La mégère apprivoisée : élaborer des stratégies pour la gestion de la résistance aux médicaments dans la grippe et l'infection par le virus de l'immunodéficience humaine

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"LA MEGERE APPRIVOISEE"

ELABORER DES STRATEGIES POUR LA GESTION DE LA RESISTANCE AUX MEDICAMENTS DANS LA GRIPPE ET L'INFECTION PAR LE VIRUS DE L'IMMUNODEFICIENCE HUMAINE

"THE TAMING OF THE SHREW"

DEVELOPING STRATEGIES FOR THE MANAGEMENT OF DRUG RESISTANCE IN INFLUENZA AND HUMAN IMMUNODEFICIENCY VIRUS INFECTION

DEDICATION

I would like to dedicate this thesis to my recently deceased father, who taught me to open my mind, to think outside the box, and to ask the right kind of questions.

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SUMMARY

RESUME EN FRANCAIS

Le développement de médicaments efficaces contre le virus de l'immunodéficience humaine (VIH) est l'une des plus grandes réussites de l'histoire médicale récente: lorsque la thérapie combinée est devenu la norme des soins après la Conférence de Vancouver en 1996, une maladie mortelle a été progressivement transformée en une maladie chronique gérable. Les décennies suivantes ont été consacrées à l'élaboration des schémas thérapeutiques consolidés pour les adultes et les enfants, à la prévention de la transmission mère-enfant (PTME) et à élargir l'accès à la thérapie antirétrovirale (ART) dans les pays en développement. Par la suite, la réussite d'un traitement antiviral de l'infection par le VIH est devenue un modèle pour l'élaboration de stratégies de traitement efficaces pour d'autres maladies virales, telles que les hépatites, les infections à *herpesviridae*, et la grippe A et B.

Ce succès est cependant menacé par la sélection rapide et la propagation de la résistance virale aux médicaments dans le monde entier, en particulier en matière de VIH-1 et de virus de la grippe A. Un énorme effort a été entrepris pour étudier les mécanismes de la pathogenèse virale, identifier de nouvelles cibles médicamenteuses, et développer des technologies innovantes de détection et d'interprétation des mécanismes de résistance des virus aux médicaments. Sur le plan de la santé publique, la lutte antivirale implique la nécessité d'un diagnostic précoce, le traitement de grandes populations de patients dans divers contextes, ainsi que le contrôle strict de l'efficacité des stratégies d'intervention antivirale dans la vie réelle. Les enfants, qui ont souvent un risque élevé de maladie grave, ont longtemps été la population la moins étudiée vis-à-vis de la sécurité et de l'efficacité de la thérapie antivirale. Il a récemment été reconnu par les autorités réglementaires en Europe et ailleurs qu'il peut être moins éthique de continuer à traiter les enfants hors AMM que de mener des essais cliniques et des études observationnelles soigneusement conçus.

Dans cette thèse nous abordons:

(1) de nouveaux modèles in vitro pour simuler un traitement combiné contre un VIH-1 multirésistant afin de promouvoir la sélection du régime de traitement le plus durable chez les patients en sauvetage thérapeutique,

(2) la meilleure approche en termes de coût-bénéfice pour la surveillance de la pharmacorésistance dans les cohortes de patients pédiatriques traités dans des milieux à faibles ressources ; et enfin

(3) Une approche translationnelle vers la gestion du traitement de la grippe et la prédiction du développement de virus résistants aux médicaments chez les enfants.

Le travail présenté ici repose sur trois études indépendantes menées aux Etats-Unis, au Pérou et en Allemagne visant à améliorer notre compréhension des mécanismes de développement de la pharmacorésistance du VIH-1 et du virus de la grippe *in vitro* et *in vivo*. Nous discutons également les limites de ces études ainsi que les leçons apprises dans l'optimisation de stratégies de traitement antiviral et la prévention des résistances contre le VIH et le virus de la grippe chez les adultes et les enfants.

