences generated presented polymorphic substitutions at the positions 72, 101 and 201 for subtype B, and 11, 50, 100, 124, 125, 251, 265, 281 and 283 for subtype C, when compared with B and C consensus obtained from Los Alamos Data Base, respectively. None of resistance mutations for raltegravir and elvitegravir listed on Stanford database was observed. Mutations associated to dolutegravir resistance, L101I, L101I/T124A and T124A, were found in 45.0%, 1.7% and 10% of subtype B sequences, respectively. Substitution L101I was found in 87.5% of subtype C.

**CONCLUSIONS:** Brazilian integrase sequences presented some degree of polymorphisms but none of polymorphic sites were related with first-generation of INIs, supporting its use in our country. However, we found some mutations have being associated to resistance to dolutegravir (L101I, L101I/T124A and T124A) in in vitro studies. In this study, L101I was observed as a polymorphic mutation for subtype B and in higher frequency in subtype C sequences.
SESSION 7
Persistence, reservoirs and elimination strategies
ABSTRACT 111

Detection of HIV-1 RNA in seminal plasma samples from treated patients with undetectable HIV-1 RNA in blood plasma on a 2002–2011 survey

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OBJECTIVE: The objective of this study was to estimate the frequency of detectable seminal HIV-1 viral load in men with repeatedly undetectable blood viral load, these last years and over a 10-year period (2002–2011) in a large cohort of HIV-1-infected men from couples requesting Assisted Reproduction Technologies (ART). We also searched for an association between HIV-1 RNA seminal viral load, HIV-1 RNA plasma viral load measured by ultrasensitive assay and blood HIV-1 DNA on a subgroup of 98 patients.

METHODS: Three hundred and four HIV-1-infected men have provided 628 paired blood and semen samples. In a subset of 98 patients for which a blood sample was available, residual viraemia, HIV-1 RNA in semen and HIV-1 DNA were studied.

RESULTS: Twenty on 304 patients (6.6%) had detectable HIV-1 RNA in semen, ranging from 135 to 2,365 copies/ml and corresponding to 23 samples, although they had concomitantly undetectable HIV-1 RNA in blood while they were under antiretroviral therapy. This prevalence was stable over time even in recent years. There was an association between HIV-1 RNA seminal viral load measured by ultrasensitive assay and blood HIV-1 DNA, but both were not associated with seminal HIV-1 RNA viral load.

CONCLUSIONS: It seems cautious individually to maintain the recommendations of safe sex and the recourse to ART, or at least to inform the couple of the residual potential risk, in serodiscordant couples desiring a child.
ABSTRACT 112

Antiviral Therapy 2012; 17 Suppl 1:A136

HIV transmitted drug resistance in paired plasma and seminal fluid: long-term persistence and potential for onward transmission

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BACKGROUND: Transmitted drug resistance (TDR) in HIV-1 infection is associated with decreased response to antiviral therapy (ART). In addition, onward transmission of resistant virus from treatment-naive patients is being increasingly recognized. The objective of this study was to compare the TDR profile in paired blood and seminal plasma samples following diagnosis and on initiation of treatment.

METHODS: ART-naive men with TDR detected at diagnosis provided paired blood and semen samples. Following initiation of ART, samples were collected at 4 week intervals until plasma viral load was <40 copies/ml. Population sequences were derived by in-house methods. In addition, some samples were tested by allele-specific PCR to detect minority populations of K103N, Y181C and M184V.

RESULTS: Sixteen subjects provided paired samples. Four of these had multidrug resistance, with the same mutations persisting in blood and semen in all the patients for the period of study (up to 7 years) except for one subject who showed K103N in plasma but not in semen. Six subjects had a single thymidine analogue mutation (T215S/D/V or M41L) which again persisted in both compartments for up to 6 years. Three subjects had a single protease inhibitor resistance mutation (L90M) and two a non-nucleoside resistance mutation (K101E or K103N) again in both compartments. TDR appeared to be lost in both compartments in one subject who showed mixed wild-type and mutant viruses (T215S/I, K219K/Q) in plasma at diagnosis and only minority mutation K103N detected in both compartments 4 months later, possibly reflecting infection with mixed wild-type and mutant virus. On starting ART, plasma and seminal TDR persisted as long as viral amplification was possible.

CONCLUSIONS: TDR persisted in both blood and seminal compartments in most patients for as long as they were followed, with little evidence of differential evolution in most cases. This provides evidence for the potential onward transmission of TDR, including multidrug resistance, from treatment-naive patients.
ABSTRACT 113

Cellular effects of Kyo II: a new compound extracted from Euphorbia sp

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BACKGROUND: Despite the therapeutic success in the treatment of HIV+ patients, the treatment still does not lead to a complete eradication of the virus from the infected individuals. This fact is due to a small pool of long-lived memory CD4+ T-cells harbouring the provirus. Several compounds have been studied removing virus from latency, however none of these molecules shows satisfactory results in the induction and cytotoxicity. We have found a new compound extracted from Euphorbia that acts as reactivators of the HIV, Kyo II.

METHODS: The antiviral assay was done infecting NL4.3 in MT-4 cells in presence of serial dilution of the compound and was revealed by cellular viability. Expression of luciferase was done in TZM-bl cells to evaluate the induction in HIV promoter. The regulation of receptor (CD4 and CD8) and coreceptors (CCR5 and CXCR4) was assessed in MT-4 and PBMC isolated from healthy donors. The assays were performed in presence and absence of the compound and were analysed by flow cytometry. The PMBC isolated were analysed for activation (CD38 and CD69) and cellular proliferation (CellTraceTMCFSECellProliferationKit) in presence of the compound.

RESULTS: Antiviral assays show an important viral inhibition by Kyo II until it reaches toxic concentration (200 μM). The assays in TZM-bl showed that the compound works as an activator of HIV promoter, since there was a dose–response of luciferase activity with increased concentration of the compound. This molecule was able to down-regulate CD4 and CD8 expression in MT-4 as well as in PBMCs with low concentration of compound (1 μM). A noticeable down-regulation of viral coreceptors (CCR5 and CXCR4) was also observed. Nevertheless, compound was not able to induce the cellular proliferation. Interestingly, this molecule (1 μM) was also capable of activate human CD4+ cells shown by double marked CD4/CD38 and CD4/CD69.

CONCLUSION: These results show that Kyo II act in HIV promoter activation and in the down-regulation of HIV receptor and coreceptor. These findings suggest that the compound can both act in proviral activation and blocking viral entrance through receptor/coreceptors down-modulation. The Kyo II can be a leading compound in a shock-and-kill strategy to be placed together with antiretroviral therapy to eliminate viral reservoir.
ABSTRACT 114

Antiviral Therapy 2012; 17 Suppl 1:A138

Switching to a raltegravir (RAL)-containing regimen in virologically suppressed patients does not impact residual HIV-1 plasma viral loads and the size of the cellular viral reservoir

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BACKGROUND: Intensification of antiretroviral therapy (ART) in virologically suppressed patients did not confer a clinical or virological benefit on ongoing replication. We investigated ongoing replication and the evolution of the cellular reservoir in patients switching to a RAL-containing regimen.

METHODS: Treatment-experienced patients from the ANRS CO3 Aquitaine Cohort switching from their current ART to RAL-containing regimens (baseline) when having a suppressed viral load (<50 HIV-1 RNA copies [cp]/ml plasma) were included. We assessed total HIV-1 DNA levels, levels of HIV-1 2-LTR circles in PBMC and ultrasensitive viral load (VL) as a measure of the viral reservoir at baseline and during follow-up (FU). Raltegravir plasma levels were also assessed.

RESULTS: We included 38 patients (M/F=27/11) with a median age of 47 (interquartile range [IQR]: 44–54) and median follow-up time on RAL was 6 months (IQR: 1.3–10.4). Of 69 viral load measurements after RAL start, 66 were strictly below 50 cp/ml and 3 patients had a detectable viral load during follow-up (2/18 at month 1 and 1/21 at month 3). Median HIV-1 total DNA/10^6 PBMCs was 193 (93–528). The slope of total HIV-1 DNA was not significantly different from zero during FU (P=0.94) as was the slope of ultrasensitive viral load during FU (P=0.21). Of 75 2-LTR HIV-1 DNA measurements all but 8 measurements from 4 patients remained undetectable. RAL plasma levels remained stable, 0.71 µg/ml (0.67–0.86) at M1 and 0.97 µg/ml (0.64–1.42) at M12.

CONCLUSIONS: Our results highlight that switching patients on stable ART to a RAL-containing regimen did not result in a decay in HIV-1 total DNA. Even though switching to RAL-containing regimen had no effect on residual plasma viral load or on the size of the surrogates for HIV-1 reservoir, patients still benefited from this strategy as virological failure were very rare.
ABSTRACT 115
Antiviral Therapy 2012; 17 Suppl 1:A139

Integrated HIV-1 DNA change after 48 weeks of raltegravir-containing therapy in patients with suppressed viraemia: a substudy of the EASIER-ANRS 138 trial

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BACKGROUND: Raltegravir-containing regimen was associated with an early increase of the 2-LTR episomal DNA but had no effect on total HIV DNA after 48 weeks in patients already virologically suppressed. However, few data exist about effect of raltegravir on the level of integrated HIV-1 DNA. Our aim was to analyse the evolution of integrated DNA HIV-1 in patients well-suppressed under raltegravir regimen.

PATIENTS AND METHODS: The EASIER-ANRS 138 trial randomized 169 previously virologically suppressed treatment-experienced patients to an early or deferral switch from enfuvirtide to raltegravir. HIV DNA was extracted from whole blood and quantified in the first 30 patients from each arm at weeks W0 and W24 and also at W48 for patients in the early-switch arm. Integrated HIV-1 DNA was assayed using an alu-LTR-based real-time nested PCR assay. Baseline total and integrated HIV-1 DNA were correlated with immunological and virological factors.

RESULTS: Baseline characteristics for the 60 patients were: CDC stage C: 60%; median HAART duration: 14 years; median enfuvirtide duration: 2.5 years; median baseline and nadir CD4 cell count: 400 cells/mm3 and 37 cells/mm3, respectively; and pVL was <50 copies/ml in 87% (52/60). Median (IQR) total and integrated DNA (log10/106 PBMC) were respectively 3.4 (3.1–3.7) and 3.4 (2.6–3.9) at baseline, 3.4 (3.2–3.7) and 3.2 (2.2–3.8) at week 24, 3.6 (3.4–3.8) and 3.4 (2.5–3.8) at week 48.

