Abstracts presented at the
International Workshop on HIV & Hepatitis Virus Drug Resistance and Curative Strategies
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Plenary abstracts
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Antiviral Therapy 2013; 18 Suppl 1:A3

Computational immunovirology of hepatitis C and implications for resistance

S Ray

Infectious Diseases Fellowship Training Program, Division of Infectious Diseases, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Hepatitis C virus (HCV) is the most genetically diverse human pathogen, posing a challenge for mechanisms of control that directly target the viral genome, whether immunologic or pharmacologic. Computational, immunologic and virologic methods have been combined to assess viral variability, tempo of evolution and selective forces. Each of these varies during the course of acute to chronic infection, by region of the genome targeted, pace of disease progression, and positive and negative pressure for change.

Rather than progressively diverging from a common ancestor, HCV shows strong evidence of reversion (purifying selection) at even the most variable sites of the genome. These phenomena are relevant to our nascent understanding of HCV resistance to direct acting antiviral (DAA) therapeutics, in particular the risk of lifelong or transmitted antiviral resistance.
ABSTRACT P2

*Antiviral Therapy* 2013; 18 Suppl 1:A4

**Immunologic strategies to cure HIV infection**

*N Chomont*

1Vaccine and Gene Therapy Institute of Florida, Port St Lucie, FL, USA

Advances in the treatment of HIV infection have dramatically reduced the death rate from AIDS and improved the quality of life of many HIV-infected individuals. Although lifelong suppression of HIV replication with ART seems possible, side effects, resistance, stigma and cost all contribute to the necessity of finding a cure. It is now clear that ART alone does not eradicate HIV: even after more than 15 years of intensive and continuous therapy, the spread of the virus resumes within a few weeks upon cessation of ART in all but exceptional cases. The failure to cure HIV infection is believed to result from low-level viral production/replication, the presence of latent replication-competent provirus in resting CD4+ T-cells, and T-cell dysfunction stemming from persistent immune activation. Current ART does not target the reservoir of long-lived latently infected cells and does not fully restore immune functions. Insights into cellular mechanisms that control HIV gene expression and chronic immune activation suggest that the modulation of immune functions may accomplish both of these goals. Several immunological strategies aimed at curing HIV infection are currently being investigated. They include the blockade of negative regulators of T-cell activation, the administration of the gamma-c cytokine IL-7, HIV immunization and many other potential therapies. Determining both the immunological and the virological impacts of such strategies is essential.
ABSTRACT P3

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RNA virus evolution: a guide for the perplexed

EC Holmes¹,²

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RNA viruses are of great biological importance because of their role as agents of disease and their presumed similarity to some of the earliest replicating molecules. I will present an overview of the ‘rules’ of evolutionary change in RNA viruses, how they shape fundamental aspects of virus design, and what this means for the response to antiviral therapy. The fulcrum of my talk is an idea that the major aspects of RNA evolution and life-history – from the way they organize their genomes to their ability to jump species boundaries – reflect an intrinsically high rate of mutation. For example, I will show that the process of genome evolution in RNA viruses is in a large part determined by a remarkably high rate of deleterious mutation, which acts to put a cap on maximum genome size. Similarly, I will show that the rates of recombination and patterns of genome organization in RNA viruses reflect aspects of their mutational burden, and that this burden also has a major impact on how viruses are able to jump species boundaries and emerge in new hosts.
Oral abstracts
ABSTRACT 1

*Antiviral Therapy* 2013; 18 Suppl 1:A9

Evolution of the HCV viral population from the one patient with S282T detected at relapse after sofosbuvir monotherapy

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BACKGROUND: Clinical Phase II studies for the NS5B nucleoside analogue sofosbuvir (SOF; formerly GS-7977) have demonstrated high efficacy in patients. To-date, resistance (S282T) was detected only in one single 2b subject who received SOF monotherapy. In this study we investigated the evolution of S282T and the HCV population within this patient.

METHODS: Deep sequencing of the NS5B gene was performed on longitudinal plasma samples at baseline, day 2 on SOF, and weeks 4, 8, 12, 24 and 48 post-SOF treatment. Intrapatient HCV evolution was visualized by maximum likelihood phylogenetic trees.

RESULTS: Deep sequencing analysis revealed very low level presence of pre-existing S282T at 0.05% (4/7,755) at baseline and 0.03% (6/23,415) at day 2 on SOF. Even though these frequencies were below the assay cutoff of 1%, they were higher than S282T frequencies detected at baseline in all other patients analysed (n=116). At the week 4 post-treatment, 99.8% of the viral population harboured S282T. The frequency of S282T quickly decreased to 27.6% at week 8 post-treatment, 0.5% at week 12 post-treatment (119/24,743). Importantly, at 24 and 48 weeks post-SOF S282T was completely replaced by wild-type (1/25,175 and 0/8,749, respectively), lower than the 0.05% detected at baseline. Phylogenetic analysis showed that post-SOF NS5B sequences can form distinct lineages. This observation together with the persistence of unique post-treatment NS5B substitutions (S79N and a synonymous mutation at R334R) in all post-SOF samples at >99% supports that wild-type outgrowth resulted from reversion and not outgrowth of the baseline wild-type population. Furthermore, the number of unique HCV variants at relapse was significantly lower than at baseline (P=0.04) suggesting that the relapse viral population underwent a genetic bottleneck.

CONCLUSIONS: Deep sequencing analysis revealed that very low level of pre-existing S282T at baseline in a single patient was enriched following SOF monotherapy. However, the enriched S282T was no longer detectable (lower than baseline) at 24 and 48 weeks post-treatment resulting from the reversion to wild-type. Our data suggest that the HCV population underwent a genetic bottleneck at relapse and SOF treatment did not appear to increase the SOF resistance population after 24 weeks post-treatment in this patient.
ABSTRACT 2

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Biochemical characterization of β-d-2′-C-methyl-2,6-diaminopurine-ribonucleoside-5′-triphosphate as a potent inhibitor of hepatitis C virus (HCV) polymerase

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BACKGROUND: β-d-2′-C-Methyl-2,6-diaminopurine-ribonucleoside (DAPN) phosphoramidate prodrugs (PD) are novel inhibitors of HCV. DAPN-PD are metabolized into two distinct nucleotide triphosphate (TP) analogues intracellularly. The first metabolite, 2′-C-methyl-GTP, is a well-characterized inhibitor of HCV NS5B polymerase. The second major metabolite, DAPN-TP, is the triphosphorylated β-d-2′-C-methyl-2,6-diaminopurine-ribonucleoside. DAPN-TP, which harbours modifications on the base moiety and the sugar ring, could behave as an A or G-like nucleoside triphosphate (NTP). This study evaluated whether 1) DAPN-TP is a substrate for NS5B, 2) it can inhibit viral RNA-dependent polymerization in vitro, and 3) if it behaves as an A or G NTP analogue.

METHODS: NS5B enzymes from various genotypes and/or containing drug resistance mutations were cloned and purified for biochemical characterization. In vitro RNA polymerization assays were employed to determine IC50 values for DAPN-TP compared to 2′-C-methyl-GTP for wild-type (WT) and mutant enzymes. Single nucleotide incorporation assays were employed to determine the catalytic efficiency for each substrate.

RESULTS: In vitro biochemical assays showed that DAPN-TP was an inhibitor of HCV RNA polymerization. Gel-based IC50 value for DAPN-TP was found to be 3.6 μM with WT genotype 1b NS5B enzyme. Similar values were obtained for WT NS5B genotype 2a (2.1 μM) and genotype 4a (1.4 μM). The S96T mutation in the 1b genotype background did not increase susceptibility to DAPN-TP (IC50=2.0 μM), while the S282T mutation showed a 10-fold increased resistance. Mechanistic studies revealed that DAPN-TP inhibits polymerization through chain-termination, consistent with the presence of a methyl group at the 2′ position of the ribose moiety. Single nucleotide incorporation assays with various RNA templates revealed that DAPN-TP behaved like an A, and not G, nucleotide analogue. Finally, the catalytic efficiency of DAPN-TP was comparable to 2′-C-methyl-GTP, confirming that DAPN-TP is an efficient substrate for NS5B.

CONCLUSIONS: DAPN-TP represents a novel anti-HCV nucleotide analogue harbouring modifications to both the sugar and base moiety. Importantly, we demonstrated that both A and G nucleotide analogue triphosphates delivered by DAPN prodrugs were active inhibitors with unique incorporation profiles. Delivering two bioactive nucleotides intracellularly could potentially have a clinical benefit through the markedly reduced possibility of resistance emergence.
ABSTRACT 3

Antiviral Therapy 2013; 18 Suppl 1:A11

HCV GT2, GT3 and GT4 NS5B polymerases exhibit increased phenotypic susceptibility to ribavirin and a nucleoside polymerase inhibitor

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BACKGROUND: Pegylated interferon alpha (PEG-IFN) and ribavirin (RBV) is the standard of care for non-GT1 HCV, and was for GT1 viruses prior to the approval of protease inhibitors in combination with PEG-IFN/RBV. Patients infected with non-GT1 viruses typically achieve higher sustained virological response (SVR) rates following PEG-IFN/RBV treatment compared to those with GT1 viruses. Better SVRs among non-GT1 compared to GT1 viruses have also been observed in clinical trials with nucleoside polymerase inhibitors (NIs). The reasons for differential responses between genotypes are unclear, but could include viral properties and relative inhibitor susceptibilities. We evaluated inhibitor susceptibility among a panel of GT1, GT2, GT3 and GT4 viruses using a replicon-based phenotypic assay.

METHODS: NS5B was amplified from patient plasma by RT-PCR and cloned into a Con1 (GT1b) luciferase-reporter HCV replicon. Replication capacity and inhibitor susceptibility relative to a Con1 reference replicon was evaluated by measuring luminescence.

RESULTS: A panel of replicons was generated that contained patient-derived NS5B regions from different genotypes. The majority of replicons exhibited a replication capacity sufficient for evaluating inhibitor susceptibility. Susceptibility to IFN, RBV and an NI was tested to evaluate biological variation between 48 GT1(a/b), 27 GT2(a/b/unknown), 19 GT3(a/unknown) and 4 GT4 viruses. Compared to the Con1 reference, GT1, 2, 3 and 4 chimeric replicons had similar susceptibilities to IFN and GT1 replicons had similar susceptibilities to RBV and an NI. On whole, GT2, 3 and 4 chimeric replicons exhibited statistically significant increases in susceptibilities to the NI and to RBV (up to approximately 4- and 15-fold, respectively). RBV susceptibilities were most variable, with replicons containing GT3 and GT4 NS5B regions particularly susceptible to RBV.

CONCLUSIONS: A panel of HCV replicons containing patient-derived NS5B sequences from GT1-4 viruses was used to document variation in HCV inhibitor susceptibility. IFN susceptibility was similar within and between genotypes. RBV and NI susceptibility was similar among replicons with GT1a/b NS5B regions, but more variable between genotypes. A number of non-GT1 viruses exhibited relatively increased susceptibility to RBV and/or NI. This observation may contribute to improved SVR rates to RBV and/or NI containing regimens among patients infected with non-GT1 viruses compared to GT1 viruses.
ABSTRACT 4

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Early detection of NS3-resistance in HCV patients with advanced disease treated with BOC/TVR-based therapy: analysis by population- and ultra-deep sequencing

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BACKGROUND: Aim of this study is to investigate the clinical relevance of early genotypic resistance test in HCV-infected patients with advanced disease treated with triple therapy.

METHODS: 119 patients (GT1a/1b/1g=46/72/1; previous non-responders/relapsers/naive/unknown =70/32/13/4; all with advanced fibrosis/cirrhosis =60/59) were treated with pegIFN-2a/ribavirin+boceprevir (n=35) or telaprevir (n=84). IL-28 genotype was available for 65 patients (12.3%=CC; 75.4%=CT; 12.3%=TT).

Baseline prevalence and kinetics of appearance of NS3-protease resistance-associated variants (RAVs) during boceprevir/telaprevir treatment were analysed by population-sequencing (and ultra-deep 454-pyrosequencing -UDPS- in a subgroup of patients), at baseline, early time points (48 h-2 weeks-4 weeks), and at virological failure. HCV RNA was evaluated at all time points. Additional data on telaprevir TDM were obtained for 16 patients.

RESULTS: In this interim analysis, the median (IQR) follow-up time for patients treated with boceprevir was 24 (20–49) weeks and with telaprevir 24 (24–38) weeks. Virological failure was observed in 19/119 (16.0%) patients, occurred always with NS3 resistance mutations, and was strongly associated with previous non-response to pegIFN/ribavirin (14/19 patients, 73.7%).

By population sequencing, 5/82 (6.1%) patients presented at baseline linear-PIs-RAVs (4=T54S and 1=V36L). Two of them (both previous non-responders) experienced early viral failure within 4 weeks of PI-based therapy: one GT1a patient with V36L failed telaprevir treatment with the emergence of R155M; one GT1g patient with T54S failed boceprevir-based therapy without additional RAVs. The other 3 patients with baseline-T54S (1=GT1a; 2=GT1b) are still under triple-therapy. Noteworthy, one of them (GT1a) after 48 h of telaprevir treatment already showed the appearance of R155K.

Of interest, baseline UDPS (on 26 patients) showed no additional minority RAVs (cutoff=0.1%).

Of 19 patients where an early time point sequence (48 h-2 weeks) was available, 4 failed, 3 showed early emergence of RAVs. Two GT1a previous non-responders failed with V36M+R155K, after showing major species (V36M48 h) or only minor species (R155K2 weeks, UDPS-prevalence=4.1%, mutational load =301 IU/ml; V36A48 h, UDPS prevalence =2.5%, mutational load =182 IU/ml). A GT1b previous relaper with A156T2 weeks, failed without additional RAVs.

Among patients tested for telaprevir TDM, the only one that failed showed plasma concentration (Cmax: 3,143 ng/ml and Cmin: 2,504 ng/ml; AUC48 h: 123,200 ng*h/ml) lower than all other 15 patients tested (mean Cmax±sd: 5,107±2,003 ng/ml and mean Cmin±sd: 1,917±917 ng/ml; AUC48 h ±sd: 148,400±4,1940 ng*h/ml), even if still within the range of telaprevir registrative studies.

CONCLUSIONS: In this interim analysis of HCV patients with advanced disease, early detection of RAVs, either at baseline or at 48 h, was always associated with PI failure in previous non-responders. This suggests the potential clinical relevance of early genotypic resistance test in predicting viral failure in selected patients.
ABSTRACT 5

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In vitro efficacy and resistance profiling of protease inhibitors against a novel HCV genotype 3a replicon

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BACKGROUND & AIMS: Third-generation NS3 protease inhibitors (PIs) exhibit potential for pan-genotypic antiviral efficacy. However, maintaining optimal PI activity against genotype (GT) 3 has been a challenge and its assessment has been hampered by the lack of a replicon-based assay. Here, we report antiviral activity and resistance for a broad panel of PIs using a novel GT3a replicon.

MATERIALS & METHODS: A replication competent GT3a subgenomic replicon based on strain S52 was established in Huh-7-1C cells. Antiviral potency of various PIs was determined in 3-day transient replication assays. Polymorphisms, resistance mutations and chimeras were created by site-directed mutagenesis and cloning. PI resistance selection studies were performed in a stable GT3a replicon cell line.

RESULTS: All PIs tested showed substantially less potency (3–1,500-fold shifts) against the GT3a replicon relative to GT1b replicons. Notably, MK-5172, a pan-genotypic PI in clinical development had an EC50=53 nM (150-fold less potent compared to GT1b). This activity was also twofold higher than that of a GT1b chimeric replicon encoding the GT3a NS3 protease domain (3aPr/1b) suggesting an interplay of HCV non-structural proteins may influence PI potency. Existing PI resistance data suggest residue Q168 in GT3a contributes to reduced susceptibility. However, introduction of D168Q to GT1b or reversion of Q168 to D in GT3a affected the susceptibility only partially relative to wild-types suggesting additional genotypic differences influence potency. Lastly, MK-5172 resistance selections in GT3a cell lines identified mutations Q41R, R155M/K/T, A156T/S/V and Q168R/P, indicating a similar resistance pathway between GT3a and GT1.

CONCLUSIONS: A robust GT3a replicon system was established in Huh-7 cells for antiviral screening. Evaluation of PI activity revealed that GT3a poses a potency challenge for PI pan-genotypic activity, and that a GT3a subgenomic replicon provides a valuable tool for PI evaluation.
ABSTRACT 6
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Simeprevir in HCV genotype-1-infected patients: deep sequencing analyses of the PILLAR and ASPIRE Phase IIb trials

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BACKGROUND: Simeprevir (TMC435) is a potent, oral, once-daily, investigational HCV NS3/4A protease inhibitor currently in Phase III clinical development for treatment of chronic infection with genotypes 1 and 4. The potential impact of NS3 baseline minority polymorphisms on treatment outcome, and the persistence of emerging mutations in patients with treatment failure, were assessed by deep sequencing (DS) in the simeprevir Phase IIb PILLAR and ASPIRE trials.

METHODS: DS results (Illumina®, 1% detection limit) were obtained for baseline isolates from 175 simeprevir treated patients and for 187 post-baseline isolates (on-treatment and follow-up). Changes at NS3 amino acid positions 43, 80, 122, 155, 156 and 168 were analysed.

RESULTS: Minority baseline polymorphisms (that is, not observed by population sequencing [PS]) were detected in 22/175 patients (12.6%). The most prevalent minority baseline polymorphisms were S122G (n=12), S122N (n=6) and S122T (n=5), which do not affect simeprevir activity in vitro. A similar proportion of patients achieving SVR (7/62, 11.3%) and patients experiencing failure (15/113, 13.3%) carried at least one minority baseline polymorphism. In patients who failed treatment, the mutations emerging at time of failure were different from the minority polymorphisms present at baseline. In 37/44 patients (84.1%) with emerging mutations at time of failure and for whom these mutations were no longer observed by PS at end of study (EOS), no emerging mutations were detected by DS. An emerging R155K was observed at time of failure in 14/44 patients and 6 of those carried at least one minority mutation at EOS: 4 patients still carried a minority R155K and 2 carried a minority D168E mutation. An emerging D168V was observed at time of failure in 23/44 patients and none of those still carried D168V as minority mutation at EOS. One of the 23 patients (with additional emerging R155K and Q80R at failure) had a minority R155K mutation at EOS.

CONCLUSIONS: Minority polymorphisms were detected at baseline by DS but did not affect outcome of simeprevir/PegIFN/RBV treatment. Mutations emerging at time of failure that were no longer detected at EOS by PS were also no longer detected by DS in the majority of patients.
ABSTRACT 7

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Structural and biochemical insights to asunaprevir’s drug resistance profile

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BACKGROUND: Asunaprevir (ASV) is a potent HCV protease inhibitor (PI) with an enzyme inhibitory constant of 0.4–1 nM and an EC₅₀ of 4 nM. ASV has demonstrated its promise and efficacy, however, just like all PIs, it is vulnerable to drug resistance especially to mutations at R155 and D168. Here, we present biochemical and structural data explaining ASV’s drug resistance profile and propose a macrocyclic analogue with enhanced efficacy.

METHODS: ASV and its P1P3 macrocyclic analogue (ASVana) were synthesized in-house. Crystal structures complexes of WT-ASV, R155K-ASV and WT-ASVana were determined. The ASV and ASVana WT complexes were superimposed onto the HCV substrate envelope for protrusion and resistance susceptibility analysis. Enzyme inhibition constants and replicon half maximum inhibitory concentrations were determined for ASV and ASVana in WT-K80, R155K, D168A and A156T to measure the effects of single active site mutations on inhibitor efficacy and resistance profile.

RESULTS: Asunaprevir protrudes outside the HCV substrate envelope at P1’ and P2 extension moieties and is characterized by significant van der Waals contacts with the catalytic histidine (H57), aspartic acid (D81) and P2 arginine (R155). This interaction favors the formation of a salt bridge between R155 and D168. In contrast, the R155K displays reduced van der Waals interactions with H57, D81 and K155 and loses electrostatic network between amino acid 155 and D168 in favor for an interaction between D168 and R123. Kinetically, ASV sees a 43-fold change in Ki with R155 and 983 with D168. ASVana’s Ki changed from 0.57 to 420.15 nM in D168A compared to ASV’s 1.97 to 1,966.06 nM. This represents a 736-versus 983-fold change in Ki with ASVana and ASV, respectively.

CONCLUSIONS: Structurally, asunaprevir strength comes from its interactions with the invariant catalytic triad, however, its weakness derives from the P2 isoquinoline’s proximity and ensuing interaction with amino acid 155, which is prone to mutations. The fold change in enzyme inhibitory constant relative to the WT and replicon IC₅₀ values confirm the resistance susceptibility of asunaprevir and also demonstrate how a P1P3 macrocycle would enhance the ASV’s potency and drug resistance profile.
ABSTRACT 8
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Viraemic control and viral coreceptor usage in two HIV-1-infected homozygous CCR5 Δ32 individuals

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BACKGROUND: Interrupting cellular expression of CCR5 and/or CCR5Δ32/Δ32 donor stem cell transplantation are promising strategies for HIV-1 control or functional cure. However, the long-term effect of the absence of functional CCR5 on HIV-1 disease is poorly understood. We therefore studied the impact of viral genotypes and coreceptor usage on virological control in two CCR5Δ32/Δ32 viraemic controllers not receiving antiretroviral therapy (ART).

METHODS: The following in-depth viral and immunological testing of patient samples were performed: 1) longitudinal sequencing of HIV-1 gag, nef, pol and env genes, 2) viral coreceptor phenotyping from pseudoviruses incorporating full-length envelope amplicons from viral RNA and DNA, and 3) high-resolution HLA typing.

RESULTS: We studied two CCR5Δ32/Δ32 patients during the time of sustained, spontaneous virological control. Pseudoviruses derived from plasma RNA from one patient without favourable HLA alleles and viral loads <800 copies/ml were only able to use CXCR4 for entry (X4 virus). HIV-1 env population sequencing from plasma RNA and cell-associated DNA amplicons used to construct the pseudoviruses revealed a 100 amino acid deletion spanning the V4 through V5 loop. The second patient with a protective B*5201 allele had persistent low-level viraemia off ART (<600 copies/ml) for 8 years prior to virological rebound. Despite predicted CXCR4 usage by V3 genotype of plasma HIV-1 RNA and cell-associated DNA, pseudoviruses derived from plasma RNA obtained prior to viral rebound used CCR5 for entry in phenotypic assays (R5 virus). A faint entry signal observed in U87 CXCR4-expressing cells was not reproducible with subsequent phenotyping. Sequence identity was confirmed between the pseudoviruses and the patient’s plasma RNA and cell-associated DNA.

CONCLUSIONS: Sustained control of X4 virus in the setting of defective CCR5 and absence of favourable HLA alleles is possible and may be due to the presence of a large env sequence deletion. Furthermore, patients who are homozygous for the CCR5Δ32 deletion can produce R5 virus, suggesting viral use of an alternative coreceptor or entry pathway. Alternatively, phenotypic assays may not be sufficiently sensitive to detect very low-levels of CXCR4 usage. If this is the case, inefficient use of CXCR4 by plasma virus may be contributing to spontaneous virological control.
ABSTRACT 9

High diversity in reverse transcriptase drug resistance mutations relative to envelope variants in early-acute HIV transmission

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BACKGROUND: We earlier described transmission of multiple low-frequency drug-resistant variants identified in very early stages of HIV infection. Here, we examined the diversity in RT relative to envelope in transmitted drug resistance cases as well as wild-type virus infection.

METHODS: We evaluated 111 longitudinal plasma samples collected every 2–7 days from 15 seroconverters who became infected with HIV-1 subtype B in the United States in 1994–2000. Viral RNA was screened using sensitive real-time PCR assays for two thymidine analogue mutations, M41L and K70R, which were prevalent during that time and, therefore, likely candidates for transmitted resistance. We also evaluated an unlikely mutation, K65R, which had not been identified by conventional genotyping for that period. The assay detection limits for M41L, K70R and K65R were 0.8%, 2.0% and 0.3% frequencies, respectively. Samples that screened positive for any mutation were further characterized by sequencing 63 clones each from RT (590 nt including codons 41–220) and env (741 nt including V1-V3).

RESULTS: Four of 15 individuals screened positive for at least one resistance mutation at 5–50 days of viral nucleic acid detection. Low-level K65R was detected in all four individuals at frequencies of 0.4–4.9%, and the mutation always co-existed unlinked with variants carrying one to five thymidine analogue mutations (RT codons 67, 69, 70, 74 and/or 219) at frequencies between 1.6–23.0%. In one individual, variants with M184V and/or NNRTI mutations were also detected. All resistance mutations present prior to seroconversion disappeared by 5 days post-seroconversion. Three persons with resistant subpopulations had evidence of only one envelope species. A fourth person who had only minority drug resistance had multivariant env diversity similar to a wild-type transmission.

CONCLUSIONS: This extended analysis of HIV RT and env in early-acute infection revealed that transmissions of multiple high- and low-frequency drug-resistant variants can be linked to a single envelope species. The mutations co-existing with K65R suggest those variants may have originated from ddi/d4T use. Conceivably, RT can diversify under antiretroviral drug pressure in the context of a limited number of transmissible envelop species. Therefore, env does not necessarily represent the spectrum of variant diversity involved in transmission.
STRUCTURAL CHANGES INDUCED BY THE PROTEASE MUTATION V82I ARE ASSOCIATED WITH A FASTER DECLINE OF CD4 CELL COUNT IN DRUG-NAIVE HIV-1-INFECTED PATIENTS

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BACKGROUND: HIV-1 protease (PR) can cause CD4+ T-cell depletion by cleaving and activating the procaspase-8, thus potentially promoting the apoptosis of infected cells. Here, we evaluated the impact of PR mutations in the substrate-binding domain (interacting with procaspase-8) on CD4-T-cell count decline in absence of therapy.

METHODS: This study includes 816 HIV-1 B-subtype infected patients at HIV diagnosis, drug-naive with genotypic testing available, and with CD4-count ≥350 cells/ul. The impact of PR mutations on CD4-decline before HAART initiation was assessed by Kaplan–Meier analysis and multivariate cox regression adjusted by patients demographics, viraemia and CD4-count at HIV diagnosis, recent versus chronic infection, viral tropism, presence of transmitted drug resistance. Structural analysis was based on molecular dynamics simulations (MDS).

RESULTS: At HIV diagnosis, patients have a median (IQR) viraemia of 4.6 (4.1–5.1) log copies/ml and CD4-count of 535 (IQR 435–688) cells/ul. Median follow-up in absence of therapy is 15.3 (IQR 5.3; 32.6) months. Median CD4 decrease during follow-up is -130 (-267; -30) cells/ul.

All the residues involving in the substrate binding domain of the PR-enzyme (R8, L23, D25, G27–D30, V32, I47–I50, L76, T80–V82, I84) show a high degree of genetic conservation in the absence of therapy (genetic variability <1%). The only exception is represented by V82I occurring in 4.5% of drug-naive patients. At HIV diagnosis, patients with V82I have median (IQR) CD4-count comparable to patients without this mutation (V82I=536 [441; 694] cells/ul versus V82wt=534 [434; 688] cells/ul). Nevertheless, V82I correlates with a higher median CD4-decrease within 12 months since diagnosis (-135 [-291; -55] cells/ul for V82I versus -90 [-175; -28] cells/ul for V82wt). V82I also correlates with a shorter time-to-achieve CD4-count <350 cells/ul (11.9 [1.2; 24.7] months versus 29.4 [24.0; 34.8] months, P=0.001). Multivariate analysis confirms the independent correlation of V82I with an increased probability to achieve CD4-count <350 cells/ul (2.4 [1.2; 4.7], P=0.01). In presence of V82I, viraemia remains stable over time (4.5 [4.0; 5.1] log copies/ml at HIV diagnosis, 4.5 [4.1; 4.9] log copies/ml at week 24, 4.4 [3.9; 4.8] log copies/ml at week 48), supporting that the CD4-depleting effect is not mediated by an increase in viraemia levels.

CONCLUSIONS: Structural modifications induced by V82I, in the highly conserved substrate-binding domain of the protease-enzyme, tightly correlate with a faster CD4+ T-lymphocytes loss. This mutation may enhance the procaspase-8-mediated cytotoxic properties of protease, and can help in identifying patients who need a more careful monitoring and/or an earlier treatment.
ABSTRACT 11

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High level of HIV-1 drug resistance in South African adolescents failing antiretroviral treatment

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BACKGROUND: HIV-1 drug resistance (HIVDR) patterns and complexity are linked to duration and antiretroviral (ARV) regimen. HIVDR patterns in adolescents and the impact of long-term ARV on future options was investigated.

METHODS: 341 adolescents (aged 8–18 years) attending Kalafong Paediatric ARV clinic in Pretoria, South Africa, were included in this study. Viral loads (VLs) and CD4 were performed 6 monthly and used to monitor treatment success on either a non-nucleoside reverse transcriptase inhibitor (NNRTI) or protease inhibitor (PI)-based regimen. Viral failure (VF) was defined as two consecutive VLs >1,000 copies/ml. Viral RNA was extracted, an RT-initiated PCR performed and full-length protease and reverse transcriptase sequenced. HIVDR and subtype were determined using the IAS-USA mutation list and Rega Subtyping Tool, respectively.

RESULTS: 76% (n=260/341) were started on an NNRTI-based regimen and found to be more likely to experience VF than those starting a PI-based regimen (P<0.05). Sixty-three adolescents experienced VF (18%), were HIV-1 subtype C, had been on treatment for a median of 5 years and 80% initiated treatment with a >WHO stage 3. Median CD4+ T-cell and VL was 484 cells/mm3 and 3.97 log copies/ml, respectively. The most prevalent mutations were M184V/I (n=50; 89%), K103N/S/R (n=41; 73%) and V106M (n=23; 41%). Two (2/63) adolescents had no resistance mutations. Eighteen (28%) had complex mutation patterns (thymidine analogue mutations [TAMS] n=16; K65R n=2). Two of the 16 adolescents with TAMS had >3 and TAM pathway 2 was most frequent. N348I was observed in 12% of subjects accessing a failing EFV-based regimen. Three subjects, on ritonavir-based regimens, had PI-associated mutations. Reduced susceptibility to ETV and RPV was observed in 31% of adolescents.

CONCLUSIONS: 96% of adolescents on a failing regimen harboured HIVDR; this is in contrast to observations in adult cohorts. A third of the cohort had high-level resistance to NRTIs, impacting on future options. Furthermore, 31% of adolescents have resistance to second generation NNRTI, and additional work is needed to determine the effectiveness of these in this population. These findings have implications for future treatment options and indicate the need for long-term treatment strategies to be developed for adolescents.
ABSTRACT 12

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Accurate prediction of response to HIV therapy without a genotype: a potential tool for therapy optimization in resource-limited settings

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INTRODUCTION: In well-resourced settings, genotypic resistance testing (GRT) plays a major role in the selection and sequencing of combination antiretroviral therapy (ART) for the long-term suppression of HIV. GRT is unavailable in most resource-limited settings (RLS) so optimal drug selection is a challenge. Here, we report the development and evaluation of the latest computer models to predict response to ART without GRT.

METHODS: Random forest (RF) models were trained to predict the probability of virological response (viral load <50 copies/ml) using baseline viral load, CD4 count, treatment history, new drug regimen and time to follow-up from 22,567 treatment change episodes (TCEs) following failure. The models were assessed during cross-validation, with an independent test set (n=1,000) and 100 TCEs from sub-Saharan Africa (100 SSA). The area under the ROC curve (AUC) was the main outcome. The predictive accuracy of the models was compared to GRT using genotypic sensitivity scores (GSS) generated by three rules-based interpretation systems. They were also used to identify potentially effective drug regimens for cases of virological failure following a treatment change in RLS.

RESULTS: The models achieved a mean AUC of 0.82 during cross validation, 0.80 with the 1,000 test set and 0.78 for the 100 SSA set. Of the 1,000 test TCEs, 346 had genotypes available and the AUC for the GSS ranged between 0.56 and 0.57, which was significantly inferior to the accuracy of the models (P<0.0001). The models identified alternative 3-drug regimens, comprising locally available drugs, with higher probabilities of response for 96% of cases in RLS where the new treatment had failed.

CONCLUSIONS: These models, trained with the largest dataset so far, were able to predict response to HIV therapy significantly more accurately than genotypes with the most commonly used interpretation systems. Despite being trained with data mostly from well-resourced settings they were accurate for cases from RLS. The models have the potential to predict and avoid treatment failure by identifying effective, alternative, practical regimens and have potential clinical utility for RLS.

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**ABSTRACT 13**

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Prevalence of transmitted drug resistance and relation to mean population viral load of treatment failing patients: a 16-year analysis within the Swiss HIV Cohort Study (SHCS)

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BACKGROUND: Incidence and prevalence of drug resistance mutations (DRM) in treated patients have declined in the SHCS, while prevalence of transmitted drug resistance mutations (TDM) remained stable. To investigate this discrepancy, we studied the population level association of DRM with TDM in recently infected, treatment-naive patients (RINP).