(1) La méthode in vitro mise au point dans l'étude 1 a été utilisée pour étudier les effets du maintien *versus* du retrait d'un médicament antirétroviral spécifique (lamivudine) lors de la commutation des schémas thérapeutiques. Ce test qui explore l'effet des combinaisons médicamenteuses fournit une occasion unique d'étudier et de comparer les effets des différentes stratégies de traitement sur les isolats cliniques provenant des patients eux- mêmes, alors que in vivo, nous n'avons qu'une seule chance de choisir le schéma thérapeutique suivant le plus approprié lors du passage d'un programme de traitement à l'autre. Ce modèle in vitro nous a permis de démontrer les interférences entre les résistances aux divers anti-rétroviraux et, en particulier, de suggérer que le maintien de la lamivudine dans le protocole thérapeutique lors du changement de ce protocole, en dépit d'une résistance de haut niveau à la lamivudine, empêchait la disparition de la mutation associée à la résistance à la lamivudine, et par ce fait retardait la sélection ultérieure de nouveaux variants résistants à d'autres molécules thérapeutiques, comme la névirapine.

(2) Avec la seconde étude, nous avons étudié l'émergence de la résistance aux médicaments in vivo dans une première cohorte d'enfants vivant avec le VIH / sida à Lima, au Pérou, dans un essai d''accès à la thérapie antirétrovirale soutenu par le Fonds mondial. Nous avons pu démontrer qu'une méthode alternative pour l'analyse de la résistance, le test de ligation d'oligonucléotides (OLA) (nécessitant moins de personnel et de matériel coûteux que la méthode standard utilisant le séquençage consensus,) avait une grande sensibilité et fournissait des informations utiles permettant le contrôle simultané périodique de la résistance aux médicaments du VIH dans des cohortes de patients suivies dans des communautés à faibles ressources.

(3) La troisième étude permet de mieux comprendre la cinétique de la charge virale et de développement in vivo la résistance aux médicaments chez les nourrissons et les enfants recevant un traitement antiviral (oseltamivir) pour une infection grippale. Avec cette étude, nous avons développé un sous-typage de la grippe et une surveillance plus étroite du développement de la résistance aux antiviraux chez les patients traités en appliquant les leçons apprises dans la gestion de l'infection à VIH-1. Nos données issues de la surveillance virologique sur des échantillons naso-pharyngés suggèrent que l'évaluation du niveau de référence et de suivi de la charge virale dans la grippe peuvent fournir des informations utiles pour estimer la durée optimale du traitement par l'oseltamivir chez l'individu malade. Une baisse moins forte que prévue de la charge virale pendant le traitement permettrait de s'orienter vers le développement d'une résistance, ou vers des problèmes d'adhérence au traitement, ou de malabsorption. Les paramètres cliniques, en comparaison, semblent être de mauvais indicateurs de la réponse au traitement antiviral.

Les résultats de ces trois études démontrent la faisabilité et l'importance de la surveillance virologique au cours du traitement antiviral, tout en soulignant la nécessité d'une détection précoce du développement de la résistance aux médicaments dans le VIH-1 et la grippe. La mise en place d'une collaboration étroite "du laboratoire au chevet du patient» est indispensable pour aider le clinicien à faire des progrès dans la gestion des infections virales chez les adultes ainsi que chez les enfants. Une meilleure compréhension des mécanismes moléculaires sous-jacents au développement de la résistance aux médicaments et aux interactions entre virus et médicaments peut aider le virologue et le spécialiste des maladies infectieuses à faire au bon moment le bon choix du médicament antiviral et / ou de la combinaison de médicaments, chez un patient donné. La viabilité à long terme des traitements antiviraux est la clé de leur succès, en particulier chez les nourrissons et les enfants, qui peuvent en avoir besoin de façon prolongée, et chez lesquels la question des risques et des avantages de la thérapie antivirale se pose avec une particulière acuité. Le traitement antiviral dans cette population particulière, qui a également un risque élevé de morbidité et de mortalité par le VIH-1 et la grippe, reste un défi en raison de la disponibilité limitée des données cliniques, des choix limités de médicaments, et les exigences particulièrement strictes pour la sécurité et de tolérance à long terme. Le présent travail apporte quelques pistes pour répondre à ce défi.

SUMMARY IN ENGLISH

The development of efficacious drugs against the human immunodeficiency virus (HIV) is one of the greatest success stories in the recent medical history: when combination therapy became standard of care after the Vancouver Conference in 1996, a deadly disease was gradually turned into a manageable chronic condition. The following decades have been dedicated to developing consolidated treatment regimens for both adults and children, to the prevention of mother-to-child transmission (pMTCT) and to expanding access to antiretroviral therapy (ART) in developing countries. Subsequently, the success story of the antiviral treatment of HIV infection has become a model for the development of successful treatment strategies for other viral diseases, such as hepatitis and infections with *herpesviridae* and influenza A and B.

