Published in final edited form as:

J Acquir Immune Defic Syndr. 2015 June 1; 69(2): 138–144. doi:10.1097/QAI.0000000000000568.

Single Genome Analysis for the Detection of Linked Multiclass Drug Resistance Mutations in HIV-1-Infected Children After Failure of Protease Inhibitor-Based First-Line Therapy

Camille Marie Lange, BSc, MSc, PhD^{*}, Stéphane Hué, PhD^{*}, Avy Violari, FCPaed[†], Mark Cotton, MMed[‡], Diana Gibb, MD[§], Abdel Babiker, PhD[§], Kennedy Otjombe, MSc[†], Ravindre Panchia, MB[†], Els Dobbels, FCPaed[†], Patrick Jean-Philippe, FRCOG^{||}, James A. McIntyre, MD[¶], Deenan Pillay, BSc, PhD, MBBS, MRCPPath, FRCP^{*,#}, and Ravindra Kumar Gupta, BMBCh, MRCP, MA, MPH, PhD^{*}

^{*}Division of Infection and Immunity, University College London, London United Kingdom

[†]Perinatal HIV Research Unit, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

[‡]Children's Infectious Diseases Clinical Research Unit, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa

[§]Medical Research Council Clinical Trials Unit, University College London, London, United Kingdom

^{||}HJF-DAIDS, a Division of the Henry M Jackson Foundation for the Advancement of Military Medicine Inc, Contractor, NIAID, NIH, Department of Health and Human Services, Bethesda, MD

[¶]Anova Health Institute, School of Public Health and Family Medicine, University of Cape Town, Cape Town, South Africa

[#]Africa Centre for Health and Population Studies, University of KwaZulu Natal

Abstract

The WHO recommends protease inhibitor (PI)-based antiretroviral therapy (ART) for vertically infected children after failed nevirapine (NVP) prophylaxis. Emergence of PI resistance on the backdrop of preexisting non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance could compromise long-term treatment options in such children. We characterized multiclass drug resistance using single genome sequencing (SGS) in children with viremia while receiving PI-based ART. We applied SGS of HIV-1 protease (PR) and reverse transcriptase to longitudinal samples from a cohort of the Children with HIV Early Antiretroviral Therapy trial with viral loads

Correspondence to: Deenan Pillay, BSc, PhD, MBBS, MRCPPath, FRCP, Africa Centre for Health and Population Studies, University of KwaZulu-Natal, P.O. Box 198, Mtubatuba, South Africa (dpillay@africacentre.ac.za) or Ravindra Kumar Gupta, BMBCh, MRCP, MA, MPH, PhD, Division of Infection and Immunity, University College London, 90 Gower Street, London WC1E 6BT, United Kingdom (ravindra.gupta@ucl.ac.uk).

Presented previously at International Workshop on Antiviral Drug Resistance 2014, June 2, 2014, Berlin, Germany.

The authors have no conflicts of interest to disclose.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.jaids.com).

>1000 copies per milliliter after 40 weeks of early ART. Bulk sequencing revealed NVP-selected resistance in 50% of these children, whereas SGS revealed NVP-selected resistance in 70%. Two children had baseline NRTI and PI mutations, suggesting previous maternal ART. Linked multiclass drug resistance after PI-based ART was detected by SGS in 2 of 10 children. In one child, the majority species contained M184V in reverse transcriptase linked to L10F, M46I/L, I54V, and V82A in PR and a triple-class drug-resistant variant with these mutations linked to the NNRTI mutation V108I. In the second child, the majority species contained M184V and V82A linked within viral genomes. We conclude that when PI-based ART is initiated soon after birth after single dose-NVP prophylaxis, PI and NRTI resistance can occur in the majority species as expected and also be selected on the same genomes as preexisting NNRTI-resistant mutations. These observations highlight a future therapeutic challenge for vertically infected children where antiretroviral drug classes are limited.

Keywords

HIV; resistance; single genome; multiclass drug resistance; children

INTRODUCTION

Children infected with human immunodeficiency virus type 1 (HIV-1) despite nevirapine (NVP) prophylaxis for the prevention of mother-to-child transmission (PMTCT) have a high prevalence of non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance.¹ Therefore, the WHO recommends protease inhibitor (PI)-based combination antiretroviral therapy within the first 12 weeks of life (early ART) in these children.² NNRTI resistance fades from the majority HIV-1 species with time³ in the absence of ART for children who acquired infection despite prophylaxis. However, in the context of early ART, the potential emergence of PI drug-resistant mutations (DRMs) on the backdrop of preexisting NNRTI resistance could compromise long-term antiretroviral efficacy.