METHODS: We included RINP with genotypic resistance tests from 1996–2011. Recent infection was defined by seroconversion dates or ambiguity score <0.5%. Time trends and risk factors (ethnicity, gender, transmission mode, subtype and infection year) associated with TDM were analysed by logistic regression.

Possible correlation of annual TDM transmission with potential transmitters (patients with replicating virus on ART) was analysed with Poisson regression. We analysed for all TDM combined and individually for the most prevalent mutation in each drug class: M184V, L90M and K103N. As transmitters in the ‘combined’ analysis we calculated the annual mean viral load (MVL) of all patients in the SHCS failing ART (VL>400 c/ml after 180 days continuous ART). For individual mutations the number of patients failing ART and carrying the corresponding mutation was used. We further limited the population for TDM to those in Swiss transmission clusters (STC) defined phylogenetically and repeated the analyses.

RESULTS: We included 2,279 RINP. TDM transmission over 16 years ranged between 2.26–12.71% to any drug, 2.26–9.62% to NRTI, 0.75–4.97% to PI and 0–5.20% to NNRTI. No time trend was observed. Subtype B was more associated with TDM than non-B (odds ratio =1.64 [95% CI, 1.07–2.52], P=0.023, multivariate analysis).

The annual TDM rate showed a positive, non-significant association with MVL of failing patients from the previous year (incidence rate ratio [IRR]=1.18 [95% CI, 0.46–2.99], P=0.73). However, transmission of M184V correlated significantly with its prevalence in patients failing ART from the previous year (IRR=3.45 [95% CI, 1.64–7.25], P=0.001). Both relations became stronger when focusing on STC (IRR=4.48 [95% CI, 0.88–22.77], P=0.07, and IRR=19.79 [95% CI, 1.03–378.74], P=0.047). No significant correlation for L90M (IRR=0.36 [95% CI, 0.12–1.08]) or K103N (IRR=1.58 [95% CI, 0.44–5.74]) was observed.

CONCLUSIONS: Transmission of mutations with high fitness cost may rely largely on failure of ART, whereas transmission of mutations with low fitness cost depends more on treatment-naive patients.
ABSTRACT 14

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X4 tropic viruses are on the rise in recent HIV-1 seroconverters

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BACKGROUND: Transmission of HIV-1 drug resistance mutations (DRM) in Spain seems to remain stable around 13%. However, the profile of new HIV-1 seroconverters has experienced significant changes. Herein, we describe the main virological features in the HIV Spanish Seroconverter Cohort, which was established 16 years ago, highlighting the major findings with respect to changes in non-B subtypes, DRM and HIV tropism.

METHODS: Retrospective, cross-sectional analyses were performed in the national database of recent HIV-1 seroconverters (proven <12 months from exposure). Demographics and laboratory parameters (plasma HIV RNA, CD4 counts, HIV-1 subtype, tropism and major DRM) were recorded from January 1997 to December 2012.

RESULTS: A total of 1,031 recent HIV-1 seroconverters were identified (92.2% male; median age 31 years; 84% MSM). Their median estimated time from infection was 7 (IQR 5–10) months. By the time of diagnosis, median plasma HIV RNA and CD4 counts were 4.5 (4.1–5.0) log copies/ml and 553 (408–737) cells/mm³, respectively. A total of 123 individuals (13.4%) carried HIV-1 non-B subtypes, increasing from 0 in the period 1997–2000 to 18.1% in the period 2010–2012 (P=0.002).

Major HIV-1 DRMs were recognized in 13.2%, being 7.2% for NRTI, 5.9% for NNRTI and 3.1% for PI. The rate did not differ much comparing B and non-B viruses (13.4% versus 11.6%, P=0.57). Whereas NRTI resistance mutations significantly decreased from 23.7% in 1997–2000 to 4.3% in 2010–2012 (P<0.01), transmission of NNRTI resistance mutations remained stable around 6%.

Overall, X4 viruses were found in 18.4% of HIV-1 seroconverters. The rate increased from 11.1% before 2003 to 23.8% since year 2010 (P=0.03). Interestingly, median CD4 counts were significantly lower in seroconverters diagnosed during the last period (691 versus 556 cells/mm³, P=0.009). This observation persisted after adjustment for potential confounders.

CONCLUSIONS: Transmission of HIV-1 drug resistance remains overall stable around 13% in Spain. However, NNRTI mutations are currently the most frequently transmitted. Moreover, non-B subtypes are significantly on the rise currently accounting for 18% of new HIV-1 infections. Finally, we found evidence for more frequent transmission of virulent strains associated with X4 tropism and more rapid CD4 depletion.
Multiple identical proviral sequences with identical integration sites demonstrate proliferation of HIV-infected cells during suppressive antiretroviral treatment

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BACKGROUND: Understanding the mechanisms of HIV persistence during suppressive antiretroviral treatment (ART) could provide insight into designing strategies to cure HIV. Identical or ‘monotypic’ HIV DNA sequences have been observed to progressively increase during effective ART, suggesting proliferation of cells with integrated proviruses. We hypothesized that monotypic HIV sequences arise due to cellular proliferation and therefore have identical chromosomal integration sites. To test this hypothesis multiple integration sites and associated env sequences were examined.

METHODS: We developed a new method that allows end point amplification of DNA fragments that extend from proviral HIV to randomly primed sites in the human genome. Sequencing of individual integration sites allows design of integration-site-specific primers for each integrant. Envelope sequence(s) linked to each integration site were amplified using integration-site-specific and env primers. The frequency of each integration site and the diversity of proviruses at each integration site were determined.

RESULTS: Fifty-five integration sites were sequenced from separate PCR reactions from specimens collected after 6.2 and 7.1 years of suppressive ART from one individual (other subjects in process). Integration sites were identified in 15 different chromosomes. As in previous studies, integration was most common in chromosome 17. Six integration sites were identified more than once, resulting in 39 unique integrations sites. The most common integration site comprised 18% (10/55) of sequences, and the associated env sequence was identical to the predominate monotypic sequence cluster previously reported to be increasing during >11 years of suppressive ART.

CONCLUSIONS: Identical env sequences with identical integration sites suggests that cellular proliferation is a significant mechanism of HIV persistence, which should be considered in the design of strategies to cure HIV infection.
ABSTRACT 16

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Persistent elevation in HIV viraemia during cART with identical WT sequences imply expansion of a clonal source

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INTRODUCTION: The source of persistent viraemia during suppressive antiretroviral therapy is unknown. We cared for an individual who developed persistent low level viraemia >50 c/ml after 11 years of cART. To investigate the origin of this viraemia, we carried out detailed quantitative and phylogenetic analysis of the HIV populations in this individual’s plasma samples.

METHODS: The patient was enrolled in a NIH natural history study of HIV infection and had received cART for over 11 years. He had 2 prior brief cART interruptions (months 72 and 108) but plasma HIV RNA returned to <50 c/ml until he developed viraemia of 200–300 c/ml persisting >6 months during which oral squamous cell carcinoma (SCC) was diagnosed; SCC contained both infiltrating CD4 and CD8+ T-cells, without distant metastases. We analysed HIV populations in plasma obtained prior to cART, during suppressive cART, and following rebound, using single genome sequencing.

RESULTS: Pre-therapy, (HIV RNA=238,000 c/ml, CD4=22 cells/μl) HIV RNA was genetically diverse with no drug resistance mutations detected by SGS. Treatment interruptions resulted in rebound viraemia containing distinct populations of identical sequences containing wild-type (WT) and drug-resistant HIV. Low level viraemia (330 c/ml; CD4=164 cells/μl) emerged after 11 y on cART; SGS revealed that the rebound contained both WT and drug-resistant HIV. The WT population consisted largely of multiple identical sequences; the resistant population comprised diverse variants encoding K103N+M184V. Switch to TDF+FTC+RTG (directly observed) produced a 10-fold reduction of the drug-resistant variants, but only a 2-fold decrease in the WT variants. SCC radiation/chemotherapy (8 weeks) reduced peripheral CD4 to 50–70 cells/μl, but viraemia persisted (60–90 c/ml) throughout, and contained only WT HIV, revealing that the source of the WT sequences was likely a stable reservoir, not ongoing replication. Viraemia decayed to <50 c/ml but rebounded after SCC recurred.

CONCLUSIONS: WT, drug-sensitive viraemia >50 c/ml, consisting of identical sequences, can arise and persist on cART. The emergence of clonal, WT viraemia on cART and its insensitivity to cART implies that the source of viraemia was an expanded clone of HIV-infected cells and/or increased HIV production from that clone.
ABSTRACT 17

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Development of a primary cell model of HIV-1 latency in naive CD4+ T-cells

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BACKGROUND: HIV-1 DNA can be readily detected in naive CD4+ T-cells from ART-naive and -experienced HIV-infected individuals. In contrast, HIV-1 DNA was rarely detected in naive CD4+ T-cells from post-treatment controllers in the ANRS Visconti Study. Taken together, these studies suggest that naive CD4+ T-cells constitute a persistent reservoir of HIV-1 infection, and that reduction/elimination of this reservoir may be important to achieve a functional cure. In this regard, the goals of this study were: (i) to develop a primary cell model of HIV-1 latency in naive CD4+ T-cells; and (ii) to compare the kinetics of virus reactivation in naive and central memory (T_CM) CD4+ T-cells.

METHODS: Total resting, naive and T_CM CD4+ T-cells were isolated from uninfected donors. These cells were then treated with 100 nM CCL19 and infected with HIV-1_LAI as described previously. Infection was assessed by qPCR of total HIV-1 DNA and 2LTRs. T-cell activation was assessed by flow analysis of CD69, CD25 and HLA-DR expression. Virus production was measured by quantification of viral RNA in the supernatant before and after exposure to 10 nM PMA+10 μg/ml PHA or anti-CD3/CD28 microbeads.

RESULTS: We found that CCL19-treated naive and T_CM CD4+ T-cells were equally susceptible to HIV-1 infection. Neither CCL19 nor HIV-1 infection promoted T-cell activation. Following HIV-1 infection of the CCL19-treated cells we observed essentially no virus production. However, virus production could be stimulated by PMA/PHA or anti-CD3/CD28. Interestingly, robust virus production was observed as early as 3 days post-stimulation in the HIV-infected T_CM CD4+ T-cells. In comparison, much less virus was produced from the HIV-infected naive CD4+ T-cells, and the kinetics of virus production was also much slower. Collectively, these data suggest that the two resting CD4+ T-cell subsets analysed here reactivate differently.

CONCLUSIONS: We have developed a primary cell model of HIV-1 latency in naive CD4+ T-cells. This model can be used to determine whether the mechanisms responsible for the establishment and reversal of latency differ in naive versus memory CD4+ T-cells.
**ABSTRACT 18**  
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Virus populations in plasma and tissues are well-mixed in RT-SHIV-infected macaques

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**BACKGROUND:** Determining which anatomic compartments contribute to plasma HIV is critical to understanding the sources of residual viraemia during cART. We analysed RT-SHIV RNA and DNA populations in the plasma and tissues from infected macaques to identify the sources of persistent viraemia.

**METHODS:** Tissues were collected at necropsy from four pigtailed macaques infected for 30 weeks with a diverse population of RT-SHIVmne. Two (6760 and 8232) were untreated and two (8030 and 8272) were treated with EFV+TNV+FTC for 20 weeks. Plasma RNA levels were measured weekly and tissue RNA/DNA levels were measured at necropsy. A total of 1,430 single-genome HIV RT DNA and RNA sequences were analysed from the plasma, PBMCs, lymph nodes, spleen, thymus, gut tissues, bone marrow and lung. Populations of viral DNA in each tissue were compared to plasma virus using phylogenetic and panmixia tests.

**RESULTS:** The levels of plasma viraemia and the number of viral DNA copies was substantially lower in 8232 compared to 6760 (untreated animals): mean 77 versus 265 DNA copies/10^6 cells. Plasma RNA levels in both treated animals were undetectable and the mean DNA levels were 41 and 113/10^6 cells (8030 and 8272, respectively). Phylogenetics showed that HIV DNA populations in tissues were not different from virus populations in plasma in either the treated or untreated animals. Sometimes, differentially expanded clones in tissues suggested genetic separation which disappeared when the clones were collapsed to a single sequence. Analyses of intracellular RNA populations revealed that the majority of proviruses in tissues from 8232 were not expressed, whereas tissues from 6760 had a greater proportion of proviruses being expressed. Few intracellular RNA sequences were detected in treated animals and most contained frame shifts or large deletions.

**CONCLUSIONS:** This study suggests that the level of plasma viraemia in untreated macaques is related to both the number of infected cells and to the proportion that express RNA, that virus populations in plasma and tissues are well-mixed in treated and untreated macaques suggesting frequent genetic exchange between plasma virus and tissues, and that intracellular RNA expressed during treatment is frequently defective.
ABSTRACT 19

HIV-1 DNA decline during long-term first-line NNRTI-based ART with consistent plasma HIV-1 RNA suppression below 50 copies/ml

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BACKGROUND: Whether HIV continues to replicate during seemingly suppressive ART remains controversial. Although HIV-1 DNA and HIV-1 RNA persistence in patients receiving long-term ART has been reported, treatment regimens and definition of virological suppression were heterogeneous in previous studies. We investigated cellular HIV-1 DNA load and residual plasma HIV-1 RNA detection in patients receiving long-term first-line NNRTI-based ART and showing a plasma HIV-1 RNA load (VL) persistently <50 copies/ml, and correlated the findings to duration of VL suppression and plasma NNRTI concentrations.

METHODS: Eligible patients started first-line ART with efavirenz or nevirapine (no change allowed) plus two NRTIs (changes allowed), achieved a VL <50 copies/ml within 6 months, and subsequently had all VL results <50 copies/ml (≥2 measurements/year) without blips or treatment interruptions. Recruitment was stratified by ART duration in 10 groups (10 patients each) spanning 1 to ≥10 years. HIV-1 DNA and HIV-1 RNA levels were measured by real-time PCR with 50%/95% detection thresholds of 20/40 HIV-1 DNA copies/10^6 PBMC and 1/3 HIV-1 RNA copies/ml, respectively.

RESULTS: The study recruited 104 adults that had started ART (84% efavirenz) in 1997–2011. HIV-1 DNA was detected in 102/104 (98%) samples at median 3.2 (range 1.5–4.4) log_{10} copies/10^6 CD4 T-cells. Higher HIV-1 DNA levels were independently predicted by Black versus White ethnicity (0.49 [0.18, 0.80]; P=0.002), higher baseline VL (0.34 [0.18, 0.51]; P<0.001), and NRTI changes (0.42 [0.07, 0.76]; P=0.019). In adjusted analyses, mean HIV-1 DNA levels declined by -0.77 log_{10} copies/10^6 CD4 T-cells (-1.50, -0.04; P=0.040) over 10 years. HIV-1 RNA was detected in 52/105 (50%) samples at median 3 (range 1–35) copies/ml; there was no association with HIV-1 DNA levels or demographic, clinical and treatment-related factors, including NNRTI concentrations. The adjusted mean HIV-1 RNA change over 10 years was -0.12 (-0.52, 0.27; P=0.531).

CONCLUSIONS: A decline in HIV-1 DNA levels was measured over time in these highly stably treated patients. HIV-1 RNA persisted in plasma at levels typically <10 copies/ml; detection was not predicted by any of the measured parameters, supporting the hypothesis that observing very low levels of HIV-1 RNA in plasma is not indicative of ongoing virus replication.
ABSTRACT 20
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Relationship and clinical significance of soluble markers of inflammation and low-level viraemia in HIV-1 elite controllers

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BACKGROUND: HIV-1 elite controllers (ECs) represent an ideal population to study the effects of HIV persistence on chronic inflammation in the absence of antiretroviral therapy (ART). We examined differences in inflammatory marker levels between ECs, ART-suppressed non-ECs, and HIV-negative controls. We also evaluated the relationship between inflammatory markers, viral load (VL), and CD4+ cell slope in ECs.

METHODS: HIV-1 ECs were identified from the International HIV Controllers Study. Soluble markers of inflammation (sCD14, sCD163, IP-10, MCP-1 and hsIL-6) were compared between ECs and cohorts of ART-suppressed non-ECs and HIV-negative controls using k-sample rank based tests. Spearman correlations between longitudinal measurements of VL and 22 soluble markers of inflammation in ECs were estimated using bootstrap analysis with 10,000 iterations; significance testing used generalized estimating equations.

RESULTS: Soluble markers of inflammation were available for 49 HIV-1 ECs, 84 ART-suppressed chronically infected non-ECs, and 49 HIV-negative participants. HIV-1 ECs had higher levels of sCD14 compared to either the ART-suppressed or HIV-negative controls (ECs versus ART-suppressed: median 1,355 ng/ml versus 416 ng/ml, P<0.001; ECs versus HIV-negative: 1,355 versus 241 ng/ml, P<0.001). Levels of sCD163 were not significantly different between the groups but both groups had significantly higher levels than HIV-negative controls (ECs versus ART-suppressed: 1,102 versus 847 ng/ml, P=0.04; ART-suppressed versus HIV-negative: 1,247 versus 847 ng/ml, P=0.002). No significant differences were noted between the groups in levels of IP-10, MCP-1, or hsIL-6. HIV-1 VL was significantly correlated with interferon-γ and interferon-γ-induced protein 10 (IP10) levels (interferon-γ: Spearman ρ=-0.11, P<0.001; IP-10: Spearman ρ=0.21, P<0.001), but not with levels of sCD14 or sCD163. Higher HIV-1 viral load, but not soluble markers of inflammation, was significantly associated with CD4+ cell decline in ECs.

CONCLUSIONS: Markers of monocyte activation were higher in ECs than HIV-negative individuals and in some cases higher than in ART-suppressed non-ECs. VL in ECs was associated with levels of interferon-γ, IP-10, and CD4+ cell slope, but not monocyte activation markers. In ECs, factors other than VL also contribute to persistently elevated soluble markers of inflammation and a multifaceted approach may be needed to optimally reduce inflammatory markers.
ABSTRACT 21

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Analysis and characterization of treatment-emergent resistance in ART-experienced, integrase inhibitor-naive subjects with dolutegravir (DTG) versus raltegravir (RAL) in SAILING (ING111762)

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BACKGROUND: SAILING was a Phase III clinical trial evaluating DTG 50 mg once daily versus RAL 400 mg twice daily in ART-experienced, integrase inhibitor (INI)-naive, HIV-1-infected subjects. Subjects also received investigator-selected background regimen of ≤ 2 drugs, at least one fully active.

METHODS: Protocol defined virological failure (PDVF) required HIV-1 RNA >400 c/ml (assessed by Abbott assay) confirmed. PDVF non-response was <1 log₁₀ c/ml decrease by week 16, unless <400 c/ml, OR ≥400 c/ml on or after week 24. PDVF rebound was ≥400 c/ml after confirmed <400 c/ml, OR >1 log₁₀ c/ml above nadir of ≥400 c/ml. Integrase genotypes and phenotypes were assessed by Monogram BioSciences. Minor variant clonal analyses were performed at baseline and PDVF. HIV-1 WT and mutant integrase proteins were used for 3H-labelled INI dissociation experiments.

RESULTS: By week 24, PDVF was more frequent under RAL (34/361; 9%) versus DTG (14/354; 4%), and fewer subjects failed with INI-genotypic or -phenotypic resistance on DTG (2/354, 0.6%) versus RAL (10/361, 2.8%), P=0.016. At PDVF, two subjects on DTG harboured HIV-1 with either R263K (and V260I) OR R263R/K substitutions; one subject had low plasma DTG levels and one had fluctuating HIV-1 RNA course on study. In each case, <2 fold-change in IC50 was observed for both DTG and RAL. Clonal data for the R263R/K mixture virus showed 100% R263R at baseline, with preferred codon usage AGG; at PDVF R263K frequency was 54.2% (# clones =96) with preferred codon usage AAA, while R263R frequency was 45.8% with AGA as the preferred codon. DTG dissociation from integrase protein:DNA complexes with V260I was equivalent to WT; for R263K or V260I_R263K DTG dissociation was faster than WT but binding remained prolonged with half of the 3H-DTG retained for 50 h.

CONCLUSIONS: Emergent integrase substitutions K or R/K at IN position R263 were observed on DTG, but conferred <2-fold increase in IC50 for both DTG and RAL. Subjects receiving DTG were significantly less likely to experience PDVF and integrase resistance than those who received RAL in this study. Together with results from other clinical trials and preclinical data, these findings demonstrate DTG’s higher barrier to resistance.
ABSTRACT 22

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Advanced mechanistic studies of GSK1265744, a new HIV integrase inhibitor (INI) dosed by oral administration or long-acting parenteral injection

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BACKGROUND: The INI GSK1265744 (GSK744, S/GSK1265744) has low nM potency and an improved in vitro resistance profile over previous generation INIs; also no resistant virus emerged in vitro passage study with wild-type virus for 112 days. GSK744 has favourable pharmacokinetic properties in early human trials following oral administration; GSK744 30 mg once daily monotherapy produced a mean 2.5 log10 copies/ml decrease in HIV-1 RNA at day 11. Additional GSK744 attributes (low solubility, low clearance) support infrequent parenteral dosing (≥Q30 days). This approach may enable improved adherence and efficacy for both HIV therapy and pre-exposure prophylaxis (PrEP). We report here further mechanism studies of GSK744 including 2-LTR measurements, in vitro passage with raltegravir (RAL)-resistant viruses, and activity against a broad panel of INI resistant site directed mutant (SDM) HIV-1.

METHODS: Quantitative PCR analysis was performed to measure synthesis of HIV DNA species in MT-4 cells in the presence of an INI or NNRTI. Wild-type and RAL-resistant SDM clones were passaged in MT-2 cells under increasing GSK744 concentrations and the IN gene sequenced. Fold change in potencies were calculated as the ratio of mutant EC50 versus WT EC50 using a HeLa-CD4 assay.

RESULTS: GSK744 inhibited integration of viral DNA with a concomitant increase in 2-LTR circles, and with no effect on viral DNA production. In passage studies starting with wild-type, E92Q, or N155H SDMs, virus could not replicate at >6.4 nM GSK744, and additional mutations were not observed during passage. However, Q148K/R/H SDM viruses could replicate at up to 160 nM drug concentrations and additional mutations accumulated dependent on the culture duration. A broad panel of resistant viruses (49 SDMs) showed GSK744 to have greater activity and a distinct resistance profile compared with previous generation INIs. However some Q148 pathway derived viruses with multiple additional mutations had reduced sensitivity to GSK744.

CONCLUSIONS: Taken together with earlier work, the mechanism of action of GSK744 was confirmed to be inhibition of HIV-1 integrase. These data further demonstrate the distinct resistance properties and next generation profile of GSK744 and support continued development for treatment and PrEP.
ABSTRACT 23

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Antiviral activity of tenofovir alafenamide (TAF) against major NRTI-resistant viruses: improvement over TDF/TFV is driven by higher TFV-DP loading in target cells

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BACKGROUND: Tenofovir alafenamide (TAF) is a new prodrug of the HIV-1 NtRTI tenofovir (TFV) that has shown improved antiviral activity at lower doses than the current prodrug tenofovir disoproxil fumarate (TDF) in clinical studies. TAF also results in lower plasma TFV levels and a fivefold increase in intracellular TFV-diphosphate (TFV-DP) in PBMCs. The TAF resistance profile in clinical settings has not been established. Therefore, TAF activity against NRTI-resistant HIV-1 clinical isolates was evaluated and compared in multiple assays.

METHODS: Drug-resistant HIV-1 clinical isolates containing NRTI mutations were selected to establish a comparative activity profile between TAF and TDF/TFV. Antiviral activity was evaluated in multiple assays, including PhenoSense™, for which TDF/TFV clinical cutoffs have been established, and in MT-2 assays. TAF resistance profile was further assessed in viral breakthrough experiments at clinically relevant drug concentrations using NRTI-resistant mutants. Intracellular TFV-DP levels were measured by LC/MS.

RESULTS: NRTI-resistant HIV-1 isolates (n=25) with complex RT mutation patterns such as K65R, 3–5 TAMs, Q151M complex, or T69ins, with or without M184V, were profiled in MT-2 assays. TAF antiviral activity fold change (FC) ranged from 1.2 to 23.7, and was highly correlated to TFV FC for each mutant (R²=0.96). In PhenoSense, these isolates showed FC for TFV of 1.63 to 27. FC between these two assays showed good correlation (R²=0.78), with assay format (multiple versus single cycle) and replication capacity potentially accounting for differences. TAF loading and TFV-DP formation were also studied in vitro with PBMCs and MT-2 cells to model PBMC loading observed in clinical studies. In viral breakthrough experiments, viruses with K65R or up to 4 TAMs were only partially inhibited with TDF/TFV, but were fully inhibited by TAF at clinically relevant concentrations. For viruses with the highest FC, TAF could achieve partial inhibition while TDF/TFV was inactive.

CONCLUSIONS: Consistent with the greater antiviral activity of TAF observed clinically, TAF demonstrated superior antiviral potency than TDF/TFV in vitro. The higher intracellular TFV-DP levels achieved with TAF can maintain antiviral activity against previously defined TDF-resistant isolates, suggesting a potential benefit of TAF usage in patients with NRTI resistance.
ABSTRACT 24

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Low frequency of amino acid changes associated with resistance to attachment inhibitor BMS-626529 in R5- and X4-tropic HIV-1 B subtypes

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BACKGROUND: BMS-626529 is a novel attachment inhibitor that targets HIV-1 gp120 and inhibits its binding to CD4+ T-cells. In the only in vivo study of BMS-626529 8 days monotherapy, administrated as its prodrug form to patients infected by HIV-1 B subtypes, env substitutions M426L and S375M were found to be strongly associated with low susceptibility to BMS-626529. M434I, S375T and M475I also contributed to loss of phenotypic susceptibility in some non-responders. Currently, there is a lack of information on primary resistance of HIV-1 B subtypes to attachment inhibitors suggesting the need for such analyses.

METHODS: Sequences of env gp120 from 109 patients infected by HIV-1 B subtypes and attachment inhibitor naive were analysed both for tropism and presence of previously described combinations of mutations linked to resistance to BMS-626529 (S375M/T, M426L, M434I, M475I). The genotypic determination of tropism was realized using Geno2pheno algorithm with a false positive rate of 10%.

RESULTS: The sequence analysis showed a total conservation for 1 out of the 4 analysed amino acid residues (position M475). 50 of 109 (45.9%) patients showed no modifications of amino acid residues on the 4 analysed positions. The M426L, considered as the key resistance substitutions, was observed in 8 viruses (7.3%); 2 M426L were associated with a S375T and 2 with S375N, respectively. The S375M, responsible for the reduced susceptibility to BMS-626529, was found in 2 viruses without variations on the other studied positions (1.8%). The S375T and M434I contributing to loss of phenotypic susceptibility in some non-responders were detected in 31 (28.4%) and 7 (6.4%) viruses, respectively. The tropism of the 109 patients was analysed and 69 (63.3%) versus 40 (36.7%) viruses were determined as R5- and X4-tropic, respectively. The frequency of primary resistance mutations was not different between R5- and X4-tropic viruses: 37 R5/22 X4 and 32 R5/18 X4 with or with no variations on the 4 positions, respectively.

CONCLUSIONS: A low prevalence of primary resistances to BMS-626529 was observed for this population of patients infected by HIV-1 B subtype. Furthermore, there was no difference of the mutation’s distribution according to the virus tropism.
ABSTRACT 25

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HIV-1 vpu and env mutations contribute to a novel mechanism of resistance to CCR5 entry inhibitors

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BACKGROUND: Entry inhibitors targeting CCR5 can reduce or prevent HIV-1 infection. However, resistance to CCR5 inhibitors can develop by at least two mechanisms. Virus entry via CXCR4 can be selected, or HIV-1 can evolve to use inhibitor-bound CCR5 for entry. Only the first mechanism has been demonstrated for macromolecular CCR5 inhibitors such as 5P12-RANTES. Here, we describe a third and novel form of resistance to 5P12-RANTES.

METHODS: HIV-1 isolate CC1/85 was passaged weekly in primary CD4 T-cell cultures containing 0.12–10 nM 5P12-RANTES. Resistant virus was isolated after 70 and 91 weeks of selection, and characterized for sensitivity to soluble CD4, mAb to CCR5, CCR5 entry inhibitors, and neutralizing mAb. Env clones were generated for sequencing and used in single cycle entry assays. Vpu clones were sequenced and used in CD4 and BST-2/tetherin downregulation assays. Cell-to-cell transmission efficiency was measured in limiting dilution assays of input infected cells.

RESULTS: Sixteen amino acid substitutions in gp120 and gp41 and four in vpu led to inhibitor resistance that correlated with more efficient use of both CD4 and CCR5 for entry and a 20–25-fold increase in the efficiency of cell-to-cell transmission. Mutations in vpu caused a loss of tetherin/BST-2 and CD4 downregulation, changes potentially associated with increased cell-to-cell virus transmission. These changes may explain the observation that single-round entry assays with cloned envelopes showed significant resistance to both 5P12-RANTES and maraviroc, but multiple round virus replication assays resulted in only marginally increased resistance. Resistance did not involve use of CXCR4 or alternative coreceptors, and CCR5 expression was required for infection of primary target cells. Resistant virus was more sensitive to neutralization with 2G12 mAb and more resistant to VRC01 and 447-52D mAb.

CONCLUSIONS: These results suggest that resistance to entry inhibitors can be achieved by increasing entry efficiency via CD4 and CCR5 and by improving cell-to-cell transmission, and that mutations of multiple HIV-1 genes can contribute to resistance. We note that resistance was acquired at a significant cost to fitness and it is unlikely that all these mutations could accumulate in virus in a patient undergoing treatment.
ABSTRACT 26

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Population dynamics of R5-tropic HIV-1 associated with maraviroc resistance

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BACKGROUND: HIV type-1 enters CD4+ cells using both CD4 and a chemokine co-receptor: CCR5 or CXCR4. The CCR5 antagonist maraviroc binds to the CCR5 receptor on the cell membrane, thus inhibiting viral entry of R5-tropic HIV-1. Resistance to maraviroc is most commonly observed through a shift in HIV tropism: a previously dominant R5-tropic population subsides to a CXCR4-using population, which may be attributable to minority CXCR4-using strains present prior to therapy commencement. In rare cases, however, drug resistance can develop such that R5-tropic HIV is able to circumvent the antagonist-bound CCR5 co-receptor. In this study we characterize the evolutionary dynamics that occur to give rise to this form of resistance, namely maraviroc resistant R5-tropic strains.

METHODS: We analysed temporally sampled envelope protein sequences from 18 patients from the clinical trials MOTIVATE 1 and 2. Maraviroc susceptibility was assessed using the Monogram Phenosense™ assay, providing a maximal percentage inhibition (MPI) reading. We performed phylogenetic analysis using the BEAST framework, including Bayesian skyline analysis to determine variance in population size.

RESULTS: Resistant viruses were found monophyletically to branch out of a previously existing population susceptible to maraviroc. Acquisition of resistance appeared to be associated with a genetic bottleneck, indicating de novo evolution of resistance as opposed to the presence of low frequency pre-existing viruses, as is the case with the emergence of CXCR4-using virus resistance. In addition, Bayesian skyline analysis indicated a genetic bottleneck occurring immediately after trial commencement in 10 out of the 18 patients, with a sudden drop in effective population size by up to an order of magnitude. A rebound was subsequently observed to pre-therapy levels after resistance develops.

CONCLUSIONS: Our study investigates the population dynamics relating to the development of resistance of R5-tropic HIV-1 to the CCR5-antagonist maraviroc while maintaining R5-tropism. In contrast to resistance developing through the emergence of a CXCR4-using population, likely attributable to pre-existing CXCR4-using strains, R5-tropic maraviroc resistance likely emerges de novo.
ABSTRACT 27

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Examining the role of C-terminal domain of HIV-1 reverse transcriptase p51 subunit in positioning and hydrolysis of RNA/DNA hybrids

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**BACKGROUND:** Previous structural studies suggest a potential role for the C-terminal domain of the p51 subunit in providing a structural support and facilitating p66 loading onto nucleic acids. However, the observation that deleting the C-terminal residues 428–440 of p51 significantly alters RNase H activity implied these residues might be actively involved in substrate recognition and hydrolysis. Recent crystallographic analysis of p66/p51 HIV-1 RT complexed with an RNA/DNA hybrid has illuminated novel and important contacts between structural elements at the C terminus of the p51 subunit and the nucleic acid duplex close to the RNase H active site.

**METHODS:** We have taken advantage of this crystal structure to examine the role of the p51 C terminus by evaluating RNase H and polymerase activities of selectively-mutated p66/p51 heterodimers carrying (i) a p51 truncation, (ii) alterations that interrupt the N348-Y427 interaction and (iii) alanine substitutions throughout the region F416–P421.

**RESULTS:** Mutants p66/p51Δ13 and p66/p51Y427A display equivalent RNase H hydrolysis patterns and in particular loss of polymerization-independent hydrolysis. In addition, a significant reduction in polymerization-independent RNase H activity for mutants p66/p51F416A and p66/p51T419A was observed. All of these mutants have reduced RNA dependent DNA polymerase activity.