This success however is endangered by the high level of adaptability of RNA- and retroviruses to a constantly changing environment and the rapid emergence of viral drug resistance worldwide, especially in HIV-1 and influenza A viruses. Enormous effort has been undertaken to investigate mechanisms of viral pathogenesis, the identification of new drug targets, as well as the development of innovative technologies for the detection and interpretation of the mechanisms of viral drug resistance development. On a public health level, this implies the need for timely diagnosis and treatment of large patient populations in diverse settings as well as the strict monitoring of the real-life effectiveness of antiviral intervention strategies. Children, who often carry a high risk of severe disease, have long been the least studied patient population with respect to the safety and efficacy of antiviral therapy. It has recently been recognized by regulatory authorities in Europe and elsewhere that it may be less ethical to continue treating children off-label than to conduct carefully designed clinical trials and observational studies in this particularly sensitive age group.

In this thesis paper we will be discussing:

(1) new *in vitro* models to simulate *combination* therapy against multidrug-resistant HIV-1 promoting the selection of the most sustainable regimen in salvage patients, to

(2) a cost-effective approach to monitoring drug resistance in treatment cohorts in low-resource settings, to

(3) a translational approach to managing influenza therapy and predicting the development of drug resistant influenza in children.

The work presented herein aims to provide a comprehensive summary of three independent studies conducted in the USA, Peru and Germany aiming to improve our understanding of the mechanisms of drug resistance development in HIV-1 and influenza *in vitro* and *in vivo*. We also discuss the limitations of the studies as well as the lessons learned in optimizing antiviral treatment strategies against HIV and influenza virus in adults and children.

(1) The newly developed *vitro* method described in Study 1 was used to investigate the effects of maintaining versus withdrawing one specific antiretroviral drug (lamivudine) when switching treatment regimens. This combination assay provides a unique opportunity to study and compare the effects of different treatment strategies in the same clinical patient isolates - whereas *in vivo* we only have one chance to pick the most suitable next drug regimen when switching from one treatment regimen to another.

The *in vitro* model showed that maintaining lamivudine while switching regimens in the situation of high-level lamivudine resistance prevented the disappearance of a "weakening" lamivudine-resistance mutation while delaying the selection of new resistant variants further down the road.

(2) With the second study we investigated the emergence of drug resistance *in vivo* in a first cohort of children with HIV/AIDS in Lima, Peru, gaining access to antiretroviral therapy supported by the Global Fund. We could demonstrate that an alternative method for resistance testing, the oligonucleotide ligation assay (OLA) (requiring less staff and costly equipment than the standard method, consensus sequencing), provides highly sensitive and useful information allowing the simultaneous periodic monitoring of HIV drug resistance across treatment cohorts in low-resource settings.

(3) The third study provides insight into viral load kinetics and *in vivo* drug resistance development in infants and children receiving antiviral therapy (oseltamivir) for *influenza* infection. With this study we make a case for influenza subtyping and closer monitoring of antiviral resistance development in treated patients applying the lessons learnt in the management of HIV-1 infection. Our data resulting from close virologic follow-up using nasopharyngeal samples suggest that the assessment of baseline and follow-up influenza viral load measurements may provide useful information to estimate the optimal duration of oseltamivir treatment in the individual patient. A less-than-expected decrease in viral load during therapy would then point toward resistance development, compliance issues or malabsorption. Clinical parameters in comparison seem to be poor indicators of response to antiviral therapy.

The results of all three studies demonstrate the feasibility and the importance and of virologic monitoring during antiviral therapy while underlining the need for timely detection of drug resistance development in both HIV-1 and influenza. The authors are making a case for a system of close collaboration "from the bench to the bedside" helping us to make progress in managing viral infections in adults as well as children. A better understanding of the underlying molecular mechanisms of drug resistance development and drug-virus interactions may help the virologist and the infectious disease specialist with the right choice and timing of antiviral drugs and/or drug combinations in the individual patient. Long-term sustainability of antiviral treatment regimens is key to their success, especially with in infants and children, who may require long-term follow-up with respect to the risks and benefits of antiviral therapy. Antiviral therapy in infants and children, who also carry a high risk of morbidity and mortality with HIV-1 and influenza, remains a challenge due to the limited availability of clinical data, limited drug choices, and particularly strict requirements for the safety and long-term tolerability. A timely and accurate diagnosis followed by successful personalized treatment under close virologic monitoring provide a promising avenue for highly specific and effective treatment of viral disease in both children and adults.