Commercial and “home-brew” population-based sequencing tends to characterize the dominant viral species in the plasma sample. The sensitivity of these assays has been reported as ~20%, which does not reflect the true diversity of the viral population and does not always provide complete information on linkages of DRMs on the same genome.⁴ Multiple methods have been developed to characterize the viral population each with its limitations. There are highly sensitive point mutation assays such as the oligonucleotide ligation assay, parallel allele-specific sequencing, and allele-specific real-time PCR. Next generation sequencing is also a highly sensitive approach with many of the advantages of the previously mentioned approaches and is also often used for whole genome reconstruction. The sensitivity range of these methods to measure HIV-1 population diversity has been reported at 0.1%–1%.^{5,6} Some clinical limitations of these methods are that point mutation assays can be limited by insensitivity of primers for their templates and analyzing thousands of sequences to confirm a rare variant.⁶ Next generation sequencing has a very high throughput of sequences, requiring specialist bioinformatics expertise to be executed to manage the data and to analyze and interpret results. Read lengths are also shorter in the case of 454, Illumina, and Ion Torrent platforms⁷ compared with Sanger

sequencing, limiting the power to determine the evolution of linked DRMs.⁵ In the future, longer read lengths with this technology will no doubt become available.

Another approach is single genome sequencing (SGS), where viral RNA is reverse transcribed, and the complementary DNA (cDNA) product is diluted so that 1 cDNA molecule is used for PCR amplification. This is followed by sequencing of a defined region of the viral genome. This is a highly sensitive method as long as a sufficient number of single genomes are analyzed,^{8,9} and the starting concentration of cDNA for the dilution series is sufficient to minimize resampling of the same viral genome.^{4,8,9} A final approach is to clone the products of one-step reverse-transcription polymerase chain reaction amplification of a region of interest in the viral genome and then randomly select clones for sequencing. This has been shown to be equally effective at measuring population diversity compared with SGS.¹⁰

In this study, we used SGS to explore the evolution of DRMs that occurred among 10 children who acquired HIV infection despite NVP for PMTCT. The cohort used for this study had experienced early virological failure after starting early ART in the Children with Early Antiretrovirals (CHER) trial.

METHODS

Participants

The CHER trial was an open-label randomized controlled trial in South Africa.^{11,12} The participants in this trial were randomized to receive deferred or early PI-based ART [40 or 96 weeks of zidovudine (AZT), lamivudine (3TC), and Kaletra (LPV/r) (Table 1) started within the first 12 weeks of life].¹¹ AZT was replaced with stavudine (d4T) if there were clinical signs of AZT intolerance. After median follow-up of 4.8 years, 12% (27/230) from the immediate therapy groups had a viral load of more than 1000 copies per milliliter.¹¹ Children experiencing treatment failure at the first time point (40 weeks) were first identified. Then, those with sufficient samples at subsequent time points of interest were selected and from these, 10 were randomly selected. The 10 children in this substudy had similar characteristics to children in the CHER trial with virological failure by week 48 of PI-based ART (see Table S1, Supplemental Digital Content, <http://links.lww.com/QAI/A659>).

Plasma remaining after viral load testing was used for our analyses and therefore was subject to availability. Plasma was available for 10/10 children before ART was started and at week 40 of ART. Two of 10 children who were viremic at week 72 of ART had plasma samples available at this time point, and 3 of 10 children had plasma samples available at week 96 of ART. We obtained plasma samples after week 96 of ART from 2 children who developed multiple linked multiclass drug resistance in the viral population by week 96 of ART (“I and J”) and from another child (“H”) for whom we did not detect such drug-resistant variants by week 96 of ART.

Continuous ART was given to 3 of 10 children (“A, C, and D”) until the end of the trial, and their viral loads were suppressed to undetectable levels (<400 copies/mL) at week 96 of

early ART and continued to be suppressed until the end of the trial. Early ART was given to 3 of 10 children (“E, F, and G”) for 0–40 weeks, 0–44 weeks, and 0–99 weeks, respectively. These children’s viral load histories did not pass the last recorded viral load time point, and they were also viremic at the last recorded time point because “E and G” died of non-AIDS-related causes and “F” defaulted from the clinic. Four of 10 children (“B, H, I, and J”) received early ART for 0–96 weeks, and only 1 child (“H”) had an undetectable viral load at week 96. At the end of an ART-free period (weeks 97–164), the viral load reached undetectable levels in only 1 child (“J”). ART was restarted in 2 of these children (“I and J”) from weeks 165 to 272 in “I” and weeks 165 to 298 in “J”. The viral load of “I” became undetectable during restarted ART at week 265 and remained suppressed until the end of the CHER trial, whereas “J” remained viremic during restarted ART. “J” received additional ritonavir (RTV) to achieve mg:mg parity with LPV from weeks 88 to 96 of early ART in response to requiring rifampicin for treatment of tuberculosis, according to local guidelines (see Table S2, Supplemental Digital Content, <http://links.lww.com/QAI/A659>). The viral load histories of each child can be viewed in Table 1.