**CONCLUSIONS:** Mutagenesis data presented here confirm and extend the crystallographic findings that structural elements close to the C terminus of p51 HIV-1 RT play a critical role in accommodating the RNA/DNA substrate for correct positioning in the RNase H active site and an intact, 440-residue p51 HIV-1 RT subunit is necessary to faithfully recapitulate activities of the RT heterodimer. Moreover, altered hydrogen bonding between N348 and Y427 affects nevirapine sensitivity, which may explain N348I-mediated nevirapine resistance.
ABSTRACT 28
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Evidence for the importance of G118R in integrase inhibitor therapy in different subtypes
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BACKGROUND: HIV-1 subtypes B, C and CRF02_A/G account for greater than 75% of all infection and it is important to understand the variations of viral control in these different subtypes. Integrase strand transfer inhibitors (INSTIs) have yet to be used on a large scale in resource-limited settings where non-B subtypes predominate. The integrase mutation G118R conferred raltegravir resistance in a CRF02_A/G HIV-1 clinical infection. We selected G118R in combination with E138K in cell culture with the experimental INSTI MK-2048. A different second-generation INSTI, dolutegravir, also selected G118R in CRF02_A/G and subtype C viruses, followed by H51Y. Our objective was to understand the mechanisms by which G118R ± H51Y or E138K affect integration and its inhibition in these three different subtypes.

METHODS: Enzymatic activities as well as INSTI inhibitory constants were measured with purified HIV-1 subtype AG, B and C enzymes harbouring G118R ± H51Y or E138K mutations using microtitre plate assays. Dose-dependent antiviral assays were performed with clonal virus containing either wild-type integrase or mutant integrase using the Monogram PhenoSense\(^\circ\) Integrase phenotypic assay. Modelling of HIV-1 integrase intasomes was performed based on structures of prototype foamy virus integrase.

RESULTS: The effects of G118R ± H51Y or E138K on integrase activity and susceptibility to dolutegravir, raltegravir and elvitegravir differs with subtype. G118R causes interference with Mg\(^{2+}\)/Mn\(^{2+}\) coordination and DNA binding. Individually, H51Y and E138K did not have a deleterious effect on integrase activity in any subtype, despite the combination of H51Y/G118R being deleterious to activity in all three subtypes. Resistance to DTG of G118R was in the order CRF02_A/G > subtype B > subtype C and resistance was increased in the presence of H51Y or E138K especially in subtypes C and CRF02_A/G. In CRF02_A/G integrase, the presence of G118R alone is sufficient to cause greater than 15-fold change in resistance to RAL.

CONCLUSIONS: In the context of ongoing INSTI-containing therapy, G118R may be clinically relevant in patients harboring subtype C and CRF02_A/G viruses, though its selection may be precluded by the related fitness cost. Longitudinal evaluation of resistance in INSTI-treated patients will be important, especially in the case of these non-B viruses.
ABSTRACT 29

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Integrase genotypic and phenotypic predictors of antiviral response to dolutegravir (DTG) in subjects with resistance to integrase inhibitors (INI)

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OBJECTIVES: Phenotypic and genotypic correlates to antiviral response produced during clinical investigation characterize an antiretroviral’s ability to inhibit HIV. The VIKING-3 study examined efficacy and safety of DTG 50 mg twice daily in patients with resistance to multiple antiretrovirals, including INIs. Baseline integrase genotypes and phenotypes were assessed to identify correlates to virological response.

METHODS: DTG was given through day 8 as functional monotherapy followed by optimization of background regimen. Day 8 categories for reduction in log_{10} HIV-1 RNA were predefined as Full (>1.0 log_{10}), Intermediate (0.5-1.0 log_{10}) or No response (<0.5 log_{10}). Integrase genotype and phenotype were evaluated by Monogram BioSciences. Baseline fold change (FC) phenotypic cutoffs in relation to day 8 responses were derived using non-linear logistic regression modelling. For integrase genotypic correlates, incidence of resistance-associated mutations and co-occurrence of mutation pairs at baseline were examined. Multivariate regression analyses of genotypic data adjusting for other covariates were performed to identify baseline mutations impacting day 8 response.

RESULTS: Derived DTG FC cutoffs (95% CI) were ≥25.99 (15.92, -) for No response and <9.4 (5.98, 15.88) for Full response. Wide confidence intervals suggest both cutoffs lack accuracy in predicting responses due to limited numbers of non-responders and few viruses with high DTG FC. Analyses of integrase genotypes showed a high correlation between mutations at position G140 and Q148. Three baseline integrase resistance mutation groups were defined in relation to day 8 response: No Q148 mutations, Q148 +1, and Q148 +≥2. Week 24 response rates (% <50 copies/ml, ‘Snapshot’) by these derived mutation groups were 79%, 45% and 11%, respectively; a similar response pattern was seen at day 8.

CONCLUSIONS: No integrase phenotypic cutoff for DTG could be defined to effectively predict antiviral responses to DTG twice daily. The three derived baseline IN genotypic groups (No Q148 mutations, Q148 +1, and Q148 +≥2) were good predictors for DTG responses in patients with INI resistance.
ABSTRACT 30

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Primary and secondary analyses of emergent drug resistance through week 48 from the STaR Study: rilpivirine/emtricitabine/tenofovir DF versus efavirenz/emtricitabine/tenofovir DF single-tablet regimens

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BACKGROUND: STaR (GS-US-264-0110) is an ongoing 96-week Phase IIIb study evaluating the safety and efficacy of two single-tablet regimens, rilpivirine/emtricitabine/tenofovir DF (RPV/FTC/TDF) and efavirenz/emtricitabine/tenofovir DF (EFV/FTC/TDF) in treatment-naive HIV-1-infected subjects. At week 48, RPV/FTC/TDF was non-inferior to EFV/FTC/TDF for HIV RNA <50 copies/ml by FDA snapshot analysis. Here, we present resistance analyses through week 48.

METHODS: Genotypic analyses of HIV-1 protease and reverse transcriptase (RT) were performed at screening (GeneSeq, Monogram); subjects with pre-existing resistance to study drugs were excluded. The primary resistance analysis population ([RAP] subjects with HIV-1 RNA ≥400 copies/ml at confirmed virological failure, discontinuation ≥ week 8, or week 48) had genotypic/phenotypic analyses at failure and baseline for protease and RT (PhenoSense GT, Monogram). Secondary resistance analyses included subjects who discontinued before week 8 and subjects at confirmed virological failure or discontinuation with HIV-1 RNA 50–399 copies/ml.

RESULTS: The primary RAP included 20/394 subjects (5.1%) receiving RPV/FTC/TDF and 7/392 subjects (1.8%) receiving EFV/FTC/TDF. In the RPV/FTC/TDF arm, 17/394 subjects (4.3%) developed NNRTI (Y181C/I [n=8], E138K/Q [n=6], K101E [n=5], H221Y [n=3], M230I/L [n=2]) and/or NRTI resistance mutations (M184V/I [n=15], K65R/N [n=3], K219E [n=3]). In the EFV/FTC/TDF arm, 3/392 subjects (0.8%) developed NNRTI (K103N [n=1], G190E/Q [n=1], Y188L [n=1]) and/or NRTI resistance mutations (M184I [n=1], K219E [n=1]).

When stratified by baseline viral load ≤ or >100,000 copies/ml, 5/260 (1.9%) versus 12/134 (9%) RPV/FTC/TDF-treated subjects and 2/250 (0.8%) versus 1/142 (0.7%) EFV/FTC/TDF-treated subjects developed resistance. Among subjects with HIV-1 RNA 50–399 copies/ml, one subject in each arm (1/2 RPV/FTC/TDF; 1/7 EFV/FTC/TDF) developed an NNRTI polymorphic site mutation (V106I RPV/FTC/TDF; V179V/I EFV/FTC/TDF) without associated phenotypic resistance.

CONCLUSIONS: Resistance development to ≥1 component of RPV/FTC/TDF was lower for subjects with baseline viral load ≤100,000 copies/ml compared to subjects with baseline viral load >100,000 copies/ml. The most common emergent mutations to RPV/FTC/TDF were Y181C/I, E138K/Q, K101E, and M184V/I in RT. Secondary resistance analyses revealed no significant additional resistance development in subjects with early discontinuation before week 8 or with HIV-1 RNA 50–399 copies/ml.
ABSTRACT 31
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Limited clinical benefit of minority K103N- and Y181C-variant quantification in addition to routine genotypic resistance testing in ART-naive patients

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BACKGROUND: Numerous studies have reported that the presence of minority NNRTI-resistant HIV-1 variants in treatment-naive patients can lead to virological treatment failure. Here, we performed a retrospective study to determine the number of treatment failures that could be prevented by implementing minority drug-resistant HIV-1 variant analyses in ART-naive patients in whom no NNRTI or NRTI resistance mutations were detected by resistance testing based on population sequencing (GRT).

METHODS: Of 1,437 patients in the Swiss HIV Cohort Study, who have initiated first-line ART with 2 NRTI and 1 NNRTI before July 2008, 519 patients fulfilled the inclusion criteria (baseline plasma sample available within 6 months prior to ART initiation, viral load >1,000/ml, and no baseline NNRTI- or NRTI resistance mutations in GRT). Key NNRTI resistance mutations K103N and Y181C were measured by allele-specific PCR in 221/519 randomly chosen patients. Virological treatment success or failure was documented for 193 and 9 patients, respectively.

RESULTS: Focusing on the 202 patients with available follow-up data, minority K103N variants were detected in 5/174 (2.9%), and minority Y181C variants in 8/185 (4.3%) patients. Virological failure occurred in 5/174 and 6/185 of those patients, respectively. None of the patients harbouring minority K103N variants experienced virological failure. One patient with pre-existing minority Y181C variants failed first-line ART, whereas the remaining 7 patients with pre-existing minority Y181C variants were successfully treated. By implementing a sensitive assay to detect minority NNRTI-resistant HIV-1 variants in more than 200 patients, one virological failure could have been prevented. In contrast, NNRTI-containing regimens would have been wrongfully withheld from 12 patients.

CONCLUSIONS: The prevalence of minority K103N- and Y181C variants was low in ART-naive patients with negative GRT in Switzerland and so was the virological failure rate. More than 200 patients would need to be tested for those mutations by AS-PCR to prevent one virological failure. In addition, 12 patients would have been wrongly denied an NNRTI-based regimen. Our study shows that the sole implementation of minority K103N- and Y181C- HIV-1 variant analysis in routine clinical settings in addition to GRT can currently not be recommended. Associated risk factors need to be discovered.
ABSTRACT 32

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HIV drug resistance occurring during low-level viraemia is associated with subsequent virological failure

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BACKGROUND: There is limited evidence that HIV resistance testing performed on samples with low-level viraemia (LLV) <1,000 copies/ml can provide useful information to guide HIV therapy. We assessed whether time to virological failure was a function of resistance at LLV.

METHODS: 4,893 resistance assays were performed on LLV samples between 50–1,000 copies/ml. 4,304 (88%) yielded successful results, ranging from 75–90% success with increasing viraemia. 2,620 LLV episodes were examined from 1,965 patients experiencing their first recorded LLV episode. Risk of virological failure ≥1,000 copies/ml after LLV was evaluated by Kaplan–Meier analysis and Cox proportional hazards model. Genotype sensitivity score (GSS) was determined from the Stanford algorithm. Patients were grouped into 4 GSS categories during LLV, corresponding to the number of active drugs prescribed: <1, 1–1.5, 2–2.5 and ≥3.

RESULTS: Of those remaining on the same therapy after LLV, 855 of 1,389 patients (62%) experienced virological failure, 411 (30%) suppressed <50 copies/ml, 82 (6%) maintained LLV, and 41 (3%) were lost-to-follow-up. There was a ‘dose-dependent’ increase in the hazard ratio for virological failure as GSS fell. Compared to GSS ≥3, hazard ratios were: 1.4 for GSS 2–2.5, 2.0 for GSS 1–1.5 and 3.0 for GSS <1.

After 6 months on constant therapy, patients with lower GSS scores had higher median viral loads: 3.4 log for GSS <1, 3.0 log for GSS 1–1.5, 2.4 log for GSS 2–2.5 and <50 copies/ml for GSS ≥3.

The proportion of patients who subsequently suppressed <50 copies was 26% for GSS ≥3, falling to 14%, 4% and 1% for GSS 2–2.5, 1–1.5 and <1, respectively. Time to virological failure was significantly faster in patients with lower GSS (mean 0.4 years for GSS <1 versus 1.1 years for GSS ≥3). Median viral load at LLV was significantly higher in patients with GSS <1 compared to those with GSS ≥3: 574 copies/ml versus 456. For 441 patients with ≥2 successive LLV episodes, 52 (12%) had a decrease in GSS.

CONCLUSIONS: Resistance genotyping can be performed successfully on samples with low viral loads between 50 and 1,000 copies/ml. The results are predictive of the risk of treatment failure.
ABSTRACT 33
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Low-frequency drug-resistant HIV-1 and risk of virological failure to first-line NNRTI-based ART: a multi-cohort European case-control study using centralized ultrasensitive 454 sequencing

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BACKGROUND: The prognostic value of pre-existing minority resistant variants (MV s) to predict the risk of virological failure (VF) to first-line NNRTI-containing ART remains to be elucidated.

METHODS: This was a retrospective case-control study nested within six observational European cohorts. HIV-1-infected adults with no pre-existing NNRTI resistance by bulk sequencing who started first-line ART including nevirapine (NVP) or efavirenz (EFV) and had 1 ml pre-ART plasma with VL ≥10,000 copies/ml available for testing were included. Cases experienced VF to first-line ART, defined as two consecutive VL >200 copies/ml after >6 months of initial ART regimen; controls were subjects from the same cohort with a VL ≤200 copies/ml at a matched time since ART initiation. 454 sequencing of RT was centralized in a single laboratory and analysed in parallel in two different laboratories using the AVA software (v2.7), blinded for clinical outcomes and the other laboratory results. Presence of MVs was defined as detection of ≥1 IAS-USA (March 2013) mutations in 1–25% of each subject’s HIV-1 population. Standard logistic regression was used to estimate odds ratios (ORs) of VF according to MV detection. Multivariable estimates were adjusted for HIV subtype, calendar year of starting NNRTI (baseline), time from sample to baseline, baseline VL, NNRTI started, NRTI backbone started and cohort.

RESULTS: 368 subjects (81 VF and 287 controls) with median (IQR) CD4 254 cells/mm³ (167–358) and viral load 4.91 (4.55–5.31) were included. 83% started EFV. Main NRTI backbones were ZDV/3TC (44%), TDF/FTC (21%) and ABC/3TC (10%). Main HIV subtypes were: 79% B, 6% C, 3% A, CRF01_AE or G. Identical MVs were detected in the two laboratories. 17.3% cases and 7.3% controls had MVs (P=0.009). Most prevalent MVs were M184I (3%), V108I (2.5%) and V75I (1%). Detection of ≥1 MVs versus no MVs was associated with the risk of VF (OR 2.49, 95% CI 1.12, 5.55, P=0.03); a trend was observed for ≥1 versus no NRTI MVs (OR 2.29, 95% CI 0.87, 6.03, P=0.09); ≥1 versus no NNRTI MVs (OR 2.14, 95% CI 0.61, 7.50, P=0.23).

CONCLUSIONS: Pre-existing MVs in reverse transcriptase more than double the risk of virological failure to first-line NNRTI-based ART.
ABSTRACT 34

Evaluation of three next-generation sequencing platforms on low frequency HIV drug resistant variants detection

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BACKGROUND: Next-generation sequencing (NGS) technologies provide powerful tools to detect very low frequency drug-resistant variants that are clinically significant. Till now, most of current NGS assays are based on Roche 454 platform, which has inherent technological limitations and experimental design issues. Other platforms such as Illumina and Pacific Biosciences carry out different sequencing chemistry and strategies, which can not only overcome some of these shortcomings, but also provide more sequence information. In this study, we compared the low frequency DR variants detection ability of three platforms (454 GS FLX, Illumina MiSeq, PacBio RS) and technical feasibility for future applications.

METHODS: First, we compared the sensitivity and accuracy of 454 with the TRUGENE HIV-1 Genotyping Kit (Bayer Inc.). Six clinical samples along with HXB2 plasmid were sequenced using two amplicons covering majority of RT and PR regions. Second, to evaluate amplicon heterogeneity and patient-to-patient variation in a broader region, 24 amplicons from 30 patients spanning in HXB2 position 1785–5590 (include PR, RT p15 RNAase and INT) were obtained using Fluidigm AccessArray and sequenced in one 454 run. Furthermore, 6 patient samples and 6 different admixture controls with known mutations are used in MiSeq and PacBio systems. For MiSeq platform, Nextera XT based shotgun method was used for 1.3 kb cDNA in RT/PR region and 2x250 bp reads were generated. For PacBio system, unamplified 1.3 kb single molecule cDNA was directly sequenced by using Circular Consensus Sequencing (CCS) protocol, by which the sequences of each complete full length cDNA were obtained.

RESULTS: Compared to TRUGENE assay, the sensitivity of 454 platform is much superior, down to 2–5%, depends on the depth around mutations and the number of samples assayed in one run. In the multiple samples/amplicons study, large variations by amplification bias were observed which resulted in variability in sequence depth. Consequently, sensitivity of low frequency variant call deteriorated in those low coverage regions. Despite having shorter read length than both 454 or PacBio, Illumina MiSeq produced much higher quality base calls that resulted in overall better quality output and significantly higher sequencing depth, which drives up sensitivity impressively to 0.8% with 100% accuracy. PacBio, on the other hand, yields 100% accuracy at 2% sensitivity level, while provides additional information such as quasispecies and recombination.

CONCLUSIONS: Among all three different platforms, they can all detect the same high frequency variants but showed variations in sensitivity to detect rare variants due to different library preparation methods and sequencing errors. 454 is sufficient for two or three amplicon sequencing with limited number of patient samples. However, MiSeq is a better platform for large sample size project in cost-effective perspective as well as sensitivity and accuracy. PacBio’s extremely long read length and single molecule sequencing technology lends itself as a unique tool for quasispecies and recombination study.
ABSTRACT 35

Characterization and validation of a deep sequencing-based HIV-1 genotypic and coreceptor tropism assay to simultaneously monitor susceptibility to maturation, protease, reverse transcriptase, and integrase inhibitors, and CCR5 antagonists

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BACKGROUND: There are 26 antiretroviral drugs, from 6 classes, approved for the treatment of HIV-1 infection. Thus, a combination of different phenotypic and genotypic tests is needed to monitor HIV-infected individuals. In this study, we developed a novel HIV-1 genotypic assay based on deep sequencing to simultaneously assess HIV-1 susceptibility to all drugs targeting the three viral enzymes as well as to quantify coreceptor tropism.

METHODS: Baseline samples (n=88) from a cohort of antiretroviral-experienced patients on a maraviroc-based regimen were used to generate gag-p2/NCp7/p1/p6/pol-PR/RT/INT and env/C2V3 PCR products. Purified amplicons were used in the construction of barcoded libraries and sequenced on the Ion Torrent PGM. Sorting, mapping and aligning of reads were done using proprietary software. Reads spanning the 3′-end of Gag, PR, RT, INT and V3 regions were extracted, truncated, translated and assembled for genotyping. HIV-1 coreceptor tropism was inferred using the 11/24/25 charge rule and Geno2Pheno. Results were compared with (i) virological response to treatment, (ii) genotyping based on population sequencing, (iii) Trofile, (iv) Trocai and (v) tropism based on population sequencing and Geno2Pheno.

RESULTS: Our HIV-1 genotypic and tropism assay consistently detected both minority drug-resistant viruses and non-R5 variants from clinical specimens with viral loads ≥1,000 copies/ml, and from B and non-B subtypes. Additional 30–60% minority variants carrying mutations associated with resistance to PI, RTI or INI, previously undetected by standard population sequencing, were reliably detected at frequencies as low as 1%. The new HIV-1 genotypic/tropism assay correlated with other HIV-1 tropism tests, that is, Trofile (79%), Trocai (81%), and population sequencing/Geno2Pheno 10% FPR (80%). Interestingly, the deep sequencing-based HIV-1 genotypic/tropism assay (83%) and Trofile (85%) showed similar concordance with clinical response following an 8-day maraviroc monotherapy (MCT).

CONCLUSIONS: DEEPGEN™HIV, our novel all-inclusive HIV-1 genotypic and coreceptor tropism assay based on deep sequencing of the protease, RT, integrase and env C2V3 region, permits the multiplexed detection of low level drug-resistant and/or non-R5 viruses in up to 96 clinical samples simultaneously. This comprehensive test, the first of its class, could be instrumental in the development of new antiretroviral drugs and, more important, will aid in the treatment and management of HIV-infected individuals.
**ABSTRACT 36**

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**In vitro** analysis of PCR founder effects in Illumina sequencing

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**BACKGROUND:** Illumina sequencing of PCR amplification products from HIV-1 RNA has great potential in providing ever-increasing amounts of data on HIV and HCV populations. In order to determine its accuracy, we investigated the founder effects (PCR bias) from amplification of samples with Illumina sequencing primers.

**METHODS:** cDNA was synthesized from mixtures of wild-type and drug-resistant HIV-1 BH10 pol transcripts (100% to 0.1% mutant). A gene-specific primer was designed to contain 10 random DNA bases (dogtag/primer ID) to label each molecule with a unique sequence during cDNA synthesis. Starting with ~100,000 cDNA molecules, the cDNA was amplified with Illumina primers containing a distinct barcode for each mixture and pooled products were sequenced using paired-end Illumina MiSeq technology. The sequencing data were used to build consensus sequences from reads that shared the identical dogtag to correct for PCR error, analysed for founder effects and for sensitivity of detecting minority drug resistance mutations.

**RESULTS:** We observed an uneven distribution of dogtag frequencies. For example, one sample resulted in 759,501 sequences but only 11,896 unique dogtags indicating amplification from only 11.89% of the ~100,000 input DNA molecules. Furthermore, approximately 50% of these unique sequences had only a single sequencing read, making consensus building powerless. The use of dogtags reduced the PCR error rate on average from 0.3% to 0.06%. The accuracy of detecting drug resistance mutations ranged from good in the 1% mutant mixture (detecting 1.8% mutant) to poor in the 0.1% mixture (detecting 5.7%). In a sampling of 200,000 sequences 52% of them contained no errors compared to a previous 454 experiment of 200,000 sequences where none were correct.

**CONCLUSIONS:** Our results show that on average >90% of template molecules did not amplify efficiently demonstrating a significant PCR founder effect. This bias limits the accuracy of Illumina technology for evaluating HIV populations. New methods that reduce PCR founder effects are clearly needed. The source of the inaccuracy in mutant detection is under investigation. Finally, errors were much reduced by inclusion of dogtags and the quality of the Illumina sequencing was greatly improved compared to our previous experience using 454 technology.
ABSTRACT 37
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Baseline polymorphisms and persistence of emergent variants from Phase II trials evaluating the HCV NS3 protease inhibitor faldaprevir

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BACKGROUND: Faldaprevir is a potent HCV NS3 protease inhibitor currently being evaluated with PegIF-Nα2a/RBV (P/R) in Phase III studies in treatment-naïve (TN) and treatment-experienced (TE) GT1 HCV-infected patients. Earlier trials tested multiple regimens that included escalating faldaprevir once daily doses in Phase Ib as well as 120 mg and 240 mg once daily or twice daily doses in Phase II, and allow for an analysis of key NS3/4A baseline polymorphisms in 966 patients, as well as a pooled analysis of the post-treatment persistence of NS3 resistance-associated variants from patients that did not achieve SVR.

METHODS: All baseline HCV NS3/4A were sequenced and changes among virological failures identified by population sequencing. Analyses of baseline polymorphisms included frequency (Phase Ib/II) and association with failure to achieve SVR (Phase II). Chimeric replicons containing NS3 sequences derived from patient samples were phenotyped using a range of faldaprevir concentrations. Persistence of major resistant variants was evaluated by population sequencing of post-treatment samples.

RESULTS: The faldaprevir resistance profile includes NS3 R155K and D168V as the predominant variants that emerge in GT1a and GT1b virological failures, respectively. The median time to outgrowth of WT virus and loss of detected resistance-associated mutations at R155 and D168 in post-treatment follow-up was 11 and 8 months, respectively. Baseline analysis of the key Q80K polymorphism showed prevalence in 23% (99/437) of GT1a patients. Q80K wasn’t significantly associated with reduced SVR in GT1a TN or TE patients treated with BI 201335. The faldaprevir EC50 values for patient-derived GT1a NS3 amplicons with K80 at baseline (range 8–42 nM) were not significantly different from the amplicons with Q80 (range 2–63 nM).

CONCLUSIONS: Post-treatment follow-up of virological failures indicated that in the majority of patients, WT virus outgrew the emergent NS3 R155K and D168V resistant variants in less than 1 year and that D168V variants were less fit than R155K. Baseline NS3/4A polymorphisms identified from pooled Phase Ib/II studies represented the heterogeneity of HCV and GT1a Q80K did not significantly reduce SVR in patients treated with BI 201335. At all BI 201335 doses tested in Phase Ib/II, GT1a Q80K did not emerge in virological failures.
ABSTRACT 38
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Roche 454 ultradeep pyrosequencing reveals complex resistance patterns post protease inhibitor failure

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BACKGROUND: Through an expanded access programme, we treated 58 chronically infected HCV patients with telaprevir or boceprevir combined with pegylated interferon/ribavirin. 21 patients failed treatment: 18 patients, all treated with telaprevir, had evidence of resistance mutations by population sequencing. We used the Roche 454 GS Junior ultra-deep pyrosequencing (UDPS) platform to quantify the presence of resistant variants in follow-up samples taken up to a year after telaprevir exposure.

METHODS: Pretreatment, at failure and post-treatment follow-up samples were tested by UDPS from 12 patients. Nested PCR using ‘bar-coded’ nested primers was followed by processing in-line with Roche 454 protocols. UDPS encompassed amino acids 9–190 of HCV protease gene in a single sequencing reaction. Roche AVA software was used to quantify the presence of wild-type and resistant variants.

RESULTS: In all 12 patients, resistant variants were detectable by UDPS up to 394 days post-telaprevir (median 199 days) at levels between 36 and 100% of the virus population. There was no correlation between time off-therapy and levels of resistant variants at follow-up. In patients where >95% of HCV sequences contained resistant variants at V36 and R155 on the same genome at failure, >90% of sequences continued to show both resistant variants on the same genome up to 293 days post-treatment (median 173 days). In patients where these resistant variants comprised <95% of the population at failure, dual-resistant variants on the same genome comprised a maximum of 15% of the population up to 293 days post-treatment (median 173 days). In patients with a single resistant variant at failure, V36 or R155, resistant variants at the same position comprised >90% of the population a median of 168 days later.

CONCLUSIONS: We demonstrate the long-term persistence of HCV expressing dual-resistance variants on the same genome, comprising >90% of the population up to 329 days post-telaprevir, even though such variants are associated with lower levels of relative replicative capacity. Persistence of these variants at such high levels has significant implications for future oral-only regimens where resistance rather than lack of response is likely to be a major cause of treatment failure.
ABSTRACT 39
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Two distinct HCV genotype 1a clades: geographical distribution and association with natural resistance mutations to HCV NS3/4A inhibitors

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BACKGROUND: HCV genotype 1a is more prone to resistance to NS3 protease inhibitors (NS3i) than 1b. We and others recently described two distinct 1a clades. We aimed at further characterizing this phylogenetical segregation and testing its association with epidemiological factors and with natural resistance to NS3i.

METHODS: Plasma samples were obtained in 2003–2012 from NS3i-naive patients chronically infected with HCV1a in Italy. NS3 was amplified by RT-PCR, sequenced and genotyped by REGA HCV Subtyping Tool. Additional HCV 1a NS3 sequences were selected from the Los Alamos repository among those with ≥1,000 bp, identifiable patient and geographical origin. Maximum likelihood phylogenies were employed using neighbour-joining as starting tree and the TBR algorithm for branch swapping. Trees robustness were confirmed with 1,000-replicate bootstrap analysis. IAS-USA 2012 resistance mutations to boceprevir/telaprevir/simeprevir were analysed.

RESULTS: 131 Italian patients were examined: 73% males, 77% HIV-coinfected, 70% IDU; median age was 47 years, time from HCV diagnosis 16 years, HCV RNA levels 6.14 log10 IU/ml. 62 sequences (47.3%) segregated in clade 1 and 69 (52.7%) in clade 2. Gender, age, transmission group, HIV status, Italian geographical region, HCV RNA levels and time from HCV diagnosis were not associated with clade. The cases from Los Alamos (n=165, 90.3% from USA) clustered in clade 1 in 72.7% and in 2 in 25.5% (3 sequences clustered externally). In the whole dataset (n=296), a lower proportion of clade 2 was observed in the Americas (versus Europe OR 0.28, 95% CI 0.17–0.47). Frequencies of resistance mutations in clade 1 versus 2, respectively, were: for simeprevir Q80K 48.9% versus 0% (P<0.001), D168E 0% versus 1.8% (P=0.07), S122G 2.7% versus 25.2% (P<0.001); for boceprevir/telaprevir, V36L 1.1% versus 3.6%, T54S 0.5% versus 1.8%, V55A 0.5% versus 1.8%, I132V 0.5% versus 0.9%, I170T 0% versus 0.9% (all P=ns). Proportions with any major boceprevir/telaprevir resistance mutations were 2.2% and 3.6% in clade 1 and 2 (P=ns).

CONCLUSIONS: The presence of two distinct HCV 1a clades is confirmed. Clade 1 is more frequent in the Americas and is associated to a high prevalence of Q80K, associated thus to simeprevir resistance.
ABSTRACT 40
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Key genetic markers in the full-length HBsAg gene correlate with HBV-driven carcinogenesis by affecting HBsAg secretion and release


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BACKGROUND: Intracellular HBsAg-retention favours the onset of hepatocellular carcinoma (HCC). Here, we define mutations in HBsAg correlated with HBV-induced HCC in vivo, and investigate their impact on HBsAg-retention and secretion in an in vitro model.

METHODS: Sixty-seven HBV chronically infected patients (74.6% genotype D, 20.9% genotype A and 4.5% others), 19 with HCC and 48 asymptomatic (as control) were analysed. Association of HBsAg mutations with HCC was assessed by Fisher test with Benjamini–Hochberg for multiple comparison correction. Mutants were constructed by site-directed mutagenesis and expressed in HuH7-hepatocytes using the expression-vectors pCHD9 with full-length HBV genome (for mutations P203Q and S210R) and pCHsAgD with full-length pre-S1/pre-S2/S-gene (for N40I and K141N). After 72 h, quantitative HBsAg was tested in cell supernatants and in cell lysates using Abbott Architect assay (experiments were performed in triplicate).

RESULTS: Novel mutations in HBsAg region (N40I, K141N, P203Q, S210R) correlate with development of HCC (P=10^-5–10^-4). 19/19 patients with HCC carry ≥1 of them (range prevalence: 12.5–37.5%), while these mutations are absent (0/48 for N40I) or occurring with low frequency (1.9% for K141N-P203Q, 7.5% for S210R) in non-HCC patients. Strong correlations are observed for P203Q+S210R (phi=0.83) and N40I+K141N (phi=0.56).

In in vitro experiments, the pair P203Q+S210R, localized in the membrane-embedded C-terminal HBsAg domain critical for HBsAg secretion, drastically decreases the supernatant release of HBsAg compared to P203Q and S210R alone, and to wild-type (9.42 [sd ±1.35] IU/ml for P203Q+S210R; 26.85 [sd ±13.01] IU/ml for P203Q; 25.95 [sd ±8.82] IU/ml for S210R; 34.40 [sd ±13.28] IU/ml for WT, P<0.01). P203Q+S210R association also decreases the ratio of supernatant to lysate HBsAg compared to wild-type (0.61 for P203Q+S210R versus 1.20 for wild-type), further supporting their ability in inducing HBsAg intracellular retention. Similarly, K141N+N40I strongly decreases supernatant HBsAg levels compared to wild-type and K141N alone (4.67 [sd ±2.31] IU/ml for K141N+N40I; 13.43 [sd ±9.68] IU/ml for K141N; 28.25 [sd ±13.12] IU/ml for WT, P<0.05). N40I resides in the HBsAg cytosolic-loop known to be important in HBsAg secretion.

CONCLUSIONS: Key genetic elements in HBsAg correlate with HCC by inducing intracellular HBsAg-retention. Their detection may help identifying patients at higher HCC risk that may deserve more intensive liver evaluation, and/or earlier anti-HBV therapy.
SESSION 1
HCV/HBV drug resistance
ABSTRACT 41

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A pan-genotype next generation sequencing assay for HCV direct-acting antiviral resistance

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OBJECTIVES: To detect NS3, NS5a and NS5b drug resistance mutations in samples containing HCV genotypes 1–6.

BACKGROUND: Pathogenica’s DxSeq™ system encompasses a highly multiplexed library of probes that may bind to a selection from dozens to thousands of nucleic acid loci that may be present in a sample, enabling the low-cost sequencing of diverse viral genomes. DxSeq probes are sequencer-agnostic and can be used on all commercially available sequencing platforms. The assay is performed in a single tube, in under 3 h, and provides significant read depth (500 to >50,000 read depth), which can be used to determine quantitative variant information.