No significant changes from baseline in total and in integrated DNA were observed between randomization group at week 24 (P>0.1, Wilcoxon test) and in the RAL arm at week 48, whatever the baseline pVL (<50 or between 50 and 400 copies/ml).

At baseline, integrated and total HIV-1 DNA were correlated (median ratio 1.00 [0.87–1.10]; Spearman coefficient 0.54; P<0.0001). In contrast, no correlation was observed with nadir CD4 cell count and baseline CD4 and CD8 cell counts, ratio CD4/CD8, 2 LTR detection and proviral tropism CCR5/CXCR4.

CONCLUSION: Proviral integrated HIV-1 DNA remains stable over 48 weeks of raltegravir-containing regimen in well-suppressed patients. High correlation between total and integrated HIV-1 DNA suggests a limited viral residual replication in successfully treated patients.
ABSTRACT 116

*Antiviral Therapy* 2012; 17 Suppl 1:A140

HIV drug resistance testing at low viral load may help differentiate between ongoing replication and release from reservoirs

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OBJECTIVES: Low-level viraemia in HIV-infected patients treated with antiretroviral therapy is a phenomenon still not fully understood. If the observed viraemia is a result of ongoing replication or if virus is released from cellular reservoirs remains unclear. We analysed our database of viral load and genotypic resistance interpretations to analyse the effect of viral load on frequency of detected drug resistance.

METHODS: A total of 1,939 viral load and protease and reverse transcriptase sequence pairs from the year 2009 to 2011 were analysed. Drug resistance interpretation was performed the HIV-GRADE resistance interpretation rule set. Pairs were split up into viral load categories of below 200 cop/ml, 200–400, 400–1,000, 1,000–10,000, 10,000–100,000 and more than 100,000 cop/ml. Resistance interpretation was stratified in the following categories: no resistance, or resistance against one, two or three drug classes. The collected data was then analysed using $\chi^2$ test.

RESULTS: A total of 1,390 sequences showed no relevant drug resistance, while 336 sequences showed resistance against one drug class (two classes: 143, three classes: 38). In 25 samples viral load was below 200 cop/ml (200–400: 29, 400–1,000: 63, 1,000–10,000: 275, 10,000–100,000: 526, above 100,000: 472). In both groups below 400 cop/ml no significant increase of resistant variants was observed compared with the mean distribution, while in the groups with 400–1,000 cop/ml viral load and 1,000–10,000 cop/ml significantly more resistant variants were found than expected ($P<0.001$). This reverses above 100,000 cop/ml, where significantly ($P<0.001$) more susceptible variants are observed.

CONCLUSION: As in most of the samples with a viral load below 400 cop/ml, no mutations leading to resistance could be found, we conclude that virus in these cases is released from cellular reservoirs. Viral loads between 400 cop/ml and 10,000 cop/ml are a clear sign for ongoing replication, and in this group the highest rate of drug resistance is observed. Viral loads above 100,000 cop/ml are a sign of missing selective pressure and thus no drug resistance is observed.
ABSTRACT 117

Antiviral Therapy 2012; 17 Suppl 1:A141

Effects of interferon-α2b on HIV-1 reservoirs in patients on suppressive antiretroviral therapy

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INTRODUCTION: Combination antiretroviral therapy (cART) suppresses but does not eliminate HIV-1 viraemia in most patients. Intensification of cART with additional potent antiretrovirals does not reduce viraemia, indicating that it likely originates from long-lived reservoirs. Cure of HIV-1 infection will require new therapies to reduce or eliminate HIV reservoirs. Interferon-α is an innate immune cytokine with broad antiviral and immune stimulatory effects. To investigate the effect of interferon-α therapy on HIV-1 persistence, we conducted a pilot study of interferon-α treatment in patients on suppressive cART.

METHODS: Individuals with HIV RNA suppressed <50 c/ml for ≥1 year on cART and persistent viral RNA ≥0.6 c/ml were enrolled. Pegylated interferon-α (PEG-IFN) 1.5 ug/kg was administered weekly for 4 weeks, with sampling for viraemia, cell associated HIV-1 DNA and immunophenotyping. Patients were followed for 48 weeks post PEG-IFN. Viral RNA levels were determined by single copy assay, and levels of total cell-associated HIV-1 DNA and 2-LTR circles determined by real-time PCR (95% quantification limits of 5 and 7.5 c/reaction, respectively). Immunophenotyping was performed for CD4 and CD8 lymphocyte subsets.

RESULTS: Four patients initiated PEG-IFN therapy and 3 of 4 completed 4 weeks of therapy (one discontinued after week 3 due to fever and severe flu-like symptoms). No consistent effect was observed on plasma HIV-1 RNA, which remained ≥0.2 c/ml in 3 of 4 patients throughout PEG-IFN therapy. Total cellular HIV DNA/10⁶ CD4 cells decreased significantly (twofold, P=0.003) in one patient, but was unchanged in the others. 2-LTR circles were not consistently detected. All patients had a 5–15% increase in the proportion of activated CD8 T-cells (CD8⁺CD38⁺ and CD8⁺CD38⁺DR⁺) during PEG-IFN therapy that resolved within 2 weeks after therapy.

CONCLUSIONS: In patients on suppressive cART, 4 weeks of interferon-α2b did not reduce persistent viraemia or cellular HIV-1 DNA levels despite large increases in CD8 T-cell activation. These observations show that the levels of immune activation and persistent viraemia can be uncoupled.
ABSTRACT 118

*Antiviral Therapy* 2012; 17 Suppl 1:A142

Long-term analysis of HIV RNA suppression <5 copies/ml in the ARTEMIS trial

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BACKGROUND: In the ARTEMIS trial, 689 antiretroviral treatment-naive patients were randomized to tenofovir/emtricitabine plus either darunavir/ritonavir (DRV/r) 800/100 mg once daily (*n* = 343) or lopinavir/ritonavir (LPV/r; *n* = 346). HIV RNA was evaluated using the Roche Amplicor Ultrasensitive assay.

METHODS: For visits from baseline to week 192, plasma samples with HIV RNA <50 copies/ml were classified as either ‘No HIV RNA detected’ (<5 HIV RNA copies/ml, optical density = background) or HIV RNA detected (5–50 copies/ml). HIV RNA suppression rates were compared between the treatment arms using different cutoff levels of HIV RNA suppression.

RESULTS: Median baseline HIV RNA was 4.86 log_{10} copies/ml in the DRV/r arm and 4.84 in the LPV/r arm. Overall, 34.4% of patients had HIV RNA >100,000 copies/ml at screening. At week 192, the percentage of patients with HIV RNA <50 copies/ml (ITT TLOVR analysis) was 68.8% in the DRV/r arm and 57.2% in the LPV/r arm (*P* = 0.002 for superiority). This result was consistent in sensitivity analyses, excluding protocol violators or early discontinuations. The percentage of patients with HIV RNA <5 copies/ml at week 192 was 54.8% in the DRV/r arm and 48.0% in the LPV/r arm (ITT missing = failure). For patients with baseline HIV RNA >100,000, the percentage with HIV RNA <5 copies/ml at week 192 was 52% for DRV/r versus 41.7% for LPV/r. For patients with baseline HIV RNA <100,000 copies/ml, the percentage with HIV RNA <5 copies/ml at week 192 was 56.2% for DRV/r versus 51.3% for LPV/r. The percentage of patients in each arm with HIV RNA <5 copies/ml rose progressively from week 2 to week 192. Across the two treatment arms, of the patients with HIV RNA <50 copies/ml, 62% had levels <5 copies/ml at week 48, versus 80.0% at week 192 (observed data analysis). No patients developed treatment-emergent primary PI mutations during the trial.

CONCLUSIONS: In the ARTEMIS trial, there were progressive reductions in HIV RNA over 192 weeks of treatment, first to 5–50 copies/ml and then <5 copies/ml. Overall, 20% of patients with HIV RNA <50 copies/ml at week 192 had traces of HIV RNA detected, in the range of 5–50 copies/ml.
SESSION 8

New resistance technologies and interpretations
Models that accurately predict response to HIV therapy are generalisable to unfamiliar datasets and settings

BACKGROUND: The optimal selection of antiretroviral drugs following treatment failure is challenging, for example, due to the complexity of resistance and range of treatment options in some settings or the unaffordability of genotyping and some drugs in others. Computer models have been developed that accurately predict response to therapy to aid decision making. However, their performance may not be generalisable to data unrepresented in the training set. Here we test recent models with independent data from unfamiliar settings.

METHODS: Two sets of random forest models, developed using data from USA, Canada, Europe and Australia, were evaluated. All predicted the probability of response from a range of variables, including baseline viral load and CD4+ T-cell count, ≤18 treatment history variables, time to follow-up and the drugs in the new regimen. The models’ unique characteristics were: Set 1: do not require a genotype, predict follow-up viral load ≤400 copies, trained using 14,964 treatment change episodes (TCEs) and achieved an AUC of 0.77 and 72% accuracy during development; and Set 2: require a genotype (62 mutations), predict follow-up viral load ≤50 copies, trained using 7,263 TCEs and achieved an AUC of 0.82 and 76% accuracy during development. The generalisability of Set 1 was assessed using 375 TCEs without genotypes from Romania and Set 2 using 375 TCEs with genotypes from clinics in North America and Europe not represented in the training data.

RESULTS: Set 1 (no genotype) achieved a mean AUC of 0.73 and accuracy of 67%. Set 2 achieved a mean AUC value of 0.87 and accuracy of 91%. Mean sensitivity and specificity values were 65% and 70% for Set 1 and 62% and 92% for Set 2. The models identified alternative regimens that were predicted to be effective in 97% and 98% of cases where the new regimen used in the clinic had failed.

CONCLUSIONS: The models were accurate for new data from unfamiliar settings and were able to identify potentially effective regimens following treatment failure. While the models that use a genotype performed significantly better than those that do not, the performance of the ‘no-genotype’ models with data from an unfamiliar setting (Romania) was encouraging.
ABSTRACT 120

Antiviral Therapy 2012; 17 Suppl 1:A146

Low-level detection and quantification of total cell-associated HIV-1 DNA and 2-LTR circles using droplet digital PCR

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BACKGROUND: Droplet digital PCR (ddPCR) is an emerging nucleic acid detection method that provides absolute quantification of target sequences without relying on the use of standard curves. We evaluated the ability of ddPCR to detect and quantify total cell-associated HIV-1 DNA and 2-LTR circles from a panel of patients on and off antiviral therapy.