Parents provided informed consent for specimens to be collected from trial participants, which were collected and stored as part of the CHER trial. All laboratory work was done at University College London.

RNA Extraction and Single Genome Amplification and Sequencing

We used the QIAamp viral RNA mini kit (Qiagen, Valencia, CA) to extract a minimum of 1000 RNA molecules from each plasma sample based on the viral load of each sample. Between 1000 and 20,000 RNA molecules were reverse transcribed from each sample to sufficiently capture the diversity of the viral population without introducing resampling bias.⁴ SuperScript III (Invitrogen Life Technologies, Carlsbad, CA) and the gene specific primers: 4505 reverse (5′-AGTCTTTCCCATATTACTATGCTTTC-3′; 3680-3707) or OPCR5 (5′-ATACCTGCCCACCAACAGG-3′; 4615-4633) were used for reverse transcription, and template RNA was degraded with RNase H (Invitrogen Life Technologies, Carlsbad, CA). 4505 reverse was an “in-house” pantropic primer. OPCR5 was designed using a multiple sequence alignment of 31 HIV-1 subtype C *pol* sequences from the Los Alamos HIV-1 Sequence Database (<http://www.hiv.lanl.gov/components/sequence/HIV/search/search.html>).

Full-length protease (PR; 99 amino acids) and amino acids 1–341 of reverse transcriptase (RT) was amplified as 1 continuous length of DNA (PR-RT ~ 1.7 kb) by nested PCR. Phusion High-Fidelity DNA polymerase and its High-Fidelity Buffer were used for PCR amplifications according to the manufacturer’s instructions. The PCR primers used with their positioning relative to HXB2 (accession number K03455) were first-round PCR primers 4505 and 2633 forward (5′-AATGATGACAGCATGYCAGGGAGT-3′; 1823-1847) and second-round PCR primers HR1 (5′-GGAAAAAGGGCTGTTGGAATGTG-3′; 2014-2038) and HR2 (5′-GGCTCTTGATAAATTTGATATGTCCATTG-3′; 3554-3583). For population sequencing, undiluted cDNA was the PCR template. For SGS, terminally diluted cDNA was PCR-amplified so that 30% of reactions were positive.¹⁰ By Poisson statistics, sequences were

deemed 80% likely to be derived from HIV-1 single genomes. We obtained 20–60 single genomes at each sample time point to achieve 90% confidence of detecting variants present at 8% of the viral population in vivo.^{4,8} PCR primers were designed using sequences from the Los Alamos HIV-1 Sequence Database (Los Alamos National Laboratory, <http://www.hiv.lanl.gov/content/hiv-db/PRIMALIGN/PRIME.html>) to amplify a wide range of HIV-1 subtypes.

PR-RT amplicons obtained from terminal dilution PCR amplification were Sanger sequenced to form a contiguous sequence using the following primers with their position relative to HXB2, sense primers were HR1, C (5'-TGGAAAGGATCACCAGCAATATTCCA-3'; 3005-3031) and B (5'-GTAAACAATGGCCATTGACAGAAGA-3'; 2609-2635) and antisense primer were HR2, HIVin-rc (5'-CACATTCCAACAGCCCTTTTCC-3'; 2014-2038), K65 (5'-TCCTAATTGAACYTCCCARAARTCYTGAGTTC-3'; 2796-2828). Sequencing primers were designed using all the population-based sequences obtained for each child. Sanger sequencing was provided by Beckman Coulter Genomics Limited.

All PR-RT sequences analyzed in this study were derived from HIV-1 subtype C. The PR-RT consensus sequences were submitted to the HIV Drug Resistance Database¹³ for HIV subtyping and DRM identification. Population sequencing and SGS were applied to samples taken at baseline and at week 40 of ART for 10 randomly selected patients from the CHER study who were viremic at week 40 of ART and also for all follow-up samples from patients "I" and "J."