METHODS: Blinded clinical samples from DAA-naive HCV-1a (n=40), HCV-1b (n=30) and HCV-2–6 (n=25) infected patients underwent HCV RNA extraction and RT-PCR. All HCV clinical samples had a viral load >50,000 copies/ml (Roche COBAS AmpliPrep TaqMan HCV RNA PCR Test). HCV genotype was confirmed by Versant HCV Genotyping Assay 2.0. Viral cDNA was generated and >400 DxSeq probes were used to target desired gene regions. Captured gene regions were sequenced using an Ion Torrent PGM and compared to sequences determined by Sanger Sequencing.

RESULTS: HCV DxSeq probes correctly identified HCV genotype 1 through 6 viral variants. Resistance locus capture size averaged 200 bases, and read depth ranged between 50 to >50,000 fold. DxSeq probes detected mutations generating both nucleotide and amino acid polymorphisms. Among detected amino acid polymorphisms in our DAA-naive clinical samples, we detected mutations reported to confer retroviral drug resistance in NS3, NS5a and NS5b proteins. Selected observed mutations include: in NS3: Q80L/K/R, D168G/E, I170T/V, 175L and E176G; in NS5a: M28T, Q30R, L31M, P58S, Y58S and Y93H/N; and in NS5b: 71V, 183I, M414L/V, L419S, Y452H, V494A and V499A.

CONCLUSIONS: HCV DxSeq probes agreed in with Versant HCV genotyping assay in clinical samples. Additionally, DxSeq detected mutations associated with resistance to antiviral drugs, such as TMC435, boceprevir, danoprevir, BI-201335, GS-9190, BMS-650032, MK-3281, VCH-916 and JTK-109. HCV DxSeq technology has the potential to be used to detect mutations caused by drug compounds and to screen patients for clinical trials and companion diagnostics.
ABSTRACT 42

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Incidence of virological failure and emergence of resistance with twice daily versus every 8 h administration of telaprevir in the OPTIMIZE study

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BACKGROUND: In the Phase III OPTIMIZE study in treatment-naive genotype 1 (GT1) HCV-infected patients, the efficacy of telaprevir (T) in combination with peginterferon/ribavirin (PR) twice daily was noninferior to T/PR every 8 h (q8h), with SVR12 rates of 74% versus 73%, respectively. Viral resistance was evaluated to compare the selective pressure of both dosing regimens.

METHODS: HCV NS3-4A population sequencing was performed at baseline and time of failure. Resistance of T variants was classified into lower-level (3- to 25-fold change [FC] in IC50; V36A/G/M, T54A/S, R155G/K/M/T and A156S), higher-level (FC>25; V36M+R155K and A156F/N/T/V) and FC<3 (V36I/L, I132V [GT1a only] and D168N). On-treatment virological failure (VF) was defined as discontinuation due to a virological stopping rule and/or having viral breakthrough. Relapse was defined as HCV RNA ≥25 IU/ml after previous HCV RNA <25 IU/ml at planned end of treatment.

RESULTS: Baseline resistance to T was low (2.4% T54S, 1.5% V36L, <0.5% V36I/M, I132V or R155K) and did not preclude successful treatment. Overall, 54/369 (15%) and 62/371 (17%) patients with T twice daily or q8h dosing, respectively, had T-resistant variants at time of failure. Variants V36M and R155K/T (GT1a) and V36A, T54A and A156S (GT1b) were identified as significantly enriched in non-SVR patients in both treatment groups. T-resistant variants were present in the majority (70% [twice daily] and 72% [q8h]) of non-SVR patients with available sequence data. 4% (twice daily) and 6.2% (q8h) of subjects had VF during, and 6.0% (twice daily) and 3.5% (q8h) had VF after the T-treatment phase. There was no significant difference in type of T-resistant variants between both treatment groups. The majority of patients with VF during treatment had higher-level variants. VF after T treatment was associated with higher-level (GT1a) and lower-level (GT1a/1b) variants, or wild-type (GT1b). Relapse was mostly associated with lower-level variants or wild-type, especially in GT1b patients.

CONCLUSIONS: In OPTIMIZE, the prevalence of T-resistant variants at baseline was low (<5%) and did not preclude successful treatment with a T/PR regimen. There was no difference between T q8h and twice daily dosing in terms of selection of resistance, and viral resistance profiles were consistent with previous observations.
ABSTRACT 43

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Genotypic and phenotypic analysis of the HCV NS3/4A-protease in patients with acute hepatitis C and HIV coinfection

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BACKGROUND: To establish chronic infection, HCV (hepatitis C virus) developed several mechanisms to evade the immune system of the host. Signal transduction pathways of the innate immunity (involving Cardif, TRIF and TC-PTP proteins) are inhibited by the HCV NS3 protease. The early NS3 evolution, which is driven by the immune system, may influence the course of acute infection with a spontaneous clearance of the virus versus a chronic infection.

METHODS: Blood was collected at diagnosis and every 4 weeks thereafter as yet from 60 patients with acute hepatitis C and HIV coinfection for the analysis of virological/biochemical parameters and for interleukin-28B (IL28B) genotyping (rs129797860). Clonal sequencing of the NS3 protease gene was performed to characterize the quasispecies heterogeneity (Hamming distance and Shannon entropy) and to analyse mutations in association with the outcome of HCV infection. Major patient NS3/4A variants were expressed in U2OS cells for the investigation of the cleavage activity regarding Cardif.

RESULTS: Persistent HCV infection was observed in 50 (83%) patients, 6 (10%) achieved spontaneous clearance and the outcome is unclear for 4 (7%) individuals. Because of the limited numbers of spontaneous clearance, no correlation with IL28B genotype was observed. NS3 quasispecies sequence analysis revealed that the baseline mean Hamming distance/Shannon entropy was comparable in patients with spontaneous clearance (0.013/0.034) versus persistent infection (0.008/0.023). Although no mutational NS3 protease patterns were detected in correlation with outcome, we observed multiple changes of the HCV quasispecies during the course of acute infection. At baseline, all tested patient NS3/4A isolates cleaved Cardif to a similar extend, which was independent of the outcome. However, expression levels of HCV NS3 variants of different patients were strongly varying, which was not associated with the corresponding HCV viral load. This indicates individual differences in NS3 protein stability and/or in Cardif cleavage activities. The underlying mechanisms are under investigation.

CONCLUSIONS: During acute hepatitis C, we observed a rapid NS3 quasispecies evolution. The cleavage of Cardif was similar in patients with different outcomes of acute hepatitis C, although NS3 expression levels were variable. This suggests an individual selection process for NS3 variants by the immune system.
ABSTRACT 44

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Surveillance for low frequency NS5B polymerase drug resistance mutations using next generation sequencing

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BACKGROUND: New direct-acting antivirals, targeting the active site of the HCV polymerase (NS5B), have been shown to be effective and have high genetic barriers to resistance. Sanger sequencing of treatment failures failed to demonstrate the development of resistance to sofosbuvir. However, the limited sensitivity of conventional sequencing may fail to identify low-frequency NS5B mutations affecting both direct-acting agents and ribavirin/interferon therapy. Tagged pooled pyrosequencing (TPP) is a cost-effective and sensitive method for identification of HIV drug-resistant minor variants. We sought to demonstrate the utility of this technique on scaled HCV drug resistance mutation (DRM) identification among nucleoside inhibitor-naive subjects.

METHODS: 133 serologically HCV+ specimens from Ottawa, Canada were conventionally genotyped. The NS5B polymerase active site for all specimens (amino acid 220~345) was sequenced in parallel using TPP. The prevalence of previously identified NS5B HCV DRM mutations was quantified above the 1% frequency cutoff.

RESULTS: HCV genotype distribution was 65% GT1a; 3.0% GT1b; 1.5% GT2a; 7.5% GT2b; 23% GT3a. There was 3,060× oversampling in the NS5B TPP reads for each subject. S282T was not observed (<1% frequency) in any HCV genotype. While V321I was observed in three genotype 1a subjects, R222Q, C223Y/H, C316Y/N/S, and L320I mutations were not otherwise detected. Inter-subtype variations were observed for ribavirin/interferon DRM. For example, D244N and Q309R, D310N were the dominant genotypes in genotype 3, but rarely detected in other genotypes. The A333E mutation, associated with SVR, was the dominant phenotype in all 12 GT2a/2b specimens but rarely seen in other genotypes.

CONCLUSIONS: TPP is applicable for large scale HCV DRM screening with the benefit of detecting low abundance DRM. Among nucleoside treatment-naive patients, no nucleoside inhibitor DRMs were found – even as minority variants. Clinical NS5B resistance to non-nucleoside and interferon/ribavirin is also predicted to be low within this cohort. Interrogation of NS5B, and other portions of the HCV genome, in prospective trials may provide valuable information that could help define treatment outcomes.
ABSTRACT 45

*Antiviral Therapy* 2013; 18 Suppl 1:A55

Implications of baseline polymorphisms for potential resistance to NS3 protease and NS5A inhibitors in hepatitis C virus genotypes 1a, 2b and 3a

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BACKGROUND: The future interferon-free combo DAA treatments could include HCV NS3 protease and/or NS5A inhibitors for potent pan-genotypic effect. We have studied the prevalence of resistance-associated amino acid variants (RAVs) in treatment-naive subjects of HCV genotypes 1a, 2b and 3a, the most common genotypes in Sweden.

METHODS: Sera from 117 and 35 treatment-naive patients were collected for the NS3 and NS5A analysis, respectively. The NS3 and the NS5A genes were each amplified by nested PCR method with degenerated primers to enable a broad genotype analysis (>80 and >85% yield). Gene sequencing was done by population sequencing method. The sequences were aligned with the NS3 and NS5 sequence from HCV genotype 1a H77 strain as reference. Interpretation of fold-change resistance to NS3 and NS5A candidate drugs were done from already published phenotypic resistance data.

RESULTS: The prevalence of known NS3 RAVs at baseline in genotype 1a was 26% (14/53); either single or combinations of V36L, T54A/S, V55A and Q80K/R mutation(s). In genotype 2b, polymorphism-like V36L, Q80G and S122R of NS3 gene were found in 100% (11/11). Similarly, V36L and D168Q were found uniquely in all 3a samples (30/30). No RAVs were found in the genotype 1a or 3a NS5A gene of 14 subjects each, whereas single NS5A polymorphisms Q30K and Q30L were found in 2 genotype 2b subjects (2/3).

CONCLUSIONS: The baseline NS3 RAVs found in genotype 1a have relatively weak resistance to the approved and the second-generation NS3 inhibitors. However, the natural polymorphisms in genotype 2b (that is, S122R) and 3a (that is, D168Q), with inherent second-generation NS3 drug resistance of up to 20- and 700-fold in comparison with a wild-type genotype 1b strain, could explain why current NS3 inhibitors are primarily effective in genotype 1 infections. The NS5A polymorphism Q30K found in one genotype 2b has been shown with a genotype 1a replicon assay a >3,000 level of fold change in EC50 to a NS5A candidate inhibitor. These preliminary results indicate that further evaluation is needed to study the role of baseline RAVs in non-genotype 1 for future interferon-free combo DAA treatments.
ABSTRACT 46

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Development of a panel of genotype-specific resistance assays for the detection of amino acid changes in domain 1 of hepatitis C virus (HCV) NS5a associated with drug resistance

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BACKGROUND: NS5a, a membrane-associated phosphoprotein of HCV, plays a crucial role in regulating viral replication and host cell interactions. NS5a inhibitors targeting domain I of NS5a protein have demonstrated promising pan-genotypic antiviral activity. We have developed a series of NS5a genotypic resistance assays specific for HCV 1a, 1b, 2, 3 and 4 to assess prevalence of NS5a amino acid (aa) changes in domain I at positions M28T, Q30H/R, L31F/M/V, P32L and Y93C/H/N, known to confer reduced susceptibility to certain NS5a inhibitors.

METHODS: HCV RNA was quantified using Abbott real-time assay and genotyped using Abbott real-time genotyping assay and primers directed towards 5′ UTR of HCV. The first 213 aa of domain I of NS5a were amplified from plasma viral RNA, using Qiagen one-step RT-PCR system, followed by a nested PCR, incorporating genotype/subtype specific primers for HCV 1a, 1b, 2, 3 and 4 in both PCRs. PCR amplicons were purified, sequenced and consensus sequences aligned in SeqScape v2.5, submitted to NCBI Blast for identification and translated using BioEdit.

RESULTS: PCR amplicons were generated from HCV viral RNA at levels of 25 IU/ml for genotypes 2 and 3; 50 IU/ml for 1a and 4 and 250 IU/ml for 1b. HCV genotyping based on 60 NS5a sequences were comparable with Abbott real-time for 59/60 samples (98%). Preliminary experiments of aa changes associated with resistance to NS5A inhibitors showed 2/29 (6.9%) genotype 1a HCV-infected patients harboured changes at either codon 30 (Q30R) or 93 (Y93H/Y), and 12.5% of individuals infected with HCV 1b harboured the mutation Y93H. Genotype 2 viruses harboured L31M in some patients; none of NS5a genotype 3 showed changes at the relevant aa positions.

CONCLUSIONS: Our NS5a genotypic resistance assays successfully amplified and sequenced a variety of HCV genotypes over a wide range of viral loads. Utilization of this assay enabled identification, albeit at low frequencies, of natural polymorphisms, which are known to confer resistance to certain NS5a inhibitors. In addition, we observed a low discordance rate regarding genotyping when NS5a and 5’UTR sequences were compared.
ABSTRACT 47
Antiviral Therapy 2013; 18 Suppl 1:A57

β-d-2′-C-Me-2,6-diaminopurine ribonucleoside phosphoramidates are potent and selective inhibitors of hepatitis C virus (HCV) and are bioconverted intracellularly to bioactive 2,6-diaminopurine- and guanosine-5′-triphosphate forms

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BACKGROUND: A unique strategy to the HCV arena for the development of anti-HCV nucleoside prodrug analogues is the ability to generate two active nucleoside triphosphates from a single nucleoside monophosphate prodrug. In addition, the novelty of the phosphoramidate technology applied in this context produces only non-toxic metabolites when it delivers the nucleoside monophosphate intracellularly.

METHODS: We used a multi-disciplinary approach, including medicinal chemistry, molecular biology/virology, rational drug design, biochemistry, cellular pharmacology, HPLC-MS/MS, bone marrow/mitochondrial toxicity and other cytotoxicity assays, to profile the 2′-C-Me-2,6-diaminopurine ribonucleoside (DAPN) prodrugs (PD) compared to a known clinically toxic purine nucleoside INX-189.

RESULTS: All of the DAPN-PD showed up to 14-fold greater potency against HCV replication in Huh7 cells compared to the parent DAPN (for example, EC50=0.26 versus 3.7 μM). The DAPN prodrugs were pan-genotypic and effective against well-defined HCV resistant mutants. Incubation of the parent DAPN in Huh7 cells at 50 μM for 4 h and subsequent HPLC-MS/MS analysis revealed a 1:3 ratio of DAPN-TP to 2′-C-Me-GTP. However, the DAPN prodrugs increased the intracellular levels of DAPN-TP in Huh7 cells up to 13-fold compared to the levels achieved with parent nucleoside DAPN. DAPN-TP was the major metabolite for DAPN prodrugs in primary human hepatocytes and was 7-fold higher than found in Huh7 cells. DAPN-TP and 2′-C-Me G-TP were substrates for genotype 1b HCV pol with an IC50=3.6 μM and IC50=0.46 μM, respectively. While INX-189 displayed cytotoxicity in Huh7, CEM, Vero and PBM cells, as well as bone marrow and mitochondrial toxicity, these new DAPN-PD were devoid of any toxicity when tested in the same assays up to 50 μM or greater.

CONCLUSIONS: DAPN-PD can deliver both DAPN-TP and 2′-C-Me G-TP intracellularly, and each was shown to inhibit HCV polymerase. The high selective potency and intracellular delivery of two active triphosphates were markedly increased by phosphate prodrugs of DAPN, which should improve their resistance profile. The DAPN prodrugs did not exhibit any of the in vitro toxicities observed with INX-189 and appear to be differentiated in their toxicity profile particularly with the release of non-toxic metabolites.
**ABSTRACT 48**

*Antiviral Therapy* 2013; **18** Suppl 1:A58

Very late HCV relapses after discontinuation of antiviral therapy for chronic hepatitis C

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BACKGROUND: Hepatitis C virus (HCV) rebound beyond 24 weeks after discontinuing a full course of hepatitis C therapy with undetectable viraemia seems to be very rare.

METHODS: All chronic hepatitis C patients treated at our institution with either interferon-based regimens or all-oral drug-acting antivirals were retrospectively examined. Very late HCV relapses were defined as serum HCV RNA rebounds beyond week 24 upon drug discontinuation being negative at week 24 post-treatment. To exclude HCV re-infection, a detailed interview about potential sources of HCV re-exposure and phylogenetic analyses was performed. Viral sequences at the E1/E2 and/or NS5B polymerase genes from specimens drawn before treatment and at the time of relapse had to exhibit high bootstrap values (>70%).

RESULTS: A total of 744 patients treated for chronic hepatitis C at our institution were examined, of whom 452 (60.7%) were coinfected with HIV. Four individuals exhibited positive HCV RNA beyond 24 weeks upon hepatitis C therapy discontinuation, being negative at the end of treatment and at week 24 post-treatment. Whereas phylogenetic analyses along with epidemiological evidence supported that HCV re-infection had occurred in two patients, the results in the remaining two cases were consistent with very late HCV relapse.

First case: 44-year old female, with cirrhosis, IL28B-CT, coinfected with HIV and HCV-3a treated for 30 weeks with peginterferon-ribavirin that rebounded 44 weeks later.

Second case: 66-year old male, non-cirrhotic, IL28B-CT, HIV-negative, infected with HCV-1b and treated with faldaprevir, BI-207127 plus ribavirin for 40 weeks that rebounded 36 weeks later.

CONCLUSIONS: The heterogeneity in these two cases along with a recent report of rebound at week 36 post-treatment with ABT-450, ABT-072 plus ribavirin in an HCV-1a monoinfected patient with IL28B-CC alleles suggest that very late HCV relapses do not exhibit a common profile and, therefore, cannot be predicted.
ABSTRACT 49

Antiviral Therapy 2013; 18 Suppl 1:A59

Impact of viral sequences beyond HCV NS5A domain I on viral susceptibility to NS5A inhibitors

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BACKGROUND: As an essential component of the viral replication complex, HCV NS5A consists of three major domains which participate in viral RNA replication (domains I and II) and virus production (domain III). Resistance studies have implicated domain I as the likely target of the antiviral activity of NS5A inhibitors. Here, we report on our studies of the effects of NS5A sequences beyond domain I on the susceptibility of HCV replicons to NS5A inhibitors.

METHODS: Transient replicons were used in the study. Chimeric replicons were constructed incorporating either domain I or the entire NS5A sequence from laboratory strains or clinical isolates into a GT-1b strain Con1 background.

RESULTS: A contribution of sequences outside of NS5A domain I to daclatasvir susceptibility was initially demonstrated when replicons derived entirely from GT-1a strain H77 or GT-2a strain JFH-1 were found to be significantly less susceptible than chimeras carrying only NS5A sequences from these laboratory strains. Additionally, although chimeric replicons carrying either domain I or the entire NS5A from GT-1a strain H77 showed similar susceptibilities to daclatasvir, a difference emerged when the known resistance mutation Q30H was introduced. The mutant chimeric replicon carrying the entire NS5A from GT-1a strain H77 was approximately 10-fold less susceptible than the chimera carrying only NS5A domain I. A similar phenomenon was observed with a panel of chimeric replicons incorporating NS5A sequences from GT-1a clinical isolates; here too, when known resistance mutations were present, chimeras carrying the entire NS5A from the clinical isolates were up to 10-fold less susceptible than those carrying only NS5A domain I from the same isolates.

CONCLUSIONS: HCV sequences outside of NS5A domain I are shown to modulate the effect of known resistance mutations on daclatasvir susceptibility. These data suggest that the full impact of resistance mutations that emerge during clinical studies could be underestimated when routinely assessed using chimeric replicons carrying only NS5A domain I from the clinical isolates.
ABSTRACT 50

First international external quality assessment programme for HBV antiviral drug resistance

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BACKGROUND: Several antiviral agents are available to treat HBV infection, most of which target the viral DNA polymerase. Antiviral drug resistance (HBVDR) has been described for all of these agents, conferred by mutations in the DNA polymerase. HBVDR mutations are mostly diagnosed using DNA sequencing or reverse line blot assays (LiPA). External quality assessment (EQA) for HBVDR determination has not yet been implemented.

METHODS: A quality assessment panel was prepared, consisting of five normal human EDTA plasma samples, spiked with clinical HBV viruses at 4.6–5.3 log IU/ml and representing HBV genotypes D, E or F with or without one or more HBVDR mutations at amino acid positions 80-173-180-181-184-194-202-204-236-250. 1 ml aliquots of each of the five samples were stored at -80°C upon preparation and distributed to participating laboratories on dry ice. Participants performed HBVDR determination according to their routine laboratory procedures. Results were reported online by laboratory code to the neutral office of QCMD as fasta files for the DNA polymerase sequences and as individual mutations for LiPA. Data analysis focused on correct detection of wild-type, mutant or mixed HBVDR codons, in relation to the consensus sequences.

RESULTS: 42 laboratories from 24 countries worldwide participated in the HBVDR EQA distribution, 38 participants reported a total of 43 datasets: 34/43 based on DNA sequencing and 9/43 based on LiPA technology. Sequencing results for the complete panel were reported for 88% of participants. There were no systematic negative results for any HBV genotypes. Main variations in performance were due to i) no results for codon 80 (12–14 datasets per sample), ii) no results for 1 or more of the panel samples (5 samples in 4 datasets) or iii) variation at individual (mixed) codon positions. LiPA results were consistent in all but one dataset. For this dataset mutations were reported at high frequency across all samples, suggesting insufficient assay specificity.

CONCLUSIONS: A worldwide EQA programme for HBVDR determination was successfully implemented. HBV drug resistance mutations were adequately detected by participants independently of the HBV genotype and resistance technology. Inter-laboratory variation was mainly observed at codons containing mixed nucleotides.
SESSION 2
HIV pathogenesis, fitness and resistance
ABSTRACT 51
Antiviral Therapy 2013; 18 Suppl 1-A63
The recognition of the HIV-1 dimeric RNA genome nucleates virus assembly

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BACKGROUND: HIV-1 must package its genome to generate infectious particles. We have shown that HIV-1 encapsidates its genome with high efficiency and most virions contain two viral RNAs. Hence, HIV-1 genome packaging is a highly regulated process; however, the mechanism that achieves this regulation is currently unknown.

METHODS: The viral components of individual particles were determined by fluorescence microscopy (single-virion analysis). Viral genomes were detected by fluorescently tagged RNA-binding proteins recognizing sequences engineered into the viral genomes. The coassembly of Gag proteins was detected by tagging Gag from each virus with a different fluorescent protein.

RESULTS: To determine how HIV-1 regulates genome encapsidation, we examined viral RNAs much larger (≈17 kb) or much smaller (≈3 kb) than that of wild-type HIV-1 (≈9 kb) and found that two copies of the RNA were incorporated into each virion independent of their sizes. However, it is possible for the virus to package just one copy of the HIV-1 RNA if the genome contains a second packaging signal that allows for the formation of intramolecular dimers (self-dimers). These results indicate that HIV-1 genome encapsidation is regulated by the packaging of one dimer.

We hypothesized that the recognition of the RNA dimer by Gag nucleates virus assembly, and the dynamics of assembly prevent the incorporation of additional RNA genomes. To test the ability of packaging signals to nucleate virus assembly, we generated an HIV-1 genome, termed HMPsi, that harbours a second packaging signal from murine leukaemia virus (MLV). The HMPsi RNA can be packaged by either HIV-1 or MLV Gag. Importantly, the presence of the HMPsi RNA promoted the coassembly of HIV-1 Gag and MLV Gag, indicating that the two packaging signals are each nucleating assembly with their corresponding Gags.

CONCLUSIONS: Taken together, our data support a model of HIV-1 assembly in which Gag recognizes dimeric RNA and this recognition serves to nucleate Gag polymerization and particle assembly. This mechanism ensures that one RNA dimer, but not more, is packaged into each nascent virion. As an absolute requirement for infectivity, and a driver of virion assembly, the viral genome should not be overlooked as a potential target for therapeutics.
ABSTRACT 52

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Concentration of pre-ART transmitted and selected HIV drug resistance mutations associated with virological failure of first-line ART in Kenya

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BACKGROUND: Transmitted and selected HIV drug resistance can undermine the effectiveness of first-line ART. Prior to initiation of ART, consensus sequencing (CS) is used in high-resource communities, but is too expensive for most global HIV treatment programmes. Testing of Kenyan pre-ART specimens by a low-cost point-mutation assay was evaluated to determine the codons and the mutant concentrations associated with virological failure of first-line ART.

METHODS: ART-naive individuals qualifying for treatment in Nairobi were enrolled in 2006 and 2010 and followed prospectively. Pre-ART specimens were retrospectively analysed by a quantitative oligonucleotide ligation assay (OLA) for mutations at reverse transcriptase codons 103, 181, 190 and 184, conferring resistance to non-nucleoside reverse transcriptase inhibitors (NNRTI) and lamivudine (3TC). Specimens from 6, 12 +/- 18 months of ART were tested for virological failure (VF; HIV RNA > 1,000 c/ml at last visit). Pre-ART specimens of subjects experiencing VF were also tested by CS and/or pyrosequencing (PS; ~1,000 amplifiable viral templates).

RESULTS: 324 ART-naive adults from 2006 and 169 from 2010, including 72 previously exposed to single-dose-nevirapine (sdNVP) in the 2010 cohort, had outcome data. OLA detected pre-ART resistance at ≥1 codon in 10/324 from 2006, and 8/97 ART-naive and 16/72 sdNVP-exposed from 2010. The median concentration of mutant in each person’s HIV population was 79%, 77% and 75%, respectively, and ranged from 3–100%. Subjects with <9% mutant (most amongst the sdNVP group) did not experience VF. Across the two cohorts HIV drug-resistance ≥9% was more common amongst subjects with virological failure (19/88; 21.6%) than those with suppression 6/405 (1.5%; P < 0.0001). Mutants were detected at all OLA codons evaluated, however, all 25 subjects with mutant concentrations ≥9% were detected by codons 103 and 190. CS of subjects with VF did not identify any additional individuals with high-level drug resistance in the pre-ART specimen. Low pre-ART concentrations of mutant (<2%) detected in additional subjects by PS were selected less frequently at VF compared to mutants detected by OLA (5/29 by PS versus 13/13 by OLA, P < 0.001).

CONCLUSION: Kenyans with pre-ART NNRTI 103 and/or 190 mutations detected by OLA at ≥9% in viral population had a high risk of VF.
ABSTRACT 53
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The drug susceptibility and viral fitness of recombinant viruses expressing patient-derived integrase genes are significantly affected by co-evolved multidrug-resistant protease and reverse transcriptase genes
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BACKGROUND: Raltegravir (RAL) is an integrase strand transfer inhibitor (INSTI) often used in salvage therapy following treatment failure with protease (PR) and/or reverse transcriptase (RT) inhibitors. We examined the effects of co-evolved drug-resistant PR and RT genes on INSTI susceptibility and viral fitness.

METHODS: We generated eight recombinant viruses expressing IN with different RAL-resistant genotypes (Y143C+E92Q, Y143C+T97A, Y143C+G163R, Y143G+G163R, Y143R+G163K, Y143R+G163R, Q148R+G140A and N155H+V151I) all derived from a patient failing RAL-containing therapy. Respective viruses expressing co-evolved multidrug-resistant PR (L10F+V32I+L33F+M46I+I47V+I54L+A71T+I84V+L90M) and RT (M41L+D67N+L74V+L100I+K103N+M184V+T215Y+K219E+N348I) genes were also generated. The susceptibility of the viruses to RAL and elvitegravir (EVG) and their replicative fitness was assessed using a phenotypic single-cycle replication assay.

RESULTS: Genotypic analysis of five sequential samples from a patient failing RAL salvage therapy showed that eight different genetically linked INSTI resistant genotypes had developed over time with Y143G+G163R eventually emerging as the dominant genotype. Phenotypic analysis of viruses expressing the patient-derived IN only showed significant decreases in RAL susceptibility compared to wild-type IN with the Y143C+G163R and Y143R+G163R genotypes having the lowest and highest fold-changes in EC50 (19- and 200-fold, respectively). The respective viruses expressing co-evolved PR+RT exhibited similar levels of RAL susceptibility with the exception of three viruses that showed significant increases of up to threefold (P≤0.032); these being Y143R+G163R, Y143R+G163K and Y143C+T97A. In contrast, only six of the viruses expressing patient-derived IN only exhibited significant decreases to EVG ranging from 5-fold (Y143R+G163R) to 227-fold (Q148R+G140A) with the susceptibility of only one genotype (Y143C+E92Q) significantly increased by the expression of co-evolved PR+RT (P=0.006). The replicative fitness of all viruses expressing patient-derived IN only was significantly decreased ranging from 8% to 56% (P≤0.01) compared to wild-type virus with the exception of Y143G+G163R virus (102%; P=0.85). Interestingly, the addition of co-evolved PR+RT significantly increased the replicative fitness of four RAL genotypes up to 73% (Y143C+G163R, Y143R+G163K, Y143R+G163R and Q148R+G140A; P≤0.024) but had no significant effect on the remaining genotypes.

CONCLUSIONS: Our data show that co-evolved PR+RT contribute to the susceptibility and viral fitness of patient-derived drug-resistant IN and that the intrapatient evolution of drug resistance is a balance between drug susceptibility and viral fitness.
ABSTRACT 54

**Antiviral Therapy** 2013; 18 Suppl 1:A66

**Contribution of HIV minority variants to drug resistance in an integrase strand transfer inhibitor-based therapy**

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**BACKGROUND:** The role of HIV minority variants on transmission, pathogenesis and virological failure to antiretroviral regimens has been explored. However, in the case of minority drug resistant variants, most studies have focused on single target regions, for example, protease, RT or integrase. Here, we used a novel HIV-1 genotypic assay based on deep-sequencing to simultaneously analyse the presence of minority variants carrying mutations associated with reduced susceptibility to PI, NRTI, NNRTI and INSTI, and to determine their coreceptor tropism.

**METHODS:** Plasma samples from twelve treatment-experienced patients experiencing virological failure while participating in a Phase II study of the INSTI elvitegravir (GS-US-183-0105) were used to generate gag-p2/NCP7/p6/pol-PR/RT/INT and env/C2V3 PCR products. Purified amplicons were used in the construction of barcoded libraries and sequenced on the Ion Torrent PGM. Sorting, mapping and aligning of reads were done using proprietary software. Reads spanning the 3′end of Gag, PR, RT, INT and V3 regions were extracted, truncated, translated and assembled for genotyping and determination of HIV-1 coreceptor tropism. Results were compared with (i) virological response to treatment, (ii) genotyping based on population sequencing, (iii) phenotyping using VIRALARTS™HIV, and (iv) tropism based on VERITROP™.

**RESULTS:** All patients had HIV-1 with extensive PR and RT resistance mutations from prior therapy. At failure in this study, new IN mutations were also detected by population sequencing (mean 9.4, 5.3 and 1.4 PI- RTI- and INSTI-resistance mutations at failure, respectively). Interestingly, additional mutations in each region were detected by deep sequencing, where the high coverage achieved in all regions (mean 7,525-fold) also allowed the accurate detection of mutations present in only 1% to 20% of the virus population. Accordingly, the mean number of mutations detected with deep sequencing increased to 14.7, 8.1 and 3.3, respectively; correlating with the phenotypic and replicative fitness results.

**CONCLUSIONS:** The potential impact of minority drug resistance variants in response to antiretroviral therapy is not completely understood. Here, we used a novel all-inclusive HIV-1 genotypic and coreceptor tropism assay to detect mutations at low frequencies and correlate their presence to phenotypic characteristics (resistance, fitness and tropism) of the virus population. Further studies, based on this innovative deep sequencing assay, will help understand the importance of the detection of HIV minority variants in the clinical practice.
ABSTRACT 55
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Is there cross talk between HIV-1 5′RT drug resistance mutations (DRMs) and connection/RNase H (3′RT) mutations?

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BACKGROUND: An increasing number of studies have described RTI-selected mutations in 3′RT. We sought to determine whether specific 3′RT mutations were significantly associated with 5′RT NRTI and NNRTI DRMs.

METHODS: We sequenced the complete RT of 308 subtype B isolates with specific patterns of 5′RT DRMs including K65R (8), L74V (23), Q151M (11), M184V (149), M184I (5), >=3 type 1 TAMs (52), >=3 type 2 TAMs (39), NNRTI DRMs (110), and no NRTI or NNRTI DRMs (91). We then supplemented this dataset with published subtype B RT sequences extending beyond codon 350 from 8,880 persons including complete RT sequences from 1,815 persons. 3′RT mutations that were >=2× more prevalent in RTI+ versus RTI- persons (false discovery rate adjusted at P<0.01) were classified as RTI-selected. Associations between 5′DRMs and 3′RTI-selected mutations were quantified using the Jaccard index (unadjusted P<0.01).