METHODS: ddPCR partitions the DNA target, fluorescently-labelled probe, and the ingredients for a PCR reaction into an emulsion of approximately 20,000 droplets. Following PCR amplification, enumeration of both fluorescing and non-fluorescing droplets is performed, allowing the absolute quantification of target molecules. To calculate the dynamic range of ddPCR for total HIV-1 DNA, 2-LTR circles and housekeeping genes, serial dilutions of DNA amplicons or episomes ranging from 1 to 10^6 were quantified by ddPCR as well as with traditional real-time PCR (RT-PCR). We also tested total HIV-1 DNA from three viraemic patients and four patients on suppressive antiretroviral therapy, and 2-LTR circles from three patients with low-level viraemia; results were correlated with RT-PCR. Genomic DNA from patient samples was digested with the Msci restriction enzyme to ensure efficient droplet packaging.

RESULTS: ddPCR accurately and precisely quantified DNA amplicons from 3 to 10^5 copies (R^2=0.989, slope=0.92) and episomal DNA from 1 to 10^5 copies (R^2=0.998, slope=0.96) as compared with RT-PCR. ddPCR also accurately quantified a CCR5 housekeeping gene used to quantify total number of PBMCs from 100 to 10^6 (R^2=0.999, slope=0.99) and mitochondrial episomal DNA from 10 to 10^5 copies (R^2=0.984, slope=0.99). Total HIV-1 DNA from digested samples from patients on and off ART ranged from 1 to 133 copies/reaction well by ddPCR and from 1 to 269 copies/well by RT-PCR (R^2=0.734); ddPCR estimated 11–58% fewer DNA copies compared with RT-PCR, but demonstrated similar detection sensitivity. Low levels of LTR circles were detected in all three patient samples by both ddPCR and RT-PCR (ranging from 2.1 to 7.5 copies/reaction well). Similar levels of CCR5 and mitochondrial DNA were detected by both platforms.

CONCLUSIONS: ddPCR is similar to RT-PCR for the quantification of HIV-1 cell-associated DNA, and is a promising novel technology for the study of HIV-1 reservoirs and persistence.
ABSTRACT 121

Antiviral Therapy 2012; 17 Suppl 1:A147

A novel assay for rapid detection of HIV-1 drug resistance mutations using suspension array technology

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BACKGROUND: To maintain the efficacy of the first-line regimens and prevent the development and transmission of HIV drug resistance (HIVDR), a resistance testing method that is rapid and easy to interpret testing results would be ideal for population-based monitoring of HIVDR. We have developed a multiplex allele-specific HIVDR mutation assay (MASHMA) using suspension array technology for the detection of HIVDR mutations.

METHODS: A total of 45 genotyping primers were designed for 20 allele-specific mutations associated with HIVDR to nucleoside reverse transcriptase inhibitor (NRTI), non-nucleoside reverse transcriptase inhibitor (NNRTI) and protease inhibitor (PI). Two DNA plasmids, one containing all the 20 relevant HIVDR mutations and the other without mutations were used to develop the multiplex assay and determine the mutant detection limit by serial dilutions of the plasmids. Following the assay standardization, we tested 96 plasma and dried blood spot (DBS) specimens collected from ART-experienced patients infected with subtype B or C viruses (62 DBS and 34 plasma specimens). The protease and RT region of the HIV-1 pol gene was first amplified from RNA or total nucleic acid extracted from the specimens by RT-PCR using an in-house assay. The DNA amplicons were then extended by multiplex allele specific primer extension (mASPE) using the 45 primers in a single reaction tube. Products were then analysed by Bio-Plex 3D suspension array system.

RESULTS: Using the plasmid DNA templates, all 20 mutant alleles were detected and the detection limit of mutant alleles was 6.3%. Of the 1,920 alleles analysed in the 96 patient samples at 20 loci (V32I, I47V, L76V, I84V and L90M in the prt gene, and M41L, K63R, K70R, L74V, Y115F, Q151M, M184V, K219E, L100I, K101E, K103N, V106A/M, Y181C, Y188L and G190A in the rt gene), 1,852 (96%) alleles were correctly detected according to conventional sequencing and 49 (3%) alleles could not be determined due to signals below the cutoff level. Of the 19 (1%) discrepant alleles, 13 (68%) were resistance-associated mutant mixture alleles which were not detected by conventional sequencing.

CONCLUSIONS: This newly developed multiplexing and low cost assay may represent an alternative HIVDR genotyping tool for HIVDR surveillance and monitoring in resource-limited settings.
ABSTRACT 122

Antiviral Therapy 2012; 17 Suppl 1:A148

Performance of tagged pooled pyrosequencing for HIV drug resistance testing over homopolymeric regions proximal to K65 and K103

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BACKGROUND: We have demonstrated that tagged pooled pyrosequencing (TPP) is a cost-effective and sensitive method for HIV drug resistance (DR) testing on plasma and dried blood spot. Limitations to wider application of this platform arise from complexities in data analysis and the base call uncertainties at DR mutation (DRM) sites adjacent to homopolymeric runs. We have developed a web-based, customizable data analysis pipeline that automates the TPP data processing for DRM and minor variant detection. We re-examined the output from the pipeline in order to characterize its performance in homopolymeric regions encompassing K65 and K103.

METHODS: The pol region of 38 pooled, multiplex (MID) identifier labelled specimens were pyrosequenced. AVA qualified reads were grouped according to MIDs and further filtered based on the individual quality score to remove false insertions. Then, using BLASTN, the reads were mapped to HXB2 and considered valid if there was 60% overlap and ≥75% identity. Based on alignment to the reference, out-of-frame insertions were removed and deletions replaced with ‘-’ within frame. A consensus sequence for each specimen was generated at specific mixture thresholds to allow minor variant identification. Specimen reads were reanalysed at the K65 and K103 codons to determine the number of valid reads providing full coverage.

RESULTS: There was 450× oversampling of each specimen theoretically allowing identification of all DRM at frequencies >5%. Analysis of reads in the 38 specimens revealed significant variation in oversampling across pol. Specifically, valid reads over the K65 codon – ATAAAGAAAAA – constituted 95% of the total reads. This is in contrast to the high error rates at the K103 codon – TAAAAAGAAAAA – which resulted in the loss of 50% of full-length reads; however, the ability to identify DRM at the 5% level remained intact.

CONCLUSIONS: Using a bioinformatic pipeline for TPP results in cost saving and improved DRM detection. However, oversampling is reduced at codons proximal to homopolymeric runs with K103 affected more than K65. Future work will evaluate the predicted paradoxical effect of K-R and K-N mutations on pyrosequencing reads which should shorten the homopolymeric length. Despite these limitations, with sufficient oversampling, TPP is able to identify minor variant DRM with high confidence.
ABSTRACT 123
Antiviral Therapy 2012; 17 Suppl 1:A149

Detection of minority HIV-1 drug-resistant variants moderately improves the prediction of salvage antiretroviral therapy outcomes

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BACKGROUND: The clinical relevance of detecting minority HIV-1 drug resistant variants in antiretroviral treatment (ART)-experienced subjects remains uncertain.

METHODS: This was a retrospective multicentre cohort study including ART-experienced adults who initiated salvage ART including ritonavir-boosted protease inhibitors, raltegravir or etravirine, had HIV-1 RNA (VL) ≥ 5,000 copies/ml and 1 ml of plasma available for testing within 12 months before treatment change (TC). Trugene® and 454 sequencing of PR and RT were used to calculate genotypic sensitivity scores (GSS-T and GSS-454, respectively) using the HIVDB Sierra interface to HIVDB/ANRS/REGA algorithms. Virological failure (VF) was defined as two consecutive VL > 200 copies/ml 90 days after TC. The ability of GSS-T and GSS-454 to predict VF was assessed by ROC curves and logistic regression, considering clinically meaningful independent variables. All possible simple logistic regression models were fitted with/without GSS covariates setting each VF definition as response variable. The association between VF and pre-TC variables was tested using χ² or Fisher’s exact test. Sensitivity analyses were performed restricting the VF definition to subjects without subsequent ART changes, and defining VF as VL > 200 copies/ml at week 24 after TC.

RESULTS: In total, 146 subjects, 26% women, with previous exposure to a median (IQR) of 13 (9–17) different ARVs were included. Overall, 94 (65%) had a previous AIDS diagnosis. The median age was 43 years (38–47). Median pre-TC VL was 4.6 log copies/ml (4.2–5.0). Pre-TC CD4+ and nadir CD4+ T-cell counts were 232 cells/mm³ (104–388) and 116 cells/mm³ (26–177), respectively. Pre-TC genotypes were obtained in a median of 48 days (0–135) before TC. Virological outcomes were evaluable in 138 individuals, 41% with VF. Using HIVDB, the areas under the ROC curve (AUC) for GSS-454 and GSS-T were 68.3% (95% CI 58.5, 78.1) and 64.4% (95% CI 54.9, 75.0), respectively (Delong test P = 0.6). Subjects with GSS-454 < 3 were more likely to develop VF than those with GSS-454 ≥ 3 (RR 1.67, 95 CI 1.03, 2.8; P = 0.036). No differences in VF were observed between different categories of GSS-T. Similar findings were obtained for ANRS and REGA and for other definitions of VF.

CONCLUSIONS: Detection of minority HIV-1 resistant variants moderately improves the prediction of salvage ART outcomes. Subjects with GSS-454 < 3 are more likely to develop VF.
ABSTRACT 124

Antiviral Therapy 2012; 17 Suppl 1:A150

A drug resistance testing pipeline for the management and analysis of HIV-1 454 sequence data

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BACKGROUND: The 454 ultradepth pyrosequencing holds great promise for the development of a high-throughput, robust and affordable HIV drug resistance test. This is particularly pertinent for resource-limited settings where the cost of conventional assays means that resistance testing is not routinely performed in clinical settings. The sheer volume of data generated by an approach using 454 pyrosequencing, however, means that there is a need for powerful, sensitive and user-friendly bioinformatics applications for management and analysis of such sequence data.

METHODS: We have developed a novel bioinformatics pipeline that receives sequence data directly from the 454 sequencing platform and outputs drug resistance information for each individual sample sequenced. The steps in the pipeline include demultiplexing of reads pertaining to different samples and amplicons, quality trimming of the sequence data using a novel algorithm (Q-trim), mapping of reads to a reference sequence and HIV drug resistance testing using the Stanford HIVdb algorithm. Furthermore, the pipeline includes an optional step to process sequence data generated using degenerate primer IDs to account for PCR resampling bias, PCR-induced recombination and sequencing error.