We used a Fisher's exact test ($P < 0.05$) to identify those codons for DRMs whose change in frequency between 2 sequential time points was significant.¹⁴

RESULTS

Detection of Baseline Drug Resistance

Eight of 10 children received perinatal NVP for PMTCT. One child did not receive this prophylaxis ("J"), whereas this information was not recorded for a second child ("H"). All mothers received a single dose of NVP at the onset of labor. Bulk sequence analyses detected baseline NVP-selected resistance in 5 of 10 children: K103N, V106M, and Y188C in 1 child each and Y181C in 2 children. However, SGS detected NVP-selected resistance mutations in 7 of 10 (70%) of these children and demonstrated a broader selection of mutations at higher frequency (Table 2).

PI and NRTI DRMs were detected by SGS only and at baseline in 2 children, "D" and "E." These were T215I (5%, $n = 21$ sequences) in "D" and the major PI DRM I50V (3%, $n = 30$ sequences) in "E." T215I is not known to confer NRTI resistance rather it is a reversion mutation of the thymidine analog mutation (TAM) T215F/Y.^{15,16} "E" died before samples could be taken after 40 weeks of ART. "D" had virological failure at week 40 (with the PI mutation M46I in 1 of 38 single genomes) but achieved a viral load less than 400 copies per milliliter by week 96 of early ART that was maintained until the end of the CHER trial. More than 1 RT DRM linked on the same genome were detected in 2 children: in "J,"

Y181C was linked with V106M (2%, $n = 49$ sequences) and K219N (2%); in “E,” Y181C linked with the accessory TAM, K219N, was detected (3%, $n = 30$ sequences) and linked dual-class resistance (Y181C with L74V) was also detected (7%) in the baseline viral population of this child.

Detection of Low-Frequency NNRTI Mutations During PI-Based Therapy

NVP-selected mutations detected at baseline were also detected after 40 weeks of ART in 2 children by SGS (Table 3) despite a treatment regimen lacking NNRTIs. In “H,” K101E was detected at week 40 of ART at a frequency of 7% but was not detected by bulk sequencing at either time point (Table 3). In “J,” Y181C was detected at a frequency of 5% at week 40 of ART with SGS but was not detected by bulk sequence analysis at this time point. The NNRTI mutation V108I was first detected at week 96 of ART at a frequency of 3% and again detected at a frequency of 5% at week 298 (Table 3). Emergence of V108I after ART initiation was not statistically supported ($P = 0.189$ for test of proportions of genomes carrying V108I between baseline and week 298).

Detection of Genetically Linked Dual-Class Resistance to PI and NRTI

Bulk sequence analysis detected dual-class DRMs in the viral populations of 2 children (“I and J”) during ART. The same mutations were detected by SGS, and SGS determined these mutations as linked on the same genome in variants from the viral populations in which they detected. The NRTI mutation M184V (selected by 3 TC) and the PR mutation V82A (selected by LPV) were observed at weeks 40 and 96 of ART for “I” (Table 4). These dual-class drug-resistant variants were detected as majority species in the viral populations at the 2 time points.

“J” received additional RTV to achieve mg:mg parity with LPV from weeks 88 to 96 of early ART in response to requiring rifampicin for treatment of tuberculosis according to local guidelines (see Table S2, Supplemental Digital Content, <http://links.lww.com/QAI/A659>). SGS revealed 29% of variants at week 96 of ART contained genetically linked M184V and V82A, but bulk sequencing did not detect V82A at this time point. Two other dual-class drug-resistant variants were detected by SGS at week 96 of ART involving M184V linked to mutations at position 46 of protease (Table 5). Despite the high frequency of M46I, it was also not detected by bulk sequence analysis. M46L could have been present at baseline given that only 1/32 genomes contained this mutation at week 96 ($P = 0.457$, Fisher’s exact test of the proportion of sequences with M46L at baseline versus week 96 of ART). ART was stopped at week 96 and restarted 45 weeks later. All SGS variants contained M184V and V82A by week 224.

Detection of Genetically Linked Triple-Class Resistance to PI, NRTI, and NNRTI

Genetically linked triple-class PI, NRTI, and NNRTI DRMs were detected by SGS in child “J” during ART. Such variants were first detected at week 96 of early ART with additional RTV (see Table S2, Supplemental Digital Content, <http://links.lww.com/QAI/A659>) as a minority species (3%, $n = 32$ sequences). The variant contained M184V, V108I in RT, and M46I in PR (Table 5). Variants with these DRMs were also detected at week 298 during

restarted ART as a minority species (5%, n = 39 sequences) and contained additional PR mutations (Table 5).