RESULTS: There were seven non-polymorphic (Y318F, G335S, N348I, K350R, A360V, Q394L and E399G) and seven polymorphic (R358K, G359S, T369V, A371V, A376S, S379C and T386A) 3′RTI-selected mutations. Y318F, N348I, Q394L and E399G were >10× more common in RTI-treated persons. The remaining 3′RT-selected mutations were only 2 to 4× more common in RTI-treated persons.

G335S, K350R, R358K, A360V and A376S were associated with type 1 and/or type 2 TAMs; Y318F and E399G were associated with K103N; N348I, A359S, A371V and S379C were associated with TAMs+NNRTI DRMs+M184V; A369V, T386A and Q394L were associated only with other 3′RT RTI-selected mutations. Two 3′RTI-selected mutations were associated with L74V. None were associated with K65R, Q151M or M184I.

CONCLUSIONS: There are few RTI-selected 3′RT mutations and most are weakly associated with RTI therapy. 11 of these mutations were associated with >=1 5′DRM. However, it was not possible to determine whether the 3′RTI and 5′DRMs were linked structurally or through shared RTI-exposure.
ABSTRACT 56

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Protease inhibitors are effective at inhibiting cell-to-cell HIV-1 transfer

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BACKGROUND: Human immunodeficiency virus-1 (HIV-1) spreads efficiently by cell-free diffusion and from cell-to-cell across a virological synapse (VS) in vitro. A recent study suggests that cell-to-cell spread of HIV-1 allows for ongoing virus replication in the presence of reverse transcriptase inhibitors (RTIs). The role of protease inhibitors (PIs) in suppressing such transfer of wild-type or PI-resistant viruses remains to be determined.

METHODS: We used a qPCR-based assay to detect de novo Pol transcripts following cell-free and cell-to-cell infection in the presence of PIs (lopinavir and darunavir) and an RT inhibitor (nevirapine) in vitro. A wild-type virus (pNL4.3_WT) and a drug resistant virus (pNL4.3_DM) containing V82A (protease) and A431V (gag) were tested. For cell-to-cell infections, donor cells infected with a standardized virus input were used to infect target cells in the presence of drug. Alternatively, virus produced by the same donor cells, in the presence of inhibitors was used to infect target cells. The 50% inhibitory concentrations (IC50) of PIs and RTIs for both modes of infection were determined. The efficiency of cell-to-cell spread of pNL4.3_WT was compared to that of pNL4.3_DM. A high-throughput LTR-driven reporter gene assay was used as an alternative method for assessing cell-to-cell versus cell-free infection in the presence of inhibitors.

RESULTS: PIs were equally effective at blocking cell-to-cell and cell-free infection (mean IC50 of LPV for cell-cell and cell-free spread =5.5 nM, DRV=2.9 nM) whereas, cell-free infection was around fourfold more susceptible to nevirapine (NVP) inhibition compared to cell-to-cell spread (mean IC50 of NVP for cell-to-cell =333 nM, and cell-free =82 nM). There was no difference in the replication capacity of pNL4.3_WT and pNL4.3_DM in a cell-to-cell assay and pNL4.3_DM maintained its drug resistant phenotype. We also found that Tat-driven reporter gene readout overestimated infection of target cells, partially due to non-specific transactivation from secretion of Tat protein, induced by the VS in co-culture. A direct measure of infection such as qPCR detection of RT products is more reliable and specific for assessing infection in vitro.

CONCLUSIONS: PIs are equally active in cell-free and cell-to-cell infections. The relative potency of antivirals for cell-to-cell spread may impact on long-term eradication strategies.
ABSTRACT 57

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Genotypic analyses of pre-existing HIV-1 drug resistance in proviral HIV-1 DNA from PBMCs in suppressed patients switching to RPV/FTC/TDF

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BACKGROUND: Transmitted HIV-1 drug resistance mutations are present in ~15% of treatment-naive patients. A regimen switch in patients with HIV-1 RNA< 50 copies/ml may be guided by a historical genotype, if available. Genotyping of archived, integrated HIV-1 DNA may have utility in this setting of viral suppression. The comparison of historical plasma HIV-1 RNA genotypes obtained prior to initiation of first-line antiretroviral therapy to proviral HIV-1 DNA genotypes obtained at the time of entry into Study GS-US-264-0106 (switch from PI/r+2 NRTIs to RPV/FTC/TDF) with a focus on patients with K103N, is presented.

METHODS: An experimental genotyping assay of the protease/reverse transcriptase region of proviral HIV-1 DNA from PBMCs generated population sequencing results (Monogram Biosciences). 81 banked PBMC samples were genotyped in a blinded fashion. Samples with K103N by historical plasma genotype and wild-type controls matched by baseline CD4 count, and viral load and CD4 count at the time of first-line antiretroviral therapy initiation were analysed. Additional samples with other key resistance mutations were also investigated.

RESULTS: Of 81 PBMC samples, 80% had proviral DNA genotypic data generated. Historical HIV-1 plasma genotypes from 21 patients with K103N and 28 patients without K103N were compared to the proviral DNA genotypes from PBMCs collected a median of 3 years later and corresponding to the time of switch to RPV/FTC/TDF. Of these patients, all historical K103N patients with proviral genotypes showed the presence of K103N at the time of switching to RPV/FTC/TDF (19/19 with data), and 0/19 patients in the wild-type control population was found to have K103N. The majority of patients with transmitted/archived K103N maintained undetectable HIV-1 RNA after switching to RPV/FTC/TDF, with only one patient experiencing virological failure with additional emergent resistance mutations. Additional RPV-associated mutations (including E138A/G/K, H221Y and M230I) were identified in the proviral genotypes that were not reported in the historical genotypes, most commonly because resistance algorithms have been extended.

CONCLUSIONS: Among virally suppressed patients, there was a high concordance for K103N by historical plasma HIV-1 RNA genotype and later proviral HIV-1 DNA genotype from PBMCs. Patients who switched to RPV/FTC/TDF with pre-existing K103N generally maintained virological suppression.
SESSION 3
Drug resistance in resource-poor countries
ABSTRACT 58
Antiviral Therapy 2013; 18 Suppl 1:A73

Genotypic resistance profiles of HIV-2-treated patients in Côte d’Ivoire, West Africa

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OBJECTIVE: To assess virological response and genotypic resistance profiles of HIV-2 antiretroviral-treated patients in care in Côte d’Ivoire.

METHODS: A cross-sectional survey was conducted among HIV-2-infected patients on antiretroviral treatment (ART). Plasma HIV-2 RNA (pVL) was performed using the recently licensed assay Biocentric™ (LOD=40 copies/ml). Protease and reverse transcriptase (RT) sequencing was performed in samples with pVL>100 copies/ml using in-house methods. HIV-2 resistance mutations were identified using the list generated by the ‘Collaborative HIV and Anti-HIV Drug Resistance Network’. Median (IQR) results were presented.

RESULTS: A total of 146 HIV-2-treated patients (46% of women) were enrolled with age of 49 years (39–54) and CD4 cell of 360/mm³ (215–528). The ART duration was 4.0 years (1.5–6.6) and the most common ART-regimen prescribed at initiation was PI-based (77%). 75% of these patients displayed pVL<40 copies/ml and pVL of the 36 viraemic patients was 3,016 copies/ml (436–5,156). Most of them (83%, n=30) were receiving a lopinavir-based regimen, and three (8%) a triple-nucleotide RT inhibitors (NRTI) regimen. Protease and RT sequencing was successful in 29/36 (81%) and 25/36 (69%) samples, respectively. HIV-2 resistance mutations to NRTIs and PIs were detected in 21/25 (84%) and 20/29 (69%) samples, respectively. The most prevalent resistance mutations to NRTIs were: M184I/V (95%, n=19), Q151M (25%, n=5), S215F/Y (25%, n=5), K65R (15%, n=3) and D67N (10%, n=2). The Q151M mutation was not specifically associated with double- or triple-NRTI regimen failure. The most prevalent PI-resistance mutations were: V47A (60%, n=12), 154M (30%, n=6) and L90M (30%, n=6). Each of the PI resistance mutations 150V, 182F, 184V, V62A and L99F were detected in three samples (15%).

CONCLUSIONS: In this series of HIV-2-treated patients in Côte d’Ivoire, 75% displayed undetectable viral load. The high rate of resistance mutations observed both to NRTI and PI drug classes, associated with the reduced number of ARV drugs active against HIV-2, is an issue, since 28% of these patients displayed virus with at least one resistance mutation to darunavir.
ABSTRACT 59
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Surveillance of transmitted HIV-1 drug resistance in newly diagnosed individuals from Rio de Janeiro, Brazil

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BACKGROUND: The transmission of HIV-1 drug-resistance has important implications for treatment outcome in infected individuals initiating antiretroviral therapy (ART). In this study, we estimated the prevalence of transmitted drug resistance (TDR) and subtype distribution, in recently diagnosed HIV-1-infected individuals from Rio de Janeiro State, in the context of the Brazilian Network for HIV-1 Drug Resistance Surveillance (HIV-BResNet).

METHODS: The evaluation of genotypic data from newly diagnosed HIV-1 infection identified in Public Health Units, during 2009 to 2012, was utilized for estimating the prevalence of TDR among ART-naive HIV-1-infected individuals from Rio de Janeiro State.

The profiles of TDR mutations were evaluated using the Calibrated Population Resistance (CPR) software available by Stanford website and subtype determination through the Brazilian Algorithm for HIV Drug Resistance Interpretation.

RESULTS: A total of 159 HIV-1-infected individuals, including 36 blood donors, were genotyped. The mean of CD4+ cell count was 416 cells/ml and viral load 89,505 copies/ml. The majority of genotyped samples were classified as subtype B (80.0%), followed by C (5.1%), F (3.1%) and unique recombinant forms (URF) composed by BF (3.1%), BC (2.5%) and FC (0.6%) sequences mosaic. The new diagnosed infection associated to subtype A1 was identified in one individual. Overall, TDR mutations were detected in 12.5% (95% CI, 6.95% to 17.05%) of the sequences analysed. Resistance to the PIs was 4.4% (95% CI, 1.21% to 7.59%), represented by the V82L mutation to atazanavir. The prevalence to the NRTIs was 5.6% (95% CI, 2.03% to 9.17%) and the remaining 2.5% (95% CI, 0.07% to 4.93%) of isolates carried NNRTIs-associated mutation.

CONCLUSIONS: The present study confirms an increase in the prevalence of the subtype C among recently diagnosed HIV-1-infected individuals, compared with previous reports. For the first time, infection with the subtype A1 was described in Rio de Janeiro, suggesting the recent introduction of this virus in Brazil.

The prevalence of HIV-1 TDR mutations is in accordance with other previous national surveys. However, our results demonstrated an accumulation of resistance associated to the NRTIs and reduction to NNRTIs, which could be a risk for the long-term usage of the analogue nucleosides in antiretroviral regimens for first- and second-line therapy in Brazil. Further research is needed to determine whether baseline resistance testing is necessary and cost-effective for these individuals.

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ABSTRACT 60
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Differential first-line antiretroviral therapy resistance in HIV-1 non-B subtypes in India, China and Thailand
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BACKGROUND: HIV drug resistance (DR) evolution is a major cause of antiretroviral treatment (ART) failure. Epidemiological comparisons of DR mutation (DRM) frequencies among subtypes can be difficult to interpret because of heterogeneous ART histories.

METHODS: We constructed a multilevel model to robustly estimate differences in DRM frequencies across HIV-1 subtypes of three single-site cohorts in India, China and Thailand. Genotyping was performed for adults failing first-line non-nucleoside reverse transcriptase inhibitor-based ART. Subtyping was required to be unambiguous by REGA. DRM were defined by the International AIDS Society-USA 2011 list. We estimated DRM frequencies in each subtype-site population and compared frequencies between populations. Mutation counts were used to estimate a multilevel beta-binomial model with mean frequency parameterized by DRM position, subtype-site and ART composition and duration. ART history effects were averaged and DRM frequencies, subtype-site frequency differences and their 95% credible intervals (CI) were estimated.

RESULTS: Of 946 sequences, 529 were evaluated: 205 India (79% male; median age 39; median CD4 145; median logVL=4.9; 100% subtype C), 177 China (73% male; median age 42; median CD4 205; median logVL=4.1; 64% AE; 36% B) and 147 Thailand (63% male; median age 38; median CD4 205; median logVL=4.0; 93% AE; 7% B). The most common DRM in all cohorts was M184V/I followed by K103N/S in B-China, B-Thailand, C-India and Y181C/I/V in AE-China and AE-Thailand. All (33/33) significant DRM frequency differences (estimates with a 95% CI excluding a null difference) between C-India and other populations were higher in C-India (log odds ratios 0.5 [0.0, 0.9] to 1.7 [1.1, 2.5]). Differences were largest at thymidine analogue mutations (TAM) 98G, 103N/S, 106M/I/A, 181C/I/V, 190A/S and 184V/I. Although VL/CD4 values did not account for DRM frequency variation across sites. DRM frequencies were lower in AE-China compared to AE-Thailand for TAM, 75I and 184VI. All (28/28) frequency differences between B-China and other populations reflected fewer DRMs in B-China; -0.6 (-1.1, 0) to -1.6 (-2.5, -0.8).

CONCLUSIONS: Using a novel application of multilevel modelling accounting for ART history, we find clear differences in DRM between HIV-1 subtypes and sites, with implications for sequential treatment regimens and ART response.
ABSTRACT 61

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Pill counts and drug level determinations do not predict therapy failure due to drug resistance in a resource-limited setting

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BACKGROUND: HIV-1 treatment management in resource-limited settings mostly involves clinical and, limited, immunological monitoring. Counselling and pill-counts are used to improve adherence, and are considered a predictor for selection of drug resistance (HIVDR), and hence a tool to guide therapy switching. We assessed the association of pill-counts and drug-levels with actual HIVDR in South African patients.

METHODS: HIVDR-genotyping and drug-level determination were performed on dried blood spots (DBS) collected from 66 patients experiencing virological failure. From DBS, calculated plasma levels >3.0 mg/l for nevirapine and >1.0 mg/l for efavirenz were considered adequate. Lopinavir levels were scored as detectable/not. Structured pill-counts over the year prior to DBS were reported as a percentage and averaged.

RESULTS: Patients were primarily female (62%), age 15–66, average 3.1 years on therapy, 64% first-line, 36% second-line therapy. All harboured subtype-C. Mean VL was 4.07 log_{10} copies/ml. 65% had resistance to reverse-transcriptase inhibitors (RTIs).

The range of average adherence over the year prior to DBS sampling was 0–100% (mean 62; median 64) in patients with HIVDR and 20–100% (mean 78; median 89) without HIVDR.

During first-line therapy, 75% of patients with and 64% without resistance had adequate drug-levels. Adherence determined by pill-counts at time of DBS did not correlate with drug-levels: 77% of adherent versus 70% of non-adherent patients had adequate levels. Resistance was present in 70% of those with an adequate drug-level, versus 64% with inadequate levels. Similarly, resistance was present in 64% of adherent and 70% of non-adherent patients.

During failure of second-line therapy, no PI mutations were found. Lopinavir was detectable in 47% of patients with and 33% without RTI resistance. Pill-counts did not correlate with drug-levels: 55% of adherent versus 31% of non-adherent patients had adequate drug-levels. RTI resistance was present in 70% of patients with detectable lopinavir versus 57% in patients without. Similarly, RTI resistance was present in 64% of adherent versus 62% in non-adherent patients.

CONCLUSIONS: We have shown that pill-counts and drug-level determination were not associated with predicting the presence of HIVDR. Albeit, pill-counts may be a good method for adherence counselling, they are not sufficient to base decisions on therapy switching.
SESSION 4
Epidemiology
ABSTRACT 62
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Molecular phylogenetic analysis of drug resistance transmissions in HIV-1 subtype B in Japan

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BACKGROUND: To better understand epidemiology of HIV-1 subtype B transmission in Japan, we conducted a phylogenetic analysis using viral pol sequences of individuals who were newly diagnosed as HIV-1 seropositive.

METHODS: Cases newly diagnosed as HIV-1 infected between 2003 and 2009 were enrolled in the study. Protease reverse transcriptase (RT) sequences were collected and each region’s subtype was determined using similarity plot analysis against subtype reference sequences. Samples classified as subtype B through whole protease-RT region (whole-B) were used for subsequent analysis. Selected sequences were aligned with foreign 322 subtype B sequences from the Los Alamos Database, and a molecular phylogeny was inferred using a distance-based method with interior branch test. Domestic transmission clusters (micro-clades: MCs) were detected from the tree using the interior branch test with 95% significance level. The monophyly of each MC was confirmed by Bayesian Marcov Chain Monte Carlo inferences. This procedure was also used to estimate the chronological phylogeny and median time of the most recent common ancestor (tMRCA).

RESULTS: During the study period, 2,150 protease-RT sequences were obtained. Overall characteristics of the sample were male (95.5%), Japanese (89.3%) and men having sex with men (MSM; 71.5%). Of these, 1,882 cases were classified as B, and 247 MCs, which showed monophyly in both tree inferences. Regarding MCs with ≤3 individuals, MSM appeared to be a major risk factor. Regarding transmission as a scale-free network, the scaling index of subtype B was approximately half that previously reported for CRF01_AE, suggesting that subtype B transmits more readily among MSM than others. The distribution of tMRCA showed that immigration of subtype B viruses into Japan began in the late 1990s and peaked in the early 2000s. Some drug resistance mutations, such as T215X, K103N and M46I/L, demonstrated MC specific patterns, suggesting that strains with the mutations were circulating in closed communities.

CONCLUSIONS: In Japan, HIV-1 subtype B transmits thorough MSM communities with over 200 distinct clusters. The geographical distribution during the network formation depends on the individual cases, suggesting that MSM communities in Japan have several subpopulations with a variety of relationships to local communities.
ABSTRACT 63

*Antiviral Therapy* 2013; 18 Suppl 1:A80


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BACKGROUND: The use of antiretroviral drugs has reduced the mortality and morbidity of patients with HIV/AIDS. More than 20 kinds of antiretroviral drugs are being used in antiretroviral drug-naive and HAART-experienced patients infected with HIV since zidovudine has been used in 1991 in South Korea. To assess the prevalence of drug resistant variants and the pattern of mutation sites by classified regular periods; 1999–2005 versus 2006–2012, we analysed the genetic variation of the HIV-1 protease and reverse transcriptase in the plasma of 1,018 antiretroviral drug-naive patients from 1999 to 2012 in South Korea.

METHODS: Specimens from 1,018 antiretroviral drug-naive patients were collected from 1999 to 2012. The protease and RT gene mutations were identified from the Stanford DB and the International AIDS Society resistance testing-USA panel (IAS-USA). Interpretation of drug resistant mutations was identified by the consensus statement from the Stanford sequence database (DB) for HIV PR (codons 1–99) and RT (codons 1–300). The intensity of drug resistance was classified as ‘SIR’ interpretation based on the Stanford DB.

RESULTS: Out of 1,018 antiretroviral drug-naive patients, 54 (5.3%) isolates showed ‘intermediate’ or ‘resistant’ by drug resistance interpretation. The predicted genotypic drug resistance to NRTIs was 2.7%, to NNRTIs was 1.3% and to PIs was 0.3% from 1999 to 2005; that to NRTIs was 2.1%, to NNRTIs was 3.5% and to PIs was 0.7% from 2006 to 2012. The phylogenetic analysis from pol gene revealed that more than 90% belongs to subtype B; and, 41 (5.7%) isolates showed at least one drug class-related drug resistance in 2006 to 2012 while 13 (4.3%) isolates showed in 1999 to 2005.

CONCLUSIONS: There has been no significant increase in the prevalence of drug resistance among antiretroviral drug-naive patients infected with HIV-1 for the last 13 years except 2012 in South Korea. But, the prevalence of antiretroviral drug resistance shows a few increases in antiretroviral drug-naive patients reported 2006 to 2012.

And, it will be continued to monitor the emergence of drug-resistant variants as a national survey.
**ABSTRACT 64**

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Viral tropism and antiretroviral drug resistance in HIV-1 subtype C infected patients initiating or failing highly active antiretroviral therapy in Johannesburg, South Africa

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**BACKGROUND:** The overall aim was to assess whether HIV-1 subtype C infected individuals failing highly active antiretroviral therapy (HAART) have a higher proportion of CXCR4-utilizing (X4-tropic) viruses compared to antiretroviral drug-naive patients. We have previously shown that up to 30% of antiretroviral drug-naive patients have CXCR4-utilizing HIV-1 subtype C. Genotypic characterization of viral tropism in HIV-1 subtype C could help inform whether CCR5 antagonists should be used as salvage therapy in patients failing HAART or as part of first-line/early regimens for maximum benefit.

**METHODS:** Samples from patients failing HAART and sent for routine genotyping between January and March 2013 to the Charlotte Maxeke Johannesburg Academic Hospital were available for this study. One hundred samples with known HIV antiretroviral drug resistance profiles were randomly selected. The full length envelope glycoprotein was RT-PCR amplified and the V3 loop region sequenced. Coreceptor utilization and viral tropism was predicted manually by assessing sequences for typical features of X4 viruses (V3 net charge and length, 11/25 rule and variable crown motif) and by using the publicly available predictive algorithms C-PSSMsinsi and geno2pheno[coreceptor]. Matched HIV-1 reverse transcriptase and protease inhibitor resistance profiles were extracted from the Viroscore database.

**RESULTS:** Overall, all patients were infected with HIV-1 subtype C and harboured virus with at least one antiretroviral drug resistance mutation. Thymidine analogue mutations (TAMs) were present in 33.3% of patient samples, with 8.3% possessing mutations associated with the TAM1 pathway, 20.8% with TAM2 and 4.2% had mutations common to both pathways. Thirty eight percent of the HAART-failing subjects were found to have CXCR4-utilizing viruses. The difference in prevalence of HAART-failing subjects having X4-tropic viruses compared to 30% of HAART-naive subjects is not statistically significant.

**CONCLUSIONS:** Interestingly, our findings contrast reports from Durban that showed a significant difference between HAART-failing subjects with X4/dual/mixed-tropic viruses (60%) compared to HAART-naive subjects ($P<0.02$). Despite the differences in proportions of X4-tropic viruses in HAART-failing subjects seen within South Africa, the high proportion of TAMs and CXCR4-utilizing HIV-1 viruses highlights the need for intensified monitoring of HAART patients and the predicament of diminishing drug options, including CCR5 antagonists, for patients failing therapy.
ABSTRACT 65

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Subtype B HIV transmission groups in Israel detected by Bayesian phylogenetics are composed predominantly of men who have sex with men (MSM) with recent HIV infection

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BACKGROUND: Characterizing local patterns of HIV transmission is useful to track epidemic spread and to focus resource allocation for testing, treatment and prevention efforts. Israel has had an ongoing HIV epidemic for >30 years with multiple introductions of HIV, but current transmission patterns are not well described. To investigate contemporary patterns of HIV subtype B spread, we conducted a retrospective analysis using clinical and epidemiological data with Bayesian reconstructions of HIV sequences.

METHODS: We reviewed data submitted to the Israeli HIV national reference laboratory during 1996–2010 to identify newly diagnosed individuals. Genotypes and demographic information were recorded. Recent infection (<1 year) was estimated using an algorithm incorporating analysis of known duration of infection, presence of ambiguous bases and CD4 count. Transmitted drug resistance codons were removed to prevent them from contributing phylogenetic signal. Sequences were aligned (MEGA 5.05) and alignments subjected to Bayesian Monte Carlo Markov Chain analyses (BEAST) to construct phylogenies and investigate ancestral relationships. Transmission clusters were defined as populations with short branch lengths and posterior probability ≥0.95 of having a recent common ancestor. The reconstructed trees were analysed with corresponding demographic and epidemiological data.

RESULTS: We identified 481 newly diagnosed individuals with HIV (risk: MSM=64.5%; IVDU=13%; heterosexual =13%; other/unknown =10%). 143 individuals (30%) were in 43 distinct transmission clusters using BEAST. Individuals in clusters were significantly skewed toward MSM risk group (MSM=80%; IVDU=9%, heterosexual =5.6%; chi-square <0.0001 compared to non-clusters). MSM in clusters were more likely to be recently infected than chronically infected (chi-square <<0.0001) than unclustered MSM. Women (11.4% of the dataset), were only infrequently members of transmission clusters (3.5%, chi-square 0.0003). Overall, 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+ heterosexual, MSM+ injection drug users).

CONCLUSIONS: Bayesian methods revealed that 30% of all newly diagnosed subtype B infected individuals in Israel can be mapped to transmission clusters. The majority of individuals in clusters were MSM with recent HIV infection who were present in both uniform and mixed risk clusters. Additional strategies will be essential to track HIV infection in women.
ABSTRACT 66

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Changes in illicit drug use practices in the greater Tel-Aviv area results in cluster transmission of the subtype A/CRF01_AE variant

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BACKGROUND: High-risk behaviour may be associated with HIV cluster transmission. We recently described an outbreak of primary HIV infection (PHI) among IV drug users (IDU) in Tel-Aviv. The aim of the current study was to identify clusters and drug resistance-associated mutations in this population.

METHODS: Active surveillance was undertaken to determine all PHI cases diagnosed from January 1 2011 to December 31 2012. Plasma samples of HIV-1 treatment-naive IDU patients diagnosed at the Tel-Aviv Medical Center were sequenced for reverse transcriptase and protease by the Trugene HIV-1 genotype kit (Siemens). Sequencing enabled determination of the subtypes of viral isolates (Stanford database). Phylogenetic relationships among viral sequences were estimated using the neighbour-joining and maximum likelihood methods (PHYLIP software package and a combination of the MAFT version 6 and the ATGC phylML programs, respectively). A bootstrap of 100 replicas was applied for both methods.

RESULTS: Twenty-six cases of IDU/PHI were diagnosed after May 1 2012. Comorbidities included hepatitis C virus (100%) and hepatitis B virus (33%) infections. Most of the patients were hospitalized due to severe skin and soft tissue infections, which were complicated with bacteremia. All HIV isolates were of a variant of subtype A/CRF01_AE that is found in most patients who emigrate from the former Soviet Union. Phylogenetic analysis showed a tight clustering suggestive of a single source of infection. There was no evidence of mutations associated with resistance to these viruses. Our investigation revealed a recent shift in illicit drug use patterns among all infected individuals from heroin to combinations of cathinone and buprenorphine. This shift was associated with increased frequency of injections and no need for boiling due to the solubility of the new compounds.

CONCLUSIONS: The ongoing emergence of an outbreak of primary HIV infection in IDUs was directly associated with a recent change in illicit drug use practices. The clustered transmission of these viruses was directly related to this example of high-risk behaviour. Although no resistance mutations had been observed, active surveillance for their presence is imperative.
ABSTRACT 67
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National sentinel surveillance of transmitted drug resistance in primary HIV-1-infected patients in France over 6 years: 2007–2012

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OBJECTIVE: As recommended by the French ANRS program for HIV-1 resistance surveillance, we estimated prevalence of transmitted drug resistance mutations (RAMs) in primary HIV-1-infected patients.

METHODS: Resistance mutations were sought in samples from 1,205 primary HIV-1-infected patients in 2007–2012 in 36 HIV clinical care centres. Genotypic resistance studies were performed on protease, reverse transcriptase and integrase from viral plasma RNA. Protease and reverse transcriptase (RT) mutations were identified from the consensus statement of the list for genotypic surveillance of transmitted HIV-1 drug resistance. As no transmitted resistance surveillance list is currently available for etravirine (ETR), rilpivirine (RPV) and integrate resistance mutations, we used the list of mutations identified from the ANRS resistance algorithm and the latest IAS-USA resistance mutations list.

RESULTS: At inclusion, median viral load and median CD4 cell count were 5.4 log_{10} copies/ml and 502 cells/ml, respectively. Prevalence of virus with protease or reverse transcriptase RAMs was 12.2% (147/1205). Integrase RAMs were observed in 1.7% (4/233, E157Q). Prevalence of PIs, NRTIs and NNRTIs RAMs was 2.7%, 5.6% and 7.1%, respectively. RAMs to RPV and/or ETR were observed in 3.8% (L100I [1], E138A [32], E138K [3], E138G [1], Y181C [8] and Y181I [1]). Resistance to two or three classes of ARV was observed in 2.2% of patients. Although HIV-1 B subtype was still predominant (67%), non-B subtypes (33%) were present in all regions of France. Frequency of RAMs was higher in patients infected with B subtype compared to non-B (15.3% [122/797] versus 6.4% [25/389], P<0.05). MSM were more frequently infected with resistant virus than heterosexual patients (12.9% [109/840] versus 8.5% [21/248], P<0.05). When compared to 2005/06 survey, the overall prevalence of resistance remained stable (12.2% versus 12.5%). However, we observed an increase in frequency of virus with NNRTIs RAMs in 2007/12 compared to 2005/06 survey (7.1% versus 4.6%) due to RAMs to etravirine or rilpivirine.

CONCLUSIONS: In France in 2007–2012, the global prevalence of transmitted drug resistant variants was 12.2% and stable compared to previous survey. MSM and B subtype infected patients are the groups with higher drug resistance prevalence.
ABSTRACT 68
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Epidemiological correlates of HIV-1 B subtype transmission and TDR in Italy according to a nationwide large-scale phylogeny

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BACKGROUND: Novel methods for large-scale phylogeny have been proven to be effective in highlighting spread correlates and characterizing epidemiological clusters, including those with TDR.

METHODS: We analysed 3,786 sequences from patients, of whom 26.5% were naive, carrying B subtype, selected with a 1:4 ratio for naive and treated subjects, enrolled between 1996 and 2012 in the Italian ARCA cohort. Clusters were inferred using PhyloPart as >3 sequences with >90% branch support and short genetic distances.

Mutations were identified according to SDRM and IAS lists. Dating phylogeny used the Beast program. Differences among variables and time trends (4-year intervals) were tested using standard parametric and non-parametric statistic methods and logistic regression analysis.

RESULTS: Males were 69.5%; 38.4% were HE, 36.3% IDUs and 22.5% MSM. Twenty-five percent of sequences formed 157 epidemiological clusters. A higher proportion of MSM (33.8%) compared to HEs (26%) and IDUs (21.3%) was detected in clusters (P<0.001). Overall clustering sequences (CS) rose from 1996–1999 to 2008–2011 (21.8% to 32.1%, P<0.001) and were more frequent among males than females (27.3% versus 22.4%, P=0.002).

Independent predictors of clustering were male gender (95% CI OR: 1.031–1.496), homosexual risk factor compared to heterosexual one (OR: 1.051–1.598) and period of sampling (OR per each period higher: 1.194–1.428).

A lower probability of clustering was age (OR per 1 year older: 0.975–0.992).

Median cluster TMRCA was 50.5 years (range 26.9–81.7). Skyline plot indicated that the effective number of infections slightly grew until 1990, remaining constant thereafter.

TDR was 12.5%; 9%, 5.2% and 2.6% for any drug, NRTIs, NNRTIs and PIs. Proportions of TDR inside or outside clusters were comparable (13.2% versus 12.2%). Both any and class TDR trends did not differ over time, as well as CS with any and class TDR.

Noteworthy, 20 clusters encompassed only patients carrying resistance mutations (12.7%), 8 of these included both drug-naive and experienced patients (5.1%). In contrast, no clusters were found involving only naive subjects with TDR.

CONCLUSIONS: MSM, males and younger individuals were strongly associated to transmission chains. Despite the increase in clustering patients, results of TDR suggest that it is stable over time and its source is represented by experienced rather than naive patients.
ABSTRACT 69
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Phylodynamics of the Montreal MSM epidemic: an ounce of prevention for a pound of cure
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BACKGROUND: The Montreal epidemic has expanded despite 80% HAART coverage and reduced population viral load. Viral phylogenetics has established the underlying structure of the transmission networks where half of the epidemic can be ascribed to one infection leading to 15 onward transmissions. Two HIV-1 testing initiatives, SPOT and Actuel sur la Rue, were designed to identify barriers to testing and increase timely diagnosis. Phylogenetic, virological and behavioural data were combined to deduce the interplay of viral, social and health-care delivery networks in the spread of the epidemic.

METHODS: Phylogenetic analysis elucidated clustering (>95% bootstrap<1% genetic distance) of primary infections (PHI<6 months, n=1,382) over the last decade. Questionnaires contrasted testing habits and risk behaviours of HIV-infected (n=37 and 49) and uninfected (1,682 and 2,441) MSM at SPOT and Actuel.

RESULTS: Viral phylogenetics identified 357 unique PHI transmissions, 104 small PHI clusters (2–4 PHI/cluster, n=275) and 60 large cluster networks (6–61 PHI/cluster, n=750). Viral loads, averaging 4.7 copies/ml, were similar in all three groups; sequence ambiguity was however, significantly lower for large clustered PHI (0.12%) than small clustered (0.24%) and unique (0.37%) transmissions. Large clusters expanded over median 11 month periods (3.5–25.5 month IQ), whereas small clustered transmissions expanded over narrow 4.8 month (1–11.5 month IQ). Large clusters were the driving force of the epidemic representing 30%, 43% and 54% of PHI in 2005, 2009 and 2012, respectively. Overall, 43/76 (57%) of newly-diagnosed MSM at SPOT and Actuel were associated with large clusters. Whereas, HIV-infection was higher for having >5 one-night partnerships three months prior to testing (OR=4.3 months), cluster size was associated with poor testing habits (OR=7.4). Size of clusters was reduced by 0.56 for each additional test in the previous 2 years (none, 1, 2 and 3). Time of last test, cluster association, sequence ambiguity and X4 tropism identified 25% of persons were primary infections (<3 months) while 30% were late presenters.