RESULTS: Evaluation of Q-trim, developed for the pipeline, on the basis of mean read length, total number of reads and the percentage of poor quality bases output, showed that it significantly outperforms all methods when applied to poor quality data. When applied to good quality data, Q-trim performs marginally better than the next best method and significantly better than all other methods. The complete pipeline has been successfully implemented on data from 641 and 475 samples sequenced using the Roche FLX and GS Junior machines, respectively. Analysis times are quick, with data from multiple patients sequenced using either approach currently taking <1 h to complete. Further, preliminary work has shown that we will be able to significantly speed up the pipeline by using graphical processing units (GPUs) for the slower mapping and resistance testing steps.

CONCLUSIONS: We have developed a high-throughput computational pipeline enabling individuals with little or no bioinformatics experience to manage and analyse HIV drug resistance sequence data generated using the Roche 454 sequencing platform.
ABSTRACT 125

Antiviral Therapy 2012; 17 Suppl 1:A151

Use of the DeepChek®-HIV system in the PRIUS study: validation of a new reliable genotyping solution to streamline the 454 sequencing analysis of HIV drug resistance in routine diagnostics and research applications

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BACKGROUND: Next generation sequencing technologies are becoming increasingly available to routine and research HIV-1 laboratories worldwide. One major limitation to a widespread access to 454 sequencing outside highly specialized laboratories is the difficulty and workload associated with bioinformatics analyses. Moreover, routine clinical diagnosis requires embedded stable analysis platforms that can be validated.

METHODS: Within the PRIUS study (NCT01346878) collaboration, we developed a software platform suitable for the automatized analysis and resistance interpretation of 454 HIV sequencing data (DeepChek®-HIV v1.1, ABL) and applied it to the analysis of baseline 454-FLX and TruGene® sequences in treatment-experienced subjects initiating salvage ART including raltegravir (RAL), etravirine (ETV) or ritonavir-boosted protease inhibitors (PIr). Genotypic information was analysed by DeepChek®-HIV with its expert system (filters for DRM [Stanford, IAS,…], low-level coverage, variants-based mutations prevalence, comparison Sanger-UDS) using a 1% frequency threshold for variant detection.

RESULTS: DeepChek®-HIV analysed in 2 h the 146 baseline samples, where 133/39/76 were from the PIR/ETV/RAL groups with an overall median follow-up on treatment of 13 years and viral load of 4.6 log copies/ml (4.2, 5.0). DeepChek®-HIV detected a median of 19 (13.75–27), 6 (4–9) and 14 (10–21) additional mutations found with UDS for reverse transcriptase (RT), protease (PRO) and integrase (INT), respectively. We compared Sanger and UDS lists of detected DRM: no difference was found for INT; one at 67N, 69G, 70R, 90I, 98S, 103N, 103R, 108I, 179E, 181C, 184V, 190A, 215F, 215I, 215S, 219E and 219R for RT; and one at 10E, 10I, 10V, 20R, 35D, 36I, 43T, 46L, 54V, 55R, 63P, 82A and 90M for PRO. We noticed elevated resistance with UDS (sensitive->intermediate or intermediate->resistant; Stanford-HIVDB) compared to Sanger: 2%, 2%, 4%, 1.5%, 1%, 2%, 1.5% and 3% for indinavir/r, saquinavir/r, nelfinavir, amrenavir/r, lopinavir/r, atazanavir/r, tipranavir/r and darunavir/r, and 5%, 3%, 2% and 1.5% for nevirapine, efavirenz, etravirine and rilpivirine.

CONCLUSIONS: DeepChek®-HIV software provides a streamlined diagnostic IT solution for 454 sequencing analysis of HIV drug resistance at least comparable to Sanger, and which can be used in routine virology laboratories as well as for clinical trials evaluating the clinical relevance of low-frequency drug-resistant HIV.
ABSTRACT 126

Antiviral Therapy 2012; 17 Suppl 1:A152

Accurate quantification of amplifiable viral templates is essential in order to determine the limit of detection of HIV drug resistance assays

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BACKGROUND: The sensitivity of assays to detect minority populations of drug-resistant HIV is often defined as the lowest percentage of mutant detected in mixture of mutant and wild-type clones. The assay sensitivity and the plasma viral load are often used to predict the expected sensitivity of the assay when testing human specimens. Because of inefficiency with RNA extraction, reverse transcription and PCR amplification, we hypothesized that the limit of detection of drug resistance assays is significantly less than predicted by the aforementioned. To determine the efficiency of our assays, the amplifiable cDNA of various lengths was compared to the clinical plasma viral load.

METHODS: A convenience sampling of plasma specimens was evaluated from four separate cohorts. Viral load was determined with one aliquot of plasma by Amplicor (Roche Diagnostics) or an in-house VQA-certified assay; both assays use an Armored RNA® quantification standard to control for inefficiency in the reverse transcription process. Nucleic acid was extracted from a second aliquot using silica (NucliSense® miniMag™, Biomerieux) and reverse transcribed (Blueprint®, Takara Bio, Inc. or SuperScript® III, Invitrogen) using either random hexamers or HIV-specific primers. The cDNA was quantified by real-time PCR of LTR and/or by single-genome amplification of pol or env.

RESULTS: The median clinical plasma viral load of 100 participants from Kenya, Thailand, Peru and Seattle was 250,050 copies/ml (IQR 13,225–757,950). The median cDNA yield quantified by real-time PCR of a short region of LTR (approximately 121 basepairs; n=84) was 26.4% (IQR 17.2–52.7%); and by end point dilution of longer regions of pol (809–1,505 basepairs) or env (908 basepairs; n=31) was 5.1% (IQR 3.1–8.1%) of the clinical plasma viral load.

CONCLUSIONS: Estimating the number of viral cDNA templates generated based on plasma volume assayed and the clinical plasma viral load overestimates the number of amplifiable viral templates by as much as 20-fold. Decreased cDNA yield of longer fragments suggests inefficient reverse transcription and/or less efficient PCR amplification. To obtain accurate estimates of the limit of detection, especially when using more sensitive modalities such as pyrosequencing, and when working with longer HIV fragments, amplifiable cDNA should be quantified.
ABSTRACT 127

*Antiviral Therapy* 2012; 17 Suppl 1: A153

**Added value of ultra deep sequencing in patients with HIV-1 transmitted drug resistance mutations in the reverse transcriptase**

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**BACKGROUND:** Ultra deep sequencing (UDS) using 454 platforms allows for an accurate estimation of the presence of minor variants. In this report we have investigated if additional mutations can be detected as viral minor mutants in a background of Sanger-detected HIV-1 transmitted drug resistance mutations (TDR) and how they can impact resistance interpretation.

**METHODS:** Plasmas from 11 treatment-naive patients (median viral load 87,900 copies/ml [15,957–1,240,000]) with documented TDR by Sanger-Sequencing (Trugene, Siemens NAD) were retested using an HIV-1 RT and Pro UDS-454 prototype from Roche. The UDS 454 information was analysed in the dedicated UDS DeepChek®-HIV (v1.1; ABL-Therapy-Edge SA) diagnostic software application, allowing analysing in routine the frequency of detection and the mutational load for each minor variant. For interpretation of the resistance mutations, the Stanford algorithm was used. Only mutations detected >1% threshold were considered for UDS analysis.

**RESULTS:** TDR to NNRTIs was detected by Sanger sequencing in 10/11 patients (with mutations in positions 90[I], 98[S], 101[Q], 103[N/S], 108[I], 138[A/K], 179[D/I] and 225[H]) and to NRTIs in 2/11 (67[N], 69[N], 215[L] and 219[Q]). The median frequency of Sanger-detected mutations was 96.7% (67.63–99.43%) and median mutational load was 136,468.5 copies/ml (6,247–1,231,072). Added UDS detected mutations were found in 8/11 patients (mutations in positions 103[N/R], 190[E], 225[H] and 230[L] for NNRTIs, and in positions 41[L], 65[R], 69[N], 77[L], 115[F] and 215[R] for NRTIs), with a median frequency of detection of 2.33% (1.01–4.94) and a median mutational VL of 1,222 copies/ml (348–24,107). When additional UDS-detected mutations were considered for resistance interpretation, we found added levels of resistance in 5/11 patients (NNRTIs, 3 patients and NRTIs, 3 patients).

**CONCLUSIONS:** Patients carrying TDR mutations detected by Sanger sequencing frequently also carry additional minor viral mutant populations >1% threshold, which can also be detected using UDS methods. In our study, almost one-half of the patients with TDR to NRTIs and or NNRTIs had resistance to additional drugs when UDS mutations were used for resistance analysis. These findings may have important implications for first- and subsequent-line therapy designs and decisions.
ABSTRACT 128

Antiviral Therapy 2012; 17 Suppl 1:A154

Assessing the use of Illumina technology for deep sequencing in virology applications

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The arrival of next-generation sequencing has brought new insights in viral dynamics and resistance upon treatment of viral infections, such as HIV and hepatitis C. Until now, 454 (Roche) has been the most popular platform for detection of low-level drug resistant variants. However, current short-read sequencing technologies have the advantage of providing a higher sequencing depth at a lower cost per sample. We evaluated the use of Illumina’s GAIIx technology for deep sequencing applications by comparing its sensitivity and accuracy with 454.

In a first experiment, five HIV plasmids were sequenced with Illumina to assess the noise level. Similar to previous 454 studies, we observed a high density of errors with frequencies <1%, introduced either during sample preparation or sequencing itself. Subsequently, we used a similar budget for sequencing 12 samples on both platforms, in order to make a fair comparison between both technologies. Part of the HIV reverse transcriptase (RT) region (amino acid position 1–400) was sequenced on Illumina and 454 resulting in an average coverage of 125,000 and 9,000 sequences per position, respectively. Among the mutations called >1% by both technologies, there was a strong correlation between the observed frequencies (R²=0.998). If we consider mutations present >1% in only one platform as putative false positives, a much lower false-positive rate was observed when using Illumina. Furthermore, the higher coverage per position of Illumina results in greater confidence when assessing low frequency mutations. Finally, the paired-end approach of Illumina also allows exploring linkage between mutations present in the same viral subspecies. The HIV-RT region was sequenced in a set of eight samples harbouring two important IAS-USA resistance-associated mutations at different frequencies. By selecting only the sequences that contained both positions (on average 40,000 per sample), we were able to show that both mutations were mutually exclusive.

In conclusion, using Illumina for viral sequencing experiments provides a cost-effective way to obtain deeper sequencing data without compromising sensitivity or accuracy. Despite the short-read length, using a paired-end approach in combination with high coverage also enables the detection of linkage between mutations.
ABSTRACT 129

Antiviral Therapy 2012; 17 Suppl 1:A155

Sequence based multiplex detection of NS mutations in HCV 1a and 1b genotypes

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BACKGROUND: The Pathogenica BioDetection™ system encompasses a library of >75,000 DxSeq probes that select and report unique DNA sequences of target genes/mutations. This technology enables the sequencing of dozens to thousands of genomic regions that may be present in a sample. The Pathogenica platform is sequencer-agnostic – the DxSeq probes can be used in conjunction with all commercially available sequencing platforms. The assay is performed in a single tube, in <3 h, and the probe technology can provide significant gene region fold coverage (500 to >50,000 read depth), which can be used to determine quantitative variant information.