Multiclass Drug Resistance During ART Is Associated With High Viral Loads

M184V was the first DRM to be detected in both “I” and “J” who had virological failure with DRMs known to confer resistance to components of ART (Tables 4 and 5). Viral load rebounds greater than 50,000 copies per milliliter during ART in these children frequently coincided with dual-class drug resistance in the majority species (Table 1).

In “I,” 92% (n = 37 sequences) of the viral population contained M184V only at week 12 of ART while the viral load fell from 750,000 copies per milliliter or greater at baseline to 7000 copies per milliliter at week 24 of ART. At week 40 of ART, there was a viral load rebound to 96,400 copies per milliliter, and the majority species (67%) contained M184V and V82A.

In “J,” the first viral load rebound was seen between weeks 24 and 40 of early ART when the viral load rebounded from 3570 copies per milliliter at week 24 of early ART to greater than 750,000 copies per milliliter at week 40 of ART without the detection of multiple linked multiclass drug resistance; the quasispecies contained wild-type virus (47%), M184V (45%), and NNRTI DRMs at a combined frequency of 8% (n = 38 sequences). The viral load later rebounded to 326,000 copies per milliliter at week 96 while receiving additional RTV, this time with dual-class drug resistance (M184V genetically linked to V82A or M46I) detected in the majority species (68%, n = 32 sequences). By week 298, during restarted ART, the VL was measured at 7440 copies per milliliter with dual-class drug resistance in 95% of the viral population (n = 39 sequences), where these variants contained M184V genetically linked to 3 major and 2 minor PI-resistant mutations (Table 5).

DISCUSSION

Our study is the first to use SGS to characterize antiretroviral resistance in children on early combination ART. The extent to which differences in sequencing approaches impact the interpretation of drug resistance has been highlighted in previous studies.^{4,6,10} In this study, SGS was advantageous because it allowed a longitudinal assessment of the evolution of population diversity in each child and allowed us to determine genetic linkages of DRMs between viral PR and RT. However, SGS remains unsuitable for routine clinical use because of the complexity of the methodology and interpretation of the data. We used Superscript III for cDNA synthesis (error rate = 1/30,000 nucleotides¹⁷) and Phusion High-Fidelity DNA Polymerase for PCR because of their low error rates (2.2% of PCR products would have 1 nucleotide misincorporation for a ~1.4 kb product and 35 PCR cycles <http://www.thermoscientificbio.com/webtools/fidelity/>). Thus, the fidelity of the PCR products was well maintained. Nonetheless, it is possible that some single instances of DRMs could be PCR artefacts.

SGS identified an additional 20% of children with NNRTI-resistant children as compared with bulk sequence analysis. SGS was also able to detect NNRTI resistance during PI-based ART in 2 of the children, which was not evident by bulk sequencing. We did not rule out the

possibility that the DRMs we observed could have been the result of natural viral nucleotide variation.

At some of the time points in each child, bulk sequence analysis did not reveal any drug resistance; however, SGS was able to detect DRMs in 25%–35% of the sequences obtained. The limit of detection of bulk sequencing approaches to detect DRMs in *pol* has been reported as 25%–35%.^{4,18-20} In our study, our SGS approach had a sensitivity that was comparable with that of Palmer et al,⁴ where any mutation detected at a frequency $\geq 35\%$ by SGS was detected with bulk sequence analysis. Similarly, we saw a variation in sensitivity at some nucleotide positions. There were some mutations or wild-type codons that were detected at a frequency between 25% and 35%, which were not detected by SGS. These discrepancies could be due to variations in primer sensitivities for their templates during cDNA synthesis and/or PCR amplification.

M184V was the first mutation selected in the majority of the viral population of the 2 children failing ART with DRMs; the viral load rebounds coincided with the majority of the viral population being replaced with dual-class drug-resistant variants, and notably no AZT-selected mutations were detected during ART. Furthermore, despite failing AZT and LPV/r-containing therapy, T215I and T219N in RT and I50V in PR were not detected by SGS or bulk sequencing during ART for the 2 children (“D and E”) who harbored these mutations in their baseline viral populations. These observations are consistent with previous data on the “protective” effect of PI on the development of NRTI resistance.^{21,22}

The presence of NNRTI resistance in one child without a history of sdNVP for PMTCT was most likely explained by vertical transmission of DRMs or maternal NVP.²³ The baseline PI DRM I50V, TAM revertant K215I, and the TAM K219N of the double mutant Y181C + K219N that were detected in “D, E, and J” may have also been vertically transmitted. Vertical transmission of drug resistance has been previously determined by phylogenetic comparison of maternal and neonatal sequences.²⁴ Unfortunately, maternal plasma samples were not available for our study.