CONCLUSIONS: Large cluster networks could be averted through timely diagnosis and earlier HAART initiation. Conversely, ongoing selection of highly transmissible variants (11% variants → 54% transmissions) has implications in introducing transmitted resistance and non-B subtypes.
ABSTRACT 70

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Sexually transmitted/founder HIV-1 cannot be directly predicted from plasma or PBMC-derived viral quasispecies in the transmitting partner

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**OBJECTIVES:** Characterization of HIV-1 sequences in newly infected individuals is important for elucidating the mechanisms of viral sexual transmission. Since most previous studies only characterized the transmitted viral population in recently infected patients, little information was available about its relationship with virus circulating in the transmitting partner. Also, few of these studies compared the viral populations identified by analysis of both RNA and DNA samples from donor/recipient pairs.

**METHODS:** We report the identification of transmitted/founder viruses in eight pairs including sexually infected patients enrolled at the time of primary HIV-1 infection (‘recipients’) and their HIV-1-infected transmitting partners (‘donors’). Using a single genome amplification approach, we compared donors’ and recipients’ quasispecies, that is, 316 and 376 C2V3 *env* sequences amplified from plasma viral RNA and PBMC-associated DNA, respectively.

**RESULTS:** We described very homogeneous viral populations in both DNA and RNA sequences in all recipients, suggesting transmission of a single variant in both heterosexual (n=4) and homosexual (n=4) routes of infection, even in cases of recent STI in donors (n=2) or recipients (n=3). In all pairs, the transmitted/founder virus was derived from an infrequent variant population within the blood donor sequences. The donor variants most closely related to the recipient sequences were derived from plasma and PBMC samples in 3/8 and 6/8 cases, respectively. Although donors were exclusively (n=4) or predominantly (n=4) infected by CCR5-tropic (R5) strains, two recipients were infected with strongly homogeneous CXCR4/dual-mixed-tropic (X4/DM) viral populations, isolated in both DNA and RNA. The proportion of donors’ X4/DM quasispecies was higher in the case of X4/DM versus R5 HIV transmission (16.7–22.0% versus 0–2.6%), suggesting that X4/DM transmission could be associated with a threshold population of X4/DM circulating quasispecies in donors.

**CONCLUSIONS:** Sexually transmitted/founder virus cannot be directly predicted by the characterization of donors’ plasma and/or PBMC-associated viral quasispecies. These suggest the selection of minor blood variants with properties favouring transmission. They also imply that viral compartmentalization between blood and genital viral subpopulations may contribute to the selection of the transmitted/founder strain.
ABSTRACT 71

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Integrase inhibitors resistance-associated mutations in multi-experienced Mexican patients failing to raltegravir

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BACKGROUND: Despite the clear benefits of ARV treatment, the development of antiretroviral resistance is still a major challenge, especially in patients that have received multiple ARV treatments that have created important cross-resistance for several drug classes. The introduction of new drugs with new targets in the viral cycle, such as the integrase inhibitors, have given a new hope for multi-experienced patients for more virological control, however resistance to these agents is developing slowly. The aim of this study was to determine the frequency of raltegravir-related mutations in Mexican patients that have failed to multiple ARV combinations.

METHODS: Using the database of the National Committee on Resistance (CORESAR) we were able to detect 191 cases with multiple ARV failures in which we prescribe raltegravir in the salvage treatment and with a follow-up of at least one year. Of those, eight cases with confirmed treatment failure were genotyped using a population sequence method (ViroSeq™ HIV-1 integrase Genotyping Kit Celera Diagnostics) and resistance was interpreted according the Stanford HIVDB genotypic resistance interpretation algorithm.

RESULTS: Only 8/191 (4.2%) of the RAL salvage treatments showed virological failure, 7 male and 1 female. All of them had more than four previous ART combinations, with viral loads between 7,000 and 880,000 copies/ml and CD4+ cell counts less than 200 cells/mm³. In three cases (37.5%) no resistance-associated mutations were found in the integrase. The mutation N155H was present in two cases, one alone and the other with E92Q. Mutations in position 148 were found in three cases, two with Q148H/G140S and one with Q148K associated to G140A. No mutation Y143R was found.

CONCLUSIONS: Failure to raltegravir-based combinations in multi-experienced patients in Mexico is rare. Close to 40% of these failures are not associated with mutations in the integrase, due to non-adherence or resistance to other components of the combination. Those cases with Q148H/G140S have decreased activity of dolutegravir.
ABSTRACT 72

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The epidemic of acute hepatitis C in HIV+ homosexual men in Madrid has emerged recently and from local intravenous drug users

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BACKGROUND: Outbreaks of acute hepatitis C in HIV+ men who have sex with men (MSM) have been reported in large cities in western countries along with increasing rates of sexually transmitted diseases. Sexually transmitted HCV spreading in this population is a relatively new phenomenon, unrecognized until ten years ago.

METHODS: All HIV+ individuals attended at a large outpatient clinic in Madrid within the last 5 years were examined. Incident syphilis was diagnosed based on RPR reactivity, being negative previously or showing >4-fold increase. Acute hepatitis C was diagnosed based on HCV-Ab seroconversion and/or positive serum HCV RNA after being negative within the last year.

RESULTS: A total of 859 episodes of syphilis and 19 of acute hepatitis C were diagnosed during the study period. Syphilis was recognized in 65/2,094 (3.1%) individuals attended in 2008 and rose up to 261/2,512 (10.4%) in 2012 (P<0.001). Acute hepatitis C was diagnosed in only one subject in 2008 but rose up to seven in 2012 (P=0.12).

All 19 HIV+ patients with acute hepatitis C were MSM. Syphilis was diagnosed concomitantly in seven. All eight individuals that were treated with peginterferon/ribavirin were cured, whereas only one untreated experienced spontaneous clearance (P=0.004). Two clusters of infections by HCV genotypes 4d (n=3) and 1a (n=4) were identified by phylogenetic analyses. No evidence of phylogenetic close relationship (bootstrap >70%) was found with previously reported clusters of acute hepatitis C in HIV+ MSM at other European large cities. Conversely, a close identity was found with sequences from intravenous drug users living in Madrid, some of whom were MSM.

CONCLUSIONS: The incidence of acute hepatitis C is low but steadily increasing in HIV+ MSM in Madrid (<1% yearly), despite very high rates of syphilis (currently 20% yearly in HIV+ MSM). Preventive measures for sexually transmitted infections and periodic HCV screening are warranted in this population since treatment of acute hepatitis C is very effective. The epidemics of acute hepatitis C in HIV+ MSM in Madrid may have arisen recently from the large local HCV reservoir of intravenous drug users.
SESSION 5
Persistence, reservoirs and elimination strategies
ABSTRACT 73

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Comparative analysis of five viral load assays in quantifying low viraemic HCV samples in the range of clinical decision points

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BACKGROUND: The objective of this study was to assess the variation between five commercial quantitative HCV RNA assays in the range of the clinical decision points recently established for treatment with protease inhibitors.

METHODS: HCV RNA standards (WHO 06/100 and PEI 3443/04) diluted to nominal concentrations of 1,000, 500, 200, 100, 25, 10 and 5 IU/ml were assayed in triplicate and single runs with *artus* HCV QS-RQG (Qiagen), COBAS Ampliprep/COBAS TaqMan (CTM) HCV v1 and v2 (Roche), RealTime HCV (Abbott) and Versant HCV 1.0 (Siemens), respectively. Specimens from patients infected with different HCV genotypes (GT1: n=6; GT2, 3 and 4: n=1) were tested in replicates of 10 each. Coefficients of variation (CV%) were calculated to assess interassay variation. Differences in quantification between HCV RNA tests were assessed by Bland–Altman analysis of results from 20 clinical samples measured in triplicate with each test.

RESULTS: Correlation coefficients of 0.82/0.61 (*artus*), 0.99/0.99 (CTM v1; levels of <= 25 IU/ml excluded due to higher test-specific detection limit), 0.92/0.82 (CTM v2), 0.99/0.97 (RealTime) and 0.91/0.97 (Versant), respectively, were determined for WHO/PEI-Standards.

Within the dilution range of 5–25 IU/ml, *artus* ‘detected’ 9/18, CTMv1 14/18, CTMv2 8/18, RealTime 16/18 and Versant 10/18, respectively. RealTime demonstrated the highest precision across all genotypes tested (8–24% CV). CTMv1 (16–58% CV) showed a high variation in quantifying genotype 4 samples. CTMv2 (17–46% CV), Versant (22–43% CV) and *artus* (18–48% CV) demonstrated lower CV% values at 1,000 IU/ml compared to those found at lower concentration (100 IU/ml). The highest difference in viral load was observed between *artus* and RealTime with a mean difference of 0.75 log IU/ml. The highest degree of consensus was observed between both versions of CTM.

CONCLUSIONS: RealTime demonstrated the highest sensitivity and the highest precision. The largest difference in quantification was observed between *artus* and RealTime with 0.75 log IU/ml and the lowest between the two versions of CTM. A switch of assays during treatment monitoring is not recommended due to the variation between the different assays. High sensitivity and accuracy in quantification of HCV RNA are crucial for reliability of clinical decisions concerning treatment discontinuation or extension.
ABSTRACT 74

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Impact of APOBEC3-driven editing on HIV evolution in diverse anatomical compartments

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BACKGROUND: Within an individual, HIV-1 exists as a population of related but distinct viral variants termed viral quasispecies. These variants can be present in distinct anatomical locations in the same individual and have the properties to evolve independently from HIV-1 found in peripheral blood. Such viral variation is a result of a combination of factors including high replication and mutation rates, recombination and APOBEC3-host selective pressure. We attempted, in this study, to determine the contribution of APOBEC3-editing in HIV-1 compartmentalization.

METHODS: We studied the level of G-to-A hypermutation in HIV-1 protease and reverse transcriptase bulk sequences among 32 HAART-treated patients for whom peripheral blood mononuclear cells (PBMCs) and body tissues or fluids were collected on the same day (16 paired PBMCs/cerebral spinal fluid [CSF]; 8 paired PBMCs/renal tissues; 8 paired PBMCs/rectal tissues). Differences in the G-to-A mutation frequencies were analysed using the Hypermut 2.0 program.

RESULTS: Overall, APOBEC3-induced hypermutated sequences were identified in 34% (11/32) of subjects in at least one anatomical reservoir. Viral hypermutation tends to be more frequent in viral compartments (total n=10; CSF n=6; renal tissue n=1; rectal tissue n=3) compared with peripheral blood (total n=4). We investigated the possibility that APOBEC3 editing might lead to the differential emergence of drug resistance mutations in anatomical compartments. APOBEC3 editing generated more G-to-A drug resistance mutations in sanctuaries: three patients’ CSF (that is, G73S in protease; M184I, M230I in RT) and two other patients’ rectal tissues (M184I, M230I in RT) while such mutations were absent from paired PBMCs. In one patient, hypermutation was observed in PBMCs sequences (including M184I in RT) while not detected in rectal tissue sequences.

CONCLUSIONS: APOBEC3-induced hypermutation observed in peripheral blood may underestimate the overall proportion of hypermutated viruses in the body. Such differential mutational process induced by APOBEC3 may favour the emergence of some drug resistance mutations in anatomical compartments in vivo. On the other side, this data suggests there is a differential degree of accumulation of defective viruses between viral compartments in patients under suppressive HAART that should be taken into account in the context of future viral eradication strategies.
ABSTRACT 75

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Falsely elevated HIV-1 viral load results observed with Roche TaqMan HIV-1 v2.0 for samples stored frozen in Vacutainer plasma preparation tubes not observed with Abbott RealTime HIV-1

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BACKGROUND: Studies have demonstrated that plasma samples collected and stored frozen using Vacutainer plasma preparation tubes (PPT) may result in falsely elevated viral load (VL) values with the Roche COBAS TaqMan HIV-1 v1.0 test. This study was designed to compare VL results obtained for patient samples stored frozen in PPT using Roche COBAS TaqMan HIV-1 v2.0 test (CTM v2.0) and Abbott RealTime HIV-1 assay (RealTime HIV-1).

METHODS: 349 samples from HIV-1-infected patients on HAART were collected and stored frozen in PPT. Samples were tested with CTM v2.0 and RealTime HIV-1. 63 samples with most discordant results (RealTime HIV-1 negative/CTM v2.0 positive) were further analysed using two nested RT-PCR assays targeting pol integrase: full-length (864 nt) and a highly conserved subregion (134 nt).

RESULTS: 260/349 samples had VL values that differed by >0.5 log10 copies/ml; 196 were detected by CTM v2.0 but not detected in RealTime HIV-1. 64 samples quantified by both assays had VL results differing by >0.5 log10 copies/ml (0.6 to 2.2 log10 copies/ml). Using two nested RT-PCR assays, HIV-1 RNA was not detected in the 63 discordant samples analysed (1.5–4.1 log10 copies/ml) confirming RealTime HIV-1 results.

CONCLUSIONS: In this study a high level of falsely elevated VLs were observed with the CTM v2.0 assay. This increase in reactivity was presumably caused by proviral DNA carryover captured by the CTM total nucleic acid extraction chemistry but not the RNA-specific extraction procedure used in RealTime HIV-1. The results suggest that using CTM with samples frozen in PPT could have significant clinical implications for HIV-1 patient management and confirms earlier findings that RealTime HIV-1 performance is not affected by PPT.
ABSTRACT 76

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Factors associated with low HIV-1 total DNA levels after suppressive antiretroviral therapy

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BACKGROUND: The objective of this study is to determine factors that influence the establishment of small reservoirs in long-term treated patients (excluding treatment since acute infection) to a level similar to HIV elite controllers (<100 HIV total DNA copies/10⁶ PBMCs).

METHODS: Cross-sectional study involving patients receiving highly active antiretroviral therapy (HAART) with plasma HIV RNA<50 copies/ml for whom total DNA measurement were performed. Patients treated since early acute infection or receiving cancer chemotherapeutic/immunosuppressive agents were excluded from the study.

RESULTS: A total of 246 patients receiving HAART with undetectable viraemia were involved in the study. Fifty eight patients had a low HIV DNA level <100 copies/10⁶ PBMCs. A low proviral DNA was associated with an ultrasensitive plasma viral load <1 copy/ml (P<0.0001), a lower plasma HIV RNA zenith (P<0.0001), a higher CD4 T-cell nadir (P=0.023), lower current CD8 T-cell counts (P=0.010), a higher current CD4/CD8 ratio (P=0.003) and a higher time spent with undetectable HIV-1 RNA (P=0.018). Total HIV-1 DNA levels were neither related to the length of time on HAART nor the duration of infection.

CONCLUSIONS: The obtaining of a low HIV DNA level, reflecting a limited pool of infected cells is associated with a high CD4 nadir and a low HIV RNA zenith, reinforcing the need to institute antiretroviral treatment early during chronic infection to control HIV reservoir. In addition, our results reinforce the idea that a small reservoir may be related to stronger control of residual viraemia that in turn keeps the immune activation system to a low activation status.

This study helps to define factors associated with low proviral DNA setpoints after long-term treatment and should be useful to identify future candidates for strategies aiming at eradicating HIV.
ABSTRACT 77
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Dual approach to HIV-1 cure: activation of latency and restoration of exhausted virus-specific T-cell function

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BACKGROUND: The persistence of HIV-1 infection in patients suppressed by combination antiretroviral therapy (cART) is due to the presence of latent reservoir(s), including that within resting CD4+ T-cells. An additional factor in persistence is the dysfunction of HIV-1-specific T-cells in infected individuals. Thus, elimination of the latent HIV-1 reservoir may require a therapeutic strategy that incorporates a combination of activation of latent HIV-1 virus as well as restoration of HIV-specific T-cell responses.

METHODS & RESULTS: We have developed multiple assays for identification of compounds that reactivate latent HIV-1. These assays involve an integrated and quiescent HIV-1 LTR-reporter present in both primary T-cells and immortalized T-cell lines. A parallel screening approach was employed. Triage of the hits from these screens has revealed compounds capable of activating latent HIV-1 reporters and virus in multiple contexts.

The ability of immunomodulatory therapies to affect human chronic viral infections also is being investigated with in vitro assays, in animal models and in clinical trials. As proof of concept, nivolumab (anti-PD-1; BMS-936558) has been tested in the context of chronic HCV in human subjects. Although this trial did not meet its primary end points, a minority of subjects experienced a significant virological response.

CONCLUSIONS: We hypothesize that a combination of approaches will be necessary for reduction of the HIV-1 reservoir leading to eradication of latent virus or functional cure. To this end, we have developed a dual approach to discover agents with complementary mechanisms of action and whose combination may eradicate latent HIV-1 infection. Future studies will be directed at a combination of these approaches.
SESSION 6
Resistance to new antiretroviral agents
ABSTRACT 78
Antiviral Therapy 2013; 18 Suppl 1:A101
Highly multi-drug resistant HIV: clonal analysis and therapeutic strategies
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BACKGROUND: Despite new antiretroviral classes and drugs, there are today HIV-infected patients in therapeutic impasses because of highly multi-drug resistant (HMDR) viruses. Having evaluated the proportion of patients carrying HMDR strains, we studied the distribution of resistance mutations between individual molecular clones, and analysed the therapeutic strategies used in such cases to obtain undetectable viral loads.

METHODS: The HMDR profile was defined as a GSS ≤ 1.5 for the two drugs etravirine and raltegravir with a full resistance to darunavir. Approximately 30 clones per gene (reverse transcriptase, protease, integrase and V3 env) have been analysed and compared with the genotypic test of plasma (bulk). HIV viral loads, CD4+ T-cell counts and trough concentrations of drugs have been used to follow the efficiency of therapeutic strategies.

RESULTS: Among 1,310 patients on treatment and with genotypic resistance testing, 25 (2%) were resistant to darunavir and 11 (0.8%) had an HMDR profile of whom 4 could be analysed. Viral loads of these four patients were 2,039 to 68,969 copies/ml and CD4+ T-cell counts 87 to 221 cells/mm³. The number of resistance mutations was 6 to 8 for NRTIs, 4 to 6 for NNRTIs, 16 to 21 for PIs, and 3 to 4 for integrase inhibitors. The only susceptible drugs were etravirine (one patient), rilpivirine (one patient) and tipranavir (two patients). HMDR profiles were harboured by the great majority of clones, and all resistance mutations located on same strains for all genes. Despite this, and a GSS regimen < 2.0 in 3 patients, they achieved viral load < 20 copies/ml. These unexpected results were obtained using different strategies: high doses of resistant drugs as darunavir or dolutegravir; combination of antiretrovirals with full or intermediate susceptibility as tipranavir, etravirine or maraviroc; and also alternative drugs as foscarnet or pegylated interferon.

CONCLUSIONS: Patients with HMDR virus were uncommon, but, in such cases, the total resistance was borne on the same predominant strains in plasma. In this study, tipranavir was the only PI with full or intermediate susceptibility. Despite very limited therapeutic options, an undetectable viraemia can be achieved by combining different strategies.
ABSTRACT 79

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Resistance associated mutations in HIV-1 subtype C primary viruses after in vitro passage with integrase inhibitors raltegravir, elvitegravir and dolutegravir

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OBJECTIVES: The prevalence of HIV-1 integrase (IN) mutations associated with resistance to the first generation IN inhibitors (INI), raltegravir (RAL) and elvitegravir (EVG), and the next generation INI, dolutegravir (DTG) have not been adequately assessed in non-B subtypes. This study evaluated the resistance mutation profiles for RAL, EVG and DTG associated with in vitro drug resistance selection experiments with HIV-1 subtype C primary virus isolates.

METHODS: Six HIV-1 subtype C primary virus isolates, including three from antiretroviral drug-naive patients (FV3, FV5 and FV6) and three from patients failing a first-line regimen (MR69, MR81 and MR89) were grown in peripheral blood mononuclear cells (PBMCs) in the presence of increasing concentrations of RAL (0.26–266.24 nM), EVG (0.26–66.56 nM) and DTG (0.26–66.56 nM) using standard methodologies. Supernatant and PBMCs were collected at baseline and at each passage. For genotypic analyses, viral RNA was extracted, and the entire pol was amplified and sequenced, by population based Sanger and high throughput (Illumina MiSeq) sequencing. Prior to INI selection, the drug-resistant primary viruses MR69, MR81 and MR89 encoded for the K103N, M184V and M184V/K103N/K238N reverse transcriptase mutations, respectively.

RESULTS: Drug-resistant primary viruses were isolated from four of the six isolates grown in increasing concentrations of RAL. The major INI mutation Q148R emerged in FV6, MR69 and MR81, and N155H in MR89. The F121Y, T66I and T66I/R263R mutations emerged in FV3, MR69/MR89 and FV6, respectively, following selection with EVG. The E92Q mutation associated with DTG resistance emerged in only one isolate, FV3. However, mutations believed to be associated with DTG resistance at positions 101 and 124 were present from baseline sequences of all six primary viruses. High throughput sequencing results of the longitudinal culture samples allowed us to track the dynamic evolution of the quasispecies in response to the drug selection pressure.

CONCLUSIONS: HIV-1 subtype C primary virus isolates grown under INI pressure mutate to contain the described major IN mutations, similar to what has been reported from patient clinical samples worldwide. DTG pressure induced a RAL and EVG drug-resistant mutation, warranting further studies to establish the benefit of DTG treatment in HIV-1 subtype-C-infected patients failing EVG or RAL-containing regimens.
ABSTRACT 80

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Primary and secondary analyses of emergent drug resistance through week 96 from the Phase III studies of elvitegravir/cobicistat/emtricitabine/tenofovir disoproxil fumarate

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**BACKGROUND:** Phase III studies of elvitegravir/cobicistat/emtricitabine/tenofovir disoproxil fumarate (Stribild [STB]) in treatment-naive subjects (GS-US-236-0102/-0103) showed non-inferior efficacy to efavirenz/emtricitabine/tenofovir disoproxil fumarate (EFV/FTC/TDF; Atripla [ATR]) or ritonavir-boosted atazanavir (ATV+RTV)+FTC/TDF for HIV-1 RNA <50 copies/ml at weeks 48 and 96. Through week 96, emergent resistance occurred in 16 STB subjects (2.3%; 16/701), 10 ATR subjects (2.9%; 10/352) and no ATV+RTV+FTC/TDF subjects. This study presents analyses of the 16 STB subjects with emergent resistance, pre-existing IN mutation effect on outcome, and analyses of subjects who discontinued before week 8 or with HIV-1 RNA 50–399 copies/ml.

**METHODS:** The resistance analysis population (RAP) had genotypic/phenotypic analyses at failure confirmation and baseline for PR/RT/IN (Monogram; Labcorp). The primary RAP studied subjects with HIV-1 RNA ≥400 copies/ml at virological failure, discontinuation, and week 48 or 96 (STB; n=36). A secondary RAP studied subjects who discontinued before week 8 and subjects with HIV-1 RNA 50–399 copies/ml at confirmed virological failure, discontinuation or week 96 (STB; n=10). Retrospective integrase genotyping was conducted on 360 STB subject baseline samples.

**RESULTS:** The 16 STB subjects with emergent resistance had median baseline HIV-1 RNA of 205,000 copies/ml and CD4+ T-cell count of 76 cells/μl. All 16 had virological rebound and adherence by pill count was <95% in 8/16 subjects. All had HIV-1 subtype B. A total of 15/16 subjects had emergent primary INSTI mutations: E92Q (n=9), N155H (n=5), Q148R (n=3) and T66I (n=2), and in RT: M184V/I (14/16) and K65R (5/16). Clonal analyses found genetic linkage between M184V/I and IN mutations. PI-based regimens were commonly used in second-line regimens in these subjects with good success. Baseline primary INSTI mutations (T97A or Y143H) or secondary INSTI mutations were rare and had no apparent effect on STB responses. The secondary resistance analysis found a high rate of assay failure due to low viral loads, but no emergent resistance in the two subjects with data.

**CONCLUSIONS:** Resistance development during first-line STB therapy was infrequent: 2.3% of STB-treated subjects through 96 weeks. Pre-existing INSTI mutations were rare and had no effect on treatment response. Secondary resistance analyses of discontinuations before week 8 or with HIV-1 RNA between 50–399 copies/ml revealed no additional STB subjects with resistance development.
ABSTRACT 81

Evolution of integrase in virus at protocol-defined virological failure from the VIKING-3 study

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OBJECTIVES: VIKING-3 examined the efficacy and safety of dolutegravir (DTG) 50 mg twice daily with 7 days functional monotherapy followed by optimization of background antiretroviral therapy in patients with resistance to multiple antiretrovirals, including INIs. Here we summarize treatment-emergent (TE) resistance detected at the protocol-defined virological failure (PDVF) time point.

METHODS: PDVF assessment with the Abbott assay was defined as any HIV-1 RNA value >400 copies/ml and meeting the following criteria: <0.5 log10 decrease at day 8, or confirmed decrease of <1 log10 copies/ml by week 16, or confirmed ≥400 copies/ml on or after week 24, or confirmed ≥400 copies/ml after prior confirmed <400 copies/ml, or confirmed >1 log10 copies/ml above a nadir of ≥400 copies/ml. IN genotypic and phenotypic data were provided by Monogram BioSciences. TE resistance was defined as mutations detected at the PDVF time point but not at baseline (day 1).

RESULTS: At this interim week 24 data-cut, 35/183 (19%) subjects enrolled met PDVF criteria and 31/35 had paired baseline and time point of PDVF samples for evaluation of TE resistance. Of these 31 subjects, 15/31 had TE mutations detected at PDVF. TE resistance was detected in virus harbouring a mutation at Q148 (at baseline or historical) for 13/15 (87%) subjects. All treatment-emergent mutations detected were well characterized raltegravir and/or elvitegravir resistance-associated mutations at positions 97 (6), 138 (5), 92 (2), 140 (2), 155 (2), 157 (1), 147 (1), 143 (1) and 148 (4). For the four subjects who had virus with TE mutations at position 148, three entered the study with historical evidence of Q148H and the fourth had Q148R at day 1 but a mixture of Q148Q/R/K at PDVF. For 12/15 with TE genotypic resistance there was also an increase in DTG FC (increase defined as >2-fold of baseline DTG FC value).

CONCLUSIONS: In this study of heavily antiretroviral-treated subjects with INI resistance, 19% of subjects experienced PDVF while receiving DTG 50 mg twice daily. Limited viral evolution was detected at PDVF and consisted of previously identified INI-associated mutations.
ABSTRACT 82

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Drug resistance mutations in HIV-2 patients failing raltegravir – extent of cross-resistance to dolutegravir

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BACKGROUND: Phenotypic studies have shown that HIV-2 isolates are susceptible to inhibition by both raltegravir and dolutegravir. Moreover, dolutegravir may exhibit in vitro limited cross-resistance to most raltegravir-resistant HIV-2 mutants, mutant G140S/Q148R being the only exception. There is scarce clinical information about the resistance profile in HIV-2 patients failing on raltegravir.

METHODS: The integrase HIV-2 coding region was sequenced using an in-house nested-PCR protocol both in raltegravir-naive and raltegravir-experienced patients with detectable plasma HIV-2 RNA. Mutations associated to raltegravir failure were characterized and mutations associated with dolutegravir resistance in HIV-1 (positions 66, 74, 92, 101, 118, 124, 138, 140, 148, 153, 193 and 263) were checked in all HIV-2 cases.

RESULTS: From a total of 279 patients recorded at the HIV-2 Spanish cohort up to December 2012, a total of 41 integrase gene sequences from 28 patients had been obtained (20 raltegravir-naive and 8 raltegravir-experienced). Six patients who failed on raltegravir selected integrase changes. Patient 1 selected for I84V+A153G+N155H+Q214H; patient 2 for I84V+E92Q+T97A+N155H+Q214H; patient 3 for I84V+A153G+N155H+Q214H; patient 4 for T97A+Y143G+Q214H; patient 5 for A153G+N155H; and patient 6 for G140A+Q148R. The following changes associated with resistance to dolutegravir in HIV-1 were identified in our HIV-2 population: E92Q (1), L101I (2), S138A (1), G140A (1), Q148R (1), A153G (3) and D193E (1). Two patients failing on raltegravir (patients 1 and 2) were subsequently treated with dolutegravir. The first achieved undetectable plasma HIV-2 RNA 6 months later and the second had only 4 weeks follow-up and had experienced >1 log reduction.

CONCLUSIONS: Failure to raltegravir in HIV-2 patients selects for new secondary changes at the integrase gene, such as I84V and Q214H, along with primary changes at positions 143, 148 and 155 typically described in HIV-1. In our experienced, N155H resistance mutation failing on raltegravir in HIV-2 in combination with other changes associated with resistance to dolutegravir does not seem to compromise their response.
**ABSTRACT 83**  
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**In vitro phenotypic susceptibility of HIV-1 non-B integrase inhibitor naive clinical isolates to dolutegravir and raltegravir**

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**BACKGROUND:** Integrase inhibitors (INI) should be evaluated across broad HIV-1 subtypes in order to evaluate the effect of genetic diversity on INI susceptibility. The aim of this study was to assess the *in vitro* phenotypic susceptibility to dolutegravir and raltegravir on various HIV-1 non-B subtypes.

**METHODS:** Phenotypic susceptibilities to both dolutegravir and raltegravir were determined using the ANRS method on co-cultivated HIV-1 isolates from the collection of the French National HIV-1 Resistance Reference Center obtained from 72 INI-naive HIV-1 non-B-infected-patients. HIV-1 subtype B BRU reference strain was used as control. A total of 43 and 29 patients were RT-inhibitor-naive and -experienced, respectively. Integrase gene direct sequencing was performed on plasma samples and on viral supernatant at time of phenotypic assay.

**RESULTS:** HIV-1 non-B subtype clinical isolates were as follows: A (n=12), C (n=8), D (n=8), F (n=5), G (n=4), H (n=2), CRF01_AE (n=4), CRF02_AG (n=16), CRF06_cpx (n=4), CRF11_cpx (n=2), CRF12_BF (n=1), CRF14_BG (n=1), CRF18_cpx (n=1), CRF25_cpx (n=1), CRF45_cpx (n=1) and unknown (U; n=2). No major key resistance mutations to INI were evidenced. The most prevalent secondary mutations involved in the development of INI resistance detected in this series were: V72I (68%), V201I (84%) and T206S (51%). Additionally, the L74M was detected in one CRF02_AG sample, the E157Q was detected in two samples (CRF01_AE and CRF02_AG) and the R263K in one CRF01_AE isolate. Phenotypic data showed that all the clinical isolates tested were susceptible to both dolutegravir and raltegravir with median IC50 values of 1.22 nM (range 0.08–3.72) for dolutegravir and of 1.53 nM (range 0.03–4.82) for raltegravir. The control HIV-1 subtype B BRU reference strain showed IC50 values of 1.86 and 2.17 nM for dolutegravir and raltegravir, respectively.

**CONCLUSIONS:** All 72 HIV-1 non-B INI-naive clinical isolates were susceptible to both dolutegravir and raltegravir with similar IC50 values than B subtype. Despite the high prevalence of polymorphic substitutions in integrase gene in non-B clinical isolates, phenotypic susceptibility to dolutegravir and raltegravir remained similar to what was observed for B subtype.
Secondary mutations in the R263K in vitro pathway of resistance against dolutegravir fail to restore integration and viral fitness

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BACKGROUND: No major resistance mutation against dolutegravir (DTG) has been observed in treatment-naive patients. The R263K mutation emerged in integrase during our in vitro selection studies with DTG and confers low-level resistance, that is, <2-fold, to this drug while decreasing integration and viral fitness. The addition of the secondary mutation H51Y to R263K further decreases HIV susceptibility to DTG but fails to restore integration and viral fitness. Although less common than H51Y, two other secondary mutations, that is, M50I and E138K, were also selected in vitro in the presence of the R263K mutation. Here we report the effects of M50I and E138K on phenotypic resistance to DTG, cell-free viral DNA integration and viral fitness.

METHODS: Various relevant integrases were produced by site-directed mutagenesis followed by bacterial expression and purification. Purified recombinant integrases were tested for strand-transfer activity in cell-free assays, and biochemical constants were calculated (Vmax, Km, Ki). The same mutations were introduced into pNL43 HIV DNA by site directed-mutagenesis and the resulting viruses were tested for phenotypic resistance to DTG and viral replication capacity. The presence of the R263K mutation was tested by allele-specific PCR in the DNA of treatment-naive patients being successfully treated with DTG.