OBJECTIVES: To develop an assay to genotype HCV and to detect NS3, NS5a and NS5b drug resistance mutations in HCV genotype 1a and 1b clinical samples using Pathogenica DxSeq probe technology.

METHODS: Clinical samples from HCV 1a- and HCV 1b-infected patients underwent HCV RNA extraction and RT-PCR. DxSeq probes where used to capture the desired gene regions from the viral cDNA. The sequence of the gene region was determined using next generation sequencing technology (Ion Torrent) and compared to the known sequence determined by Sanger Sequencing.

RESULTS: The HCV DxSeq assay enabled genotyping of HCV viral variants present within the sample, correctly identifying HCV 1a and HCV 1b infections. DxSeq probes were able to sequence regions previously reported to confer antiviral drug resistance, in both NS5a and NS5b. Resistance locus capture size was between 150 and 250 bases, and read depth ranged between 50- to 4,756-fold for 215 probes. In total, >20 nucleotide variants resulting in amino acid substitutions within viral proteins were identified, including codons predicted to modulate antiviral drug resistance, and >50 nucleotide variants generating synonymous codons were sequenced. Quantitative variant information provided by DxSeq probes allowed for precise percentage estimates for codon usage by viral variants within these patient samples.

CONCLUSIONS: The HCV DxSeq assay enables the sensitive sequencing of a broad range of genotype and resistance loci simultaneously from a patient sample, with a single tube assay. This low-cost technology will enable the broader application of new sequencing platforms to clinical genotyping.
ABSTRACT 130

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V3 loop evolutionary dynamics in ACTG A5211 vary by CCR5 antagonist treatment outcome

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BACKGROUND: Large shifts in HIV-1 gp120 V3 loop populations occur in chronically HIV-infected subjects in whom CCRX4-using (X4) virus emerges with failure of CCR5 antagonist (vicriviroc [VCV]) therapy. To investigate further V3 evolutionary dynamics, we performed deep sequencing on plasma from subjects with other treatment outcomes.

METHODS: We selected subjects from ACTG A5211, a Phase IIb study of VCV in subjects with CCR5-using (R5) virus only by Trofile, with HIV-1 RNA levels >1,000 copies/ml during the time interval studied. Three time points were analysed for each subject: study entry, an intermediate time point, and at or after protocol-defined virological failure (VF; <1 log₁₀ decrease in HIV-1 RNA level at week 16). A total of 43 subjects met criteria: subjects who received VCV and had VF with R5 virus (n=9), subjects who received VCV without VF (n=5), subjects with detectable X4 virus (n=12) and placebo subjects who did not receive VCV (n=17). HIV-1 RNA was extracted from plasma and degenerate consensus primers were used to reverse transcribe and amplify V3 loop-coding regions. Amplicons were submitted in a blinded fashion for 454 deep sequencing and custom analysis.

RESULTS: At least 1,000 sequences were obtained per time point per subject. Placebo samples demonstrated limited changes in V3 diversity; minority predicted X4 variants were not detected in this prescreened population. Subjects who completed VCV treatment with R5 virus demonstrated modest diversification around the dominant pre-treatment V3 loop forms. Preexisting minority X4 variants were not detected in this group and did not emerge during therapy. In a subset of these patients, purifying selection was observed. For subjects in whom X4 virus did emerge, the prevalence of X4 V3 forms increased rapidly after VCV treatment. The removal of VCV was associated with the re-emergence of R5 forms.

CONCLUSIONS: Chronically HIV-infected subjects not receiving VCV and the majority of participants we analysed on VCV demonstrated drift in V3 forms. V3 population shifts were observed in VCV-treated subjects in whom HIV-1 escaped drug pressure through the emergence of CXCR4-using virus or VCV resistance. Clinically relevant minority X4 variants were not highly prevalent among patients with phenotypically R5 virus.
ABSTRACT 131
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Genotypic tropism determination compared to phenotypic methods to screen HIV-infected, treatment-naive subjects for Cenicriviroc Study 202

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BACKGROUND: Ultradeep sequencing (UDS) technologies can detect minor viral populations and have been shown in retrospective analyses to perform equivalently to a sensitive phenotypic assay to identify appropriate candidates for CCR5-antagonist-based therapy. We describe tropism screening results from genotypic tropism testing (GTT) using triplicate population sequencing (TPS) combined with UDS and a sensitive phenotypic tropism assay in a Phase 2b trial.

METHODS: Cenicriviroc (CVC) is a once-daily oral CCR5/CCR2 antagonist currently in development for treatment of HIV infection. Study 202 (652-2-202; NCT01338883) is ongoing and excludes treatment-naive subjects with detectable non-R5 (X4/DM) virus by either GTT or phenotypic tropism testing. Phenotypic testing is conducted by Monogram Biosciences using the enhanced sensitivity Trofile assay (ESTA). GTT is conducted by Quest Diagnostics using TPS of the V3 loop with interpretation by the geno2pheno (FPR 5.75%) and PSSM_{ext} (X4 cutoff ≥4.75) algorithms, followed by UDS (Roche/454 Life Sciences) for R5 samples.

RESULTS: Screening results were available for 194 subjects as of January 2012. Concordance between GTT and ESTA was 80%. Relative to ESTA, GTT sensitivity was 72% and specificity was 82% (κ=0.42). GTT classified 26% of subjects as X4/DM versus 15% for ESTA; 30% of subjects were classified as X4/DM by either assay, versus 11% by both assays. Overall, 15% of subjects were classified as X4/DM by GTT but R5 by ESTA, versus 4% classified as X4/DM by ESTA but R5 by GTT (McNemar P=0.0005). The median (IQR) percentage X4-tropic virus was 10% (3–69%) in samples classified as X4/DM by UDS and R5 by ESTA versus 39% (12–91%) X4-tropic virus in those classified as X4/DM by both UDS and ESTA. Relative to ESTA, TPS alone had 60% sensitivity and 86% specificity (κ=0.39). The relative performance of TPS also varied with the interpretation algorithm (for example, geno2pheno versus PSSM) and selected FPR (for example, 5.75% versus 10%).

CONCLUSIONS: GTT and ESTA demonstrated good concordance in tropism determination. In the discordant subset, GTT identified X4-tropic virus in more subjects than ESTA. Therefore use of GTT for tropism determination could lead to fewer subjects with X4-tropic virus being classified as candidates for CCR5 antagonists.
ABSTRACT 132
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Factors related to predominance of non-R5 tropism in proviral genotypic tests
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BACKGROUND: Tropism determination in provirus has been used to switch ARV treatment, using CCR5 inhibitors, in patients with undetectable viral load. Proviral genotypic tests have shown an elevated frequency of non-R5 tropic viruses, but the reason for this is not known. Our objective was to determine those factors related to non-R5 tropism in proviral sequences.

METHODS: We evaluated 59 proviral genotypic tropism tests, performed with an in-house method, with an FPR of 10%, in order to determine factors related to a non-R5 result. Gender, age, years of infection, number or ARV combinations used, as well as CD4+ T-cell nadir and in the last determination were evaluated.

RESULTS: Overall, 8 out of 59 (13.55%) proviral tests had R5 tropic viruses. Gender, age and recent CD4+ T-cell counts were not different between patients with R5 and non-R5 viruses. Patients with non-R5 viruses had a significantly longer time with HIV infection: 13.46 (range 3.25–23.2) versus 7.5 (range 0.7–13.2) of those with R5 viruses (P<0.01). As a consequence of this, more patients with non-R5 viruses had used ≥4 ARV combinations. By contrast, CD4+ T-cell nadir count was significantly lower in patients with R5 viruses (129 [range 6–210]) than in those with non-R5 viruses (205 [range 2–726]; P=0.04).

CONCLUSIONS: Genotypic proviral tropism tests usually find more non-R5 viruses than those performed in viral sequences. While this phenomenon is related to a better detection of non-R5 sequences in provirus, it is also related to a longer time on HIV infection as well as number of ARV treatment combinations, but not to nadir for CD4+ T-cell counts. The real predicted value of proviral results in the response to CCR5 inhibitors treatment should be determined.
ABSTRACT 133

*Antiviral Therapy* 2012; 17 Suppl 1:A159

Mutations at position 16 of the V3 related to non-R5 genotypic tropism


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**BACKGROUND:** While genotypic tropism is mainly related to changes in amino acids 11 and 25 of the V3 envelope sequences, some other amino acids could be related to tropism selection. Our objective was to find some other amino acid changes that could be related to a specific tropism.

**METHODS:** 177 patients were tested with an in-house method for genotypic tropism determination according to a previously published technique. Amino acid changes were evaluated in relation to R5 or non-R5 tropism determined through geno2pheno algorithm. Sequences were grouped phylogenetically to test relatedness.

**RESULTS:** We evaluated 119 cases with R5 and 58 with non-R5 tropic sequences. Of all the V3 sequences collected, we could only correlate changes at position 16 with differences in the final tropism classification. In all R5 cases, the amino acid at position 16 was a proline, while 13/58 (22.4%) had some change at amino acid 16 in non-R5 sequences. Change to G was the most frequently found (n=3), followed by F and T (2 cases) and two cases of deletions at this position, and one case each to amino acids E, I, R and L. In total, 10 of the cases with any mutation had FPR values <2%. Phylogenetic trees of the non-R5 sequences did not show any particular branching between those with and without any amino acid change at position 16.

**CONCLUSIONS:** Mutations in V3 amino acid at position 16 are frequent in those sequences belonging to non-R5 viruses, and are related to lower FPR values. These changes can be compensatory, but should be evaluated as a part of the interpreting algorithms for genotypic tropism determination.
ABSTRACT 134

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Comparison of the Antivirogram and PhenoSense assays to determine phenotypic susceptibility to rilpivirine in patient samples from the Phase III ECHO and THRIVE trials

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BACKGROUND: HIV-1 phenotyping is a quantitative method to directly assess the impact of individual and complex mutational patterns on antiviral drug susceptibility. Assay variability complicates interpretation of results, especially around the assay- and drug-specific cutoff values. Samples from rilpivirine (RPV)-treated patients experiencing virological failure (VF) were collected at baseline and failure in the Phase III studies ECHO and THRIVE and tested using either Antivirogram® (AVG; Janssen Diagnostics BVBA) or PhenoSense™ (PS; Monogram Biosciences, Inc.) to gain insight into the effect of mutations on phenotypical susceptibility to RPV. Here we report a comparison of the results obtained with both methods.