Age adjusted full-dose RTV can select M46I, I54V, and V82A in children,²⁵ as well as L10F, M46L, and Q58E in adults.²⁶ We detected genetic linkage of all these mutations in J after RTV super-boosted LPV/r, conferring greater PI resistance and reducing treatment options as compared with “I.”¹³ Currently, there are no published data on the selection of resistance after RTV super-boosting of LPV/r; therefore, our findings, although novel and important, require verification in a larger sample cohort.

The current virological understanding of drug-resistant reservoirs suggests that multiclass drug-resistant viruses can compromise ART in the future, although this requires formal testing in children where drug class options are limited. We consider the possibility that V108I was selected by NVP for PMTCT because it is not usually found in patients infected with subtype C who were not exposed to this drug.¹³ Our evidence also points to the triple-class drug-resistant variants detected in “J” to be a result of genetic “hitchhiking” by V108I. V108I is known to confer modest reductions in NVP/EFV susceptibility in vitro,¹³ but its clinical significance is not known.¹³

Although multiclass drug resistance may be more common than previously believed, it should be emphasized that the CHER trial demonstrated excellent outcomes overall with 84.6% (280/331) of the children on ART achieving viral loads <400 copies per milliliter at the end of the trial. These include 2 of 10 children from our study cohort (“C” and “I”) who achieved and maintained viral loads <400 copies per milliliter at weeks 164 and 265 respectively, until the end of the CHER trial. Further large-scale studies are needed to address long-term implications in these NVP exposed vertically infected children with virological failure on LPV/r, and in particular, the efficacy of the second-generation NNRTI etravirine and the PI darunavir warrant investigation. Finally, these data reinforce the urgent requirement for appropriate pediatric formulations of a wider array of antiretroviral medications in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors thank Wendy Snowden of GSK/ViiV Health-care, Paul Grant of UCLH, and the families and children who participated in the CHER trial and the study team.

Supported by University College London Grand Challenges Global Health Program, US National Institutes of Health, and Abbvie Laboratories. R.K.G. is funded by a Wellcome Trust Fellowship (WT093722MA). Funding for the CHER study was provided by the National Institute of Allergy and Infectious Diseases of the US National Institutes for Health, through the Comprehensive International Program of Research on AIDS network (Grant number U19 AI53217). Additional support was provided by the Departments of Health of the Western Cape and Gauteng Provinces, South Africa, and GlaxoSmithKline.

REFERENCES

1. Arrive E, Newell ML, Ekouevi DK, et al. Prevalence of resistance to nevirapine in mothers and children after single-dose exposure to prevent vertical transmission of HIV-1: a meta-analysis. *Int J Epidemiol.* 2007; 36:1009–1021. [PubMed: 17533166]
2. World Health Organization. Use of Antiretroviral Drugs for Treating Pregnant Women and Preventing HIV Infection in Infants. Programmatic Update. Apr. 2012 Reference number; WHO/HIV/2012.6 Available at: http://www.who.int/hiv/pub/mtct/programmatic_update2012/en/
3. Eshleman SH, Mracna M, Guay LA, et al. Selection and fading of resistance mutations in women and infants receiving nevirapine to prevent HIV-1 vertical transmission (HIVNET 012). *AIDS.* 2001; 15:1951. [PubMed: 11600822]
4. Palmer S, Kearney M, Maldarelli F, et al. Multiple, linked human immunodeficiency virus type 1 drug resistance mutations in treatment-experienced patients are missed by standard genotype analysis. *J Clin Microbiol.* 2005; 43:406–413. [PubMed: 15635002]
5. Wang C, Mitsuya Y, Gharizadeh B, et al. Characterization of mutation spectra with ultra-deep pyrosequencing: application to HIV-1 drug resistance. *Genome Res.* 2007; 17:1195–1201. [PubMed: 17600086]
6. Halvas EK, Aldrovandi GM, Balfe P, et al. Blinded, multicenter comparison of methods to detect a drug-resistant mutant of human immunodeficiency virus type 1 at low frequency. *J Clin Microbiol.* 2006; 44:2612–2614. [PubMed: 16825395]
7. Gibson RM, Schmotzer CL, Quiñones-Mateu ME. Next-generation sequencing to Help Monitor patients infected with hiv: Ready for clinical Use? *Curr Infect Dis Rep.* 2014; 16:1–9.
8. Keele BF, Giorgi EE, Salazar-Gonzalez JF, et al. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc Natl Acad Sci U S A.* 2008; 105:7552–7557. [PubMed: 18490657]