RESULTS: Similar to the H51Y mutation, M50I and E138K slightly increased resistance to DTG (<3- and <4-fold, respectively) but failed to restore the catalytic activity of the R263K integrase as well as the defect in viral replication associated with this mutation. M50I and E138K had more neutral effects than H51Y on R263K, with M50I decreasing DNA binding, while modelling using I-TASSER predicted that E138K changes the conformation of the catalytic site. The use of an AS-PCR assay did not reveal the presence of the R263K mutation in drug-naive patients who were treated with DTG.

CONCLUSIONS: None of the secondary mutations associated with R263K restored HIV integrase activity or viral replication capacity, suggesting that the R263K resistance pathway might be an evolutionary dead-end for the virus. This may in part explain the absence of resistance to DTG to date in treatment-naive populations.
ABSTRACT 85

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Epidemiology of dolutegravir (DTG) resistance in ~700 raltegravir-resistant isolates

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BACKGROUND: The integrase inhibitor (INI) dolutegravir (DTG) has low fold-change (FC) in IC50 against HIV-1 with raltegravir (RAL)-resistant Y143 and N155 genotypes and Q148H/K/R single mutations; FC generally increases for Q148H/K/R as additional RAL-associated mutations accumulate. Continuing RAL therapy at increased viral loads can lead to additional mutations or pathway evolution (for example, N155H to Y143 or Q148) and increased chance of higher DTG FC. Monogram Bioscience’s RAL-resistant clinical isolate library was queried to evaluate frequency of INI resistance patterns in the clinic and the extent that they may be changing over time. Additionally, DTG and RAL phenotypic testing was performed.

METHODS: Monogram Bioscience’s RAL-resistant library (n=1,079) was separated into populations by collection date windows August 2008–December 2009 (n=273) and January 2010–December 2011 (n=806), which overlapped with recruitment periods for VIKING study cohort 1 (31 August 2009–12 October 2009) and cohort 2 (27 May 2010–2 November 2010). Proportions of RAL individual primary, secondary, and also Q148H/K/R plus secondary mutation genotypes, were compared across windows. DTG and RAL FC resistance generated for 705 RAL-resistant isolates selected at random from the 806 January 2010–December 2011 population isolates was characterized by RAL resistance pathway.

RESULTS: From August 2008–December 2009 to January 2010–December 2011 the change in proportions for a subset of evaluated substitutions were: Q148H, 41.8% to 31.4%; Q148K, 2.2% to 0.5%; Q148R, 11% to 9.7%; G140A/C/S, 50.6% to 37.5%; Q148 plus Y143C/H/R and/or N155H, 4.4% to 3.3%; N155H, 31.5% to 34.5%. For RAL mutation categories within the January 2010–December 2011 window the proportions and median DTG and RAL FCs (percentage [DTG-FC, RAL-FC]) were: all 705 isolates (100% [1.8, 89]); Q148+≥2 secondary resistance mutations (13.0% [6.0, >max]); Q148+1 secondary resistance mutation (26.1% [4.8, >max]); Y143 (13.9% [1.2, 100]); N155 (28.9% [1.5, 25]); ≥2 primary (6.0% [1.9, 79]); and primary not detected (12.1% [0.9, 4.0]).

CONCLUSIONS: Decreases in the proportion of Q148 pathway virus (and the tightly associated G140 secondary mutation) were observed from the early to late window, as would be expected if subjects experiencing virological failure on RAL were discontinuing RAL more promptly over time. The composition of these ‘real world’ clinical practice samples is consistent with contemporaneous VIKING-3 study participants, supporting the translation of VIKING-3 results to broader clinical practice.
ABSTRACT 86
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Identification of novel HIV-1 integrase inhibitors with excellent resistance profiles from natural products

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BACKGROUND: Two integrase (IN) inhibitors raltegravir and elvitegravir are currently approved for therapy, while dolutegravir is now in advanced clinical trials. The IN strand transfer inhibitors (INSTIs) seem to share similar structures and they all specifically target the strand transfer step of integration. This may be the reason for the extensive cross-resistance that exists among members of this class. Thus, it is important to identify novel IN inhibitors that are structurally or mechanistically different from the current INSTIs. IN possesses multiple sites, distinct from the IN active site involved in the strand transfer step, that could be targeted to develop new HIV-1 IN inhibitors. Since IN must bind to viral DNA prior to the 3'-processing step, the inhibition of IN binding to viral DNA represents an interesting target.

METHODS: We have developed a fluorescence HIV-1 IN DNA binding assay that can identify small molecules termed IN binding inhibitors (INBIs) that inhibit IN binding to viral DNA.

RESULTS: Using this biochemical assay, we have identified 5 INBIs from a collection of 60 known or new natural products, including triterpenoids, sesquiterpenoids and phenolic compounds isolated from plants of the Schisandraceae family. These five INBIs showed abilities to block binding of IN to viral DNA with IC₅₀s ranging from 2–10 μM. The compounds also inhibited 3’-processing and strand transfer with similar IC₅₀s. One of the five INBIs is a known compound named nigranoic acid, which was previously reported to moderately inhibit the cytopathic effects of HIV-1 in C8166 cells at EC₅₀ values between 22 to 35 μM. The structures of these compounds will be shown. These compounds show activity against IN enzymes containing known INSTI mutations.

CONCLUSIONS: We have identified five new INBIs that might have better resistance profiles than the current INSTIs. These novel IN inhibitors from natural products may turn out to have clinical utility.
ABSTRACT 87
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Complexity of cross-resistance mutation patterns in diarylpyrimidine non-nucleoside reverse transcriptase inhibitors rilpivirine and etravirine in clinical isolates

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BACKGROUND: Rilpivirine (RPV) and etravirine (ETR) are diarylpyrimidine non-nucleoside reverse transcriptase inhibitors (NNRTI) with a broad spectrum of anti-HIV activity against viruses resistant to nevirapine and efavirenz (EFV). To better understand the mechanism of diarylpyrimidine resistance, we analysed cross-resistance mutation patterns of RPV, ETR and EFV using an in-house enzymatic reverse transcriptase (RT) assay.

METHODS: HIV RT DNA templates were prepared from 19 NL4-3 based clones with different combinations of 11 NNRTI resistance-associated mutations (RAM) introduced by site-directed mutagenesis and 31 patient-derived isolates with different patterns of NNRTI RAM. Active recombinant RT heterodimer complexes (p66/p51) were synthesized from RT DNA templates by a cell-free in vitro translation system. To measure RT activity, we mixed digoxigenin (DIG)-labelled primer and artificial ssDNA template, and biotinylated substrate-nucleotide analogues with the synthesized RT and dNTPs. Incorporation of biotinylated substrate-nucleotide analogues into RT product was quantified by emitted light intensities using AlphaScreen™, and IC50 values of 3 NNRTIs (RPV, ETR and EFV) were calculated. Fold changes in IC50 for mutated RTs were compared to wild-type NL4-3 RT. To clarify the effects of these additional mutations on RPV susceptibilities, we performed molecular modelling studies.

RESULTS: Among 50 mutated RTs, two patterns (K103S and V179D+G190S) showed high-level resistance to EFV (14.7- to 534.9-fold resistance) but remained susceptible to ETR (1.0- to 3.3-fold) and RPV (0.7- to 1.4-fold). Interestingly, four patient-derived RTs with K103N demonstrated low-level resistance to RPV (3.3- to 6.2-fold) but remained susceptible to ETR (0.7- to 1.3-fold). These RTs had the following additional mutations: I135L, V179I, G198E, Q199K, T286A and A288G. One patient-derived K103N RT without these mutations remained susceptible to RPV, suggesting that these mutations are responsible for low-level resistance to RPV.

CONCLUSIONS: The complexity of cross-resistance patterns of new NNRTIs, ETR and RPV, was shown by enzymatic RT assay. Because of the flexible structures of these two new NNRTIs, understanding their binding mode requires further mapping of minor resistance mutations.
ABSTRACT 88

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Genetic analysis and natural polymorphisms in HIV-1 gp41 isolates from Maputo, Mozambique

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BACKGROUND: Enfuvirtide was the first fusion inhibitor approved by the FDA in 2003 for HIV-1 infection in treatment-experienced patients. It is the first approved antiviral agent to attack the HIV life cycle in its early stages. For HIV fusion to occur, the HR1 and HR2 domains in the gp41 region need to interact. Enfuvirtide is a synthetic peptide that corresponds to 36 amino acids of the HR2, which competitively binds to HR1 inhibiting the interaction with the HR2 domain, thus preventing fusogenic conformation, inhibiting viral entry into host cells. Resistance to enfuvirtide is conferred by mutations occurring in the HR1 region involving residues 36–45. Mozambique, a sub-Saharan country, with an HIV prevalence of 11.5%, provides first-line and second-line HAART-based treatment. In poor resource settings, such as Mozambique, the lack of adequate infrastructures, high costs of viral load tests and the availability of salvage treatment have hindered the intended objective of monitoring HIV treatment, suggesting an important concern regarding the development of drug resistance. The general aim of this study was to evaluate natural occurring polymorphisms and resistance-associated mutations in the gp41 region of HIV-1 isolates from Mozambique.

METHODS: The study included 78 patients naive to ARV treatment and 28 patients failing a first-line regimen recruited from Centro de Saúde Alto-Maé situated in Maputo. The gp41 gene from 103 patients was sequenced and resistance-associated mutations for enfuvirtide were screened.

RESULTS: Subtype analysis revealed that 93% of sequences were classified as subtype C, 2% as subtype G, 1% as subtype A1 and the other 4% as URFs. No ENF resistance-associated mutations in HR1 of gp41 were detected. The major polymorphisms in the HR1 were N42S, L54M, A67T and V72I.

CONCLUSIONS: This study suggests that this new class of antiviral drug may be effective as a salvage therapy in patients failing first line regimens in Mozambique. However, further phenotypic studies are required to determine the clinical relevance of the polymorphisms detected in this study.
SESSION 7
Mechanisms of HIV drug resistance
ABSTRACT 89

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Contribution of raltegravir selected secondary mutations to reduction in elvitegravir susceptibility of patient-derived HIV integrase containing Y143 mutations

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BACKGROUND: The mutations at positions 143, 148 and 155 of HIV integrase (IN) define major mutational pathways to raltegravir (RAL) resistance. IN containing N155 or Q148 mutations have showed cross-resistance to elvitegravir (EVG), whereas Y143 mutations have been reported to be susceptible to EVG in some studies. In the current study, we have investigated EVG susceptibility using a large panel of patient-derived viruses and site-directed mutants containing Y143 mutations.

METHODS: 77 patient-derived viruses containing RAL selected Y143 mutations (without mixtures) were included in the study. IN region of these viruses was sequenced using conventional method. A large panel of SDMs containing Y143 mutations alone and combination with RAL secondary mutations (L74I/M, T97A, G163R and S230R) were constructed. EVG and RAL susceptibilities were determined using Phenosense Integrase Assay.

RESULTS: RAL selected resistant viruses contained different substitutions at position 143, including well-known substitutions Y143C/R, and less frequent substitutions Y143A/H/G/S. Sequence analysis revealed most viruses had at least one or more RAL secondary mutations, with the high frequency of T97A, S230R and L74I/M. Y143 mutation containing viruses (no mixtures) exhibited cross-resistance to EVG, fold change in IC50 varied from 1.9 to >150 (median FC=20). SDM analysis demonstrated that a single mutation at position 143 had minimal or no effect on reduction in EVG susceptibility, with FC 1.4 to 3.0 for different Y143 substitutions. However, the incremental addition of other IN mutations (T97A, S230R, L74I/M and G163R) to Y143 mutations together can confer large reductions in EVG susceptibility (FC=>20). The impact on EVG susceptibility of patient viruses and SDMs correlated strongly with the impact each mutation profile had on RAL susceptibility, although the magnitude of reductions in EVG susceptibility was lower compared to RAL susceptibility.

CONCLUSIONS: In this study, we demonstrated the patient-derived viruses containing Y143 mutations selected by RAL are cross-resistant to EVG. The secondary mutations play a critical role for Y143 mutation viruses to escape under EVG pressure.
ABSTRACT 90

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Reduced HIV-1 integrase flexibility 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 catalyses the insertion of the viral genome into the host chromosome. Raltegravir (RAL) was the first integrase inhibitor approved by the US 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 139–150 loop is increased and the length of this helix is extended from three residues to four 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. The most rigidity is observed in the 148R/140A mutant.

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 139–150 loop, therefore, decreasing the flexibility of the HIV-1 integrase protein. This loss of protein flexibility may serve to decrease the residence time observed in the RAL-resistance mutants compared to wild type.

ACKNOWLEDGEMENTS: This work was generously supported by a grant (IISP 37125) from Merck Research Laboratories.
BACKGROUND: It has been demonstrated for some drugs (raltegravir, elvitegravir, dolutegravir and protease inhibitors) that the genetic barrier, defined as the number of genetic transitions and/or transversions needed to get a resistance mutation, can differ between HIV-1 subtypes. The genetic barrier for the evolution of resistance to non-nucleoside reverse transcriptase inhibitors of second generation including etravirine and rilpivirine was compared between HIV-1 subtypes CRF02_AG and B. Subtype CRF02_AG is highly prevalent in West Africa and is becoming more frequent in Europe.

METHODS: Analysis of 25 substitutions associated with etravirine and rilpivirine resistance at 12 amino acid positions (90, 98, 100, 101, 106, 138, 179, 181, 190, 221, 227 and 230) in 267 nucleotide sequences (136 HIV-1 subtype B and 131 HIV-1 subtype CRF02_AG) of reverse transcriptase gene derived from antiretroviral-naive patients. The genetic barrier was calculated as the sum of transitions (scored as 1) and/or transversions (2.5) required for evolution to drug resistance substitution.

RESULTS: The majority (7/12) of studied amino acid positions were conserved between HIV-1 subtypes CRF02_AG and B, leading to a similar genetic barrier. At each position we always found a predominant codon that represented >72% of the population, except at position 98 for subtype CRF02_AG, where the codon GCG accounted for 63.3%. Different predominant codons between subtypes B and CRF02_AG were observed in 5/12 positions (90, 98, 179, 181 and 227). These differences have an effect on the calculated genetic barrier only at positions 179 for the V179D and V179F codons (2.5 versus 3.5 for V179D, 2.5 versus 5 for the V179F respectively for subtype B versus CRF02_AG). CRF02_AG subtype viruses had a higher genetic barrier for the etravirine resistance associated mutations V179D and V179F than B subtype viruses.

CONCLUSIONS: A majority of studied amino acid positions including all corresponding to etravirine and rilpivirine primary resistance mutations showed a high level of conservation. For rilpivirine, genetic barrier was the same between the subtype B or CRF02_AG. Nevertheless, subtype CRF02_AG showed a higher genetic barrier to acquire mutations V179D and V179F as compared with subtype B, which could play a role in the resistance to etravirine.
ABSTRACT 92

High prevalence and early selection of G→A HIV-1 minority species in drug-naive populations

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BACKGROUND: Curiously, E138K, a G→A mutation in HIV-1 reverse transcriptase (RT), is preferentially selected by TMC125 (etravirine, ETR) and TMC278 (rilpivirine, RPV) over other mutations at position E138 that offer greater drug resistance. HIV-1 RT is biased towards generating G→A mutations, and host-factors like APOBEC3 enzymes force G→A mutations into the viral genome. We hypothesized that due to these factors E138K may have a relatively lower genetic barrier to emergence than other E138 mutations.

METHODS: We designed an allele-specific PCR to monitor the emergence of E138A/G/K/Q/R/V during ETR selection experiments. We also analysed drug-naive patients for E138 mutations via AS-PCR. Results were confirmed by ultradepth sequencing (UDS).

RESULTS: E138K, as well as E138G, consistently emerged first during ETR selection experiments, followed by E138A and E138Q; E138R was never selected. E138K minority species emerged very early prompting us to screen drug-naive patients for E138 mutations. Surprisingly, E138K was identified as a tiny minority in 23% of drug-naive subtype B patients; a result confirmed by UDS. UDS revealed that this was not specific to E138K by revealing many other small minority species in a pattern consistent with the mutational bias of HIV RT. No viral sequences, including those linked to E138K, were identified to be APOBEC3-hypermutated in these selection experiments and in the drug-naive subtype B patients.

CONCLUSIONS: Our results elucidate the selection of codon E138 mutations, confirm the mutational bias of HIV-1 in patients with more certainty than bulk-sequencing based approaches and question the role of APOBEC3 in the development of drug resistance.
ABSTRACT 93
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In vitro selection experiments with rilpivirine and/or emtricitabine using clinical isolates, with E138K and/or M184I, obtained from patients with virological failure

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BACKGROUND: In patients with virological failure (VF) on rilpivirine treatment in the Phase III studies, frequent emergence of E138K+M184I, which showed more rilpivirine resistance than E138K alone, was observed. Occurrence of this particular combination of mutations may be related to fitness, effects on polymerization or increased dNTP usage. However, most in vitro mechanistic studies have been performed using site-directed mutants. We examined clinical isolates, derived from VFs, harbouring E138K or M184I, alone or in combination, with or without selective drug pressure, to assess emergence of additional mutations or reversion to wild type.

METHODS: Seven recombinant clinical isolates, containing patient-derived protease/reverse transcriptase, were tested: E138K (n=2), M184I (n=2) and E138K+M184I (n=3). Fold change in EC₅₀ (FC) values for rilpivirine and emtricitabine ranged from 0.5–8.8 and 0.6–34.8, respectively, depending on the mutations present, and these values were used to set the starting drug concentration. MT4 cells were infected at low multiplicity of infection and passaged in the absence/presence of increasing concentrations of rilpivirine and/or emtricitabine. Cells were examined for signs of viral replication; resistance testing of the selected viral strains was performed.

RESULTS: After culturing for up to 4 months without drug, reversion to wild type at M184 was observed in only one of the E138K+M184I isolates; accumulation of compensatory mutations was limited. With rilpivirine, the E138K isolates acquired additional resistance-associated mutations (RAMs): A62V+V189I+G190E in one and V106I+M230I in the other; the M184I isolates acquired V106I+K219N+H221Y+M230I or E138K; and the E138K+M184I isolates acquired V90I+V189I+N348I. With emtricitabine, one E138K isolate acquired M184T; one M184I and E138K+M184I isolate each acquired M184V; the remaining isolates did not accumulate RAMs. With rilpivirine + emtricitabine, the E138K isolates acquired V90I+V189I+N348I; the E138K+M184I isolates acquired V90I or V90I+V189I+N348I.

CONCLUSIONS: In vitro selection experiments using clinical isolates, from VFs, which harbour E138K and/or M184I, provided insights into the mechanisms of resistance under selective pressure of rilpivirine and/or emtricitabine. Mutations acquired under selective pressure were similar to those observed in vivo. In the absence of drug, strains with E138K, with M184I or with E138K+M184I appeared to represent stable viral populations.
ABSTRACT 94

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Different mutations at E138 in HIV-1 reverse transcriptase and interactions with M184I in defining resistance patterns to rilpivirine

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BACKGROUND: Several emergent drug resistant mutations at codon E138 (A/G/K/Q/V) of HIV-1 reverse transcriptase (RT) are associated with rilpivirine and etravirine, but E138K over others is the most frequent mutation together with M184I in patients of the Phase III studies (ECHO and THRIVE) who failed rilpivirine therapy together with co-formulated emtricitabine/tenofovir. Biochemical studies have shown that the E138K and M184I mutations can mutually compensate each other to restore enzymatic fitness. Phenotyping studies have demonstrated that M184I enhanced rilpivirine resistance in tandem with E138K more than E138K alone. In this study, we compared the impacts of mutations at E138 (A/G/K/Q/R/V) alone or in combination with M184I on susceptibility to rilpivirine and etravirine, and enzymatic fitness. We also compared the interactions of these mutations with M184I on viral replication capacity to determine why E138K over others with M184I was the most prevalent mutation in patients failing rilpivirine-containing combination therapy.

METHODS: We tested 12 recombinant RT enzymes and viruses containing each mutation at E138 (A/G/K/Q/R/V) in the background of wild-type and M184I in regard to enzyme processivity, efficiencies of processive DNA synthesis, replication capacity and susceptibility to rilpivirine and etravirine.

RESULTS: Each of the E138 A/G/K/Q/R mutations, alone or in combination with M184I, confers cross-resistance to rilpivirine and etravirine, among which E138R conferred the highest level of resistance. Fold-changes for resistance to rilpivirine and etravirine, by recombinant RT assay/cell-based assays, were, respectively, E138R (3.0/3.5; 3.1/4.2), E138Q (2.9/4.3; 2.5/3.7), E138G (2.2/3.7; 2.2/3.1), E138A (2.1/2.2; 1.6/3.2), E138K (1.8/2.7; 2.3/3.3), E138V (1.0/0.9; 1.1/1.1), E138Q/M184I (3.1/4.8; 3.0/4.4), E138R/M184I (3.0/4.7; 3.3/4.9), E138G/M184I (2.6/4.4; 2.9/4.1), E138K/M184I (2.5/4.3; 2.8/4.1), E138A/M184I (2.2/3.0; 2.5/3.1), E138V/M184I (0.4/0.8; 0.7/0.8). The E138K/Q/R mutations can compensate M184I in regard to both enzymatic fitness and viral replication capacity.

CONCLUSIONS: The favoured emergence of E138K over other mutations at position E138, together with M184I, is not due to an advantage in either level of drug resistance or viral replication capacity but may reflect the fact that E138K requires only one favourable G to A retroviral hypermutation, although other factors may also affect the barrier to resistance. We also suggest that E138R should be recognized as an etravirine resistance-associated mutation.
SESSION 8
Clinical implications of resistance
ABSTRACT 95

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Simplification strategy in heavily pretreated patients with multi-drug-resistant virus: the STOPNUK study

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BACKGROUND: Salvage therapy in heavily pretreated patients harbouring multi-drug-resistant (MDR) virus usually includes nucleos(t)ide reverse transcriptase inhibitors (NRTIs), despite their having null or low level of activity, particularly when the regimen does not include three fully active agents. Reducing HIV-1 fitness by maintaining some RT mutations or residual activity of NRTIs are the reasons argued for maintaining NRTIs. A simplification treatment strategy based on removing NRTIs for different reasons in long-term virologically controlled patients is assessed in this 48-week study.

METHODS: Heavily pretreated MDR patients with undetectable viral load (VL) on a stable regimen containing ≥3 drugs, including ≥1 NRTI with low or null activity, were enrolled in a simplification study by removing all NRTIs. Genotypic sensitive score (GSS [4: sensitive; 3: potential low-level; 2: low-level; 1: intermediate; 0: resistant]) was defined according to the HIVdb Stanford Genotypic Resistance Interpretation Algorithm. Mutations were assessed using the 2013 IAS-US HIV Drug Resistance Mutations list.

RESULTS: We included 31 patients treated with a median of 10.5 previous regimens (IQR: 7–14). 19 patients were previously exposed to T20 and 1 patient to RAL. Baseline mutations (IQR) were: NRTI 7 (7–8.5); NNRTI 1 (1–2); PI 10 (10–11). Median time with virological suppression before entry was 51 months (12–83). 2 NRTIs were removed in 27 patients (87%) and 1 NRTI in 4 patients (13%). 20 patients maintained at least 2 fully active drugs and 11 patients 1 fully active drug, plus another drug with at least moderate activity (GSS ≥2). 1 patient (3%) interrupted ART voluntarily after week 24, at which time the VL was undetectable. 30 patients (97%) had VL < 37 copies/ml at week 48. The two patients (6%) with VF, both with two fully active drugs, successfully resuppressed following the reinduction of prior nucleos(t)ides.

CONCLUSIONS: Most heavily pretreated MDR patients with viral suppression of long duration (≥12 months) remain fully suppressed after simplifying therapy by removing low/inactive NRTIs while maintaining at least two active drugs. The few failures successfully resuppressed following the reinduction of prior nucleos(t)ides.
ABSTRACT 96

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The role of viral phylogenetic clustering in transmitted resistance to first- and second-generation non-nucleoside reverse inhibitors

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BACKGROUND: The Montreal MSM epidemic has expanded despite 80% HAART coverage. Viral phylogenetics has established that half of the ongoing spread of the epidemic occurs through transmission clustering where 1 infection leads to 15 onward transmissions, including large clusters harbouring resistance to first-generation NNRTIs, including efavirenz (EFV) and nevirapine (NVP). This study assessed the impact of phylogenetic clustering in transmitted resistance and response to second-generation NNRTIs.

METHODS: The provincial genotyping programme has provided virological and drug resistance data over the last data. Phylogenetic analysis on sequence datasets elucidated clustering patterns of transmitted resistance in antiretroviral therapy (ART)-naive and ART-experienced populations. Four viruses from a large transmission cluster harbouring G190A were expanded in culture and selected for resistance to second-generation NNRTIs, including etravirine (ETV), rilpivirine (RPV) and dapivirine (DPV).

RESULTS: There has been a steady increase in viral suppression (VL<50 copies/ml) at a population level from 34% in 2002 to 68% in 2011. This has been accompanied by an overall reduction in drug resistance in the genotyped population from 55% in 2002 to 21% in 2011. Nevertheless, transmitted resistance to the NNRTI drug class has remained steady at 20% due to episodic expansion of transmission clusters harbouring G190A (n=61), K103N (n=3, 3, 6, 7, 9, 33) and V108I/K103R (n=21). Large clusters harbouring NNRTIs show higher viral set points than wild-type (wt) virus. Viruses harbouring G190A were hypersensitive to ETV, RPV and DPV, showing a delayed acquisition of E138K than wt viruses. Extended selection of wt viruses developing E138K led to complex second-generation NNRTI resistance pathways ([E138K, V179D, Y189I], [E138K, V90I, K101Q, V189I] and [E138A, K103N, V108I, V189I, Y181C]), whereas viruses harbouring G190A viruses only developed E138K. The Stanford database predicts potential low-level RPV and ETV resistance for G190A as a single-point mutation, whereas Virco and ANRS algorithms predict ETV and RPV drug susceptibility.

CONCLUSIONS: Transmitted resistance to NNRTIs remain a concern in the post-HAART era. Prevention and treatment as prevention interventions are necessary to avert the development of NNRTI-resistant sub-epidemics. G190A present as a single-point mutation remain susceptible to RPV and ETV, although resistant to EFV and nevirapine.
HIV-1 tropism switch in PBMC despite plasma virological success in patients receiving intensification with enfuvirtide

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OBJECTIVE: HIV-1 DNA tropism rarely evolves during the first 2 years of potent and successful combined antiretroviral treatment (cART). The objective of this study was to describe the evolution of HIV-1 DNA tropism in patients initiating enfuvirtide (ENF)-based regimens in different clinical settings.

METHODS: Patients from two randomized clinical trials with an ENF arm were analysed: (i) ANRS130-APOLLO study, which enrolled severely immunocompromised cART-naive patients \( n=101 \) in ENF arm), and (ii) INNOVE study, which enrolled cART-experienced patients with virological failure \( n=14 \) in ENF arm), receiving ENF in addition to cART for a period of 6 and 3 months, respectively. HIV-1 DNA tropism from PBMC was performed at baseline and W24 by V3 loop sequencing and interpreted using Geno2Pheno algorithm (false positive rate [FPR]=10%).

RESULTS: HIV DNA tropism determination was available in both baseline and W24 PBMC samples in 48/101 and in 14/14 patients in the ANRS130-APOLLO and INNOVE studies, respectively. A tropism switch in HIV-1 DNA from R5 at baseline to X4 at W24 was observed in 9/48 (19%) and 4/14 (29%) patients, respectively. Plasma viral replication was fully suppressed (HIV RNA<50 copies/ml) in 8 out of the 9 patients and in all 4 patients with a R5 to X4 switch in HIV-1 DNA between baseline and W24 in the ANRS130-APOLLO and in the INNOVE study, respectively. FPR values at baseline ranged between 10–20% in 6/13 patients (46%). The 11/25 rule predicted X4 tropism at baseline in two samples of the series, both with a limit FPR value (11.5%). No specific pattern in V3 loop sequence could be evidenced in viruses with HIV-1 DNA tropism switch. Nucleotidic changes at position 25 of V3 loop observed between baseline and W24 were APOBEC-mediated G to A mutations, in the GA dinucleotide context, in 7/13 (54%) patients.

CONCLUSIONS: In two randomized clinical trials assessing ENF intensification, both in cART-naive and in cART-experienced patients, we observed frequent and rapid changes in HIV-1 DNA tropism from R5 to X4, in spite of virological success. Further studies are needed to explore the dynamics of DNA tropism change and the underlying mechanism under ENF drug pressure.
ABSTRACT 98

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Time on drug analysis based on EuResist data
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BACKGROUND: While the health condition of HIV-1-infected patients has improved during the last years, the need of live long antiretroviral treatment and multiple side effects of antiretroviral drugs challenge the initiation of long-lasting regimens. The average running time of antiretrovirals composing the cART may be seen as an indicator of tolerability and durability.

METHODS: To evaluate the running time of different drugs used in HIV-1 treatment, we analysed data from the EuResist database. The evaluation period was from 2006 to 2012. We excluded all patients lost-to-follow-up and also drugs, for which we didn’t reach at least 100 cases. To obtain accuracy in the first 2 years of survival time, we considered only therapy start dates up to 2 years before the end of observation period. The cARTs were then transformed to single drug treatment lines, and intake interruptions of less than 5 days were ignored.

RESULTS: We obtained 31,826 drug lines from 9,666 patients. The average and median times on drug were 13.9 and 9.0 months. For the subgroup of therapy-naive patients (n=6,574) 15.3 and 10.3. Overall survival rates for all/naive patients were: after (n) months, (1) 0.90/0.91, (3) 0.77/0.80, (6) 0.62/0.65, (12) 0.41/0.46, (24) 0.20/0.24. For each drug we obtained following running times in days (n, average): 3TC (4,223, 421.1); ABC (2,389, 429.6); d4T (278, 373.5); ddi (390, 376.2); FTC (4,289, 460.4); TDF (4,502, 453.3); ZDV (2,762, 383.2); EFV (2,360, 367.8); ETR (278, 470.9); NVP (945, 374.0); ATV (2,415, 394.2); DRV (877, 424.5); FPV (515, 430.3); LPV (3,707, 452.9); NFV (100, 189.8); SQV (401, 427.1); RAL (803, 424.2); MVC (159, 405.6); T20 (433, 255.7).

DISCUSSION: In this cohort, the level of therapy changes remain high. After 2 years, 80% of the patients switched their treatment regimen. Switches due to side effects and availability of alternatives may explain this result.

Consideration of viral load measures could provide further insight in switching reasons. For the NRTIs emtricitabine had the highest average time on drug, for the NNRTIs etravirine and for the PIs lopinavir.
ABSTRACT 99

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Lower maraviroc plasma levels in combination with darunavir than with other protease inhibitors was associated to virological failure – 24 week analysis of the MITOX study

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BACKGROUND: The MITOX study was initiated to address toxicity and safety of switch strategies from NRTI-containing to NRTI-free and maraviroc (MVC)-containing regimen. At week 24, only safety analyses were performed.

METHODS: In this two-armed, randomized, prospective study, 80 HIV-infected men and post-menopausal women with undetectable HIV plasma load <50 copies/ml and receiving two NRTI+PI/r were randomized either to continue ART or to switch to MVC+PI/r regimen. Inclusion criteria were no contraindication to MVC, X4 tropism at screening from proviral DNA in unicate (FPR >15%) and the absence of historic genotypic drug resistance to the current PI/r. Virological failure was defined as any viral load >50 copies/ml at week 24.

RESULTS: From 124 screened patients, 40 for each arm were included. Three (NRTI) and six (MVC) patients were lost to follow-up, eight after screening and one after week 4, respectively. In week 24, six (MVC) and two patients (NRTI) were viraemic with >50 copies/ml, four of those (MVC) had >200 copies/ml. Tropism testing during failure was performed successfully in five cases, one switch to X4 tropism could be observed. No drug resistance was detected. Two patients, one in each study arm, had continuous low level viraemia, one despite several changes of ART. All others went <50 HIV RNA copies/ml, four after a change of regimen, two without any change of ART. MVC and PI drug levels were available for five viraemic patients and showed insufficient drug levels in all three cases with the combination of MVC (twice daily) and DRV/r (once daily). Therefore, a look-back analysis of all PI/r and MVC plasma levels in the MVC study arm was performed, exhibiting mean (median) MVC levels of 234 ng/ml (172 ng/ml, combined with DRV/r) and 410 ng/ml (345 ng/ml, combined with other PI/r; P=0.045). PI/r plasma level did not show any significant deviations.

CONCLUSIONS: In this study, failure at week 24 was caused frequently by insufficient MVC levels, but very rarely due to occurrence of X4 virus or drug resistance. The most frequently used combination, DRV/r+MVC, was associated with lower plasma levels of MVC. This finding was unexpected and needs further investigation. DRV/r+MVC regimen should be monitored by therapeutic drug monitoring.
ABSTRACT 100

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Reliability of HIV-1 drug-resistance test in patients with low viraemia levels

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OBJECTIVES: We evaluated reliability and usefulness of genotypic resistance-testing (GRT) in patients failing cART with viral load (VL) <500 copies/ml for whom GRT is not recommended by current guidelines (DHHS-2/2013).