METHODS: Plasma samples from 61 VFs obtained at baseline and failure were subjected to phenotypic susceptibility testing using AVG and PS (n=122, using a single determination per sample). In parallel, 17 site-directed mutants (SDMs) with relevant mutations were subjected to both assays. The biological cutoff (BCO) for RPV was 3.7 for AVG and 2.0 for PS, both based on assay variation using wild-type isolates.

RESULTS: AVG and PS were both successful in 108/122 (88.5%) clinical samples and in all 17 SDMs. The overall correlation between RPV AVG and PS fold changes in EC50 (FC) values was high (R²>0.7), with a trend towards lower RPV FCs with PS versus AVG. Concordant resistance calls for RPV were obtained in 97/108 (89.8%) clinical samples and for 14/17 (82.4%) SDMs. Most (9/11) discordant calls were observed in the failure samples, without a clear association with a specific mutational pattern, and generally with FC values around the cutoff of either assay. The three SDMs with a discordant resistance call harboured K101E or E138Q (both susceptible in AVG and resistant in PS), or E138K+M184I (resistant in AVG and susceptible in PS).

CONCLUSIONS: Analysis of phenotypic susceptibility to rilpivirine using AVG or PS resulted in a high level of concordance, with a general trend towards lower RPV FC values with PS. Discordance was most frequent in failure samples harbouring multiple resistance mutations and with FC values around the cutoff value of either assay.
ABSTRACT 135

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Molecular characterization of ambiguous mutations in HIV-1 polymerase gene: implications for monitoring HIV infection status and drug resistance

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BACKGROUND: Estimations of transmitted HIV drug resistance (HIVDR) and HIV incidence are based on the cohort of recently HIV-infected individuals. However, accurate identification of recently infected individuals has been challenging due to the limitations of current serological tests. HIV has been characterized as the fastest evolving RNA virus with a high mutation rate. The amount of mutations accumulated in the viruses reflects the length of infection, thus indicating the infection status. This study aimed to explore a molecular approach for detecting recent infections and to provide a tool for monitoring HIVDR development at early stage of infection.

METHODS: Ambiguous nucleotides accumulated in the pol gene of HIV-1 variants were extracted and calculated for frequency with the dataset of recent (HIVDR Threshold Survey [HIVDR-TS]) and established (HIVDR monitoring survey at baseline) infections. The pol-sequences were then translated into putative amino acid sequences in which the amino acids that differed from wild type at the HIVDR sites were identified for characterizing the proportional score of the ambiguous or fitted mutations.

RESULTS: We detected an average of ambiguous mutations of 2.04×10^-3 nucleotides/site and 14.41×10^-3 nucleotides/site in HIV-1 recent and established infections, respectively. With the defined HIV-1 substitution rate of 2×10^-3 nucleotides/site/year, 75.2% of subjects in HIVDR-TS and 3.3% in HIVDR baseline survey were classified as being infected within 1 year. To monitor the development of HIVDR, we measured the mutation scores at each of HIVDR sites and identified sites with scores that increased, remained stable or decreased across the recent to established infections. Importantly, we detected that the HIVDR caused by ambiguous mutations increased from 0.6×10^-2 to 3.69×10^-2/DR-site but those by fitted mutations remained at the level of 6.34–6.98×10^-2/DR-site across the recent to established infections, illustrating that the transmitted HIVDR background existed in drug-naive populations of both cohorts, and under such background new HIVDR continued to develop.

CONCLUSIONS: Overall, our findings suggest that molecular characterization of ambiguous mutations in HIV may serve as an additional tool to differentiate recent from established infections, evaluate infection status and monitor the development of drug resistance.
ABSTRACT 136

Antiviral Therapy 2012; 17 Suppl 1:A162

HIV2EU – supporting standardized HIV-2 drug resistance interpretation in Europe

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BACKGROUND: Various items are complicating the treatment of HIV-2-infected patients. Compared to HIV-1, there is much less treatment experience, no evidence from randomized control trials, a reduced number of effective drugs and no broadly available test for viral load monitoring. In case of treatment failure there is only limited guidance and presently no easy accessible tool for nucleic acid sequence interpretation available. To solve this problem, we initiated an expert workshop to address some of these problems.

METHODS: A panel of experts from four different European countries voted on a rule set for interpretation of mutations in the HIV-2 protease, reverse transcriptase and integrase. Rules were proposed by each member and were then modified during discussion by considering data gained from HIV-1 and accumulated experience of the follow-up of HIV-2-infected patients. Based on the HIV-GRADE internet tool, an online tool was developed to make the rule set easily accessible and usable.

RESULTS: Rules were laid down for the interpretation of HIV-2 drug resistance to NRTIs, PIs and integrase inhibitors (INIs). Due to natural resistance of HIV-2, usage of NNRTIs and T-20 was not recommended as part of an antiretroviral regimen for HIV-2. These rules were then translated in a machine interpretable format (algorithm specification interface [ASI]) and the HIV-GRADE tool was extended for usage of HIV-2 sequences. Further consensus sequences were generated from the reference sequence data set provided by Los Alamos National Laboratories. In contrast to HIV-1, mutations were compared to a group specific consensus sequence (Group A or Group B) and not to a consensus sequence from the most predominant HIV-2 Group A. This change was necessary due to significant differences between the various HIV-2 strains.

CONCLUSIONS: We developed a rule set and an automated tool for HIV-2 drug resistance analyses. This tool and the rules will be freely available on the internet. Access to the pre-publication versions can be granted by each of the group members. To keep the algorithm rules up-to-date it will be actualized on a yearly basis.
SESSION 9
Epidemiology
ABSTRACT 137

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Elevated prevalence of the E138A mutation in reverse transcriptase of HIV-1 subtypes A and C from recently infected, untreated subjects

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BACKGROUND: HIV-1 susceptibility to second generation non-nucleoside reverse transcriptase (RT) inhibitors (NNRTI) is affected by several mutations in RT, including at position 138. The E138A mutation has been found in viruses from NNRTI-naive patients, and is associated with low-level reduced NNRTI susceptibility in vitro that is of unknown clinical significance. The prevalence of E138A in viruses from drug-naive subjects infected with non-subtype B viruses from diverse geographical origins is incompletely characterized. These data are needed to interpret genotype data from surveys of transmitted drug resistance, especially in resource-limited settings.

METHODS: RT sequences from specimens gathered during surveillance of transmitted HIVDR (n=2,732) in 23 countries in Africa, Asia and Central America were generated by WHO genotyping labs. Sequences were analysed using the Stanford HIV Drug Resistance Database CPR tool (version 5). The prevalence of E138A and other mutations for surveillance of HIV drug resistance (SDRM) was determined according to subtype. Results were compared to those from a cohort of patients initiating therapy in Africa and Asia (n=3,562) and to sequences from drug-naive subjects in the Stanford database (n=32,891).

RESULTS: The most prevalent subtypes were C (47%), CRF01_AE (20%), CRF07_BC (9.3%), CRF02_AG (7.5%), B (4.8%) and A (3.7%). NNRTI SDRM were detected in 1.8% of all specimens; E138A was found in 4.1%. E138A prevalence was relatively high in subtypes C (7.5%) and A (6.0%) compared to B (0%), CRF01_AE (0.4%), CRF02_AG (2.0%) and CRF07_BC (0%). Comparisons of prevalence between subtype A and B, CRF01_AE or CRF07_BC, and between subtypes C and CRF01_AE, CRF02_AG, and CRF07_BC were statistically significant (Fisher Exact test with adjustment for multiple comparisons, P<0.05). Similar trends were observed in patients initiating therapy and in the Stanford database: E138A prevalence was always highest in subtypes A and C (2.7–9.0%) and lowest in CRF01_AE (0–1.0%).

CONCLUSIONS: The E138A mutation was found frequently in viruses from untreated subjects and is over-represented in subtypes A and C. E138A is polymorphic and should not be considered when estimating prevalence of transmitted drug resistant HIV or when defining populations of viruses as being unexposed to antiretroviral drugs.
ABSTRACT 138

**Antiviral Therapy** 2012; 17 Suppl 1:A166

**Effect of frequently transmitted M46I/L mutations on the in vitro selection of drug resistance against lopinavir**

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**BACKGROUND:** Approximately 10% of newly diagnosed HIV-1 patients are infected with a drug-resistant variant. M46I/L are the most frequently detected solitary protease-resistance mutations in these patients. It is debated whether these variants should be considered resistance-related polymorphisms or indicators of drug-exposure in a previous host. We have identified a large transmission cluster of viruses containing M46L, suggesting ongoing transmission of this variant. We investigated the impact of baseline M46 mutations on the drug-resistance pathways of lopinavir (LPV).

**METHODS:** Viruses were selected from two therapy-naive patients: one from the transmission cluster containing M46L and one harbouring M46I. The C-terminus of gag and protease gene from both patient viruses were cloned into the subtype B reference strain HXB2. Additionally, site-directed mutants containing M46I or M46L were constructed. First, LPV susceptibility was determined using a multiple cycle assay. Subsequently, multiple in vitro selection experiments were conducted and compared to wild-type subtype B, C and AG selections. The C-terminus of gag and the protease gene were sequenced to identify genotypic changes.

**RESULTS:** At start, all viruses were fully susceptible to LPV (IC_{50} range 3.4–8.6 nM). Under pressure of LPV, the dominant resistance pathway of subtype B (±M46 variants) and subtype C viruses was characterized by selection of L10F and V82A. In the viruses lacking the M46 variants at start, M46I/L (8/14) and/or I54V (7/14) were frequently selected as well. Viruses with an M46 variant at baseline had a tendency for selection of I50V (3/10) in addition to L10F and V82A. In subtype AG a different pattern with V82F in absence of L10F (3/3) was observed. In 2/3 cultures, M46I was also selected. Gag mutations were commonly observed for all subtypes (19/36); mainly A431V (7/36) and I437V/T/S (8/36) were selected.

**CONCLUSIONS:** Baseline M46 variants have no major impact on development of LPV resistance since the dominant resistance pathway of all subtype B (wild-type and M46 variants) and C viruses was characterized by L10F and V82A. Presence of M46 variants did result in preferential selection of I50V, while I54V was often selected in wild-type viruses. The dominant resistance pathway of subtype AG differs from the pathways of subtype B and C.
ABSTRACT 139

Antiviral Therapy 2012; 17 Suppl 1:A167

Persistence of transmitted HIV drug resistance mutations

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BACKGROUND: There is little quantitative data on the persistence of transmitted drug resistance (TDR) mutations, particularly on whether this varies according to individual mutation. Such information is critical for HIV transmission models and other applications.