9. Salazar-Gonzalez JF, Bailes E, Pham KT, et al. Deciphering human immunodeficiency virus type 1 transmission and early envelope diversification by single-genome amplification and sequencing. *J Virol*. 2008; 82:3952–3970. [PubMed: 18256145]
10. Jordan MR, Kearney M, Palmer S, et al. Comparison of standard PCR/cloning to single genome sequencing for analysis of HIV-1 populations. *J Virol Methods*. 2010; 168:114–120. [PubMed: 20451557]
11. Cotton MF, Violari A, Otwombe K, et al. Early time-limited antiretroviral therapy versus deferred therapy in South African infants infected with HIV: results from the children with HIV early antiretroviral (CHER) randomised trial. *Lancet*. 2013; 382:1555–1563. [PubMed: 24209829]
12. Violari A, Cotton MF, Gibb DM, et al. Early antiretroviral therapy and mortality among HIV-infected infants. *N Engl J Med*. 2008; 359:2233–2244. [PubMed: 19020325]
13. Liu TF, Shafer RW. Web resources for HIV type 1 genotypic-resistance test interpretation. *Clin Infect Dis*. 2006; 42:1608. [PubMed: 16652319]
14. Kearney MF, Spindler J, Shao W, et al. Lack of Detectable HIV-1 Molecular evolution during suppressive antiretroviral therapy. *PLoS Pathog*. 2014; 10:e1004010. [PubMed: 24651464]
15. Riva C, Violin M, Cozzi-Lepri A, et al. Transmitted virus with substitutions at position 215 and risk of virological failure in antiretroviral-naïve patients starting highly active antiretroviral therapy. *Antivir Ther*. 2002; 7:S136–S136.
16. Violin M, Cozzi-Lepri A, Velleca R, et al. Risk of failure in patients with 215 HIV-1 revertants starting their first thymidine analog-containing highly active antiretroviral therapy. *AIDS*. 2004; 18:227–235. [PubMed: 15075540]
17. Roberts JD, Bebenek K, Kunkel TA. The accuracy of reverse transcriptase from HIV-1. *Science*. 1988; 242:1171–1173. [PubMed: 2460925]
18. Hall, JC.; Hall, BJ.; Cockerel, CJ. Laboratory testing for HIV/AIDS. In: Tang, Y-W., editor. *HIV/AIDS in the Post-HAART Era: Manifestations, Treatment, Epidemiology*. 1 Har/Dgd edition. McGraw-Hill Medical; 2011. p. 128-129.
19. Günthard HF, Wong JK, Ignacio CC, et al. Comparative performance of high-density oligonucleotide sequencing and dideoxynucleotide sequencing of HIV type 1 pol from clinical samples. *AIDS Res Hum Retroviruses*. 1998; 14:869–876. [PubMed: 9671215]
20. Shafer RW, Winters MA, Palmer S, et al. Multiple concurrent reverse transcriptase and protease mutations and multidrug resistance of HIV-1 isolates from heavily treated patients. *Ann Intern Med*. 1998; 128:906–911. [PubMed: 9634429]
21. Gupta R, Hill A, Sawyer AW, et al. Emergence of drug resistance in HIV type 1-infected patients after receipt of first-line highly active antiretroviral therapy: a systematic review of clinical trials. *Clin Infect Dis*. 2008; 47:712–722. [PubMed: 18662137]
22. Hill A, McBride A, Sawyer AW, Clumeck N, Gupta RK. Resistance at virological failure using boosted protease inhibitors versus nonnucleoside reverse transcriptase inhibitors as first-line antiretroviral therapy—implications for sustained efficacy of ART in resource-limited settings. *J Infect Dis*. 2013; 207(suppl 2):S78–S84. [PubMed: 23687293]
23. Benaboud S, Ekouevi DK, Urien S, et al. Population pharmacokinetics of nevirapine in HIV-1-infected pregnant women and their neonates. *Antimicrob Agents Chemother*. 2011; 55:331–337. [PubMed: 20956588]
24. Johnson VA, Petropoulos CJ, Woods CR, et al. Vertical transmission of multidrug-resistant human immunodeficiency virus type 1 (HIV-1) and continued evolution of drug resistance in an HIV-1-infected infant. *J Infect Dis*. 2001; 183:1688–1693. [PubMed: 11343221]
25. van Zyl GU, van der Merwe L, Claassen M, et al. Protease inhibitor resistance in South African children with virologic failure. *Pediatr Infect Dis J*. 2009; 28:1125–1127. [PubMed: 19779394]
26. Molla A, Korneyeva M, Gao Q, et al. Ordered accumulation of mutations in HIV protease confers resistance to ritonavir. *Nat Med*. 1996; 2:760–766. [PubMed: 8673921]