METHODS: A retrospective analysis was performed on 10,788 HIV-1 clinical samples that were genotyped using the commercial ViroSeq HIV-1 Genotyping System or a homemade system. Samples were stratified in six groups according to different VL levels (50–200; 200–500; 500–1000; 1000–10,000; 10,000–100,000; >100,000 copies/ml). Genotyping success rate was evaluated. Phylogenetic analysis was performed to test reliability and reproducibility of genotypic test also at low VL. Major resistance mutations (MRMs, IAS-list 2013) were evaluated in 2,465 samples from 2,134 in treated patients with VL >50 copies/ml. Finally, 21 patients having more than one GRT with VL <500 copies/ml under the same treatment were analysed for genotypic sensitive scores (GSS; Stanford HIVdb 6.2.0) and genetic diversity (dN and dS).

RESULTS: Overall, genotypic success rate was 96%; for VL 50–200 and 200–500 copies/ml it was 68% and 87%, respectively, reaching 95% for VL 500–1,000 copies/ml and 99% for VL >1,000 copies/ml. Phylogenesis revealed a high homology among sequences belonging to the same subject for 97.2% of patients analysed. The overall prevalence of resistance in the 2,465 GRTs analysed was 71%. This prevalence significantly varied according to VL. Detection of ≥1 MRM was as follows: 50–200 copies/ml =52%; 200–500= 74%; 500–1,000=75%; 1,000–10,000=85%; 10,000–100,000=74%; >100,000=58% (P<0.001, Chi-square test). PI resistance mutations were less frequent than NRTI/NNRTI-resistance mutations (41% versus 76% versus 77%; P<0.001). In PI-failing patients (boosted-unboosted), PI resistance was: 50–200=44%; 200–500=73%; 500–1,000=80%; 1,000–10,000=93%; 10,000–100,000=78%; >100,000=57% (P<0.001). Analysing the 21 patients with two GRTs at VL 50–500 copies/ml, the median GSS of the last GRT decreased compared to the first (VL 50–500: 3.0 [2.5–3.0] versus 3.0 [1.5–3.0]; P=0.048), confirming a resistance evolution in patients maintaining low VL. Moreover, genetic diversity between the first and last GRT increased over time (mean dN: 0.002 versus 0.005; mean dS: 0.007 versus 0.030), suggesting that HIV-1 virus rapidly evolved also at VL <500 copies/ml.

CONCLUSIONS: In patients failing cART with VL ≤500 copies/ml, HIV-1 genotyping provides reliable and reproducible results. Resistance information can be helpful for the optimization of therapy in patients under virological failure to avoid a viraemia increase and resistance mutations accumulation.
ABSTRACT 101

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Resistance analyses in highly treatment-experienced patients treated with etravirine in a roll-over study confirm the results from the Phase III DUET studies

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BACKGROUND: Highly treatment-experienced HIV-1-infected patients with few treatment options were enrolled from DUET into TMC125-C217 and received 200 mg twice-daily etravirine and a background regimen (BR), including 600/100 mg twice-daily darunavir/ritonavir with ≥1 approved antiretroviral drug.

METHODS: Etravirine resistance was explored in the group of etravirine-naive patients who enrolled from the DUET placebo group (that is, darunavir/ritonavir +BR; n=256); patients either discontinued (n=217) or completed (n=39) DUET. Most (32/39) DUET-completers enrolled in TMC125-C217 with undetectable viral load (VL) and without available resistance information at baseline. Therefore, this analysis focused on DUET-discontinuers; development of resistance to etravirine was analysed in virological failures (VFs) with available genotypic and phenotypic data.

RESULTS: The TMC125-C217 week 24 (week 48) response rates (snapshot VL<50 copies/ml) were 79.5% (76.9%) in DUET-completers and 36.4% (27.6%) in DUET-discontinuers, respectively. Limited data were available at week 96 due to an increased discontinuation rate beyond week 48 (including patients with a last VL<50 copies/ml switching to commercial etravirine). In the DUET-discontinuers, median baseline VL was 4.56 log10 copies/ml, including 18/217 (8.3%) patients with undetectable VL. High-level baseline resistance was observed: 73.3% (159/217) had ≤1 sensitive drug in the BR, 97.2% had ≥3 primary protease inhibitor mutations, 86% had a darunavir fold change (FC) ≥10, 92.2% had ≥4 NRTI RAMs, 29.5% had ≥4 NNRTI RAMs and 16% had an etravirine weighted genotypic score ≥4. VF was observed in 131/217 (60.4%) DUET-discontinuers with more non-responders (110/131) than rebounders (21/131). Median etravirine FC increased from 1.9 at baseline to 224.2 at end point (n=82 with paired data). The most frequently emerging NNRTI RAMs in VFs with paired baseline/end point genotypic data (n=87) were V179I (n=23), Y181C (n=15), Y181I (n=15), L100I (n=11), V179F (n=11) and V108I (n=10). All these mutations were previously reported as emerging mutations upon VF with an etravirine-containing regimen in DUET. L100I, V179F, Y181C and Y181I are also included in the etravirine weighted genotypic score.

CONCLUSIONS: Study TMC125-C217 enrolled highly treatment experienced patients who started on an etravirine-containing regimen as one of few treatment options. In patients with VF, the emerging etravirine resistance profile was consistent with the one described previously in the DUET studies.
SESSION 9
New resistance technologies and interpretations
ABSTRACT 102

Antiviral Therapy 2013; 18 Suppl 1:A133

Deep sequencing of HIV: variant detection by Illumina MiSeq and Roche 454 GS FLX+

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BACKGROUND: Numerous studies of HIV nucleotide variation utilizing next-generation sequencing have been reported in the literature coincident with the first ultra deep sequencing systems from 454 Life Sciences. Now that the Illumina MiSeq can generate 250-base read lengths, paired-end sequencing on this platform allows for full-length sequencing of the same 400–500-base targets previously only possible with the Roche 454 technology.

METHODS: We evaluated the HIV variant detection capabilities of the Illumina MiSeq and the Roche 454 GS FLX+ on a set of 48 uniquely indexed amplicons, each defining 465 bases of the HIV reverse transcriptase (RT) gene, each from a different HIV RNA specimen, with all RNA specimens obtained from seroconverters in PrEP studies. The Roche 454 AVA software version 2.6 was used to report variant frequencies from the 454 sff files, while the CLC Genomics Workbench 5.5.1 was employed to identify variants in the reads contained in the Illumina fastq files. Sequence reads were aligned to subtype-specific references obtained from the LANL HIV Sequence database.

RESULTS: Overall, variant frequencies were highly comparable and, in some cases, identical. For example, in tracking reversion of the drug resistance mutation M184V in one patient removed from pre-exposure prophylaxis therapy (FTC/TDF) and sampled longitudinally from -4 weeks to 52 weeks post-serconversion, the frequency of M184V detected by both sequencing methods at 4 weeks =88.14%, at 24 weeks =2.47% by 454, 2.57% by Illumina and at 52 weeks =0.42% by 454, 0.53% by Illumina.

CONCLUSIONS: Variant detection capabilities achievable by both platforms were very similar. Generally, variant detection sensitivity is higher with the Illumina MiSeq, due to the 10-fold higher read depths. However, lower quality basecalls in the region of overlap between paired-end reads (35 bases) reduces the read coverage in this region. At the same time, inaccuracies in 454-based variant frequency determination at critical drug resistance sites, such as K65R in HIV subtype C, located in homopolymer stretches, are absent in the MiSeq data. The results here further confirmed the low prevalence of minor sequence variants in PrEP trials, even when analysed using deeper sequencing on the Illumina MiSeq.
ABSTRACT 103
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Improved detection of HIV-1 co-receptor usage from sequence variation and V3 structural information
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BACKGROUND: HIV type 1 enters CD4+ cells using both CD4 and a chemokine co-receptor: CCR5 or CXCR4. Determining tropism of HIV-1 is required before administration of CCR5 antagonists, which inhibit R5 HIV-1. Non-R5 virus is naturally insensitive to CCR5 antagonists. Currently, genotypic and phenotypic methods are used to screen for tropism. A new and novel genotypic tropism prediction algorithm for HIV-1 subtype B, TroGen, is described. Based on 35 residues in close structural proximity in the V3 loop sequence of the envelope gene and its structure, it classifies viral sequences as R5 or non-R5.

METHODS: The TroGen algorithm, together with the charge rule, PSSM and SVM algorithms, the methods most commonly applied to tropism prediction, were used in the evaluation, firstly, using the phenotypic RLU measurements for clonal data from 20 patients then the clinical outcomes at 8 weeks of 317 patients enrolled in maraviroc (MVC) studies MOTIVATE -1, -2 and A4001029. The baseline virus from these patients was classified by the three algorithms using the population sequence data and then tested to see whether this genotypic determination could be used to predict poor response (no suppression of viral load following 8 weeks of MVC therapy). The TroGen algorithm was further refined using the SVM predictor when TroGen could not make a confident call.

RESULTS: In classifying sequences relative to phenotypic RLU measurements, TroGen had sensitivity and specificity of 0.86/0.96, compared to 0.81/0.95 and 0.86/0.87 for WebPSSM and Geno2Pheno, respectively. Analysis of population sequence data for 317 patients called 230 patients as R5-tropic with a 2.3 median log reduction of RNA, and 74 as non-R5 with a 1.2 median log reduction of RNA. By combining TroGen with SVM, we achieved highly significant prediction of therapy outcome (no week-8 suppression: 46/323, 14.2%; P<0.001, Wilcoxon test), with sensitivity and specificity of 0.83/0.33.

CONCLUSIONS: We present a novel tropism prediction algorithm that uses a combination of genetic and structural information and demonstrate that our method provides equal or higher accuracy in detecting non-R5 HIV-1 subtype B variants than existing genotypic methods. This algorithm can be used as an additional step in next generation sequence analysis.
ABSTRACT 104

*Antiviral Therapy* 2013; 18 Suppl 1:A135

**Evaluation of the Roche 454 HIV-1 drug resistance assay in a clinical laboratory**

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**BACKGROUND:** Studies have found that low-frequency mutations are associated with virological failure to first-line HAART. However, the lack of a standardized high-throughput assay has precluded the detection of low-frequency mutants in diagnostic laboratories.

**OBJECTIVE:** To evaluate the performance of the Roche prototype ultra-deep sequencing (UDS) HIV-1 drug resistance assay.

**METHODS:** 50 plasma samples characterized by population sequencing were retrospectively tested by the UDS assay, including different subtypes (A, n=13; B, n=18; C, n=6; D, n=2; CRF01, n=3; CRF02, n=5; CRF06, n=1 and G, n=2), viral loads between 114–1,806,407 cp/ml, drug-naive (n=29) and drug-experienced (n=21) individuals. Analysis of resistance-associated mutations (RAMs) was based on the Stanford algorithm.

**RESULTS:** The UDS assay was successful for 44/50 (88%) samples. It detected all RAMs detected by population sequencing, including RAMs affecting NRTI (n=14), NNRTI (n=14) and PR (n=25), at frequencies between 36–100%. In addition, 39 low-frequency RAMs were exclusively detected by the UDS assay affecting NRTI (n=6), NNRTI (n=14) and PR (n=19). Low-frequency mutations were seen in both drug-naive (20/28, 71%) and drug-experienced (9/15, 60%) individuals. Analysis of resistance-associated mutations (RAMs) was based on the Stanford algorithm.

**CONCLUSIONS:** The UDS HIV drug resistance assay performed well across a wide range of subtypes and viral loads; RAMs detected by population sequencing were also detected by UDS at frequencies above 20%; the UDS detected additional mutations at frequencies below 20%, which correlate with patients’ treatment history and had, in some cases, important prognostic implications.
ABSTRACT 105

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Subtyping analysis of hepatitis C virus using the Inno-LiPA assay and NS5B sequencing

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BACKGROUND: Accurate subtyping of hepatitis C virus (HCV) is important for interpreting treatment outcome of the specific subtypes. The 5′UTR and core region targeted by INNO-LiPA is the standard method for classifying HCV genotype and subtype. Although the accuracy of the assay is well characterized for HCV subtypes 1a and 1b, the accuracy for other subtypes is less defined. We compared INNO-LiPA subtyping to NS5B sequencing to assess assay performance across HCV genotypes 1–6.

METHODS: HCV samples were subtyped using the Siemens VERSANT® HCV Genotype INNO-LiPA 2.0 Assay. Population sequencing of HCV NS5B was performed using Sanger sequencing. Nucleotide sequences of NS5B were BLAST aligned to determine the most similar HCV subtype. The results were compared to the INNO-LiPA determination. Phylogenetic analysis was used to confirm discrepancies between BLAST and INNO-LiPA.

RESULTS: INNO-LiPA results were obtained for 1,297/1,305 samples (1 genotype 1, 225 genotype 1a, 66 genotype 1b, 354 genotype 2, 616 genotype 3, 28 genotype 4, 1 genotype 5, 6 genotype 6). NS5B sequencing was successful for 1,292 samples. NS5B sequencing analysis for 297 genotype 1a and 1b samples revealed no discrepancies from INNO-LiPA. One sample was determined by INNO-LiPA as genotype 1; however, sequence analysis determined coinfection of 1a and 1b. One additional undetermined sample was determined as 1a by sequencing. There were 94/346 genotype 2 discrepancies (27.2%): 9 samples were identified as a different genotype or undetermined by INNO-LiPA, 39 were identified as the correct genotype, with subtype specification obtained by NS5B sequence and 47 needed subtype correction. There were 27/613 genotype 3 discrepancies (4.4%), 5 undetermined samples were subtyped, 22 were identified as the correct genotype but lacked subtype and 5 required subtype correction. All 28 genotype 4 samples and 6 genotype 6 samples were further defined with subtype information. The single 5a subject was concordant between INNO-LiPA and sequencing.

CONCLUSIONS: INNO-LiPA and NS5B sequencing HCV subtyping discrepancies were observed in 164/994 (16.5%) of non-genotype 1 HCV samples. Although INNO-LiPA shows reliable subtyping results for HCV genotype 1, sequence analysis of the NS5B was able to provide correct and refined subtype for other genotypes.
ABSTRACT 106

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Inhibitory slopes show minimal variation within and across mechanistic classes of HIV-1 antiretroviral agents

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BACKGROUND: HIV-1 antiviral assays are usually end point assays measuring the amount of virus generated in cell culture following single or multiple cycles of infection to determine drug potencies. Inhibitor potency, in addition to pharmacological and physical properties around PK and safety, has often been a main driver of compound selection for clinical development. Recent reports, however, have also implicated the slope of concentration inhibition curve (hill coefficient) as an important factor in pharmacological control of viraemia, with different classes of inhibitors exhibiting different slopes. Here, we re-examine these findings using a kinetic assay that measures the number of infected cells in culture, which provides a direct measure of the effects of inhibitors on the spread of infection.

METHODS: The kinetic viral-spread assay employs fluorescent reporters encoded either in the target cell line or in the virus to measure the number of infected cells in culture over time. We used this assay to determine potencies and slopes of structurally diverse inhibitors that target HIV integrase, protease and reverse transcriptase over a large potency range.

RESULTS: Data obtained from evaluation of an extensive collection of HIV replication inhibitors demonstrate that there is little variability in slopes within a mechanistic class despite utilizing compounds that exhibit a wide range in potencies and high chemical diversity. Integrase strand transfer inhibitors and non-nucleoside reverse transcriptase inhibitors both had slopes of approximately 1.4, while protease inhibitors exhibited slopes of 2.2. The presence of resistance-conferring mutations, while exhibiting the potential to affect potency, had no effects on the slopes of the concentration response curves.

CONCLUSIONS: Slopes of concentration versus inhibition curves show little variability either within or between different mechanistic classes of HIV replication inhibitors. Consistent with clinical experience, the slopes of dose response curves are not likely to be a significant factor in determining clinical efficacy under typical dosing scenarios.
ABSTRACT 107
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Simplified multiplex mutation-specific PCR screening for HIV drug resistance

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BACKGROUND: We have reported on the development, validation and application of sensitive mutation-specific PCR assays for detecting HIV drug resistance mutations at frequencies of 0.3–2% in clinical specimens. While these are powerful research tools, the method needs further simplification and reduction in cost to increase utility in routine mutation screening for identifying the minority of individuals who harbour drug resistance. We modified the original PCR method to suit a multiplex format that can be used as first-line detection for drug resistance.

METHODS: We designed a multiplex drug resistance screening approach that accommodates up to four mutation-specific reactions, one in protease and three in RT. The original assay amplicon lengths for each reaction were shortened to less than 200 bp to minimize background fluorescence and prevent interference from overlapping amplicons. Specific FAM-quencher probes were replaced with a DNA-intercalating dye (EvaGreen). As an example for use in US domestic surveillance of newly diagnosed persons, we selected four of the most commonly transmitted mutations, PR L90M and RT M41L, K103N and T215Y. Multiplex reactions in the positive range were directly sequenced to verify that the mutations were present and to examine resistance mutation linkage.

RESULTS: The quadruplex mutation reactions maintained specificity while losing some sensitivity, likely due to reagent competition and background fluorescence with the nonspecific dye. Lower limits of detection for the different mutations were in the range of 1–10%. All newly diagnosed individuals with single and multiple target mutations were correctly amplified, and linkage with non-targeted mutations (for example, T215Y→M184V) were identified.

CONCLUSIONS: The multiplex screening approach successfully identified commonly transmitted mutations in newly diagnosed infections; for patient management, those positive specimens can be selected for complete genotyping by conventional or deep sequencing. The method allows for exchanging primer sets to target other specific mutations of interest (for example, a K65R-K103N-M184V +/-PI panel). The reduction in sensitivity was nominal and maintains value for drug resistance surveillance and treatment monitoring. Substituting an intercalating dye for complex and expensive probes and combining test reactions in one well decreases both the effort and cost of identifying the fraction of HIV-positive individuals with drug resistance mutations.
ABSTRACT 108

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Comparison of 454 and Illumina deep sequencing in participants failing raltegravir-based antiretroviral therapy

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BACKGROUND: The impact of raltegravir-resistant HIV-1 minority variants (MVs) on raltegravir treatment failure is still unknown. For MV detection, Illumina sequencing offers significantly greater throughput than 454, but sequence analysis tools and validation for viral sequencing are needed. Using a novel Illumina sequence analysis system, we evaluated the performance of Illumina and 454 in the detection of HIV-1 integrase-resistant MVs.

METHODS: ACTG A5262 was a single-arm study of raltegravir and darunavir/ritonavir in treatment-naïve patients. Pretreatment plasma was obtained from five participants with detectable raltegravir resistance at the time of virological failure. At baseline, all participants had HIV-1 RNA>100,000 copies/ml and no evidence of raltegravir resistance by conventional genotyping. A control library was created by pooling integrase clones at proportions down to 0.1%. Multiplexed sequencing was performed on the Roche/454 GS-FLX and Illumina HiSeq 2000 platforms at comparative costs. The Illumina sequence analysis pipeline is based on a random-forest classifier optimized through a crowdsourcing competition. 454 sequence analysis was performed with the V-Phaser algorithm.

RESULTS: Illumina sequencing resulted in significantly higher sequence coverage and an MV limit of detection of 0.095%. Illumina accurately detected all MVs in the control library at ≥0.5% and 7/10 MVs expected at 0.1% with 1 false-positive variant call. 454 sequencing failed to detect 2/10 MVs at 1% and all 10 MVs at 0.1% with 5 false-positive calls. For MVs detected by both 454 and Illumina in the patient samples, the correlation in the detected variant frequencies was high (R²=0.92, P<0.001). Illumina sequencing detected 2.4-fold greater nucleotide MVs and 2.9-fold greater amino acid MVs compared to 454. The only raltegravir-resistant MV detected was an E90K mutation in one participant by Illumina sequencing, but not by 454. Down-sampling analysis showed that similar Illumina assay characteristics can be obtained with <10% of the observed nucleotide coverage.

CONCLUSIONS: In participants of A5262 with raltegravir resistance at virological failure, baseline raltegravir-resistant MVs were rarely detected. At comparable costs to 454 sequencing, Illumina demonstrated greater depth of coverage, increased sensitivity for detecting HIV minority variants and fewer false-positive variant calls.
ABSTRACT 109

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Proof of concept using a novel ambient storage and transport device: ViveST™

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BACKGROUND AND AIMS: Monitoring HCV viral load is a key diagnostic tool for accessing patient response to direct-acting antiviral therapy. Use of frozen plasma has logistic and cost limitations. Herein, we describe a novel ambient storage and transport device, which can be utilized for management of HCV-infected patients undergoing therapy and provide a global solution for infectious disease testing.

METHODS: HCV infectious plasma (1 ml) was loaded onto ViveST, dried and stored at ambient temperature. Samples were recovered with 1 ml of recovery buffer and analysed using Abbott real time HCV assay (RT). To assess inter and intra precision, specimens with varying viral loads (vl) were analysed in triplicate on three separate runs (n=27). High titre samples were diluted and tested in triplicate to assess analytical range. Four levels (n=23 each) of HCV plasma were tested to assess limit of detection. Proof of concept ViveST was performed prospectively using an HCV patient (genotype 1a, IL23B genotype CT) prior to and during therapy (PEG/interferon, ribavirin and telaprevir). To date, baseline, week 2, 4, 8, 12 and 20 time points were analysed using Roche COBAS TaqMan assay (frozen plasma only) and RT (frozen and plasma processed through ViveST).

RESULTS: HCV samples were processed through ViveST, sd for precision < +/- 0.1 log IU/ml, 95% CI of <0.05% intra-assay and <0.06% inter-assay. Analytical range was 1.3–6.6 log IU/ml with R²=0.9979. When a nominal concentration of 1.57 log IU/ml of HCV plasma was processed through ViveST, 91% (21 of 23) were detected and average calculated vl was 0.61 log IU/ml. To date, frozen and ViveST samples from patient yielded similar >5 log reduction with no HCV RNA detected through week 20.

CONCLUSIONS: ViveST processed samples yielded 95% CI of <0.06% inter- and <0.05% intra-assay precision with broad analytical measurement range. HCV patient specimens through ViveST and tested produced a similar vl profile as specimens processed with frozen plasma. ViveST device has great potential to offer a global solution for HCV vl access and reduce costs in both developed and developing countries.
ABSTRACT 110

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Clinical efficiency of a subtype-specific genotyping assay targeting the HCV NS5B polymerase region on a large panel of clinical trial isolates

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BACKGROUND: HCV clinical trials are in progress investigating protease and polymerase inhibitors either alone or in combinations, using multiple DAAs +/- SoC. We report on the clinical performance of the subtype-specific genotyping assay for NS5B polymerase (591 AA) that was determined on clinical isolates.

METHOD: Population sequencing of NS5B gene (1,773 bp) was performed on 1,239 isolates. The majority of the samples were G1 (n=1,228; G1a=738 and G1b=490), with 9 G3a and 2 lacking genotype information. All G1a and G3a samples amplified the complete gene. For G1b samples, the last 10 amino acids at the C-terminal are unavailable (1,743 bp: amplification primer lies within NS5B end). The G1b subtype-specific primers were provided by Gilead Sciences. Externally derived subtype information, viral load (VL) and sample source was available for most samples.

RESULTS: Positive sequences were obtained for 1,156/1,239 samples (93.3%). Complete NS5B was available for 687/747 (92%) G1a and G3a samples, while 469/490 (95.7%) G1b samples were positive for 581 amino acids. Sequencing success based on VL was 33.3% (7/21; 0–1,000 IU/ml); 93.9% (46/49; 1,001–10,000 IU/ml); 93.3% (56/60; 10,001–100,000 IU/ml) and 94% (781/931; >100,000 IU/ml). VL information was unavailable for 278 samples, and 266/278 (95.7%) samples yielded a positive sequence. A 100% success was achieved for 7/7 samples with VL between 550–1,000 IU/ml. Among the low VL samples (<550 IU/ml) that failed (n=14), 13 were from 6 Swiss patients. Sequencing success based on subtypes were 92.4% for G1a (682/738); 95.7% for G1b (469/490) and 55.6% for G3a (5/9). External subtype was unavailable for 14 internally subtyped samples. Sequencing success based on geographical distribution was 95.1% for Americas (645/678), 90.5% for Europe (480/530) and 100% for South Korea (29/29). Sample source was unavailable for two positive isolates.

CONCLUSIONS: An overall success of 93% was observed for the subtype-specific NS5B genotyping assay and efficiency improved with increasing VL (33–94%). The NS5B assay was optimal for genotyping all G1 strains >1,000 IU/ml. The diversity of the NS5B database (containing clinical isolates from ongoing clinical trials) for G1 samples could identify resistant mutation(s) pathways that may provide treatment optimization guidance with respect to potential DAA combinations.
ABSTRACT 111

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In silico analysis of the effects of sequencing errors on geno2pheno_{coreceptor}

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BACKGROUND: HIV-1 genotypic coreceptor tropism determination (CTD) with geno2pheno_{coreceptor} has been validated by several studies. Nonetheless, a systematic in silico study on the effects of sequencing errors on geno2pheno_{coreceptor} is still missing.

MATERIALS AND METHODS: A dataset of 70,644 HIV-1 nucleotide sequences containing non-duplicated V3 regions of the env gene were downloaded from the Los Alamos National Laboratory Sequence Database. From each V3 sequence in the dataset, 1,470 sequences were generated in the following way: each of the sequence’s 105 nucleotides was replaced with each of the 15 possible nucleotides and nucleotide ambiguity codes. The geno2pheno_{coreceptor} false-positive rate (FPR) was computed for each of the resulting 111,264,300 sequences. Sequences producing alignment errors in geno2pheno_{coreceptor} were discarded; alignment errors in original sequences triggered the deletion of the sequences derived from it. After evaluation with geno2pheno_{coreceptor}, proportions of changes in predicted tropism (CIPT) were quantified. For the analysis, the following classification cutoffs were used: \{5,15\}, \{10\} and \{20\}.

RESULTS: After sequences with alignment errors were discarded, 65,309 FPRs corresponding to the unaltered dataset (U), and 95,044,382 FPRs corresponding to the altered dataset (A), remained. Dataset U had an average FPR of 44.65 (sd=33), while in dataset A, the average FPR shifted to 42.18 (sd=34). Position-wise average FPR-shifts ranged from -18.70 to 11.02, while they ranged from -4.76 to -1.42 when averaged by nucleotide or nucleotide ambiguity code. The highest absolute average FPR-shifts could be found around nucleotide positions 20, 32 and 70, and they were all negative. CIPT-probabilities conditioned on original tropism showed for all cutoff sets a more frequent change in predicted tropism from R5 to X4 than from X4 to R5. Disregarding 'intermediate' tropism predictions for cutoff set \{5,15\}, the most likely event was no change in predicted tropism with probability >0.9.

CONCLUSIONS: Low CIPT-probabilities corroborated geno2pheno_{coreceptor}’s robustness in CTD, and showed a propensity to lower FPR with altered sequence. In a low proportion of cases, even single sequencing errors might result in a CIPT. However, FPR-shifts vary depending on the nucleotide position the sequence change occurs at, emphasizing that base calling should be especially accurate at these FPR-shift hotspots.
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HIV-1 drug-resistance genotyping by ultra-deep sequencing increases the sensitivity of detection of minor and intermediate resistant viral populations

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BACKGROUND: HIV-1 resistance genotyping is recommended prior to and during HAART therapy to detect the presence of resistant viral populations associated with treatment failure. Genotyping is routinely based on bulk sequencing, which detects only substitutions that represent at least 20% of the viral quasispecies. The recently developed ultra-deep sequencing technology (UDS) is claimed to be able to detect such variants with a sensitivity cutoff of 1%, which appears to be clinically relevant. The goal of this study was to compare the results of UDS-based genotyping of the HIV-1 protease (Pr) and reverse transcriptase (RT) genotyping.

METHODS: We compared the new kit developed by Roche Diagnostics on GS Junior Sequencer to two bulk sequencing methods: ViroSeq HIV-1 Genotyping System (Abbott Molecular) on ABI Prism 3130XL sequencer (Life Technologies) and TRUGENE HIV-1 (Siemens Healthcare).

RESULTS: We studied 25 HIV-1-infected naive and treated patients and six samples from the French National Quality Controls (NCQ) from the last 2 years. The UDS assay is designed to explore Pr and RT sequences by means of four overlapping amplicons, for which we obtained a median of 1,723 sequences per amplicon and per patient, with a good mean quality of 33.9 ±1.8 (Phred Score). The Roche UDS kit was unable to amplify HIV RNA in three samples with a low viral load (range: 345–1,168 copies/ml), which were amplified by the other methods. The three techniques (UDS, ViroSeq and Trugene) were fully concordant for all mutations that represented more than 20% of the viral quasispecies in the patients and NCQ samples. In 7 and 11 patients, UDS detected 1 or more mutations representing 1–16% and 1–19% for Pr and RT, respectively.

CONCLUSIONS: The UDS-based method developed by Roche was less efficient than the other methods in amplifying samples with a low viral load. However, UDS was substantially more sensitive than bulk sequencing methods and could detect mutations that represented as few as 1% of the viral quasispecies. Thus, UDS appears as a promising method for HIV resistance genotyping and prediction of resistance emergence.
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Provirial DNA testing of HIV tropism in the Maraviroc Switch Collaborative Study (MARCH) – results of a three-phase quality assurance (QA) programme

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INTRODUCTION: MARCH is a maraviroc (MVC) switch study in virologically suppressed subjects on stable PI-based therapy. HIV-1 tropism is determined using population-based sequencing of proviral-DNA in aviraemic patients. Before initiating the clinical trial, we implemented a three-phase proviral-DNA tropism QA programme to ensure consistency in test outcome across multiple MARCH laboratories.

METHODS: MARCH QA began with two pre-study phases and a 6-monthly on-study phase. Phase 1 consisted of chromatogram interpretation (n=10) – ‘competency’ was 100% concordance with reference laboratory output. Phase 2 required triplicate testing of 20 DNA samples from HIV-positive volunteers (VL < 50 cp/ml (n=18); 10/15 X4-tropic on prior phenotypic testing), with the lowest FPR of any replicate defining overall tropism as determined by Geno2Pheno. The FPR was set to 10%. An abbreviated repeat of Phase 2 – Phase 2/3 was undertaken in order to certify more laboratories. This Phase and the on-study Phase 3 involved 2 clonal and up to 10 of the samples used in Phase 2. Laboratories were accredited and passed Phase 2 and thereafter if ≤2 R5 and ≤1 X4 were miscalled versus a consensus interpretation.

RESULTS: All 13 laboratories were competent in Phase 1. In Phase 2, 7/13 laboratories were competent and following Phase 2/3, all were approved to test patient samples in MARCH. A reduction in FPR from 20% to 10% occurred following assessment of Phase 2 results and how these aligned with the clinical cutoffs for response to MVC generated in the MOTIVATE studies. In Phase 3, 11/12 laboratories were competent and 1 laboratory miscalled 2 X4 as R5 and is undergoing further investigation.

CONCLUSIONS: An international QA programme revealed high inter-laboratory variability in tropism determination from proviral DNA. This variability would have been missed had a single or duplicate sequencing approach been used and highlights the importance of intensive QA of laboratories performing tropism testing before embarking on clinical studies and/or routine clinical care where MVC is used.
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Cost-effective pyrosequencing strategies for HIV-1 drug resistance detection

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BACKGROUND: As the duration and size of HIV antiretroviral treatment programmes increase in resource-limited settings, the emergence of transmitted and acquired HIV drug resistance is concerning. This study examined the power of pyrosequencing (PS) on the 454 GS Junior platform to monitor HIV drug resistance patterns via large-scale individual testing or surveillance studies. We demonstrate that 1) a PS-based prototype assay is superior to SS and 2) a PS sample pooling strategy can decrease cost while maintaining accurate surveillance.

METHODS: 80 HIV-1 plasma samples (median VL 66,786 cp/ml) were subjected to Sanger sequencing (SS) and individual PS using prototype primer plates, containing barcoded primers covering PR codons 10–99 and RT codons 1–251. For the pool-PS protocol, the same 80 samples were subdivided into the eight-sample pools. All PS samples were analysed using 454 AVA software and considered positive if an IAS-USA mutation was detected at ≥5 reads. Mutational profiles from the two PS protocols were compared to determine specificity and sensitivity. SS results were also compared to pool-PS results to determine concordance for surveillance studies.

RESULTS: Individual-PS detected 464 mutations, 44 below 20%. Of the 427 mutations detected by SS, 7 were not detected by PS. Four of these discrepancies were due to incorrect combinatorial expansion of SS ambiguity characters, demonstrating the strength of haplotypic PS. Suboptimal alignment or sequencing coverage in PS accounted for the remaining cases. When using individual-PS as the gold standard, 450 mutations ≥20% were detected by both individual PS and pool-PS, which also detected additional 17 and 15 mutations, respectively, resulting in 92.9% specificity and 96.4% sensitivity for the pool-PS protocol. The per-sample cost of the pool-PS protocol was ~50% of individual-PS and ~25% of SS.

CONCLUSIONS: There was a strong correlation between the three protocols. Even a PS mutation detection level of ≥20% showed a distinct advantage over SS for haplotype detection. The pool-PS protocol combined with sample barcoding is an affordable high-throughput option for resistant variant detection. Furthermore, PS on samples pooled pre-amplification is a cost-effective option for regional/institutional surveillance.