METHODS: Patients (seroconverters and chronic ART-naive) with TDR mutations (according to the 2009 surveillance drug resistance mutations list) present at their first resistance test and with subsequent tests performed whilst ART-naive were identified from the UK HIV Drug Resistance Database. The rate at which mutations became undetectable (‘lost’), as determined by population sequencing from plasma virus, was estimated using exponential regression (that is, assuming a constant rate of loss after the initial resistance test) accounting for interval censoring.

RESULTS: In total, 313 patients (670 resistance tests; 89% had 2 tests, 11% had >2 tests) were identified. Overall, 79% had subtype B virus, 13% non-B and 7% unknown subtype. The median (IQR) time between consecutive tests was 40 weeks (10–96). In univariate analysis, the rate of loss (per 100 person-years; 95% CI) of nucleoside reverse transcriptase inhibitor (NRTI; n=404), non-NRTI (n=154) and protease inhibitor (PI; n=162) mutations was 15 (11, 21), 25 (17, 38) and 21 (14, 31), respectively. However, there were differences within class. The rate of loss of thymidine analogue mutations (TAMs) was 8 (4, 15), 12 (4, 15), 14 (6, 33) and 4 (1, 19) for M41L (n=77), D67N (n=27), L210W (n=25) and K219Q (n=25), respectively, which was similar to T215 revertants: 5 (3, 11; n=106; P=0.2). The rate of fading of T215Y (n=25) and T215F (n=9) was 41 (20, 84) and 58 (15, 224), respectively. As expected M184V reverted quickest: 71 (34, 149; n=34). The rate of fading of non-NRTI mutations was 18 (10, 34), 54 (26, 113) and 19 (6, 56) for K103N (n=73), Y181C (n=20) and G190A (n=17), respectively, and 12 (5, 31) for PI mutation L90M (n=32). In multivariate analysis, mutations in patients with non-B subtype virus were less persistent than subtype B virus (P=0.002).

CONCLUSIONS: TAMs and T215 revertants appear to be highly stable, with non-NRTI mutations being relatively less persistent, contrary to conventional wisdom. However, the quickest reverting mutations may not be detected even in the first sample tested, and thus might be excluded from this analysis.
ABSTRACT 140

Antiviral Therapy 2012; 17 Suppl 1:A168

Community HIV-1 drug resistance is associated with transmitted drug resistance

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BACKGROUND: Community viral load (CVL) measurements are associated with incidence of new HIV-1 infections in a population. We hypothesized that similarly measured community drug resistance (CDR) could predict incidence of transmitted drug resistance (TDR).

METHODS: CDR was measured as the prevalence of HIV-1 drug resistance (Monogram Biosciences) between 2001 and 2011 for patients receiving care at UCSD Owen Clinic, which provides care to approximately 40% of those receiving care for HIV/AIDS in San Diego County. Drug resistance was defined as a mutation at a key resistance position in HIV-1 reverse transcriptase (RT) or protease (pro) according to the WHO 2009 surveillance TDR mutation list. At each position, CDR was evaluated both as the overall prevalence of resistance and by weighting each resistance position by its proportion to the calculated CVL. CVL was the total viral load of patients from the Owen Clinic each year, and the weighting was the proportion of the CVL associated with patients identified with resistance at each interrogated position. TDR was similarly measured (Monogram Biosciences) for patients in the San Diego Primary Infection Cohort during the same period but without adjusting for viral loads. Spearman ranked correlation coefficients were used to determine the association between CDR and TDR.

RESULTS: We analysed 1,088 resistance test results from 971 Owen Clinic patients with a median viral load of 100,000 copies/ml. Baseline resistance results were available for 542 newly infected patients in the primary infection cohort. Between 2001 and 2011, CDR and TDR varied widely from 0% to 50% depending on the year. CDR at position 46 in pro was associated with TDR between 2001 and 2011 (P=0.01). When CDR was weighted by viral load of patients, CDR was associated with TDR at position 188 in RT (P=0.08) and 46 and 82 in pro (P=0.02 and P=0.04, respectively).

CONCLUSIONS: Despite representing a limited percentage of chronically infected patients in San Diego County and perhaps underutilization of resistance testing for patients with treatment failure, CDR measurements correlated with TDR at key resistance positions and therefore may be a useful tool to predict TDR.
ABSTRACT 141

*Antiviral Therapy* 2012; 17 Suppl 1:A169

Trends in HIV-1 resistance prevalence at the community level following the expansion of access to antiretroviral treatment in British Columbia, Canada

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BACKGROUND: Several Canadian provinces have reported recent increases in the prevalence of primary drug resistance, particularly to NNRTIs.

METHODS: Resistance genotypes were obtained from plasma samples collected from patients enrolled in the British Columbia Drug Treatment Program (DTP) between 2000–2011, a period in which DTP enrolment nearly doubled (from 3,266 to 6,345 active treated patients). All patients with plasma viral load (pVL) data in British Columbia are included regardless of their antiviral therapy history. The known ‘community’ pVL for the whole province was calculated as the sum of each patient’s maximum pVL that year. Sequences were scored for the presence of resistance mutations (2009 WHO surveillance list) and were grouped by the number of drug resistance categories. The viral load of those infected individuals not linked to HIV care is not known nor included.

RESULTS: In British Columbia, the drug-resistant proportion of the total known provincial pVL decreased substantially over the course of study. In 2000, 35% of the provincial pVL could be attributed to drug-resistant variants, mostly from pretreated patients, declining to a low of 15% in 2009. Concomitantly, the proportion of individuals achieving pVL suppression to undetectable levels increased. HIV drug resistance levels may have reached a nadir (especially for NNRTIs), as no further reductions in resistant pVL were observed in 2010 and 2011. In 2011, successfully suppressed patients (n=4,393 individuals) contributed <0.05% of the total known provincial viral burden; a combined pVL equal to that of approximately four untreated individuals. Among new antiretroviral-naive DTP enrollees, the overall prevalence of primary resistance declined from 10% in 2000 to 5% in 2008 driven largely by decreases in primary NRTI resistance. No further decrease in primary resistance was observed since 2009.

CONCLUSIONS: Drug resistant HIV variants currently represent a significant minority (approximately 18%) of all known circulating HIV variants in British Columbia. Expansion of antiretroviral therapy in British Columbia has not led to increased prevalence or transmission of drug-resistant HIV. However, the lack of further decline in NNRTI-resistant HIV pVL in the community since 2009 may be of concern.
ABSTRACT 142

Antiviral Therapy 2012; 17 Suppl 1:A170

Increase of the HIV-1 non-B subtypes frequency in patients during primary infection in France and effect of HIV-1 subtypes on the virological and immunological response to cART

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BACKGROUND: The objectives were to describe HIV-1 diversity in patients diagnosed in France at the time of primary infection (PHI) between 1996 and 2010, the time trends in the distribution of the subtypes according to gender, risk group, and geographical origin. To study virological and immunological responses after combined antiretroviral therapy (cART) initiation according to HIV-1 subtypes.

METHODS: Study population comprised patients enrolled during PHI in the PRIMO cohort. Subtypes were determined by phylogenetic analysis of reverse transcriptase sequences. Viral suppression (VL<400 copies/ml) was assessed at M6, M12 and M24 in patients who initiated cART during primary infection. Trends in CD4+ T-cell count were modeled using a linear mixed-effects model with three slopes, adjusted for gender and baseline viral load.

RESULTS: From 1996 to September 2010, 1,128 patients were enrolled. Although HIV-1 B subtype was still predominant (74.7%), non-B subtypes (25.3%) were present in all regions of France with an increasing frequency over time. Although CRF02_AG was predominant (56%) among the 283 non-B strains, we found a wide diversity including 6 subtypes, 10 CRF and several URF. Overall, 488 out of 1,128 patients started cART following PHI diagnosis and had a follow-up of ≥1 year. Patients infected with a non-B virus had a virological response at M6, M12 and M24 that was similar or even better compared to those infected with a B subtype strain. We observed a better immunological response between M1 and M18 both in patients infected with a CRF02_AG strain and those with other non-B viruses, compared to those with a B subtype. Similar results were observed when restricting the analysis to non-African patients or to patients initiating a boosted-PI containing regimen.

CONCLUSIONS: The circulation of non-B subtypes has significantly increased in France over the last 15 years. Patients infected with a CRF02_AG or a non-B virus had a trend to a better virological response at M12 compared with patients infected with B subtype and a better immunological response between 1 and 18 months. Our results are encouraging for countries where CRF02_AG strains predominate in view of the increasing availability of ART in such countries.
Drug resistance and phylogenetic analysis of HIV-1 strains sampled from newly diagnosed untreated patients in Paris restricted area, France

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BACKGROUND: There is evidence of high levels of HIV transmission among men who have sex with men (MSM) in France and especially those living in the Paris area. Our objective was to characterize the current spread of HIV in Paris restricted area using a molecular epidemiological approach.

METHODS: We included untreated patients who were newly diagnosed between 2008 and 2011, and followed by private physicians, in Paris. Genotypic resistance tests were performed at baseline. HIV pol sequences were analysed using a population-based phylogenetic approach in order to characterize HIV transmission dynamics.

RESULTS: This study included 547 patients (97% men). Median age was 37 years. Median CD4+ T-cell count was 448 cells/µl and HIV-1 plasma viral load was 47,280 copies/ml. These patients were predominantly infected with viruses belonging to subtype B (76%) or CRF02_AG (14%). Viruses from 108 (20%) patients cosegregated into 49 transmission chains with increasing frequency (14% in 2008, 20% in 2009, 23% in 2010 and 19% in 2011). The mean number of patients per cluster was 2.2. Primary drug resistance mutations were observed in 52/547 (9.5%) of samples. Eight resistant strains were involved into clustered events. Overall, the prevalence of nucleoside reverse transcriptase inhibitor, non-nucleoside reverse transcriptase inhibitor and protease inhibitor resistance-associated mutations was 4.2%, 5.5% and 3%, respectively. The frequency of viruses resistant to ≥2 and 3 classes of ART was 2.3% and 0.7%, respectively. Predictions of viral tropism were determined for 38 individuals. X4 or X4 dual or mixed viruses were seen in 7 (18%) subjects. Gender, CD4+ T-cell counts, viral load and subtype were similar in clustered and non-clustered transmissions. However, the patients included into transmission clusters tended to be younger (median age 34 years) and to be infected with a lower proportion of transmitted-drug resistance (7.4%). But two strains in the same cluster showed multidrug resistance (MDR) to reverse transcriptase, protease and integrase inhibitor classes.