TABLE 1

Viral Load Histories of the 10 Children in This Study

Study ID	Weeks										Therapy Status	
	0	24	40	72	96	164	224	265	281	298	Early ART	Restarted ART
	Viral Load (Copies RNA per mL Blood)										Duration, wk	
A	>750,000	<50	58,200	<400	<400	<400	<400				0–273	
B	>750,000	<400	729,000	<400	11,040	6050					0–96	97–164
C	>750,000	<400	4890	640,000	<400	<400	<400			<400	0–313	
D	318,000	44,000	295,000	56,600	<400						0–316	
E*	>750,000	<400	4100								0–44	
F	617,000	>750,000	>750,000								0–40	
G*	>750,000	5400	>750,000	66,300							0–99	
H	>750,000	3050	281,000	<400	<400	503,000					0–96	97–164
I	>750,000	7000	96,400	852,000	152,200	181,000	194,000	<400	<400		0–96	165–272
J	535,000	3570	>750,000	40,200	326,000	<400	88,400		402	7440	0–96	97–164
												165–298

Plasma samples were taken at baseline and then every 4 weeks until week 24, then every 8 weeks until week 48, and then every 12 weeks until the end of the CHER Trial.

Bold viral loads indicated those samples that were used for sequence analysis in this study (for full details of the therapeutic history of each child, see Table S2, Supplemental Digital Content, <http://links.lww.com/QAI/A659>).

* Those children who died by the end of the trial from non-AIDS-related causes.

TABLE 2

Frequencies of Baseline Antiretroviral Drug-Resistant Variants by SGS

Study ID	A	B	C	D	E	F	G	H	I	J
No of variants analyzed (n)	49	30	33	21	30	57	26	34	30	50
Wildtype, %	100	93	45	95	37	42	96	47	100	44
NNRTI resistance, %										
A98G							4			
K101E			3					12		
K103N			36		7					4
V106A		7	3							
V106M						37		3		4
Y181C					40	21				44
Y188C			3					38		
G190A			9		3					
Y181C + V106M										2
NNRTI + NRTI resistance										
Y181C + L74V					7					
NNRTI resistance + TAM										
Y181C + K219N					3					2
TAM revertants										
T215I				5						
PI resistance										
I50V					3					

Frequencies in bold represent variants detected by both SGS and bulk sequence analysis.

TABLE 3

Frequencies of NVP-Selected NNRTI-Resistant Mutations Detected by SGS in 2 Children, Study IDs: H and J

Therapy Status	Early ART			No ART			Restarted ART	
Week	0	40	96	164	224	298		
Number of variants analyzed for study ID H (n)	34	29		39				
Number of variants analyzed for study ID J (n)	31	38	32		34	39		
Study ID H								
K101E, %	12	7						
Study ID J								
Y181C, %	48	7						
Y188C, %		3						
V108I, %			3			5		

The sampling time points for SGS are indicated.

Frequencies in bold represent variants detected by both SGS and bulk sequence analysis.

TABLE 4

Dual-Class Resistance Detected by SGS During First-Line PI-Based Therapy for Study ID I

Therapy Status	Early ART					No ART	Restarted ART
Week	0	12	40	72	96	164	224
Number of variants analyzed, n	33	37	28	44	47	38	22
Wildtype, %	100	8				100	100
M184V, %		92	32	9	21		
V82A + M184V, %		68	91	79			

Frequencies in bold indicated variants detected by both SGS and bulk sequence. "+" indicated linkage on the same genome.

Early ART was stopped at week 96 and restarted at week 165.

TABLE 5

Dual- and Triple-Class Resistance Detected by SGS During First-Line PI-Based Therapy in Study ID J

Therapy Status	Early ART			Restarted ART	
Week	0	40	96	224	298
Number of variants analyzed, n	50	38	32	34	39
Frequencies, %					
Wildtype	44	47			
M184V		45	28		
M184V + V82A			34	71	
M184V + M46I			31		
M184V + M46L			3		
M184V + M46I + V108I			3		
M184V + V82A + L10F				29	
M184V + V82A + I54V + L10F					3
M184V + V82A + I54V + M46I + Q58E + L10F					92
M184V + V82A + I54V + M46I + Q58E + L10F + V108I					5

Frequencies in bold indicate variants detected by both SGS and bulk sequence analysis.

“+” indicated linkage on the same genome.

Early ART was stopped at week 96 and restarted at week 165.