CONCLUSIONS: In this Parisian population, in the majority of MSM, our results suggest a significant source of onward transmission. The overall frequency of resistant viruses was similar to those reported in previously studied populations in France with nevertheless a transmission of MDR viruses.
ABSTRACT 144

Antiviral Therapy 2012; 17 Suppl 1:A172

Identification of HIV transmission networks in Israel

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BACKGROUND: HIV has spread throughout the world and is now endemic in most developed countries. Identifying local patterns of HIV transmission is useful in order to focus resource allocation for testing, treatment and prevention efforts. Israel has had an ongoing HIV epidemic for >30 years; there have been multiple introductions of HIV through indigenous Israeli and immigrant populations, but current transmission patterns are not well described. To investigate contemporary patterns of HIV spread, we conducted a retrospective analysis using epidemiological data combined with phylogenetic reconstructions of HIV sequences of recently diagnosed individuals.

METHODS: We reviewed data submitted to the Israeli HIV national reference laboratory during 1996–2010 to identify newly diagnosed individuals. Genotypes and demographic information including gender, year of diagnoses and HIV risk were recorded. Recently infected (<1 year) individuals were identified by recent previous negative HIV ELISA infections. Codons for transmitted drug resistance mutations were removed to prevent transmitted drug resistance mutations from contributing phylogenetic signal. Sequences were aligned using MEGA 5.05, and alignments were subjected to Bayesian Monte Carlo Markov Chain analyses using BEAST to construct phylogenies and investigate ancestral relationships. Transmission networks were defined as distinct populations with short branch lengths and posterior probability ≥0.95 of having a recent common ancestor. The reconstructed phylogenies were analysed with corresponding demographic information and drug resistance mutation profiles.

RESULTS: We identified 560 individuals with newly diagnosed HIV infection (subtype B 273, subtype A/ AE 287) including 36 seroconversions. Phylogenetic analysis and Bayesian reconstructions revealed that sequences from 121 (26%) patients could be classified into 34 distinct transmission groups. Transmission groups had a median size of 3 individuals (range 2–12); 18/36 (50%) of all seroconvertors could be assigned to a transmission cluster. Clusters consisted of individuals with a common risk group (for example, men who have sex with men [MSM]), but groups also consisted of individuals of differing risk (for example, MSM and heterosexual, MSM and injection drug users). Clusters included individuals with common drug resistance mutations.

CONCLUSIONS: Phylogenetics identified transmission networks for 25% of all newly diagnosed individuals and half of all seroconverters. HIV, including drug resistant HIV, spreads through homogeneous and heterogeneous risk groups.
Impact of the HIV subtype in the resistance mutation profile in immigrant populations on virological failure in Spain

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BACKGROUND: In recent years, an increased number of patients infected with HIV-1 non-B subtypes (nBS) are recorded in resistance databases mainly due to immigrant populations from regions with a high prevalence of non-B variants. Changes over time in the prevalence of HIV clades and modern antiretroviral regimens (ARVs) may result in a different resistance profile in Spain.

METHODOLOGY: All HIV patients recorded in the ResRIS from 22 different HIV clinics were analysed. Sequences from the pol gene were used to assign HIV subtype using Stanford and Rega algorithms along with phylogenetic analysis by MEGA4. In addition, the prevalence of ARV RAMs were reported using the National algorithm and were compared with treatment exposure and virological information. Fisher exact test or Mann–Whitney was used for statistical analyses.

RESULTS: From 1,225 patients, 211 (17.2%) were immigrants. Immigrant populations were younger (<36 years 30.3% versus 25.9%; P<0.05) and had a lower median of CD4 T-cell counts at virological failure (VF; 258 versus 300). Immigrants had a higher nBS proportion (26.1% versus 3.6%; P<0.0001) and the most predominant variants were CRF02_AG (25%), C (9.1%), CRF01_AE (8.0%) and G (6.8%). CRF02_AG was seen among immigrants (20.4%) and native Spaniards (30.6%), whereas the rest were mainly seen in immigrants. The NRTI RAMs profile was as follows: lower prevalence of TAMs among immigrants (19% versus 36.4%; P<0.0001), M184V (25.1% versus 33.9%; P<0.05) and L74V (3.8% versus 7.7%; P<0.05). This observation correlates with a lower exposure to 3TC and ddi (12.9% versus 32.7% and 17.1% versus 26.4%, respectively; P<0.01) and higher exposure to FTC (45% versus 27.5%; P<0.001) in immigrant populations. The PI RAMs profile was as follows: lower prevalence for L90M (4.7% versus 13.0%) and I84V (0.95% versus 3.5%), with a lower exposure to indinavir (0.5% versus 3.3%) and higher exposure to darunavir (8.5% versus 4.1%; P<0.05 in all cases) in immigrant populations.

CONCLUSIONS: There are immunological and subtypes differences among HIV-positive patients with VF according to country of origin. Among immigrant populations, lower prevalence of RAMs to NRTIs and PIs is associated with different exposure to ARVs. These findings might be useful for therapeutic management of individuals infected with HIV-1 nBS.
ABSTRACT 146

Antiviral Therapy 2012; 17 Suppl 1:A174

Contact tracing and TDR of F1, C and A1 subtypes circulating in Italy are associated with modality of infection and geographical origin of patients

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BACKGROUND: Italian HIV-1 epidemic due to non-B variants is sustained mainly by the F1, C and A1 clades, which circulate at high prevalence in South America and Eastern Europe, in Africa, South East Asia and South America, and in Africa and East Europe, respectively. The aim of this study was to define the epidemiological networks and transmission clusters of transmitted drug resistance for these strains, through phylogenetic approaches.

METHODS: We analysed pol sequences of 343, 285 and 144 patients carrying subtype F1, C and A1 virus stored in the ARCA database and encompassing the 1997-2011 period. The transmission networks were identified by MrBayes and Beast programmes (GTR+I+G model). Mutations related to drug resistance were analysed with the SDRM list for naive individuals.

RESULTS: TDR was evaluated in 169, 120 and 36 patients naive for antiretrovirals carrying F1, C and A1 subtype, respectively. The prevalence of any resistance was 13.4% (11.8% for NRTIs, 6.5% for NNRTIs and 7.1% for PIs), 10% (1.7% for NRTIs, 6.7% for NNRTIs and 2.5% for PIs) and 11.1% (0% for NRTIs, 5.6% for NNRTIs and 5.6% for PIs), respectively.

Among patients with F1 clade, HEs had a higher probability to be detected in transmission networks (78.6% in HEs versus 63.5% in MSM; P=0.03) and among those with TDR (n=26) the proportion of clustering sequences was 57.6% (n=15). TDR was higher in South Americans (23.5%) compared to Italian and Romanian patients with F1 subtype (15% and 8.3%, respectively). Among patients with C subtype, MSM were associated with a higher probability to be included in transmission networks (70% for MSM versus 30.3% for HEs; P=0.002) and among those with TDR (n=12) the proportion of clustering sequences was 16.7%. TDR was higher in Africans (16.4%) compared to Italians (5.3%) and South Americans (0%). Among patients with A1 variants, the proportion of clustering sequences was 51.4% (n=74), slightly higher in IDs (85.7%) and HEs (59.3%) compared to MSM (25%; P=0.056). TDR was 12.5%, 16.7% and none in Italians, Africans and patients from Eastern Europe, respectively.

CONCLUSIONS: A striking proportion of epidemiological networks could be identified in individuals carrying F1, C and A1 subtype residing in Italy. The contact tracing revealed an unexpected burden of resistant isolates that was also associated with heterosexual or homosexual contacts for F1 or C subtype and with injecting drug use for A1 subtype. These findings indicate a complex picture of non-B subtype circulation in Italy underlying the need of public interventions to control the spread of such variants and transmitted drug resistance.
ABSTRACT 147

Antiviral Therapy 2012; 17 Suppl 1:A175

Transmitted drug resistance in HIV-2-infected antiretroviral-naive patients from the French National HIV-2 Cohort ANRS CO5 between 2007 and 2011

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BACKGROUND: The objective was to assess the prevalence of transmitted drug resistance mutations (DRM) and to determine viral tropism in HIV-2-infected patients included in the French National HIV-2 Cohort (ANRS-CO5).

METHODS: A total of 68 HIV-2-infected patients diagnosed between 2007 and 2011 issued from the French National HIV-2 Cohort were studied. All patients were antiretroviral-naive with detectable HIV-2 RNA level (>100 copies/ml). Protease and RT were sequenced with an in-house method, and interpreted with HIV-2 ANRS, Rega and CHAIN-WP2 mutations lists. HIV-2 tropism was assessed by gp105 sequencing, and interpreted with gp105 major determinants of CXCR4 coreceptor use we described (L18X, V19K/R, V3 global net charge, insertions at position 24). Absence of antiretroviral drugs in samples harbouring DRM or polymorphic substitutions was confirmed using UPLC-MS/MS.

RESULTS: Median age of the patients was 42 years (IQR 34–47), 60% were women, 74% were from West Africa and 12% were in CDC-C disease stage. Median CD4+ T-cell count was 412/mm³ (IQR 300–555). Median HIV-2 RNA level was 674 copies/ml (IQR 295–2,522). Protease, RT, and gp105 sequencing was successful in 60 (88%), 47 (69%) and 40 (61%) samples. In total, 65% of patients were infected with HIV-2 group A and 35% with HIV-2 group B. HIV-2 RNA tropism was as follows: 36 (90%) R5 and 4 (10%) X4/dual-mixed. No NRRTI DRM was found. PI DRM was found in three samples (5.0%; 95% CI 0.1, 9.9), evidencing the V47A in two cases and the I82F in the remaining one. In addition, we observed the N69D/S, V11I and E219D RT substitutions in 2 (4.2%), 4 (8.5%) and 1 (2.1%) samples, and the V71I, I89V and L99F protease substitutions in 5 (8.3%), 7 (11.7%) and 3 (5.0%) samples. These substitutions are known to be polymorphic but were described as possibly playing a role in increasing level of drugs resistance. Antiretroviral drugs plasma concentrations were below the limit of quantification in all cases.

CONCLUSIONS: A prevalence of 5.0% of transmitted DRM was observed in these antiretroviral-naive viraemic HIV-2-infected patients issued from the French National HIV-2 Cohort. This prevalence was in similar range to that reported in Côte d’Ivoire or Portugal. Tropism analysis showed that 10% of HIV-2-infected patients displayed X4/dual-mixed virus.