LES MOTS CLES EN FRANCAIS

VIH-1, grippe, traitement antiviral, thérapie antirétrovirale, résistance aux médicaments, surveillance virologique, lamivudine, névirapine, zidovudine, oseltamivir

KEYWORDS IN ENGLISH

HIV-1, influenza, antiviral therapy, antiretroviral therapy, drug resistance, virologic surveillance, lamivudine, nevirapine, nelfinavir, oseltamivir

TABLE OF CONTENTS

1. INTRODUCTION

INTRODUCING THE CONCEPT OF ANTIVIRAL THERAPY

The advent of the AIDS epidemic in the early 1980s has marked the beginning of a significant international research effort aimed at understanding and treating an entirely new disease. The discovery and development of multiple active compounds to treat infections with the human immunodeficiency virus (HIV) has set an example of what can be accomplished with successful international collaboration and new treatment strategies aimed at viral targets.

The Vancouver Conference in 1996 marked the beginning of a new dynamic: monotherapy became obsolete and was replaced by antiviral drug combinations with the aim to prevent (or delay) the emergence of HIV drug resistance. This new approach made it possible to sustain the success of anti-retroviral therapy (ART) over prolonged periods of time. With the development of additional drugs, drug combinations and treatment strategies, this ongoing process eventually helped to turn HIV infection from a deadly disease to a manageable condition.[1,2] Fixed-dose regimens combining several antiviral drugs in one pill may help to contain resistance development over time.[3] An overview of approved antiretroviral drugs and current recommendations for initiating ART can be found in Tables 1A and 1B:

Table 1 A: Overview of currently licensed antiretroviral drugs and monthly average wholesale price (in US$; Status: March 27, 2012. Source: AIDS Info 2012[4])

Antiretroviral Drug Generic (Brand) Name	Strength	Dosing	Tabs/Capsules/ mLs per Month	AWP[a] (Monthly)
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)				
abacavir (Ziagen)	300-mg tab 20-mg/mL soln	2 tabs daily 30 mLs daily	60 tabs 900 mL	$641.50 $674.60
didanosine DR (generic product) (Videx EC)	400-mg cap 400-mg cap	1 cap daily 1 cap daily	30 caps (≥ 60 kg) 30 caps (≥ 60 kg)	$368.72 $460.14
emtricitabine (Emtriva)	200-mg cap	1 cap daily	30 tabs	$504.37
lamivudine (generic) (Epivir) (Epivir)	300-mg tab 300-mg tab 10-mg/mL soln	1 tab daily 1 tab daily 30 mL daily	30 tabs 30 tabs 900 mL	$429.66 $477.41 $509.28
stavudine (generic) (Zerit)	40-mg cap 40-mg cap	1 cap twice daily 1 cap twice daily	60 caps 60 caps	$411.16 $493.38
tenofovir (Viread)	300-mg tab	1 tab daily	30 tabs	$873.28
zidovudine (generic) (Retrovir)	300-mg tab 300-mg tab	1 tab twice daily 1 tab twice daily	60 tabs 60 tabs	$360.97 $557.83
Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)				
delavirdine (Rescriptor)	200-mg tab	2 tabs three times daily	180 tabs	$365.45
efavirenz (Sustiva)	200-mg cap 600-mg tab	3 caps daily 1 tab daily	90 caps 30 tabs	$689.52 $689.52
etravirine (Intelence)	100-mg tab 200-mg tab	2 tabs twice daily 1 tab twice daily	120 tabs 60 tabs	$978.64 $978.64
nevirapine (Viramune) nevirapine XR (Viramune XR)	200-mg tab 400-mg tab	1 tab twice daily 1 tab daily	60 tabs 30 tabs	$723.08 $632.68
rilpivirine (Edurant)	25-mg tab	1 tab daily	30 tabs	$804.38
Protease Inhibitors (PIs)				
atazanavir (Reyataz)	150-mg cap[b] 200-mg cap 300-mg cap[b]	2 caps daily 2 caps daily 1 cap daily	60 caps 60 caps 30 caps	$1,176.23 $1,176.23 $1,165.12
darunavir (Prezista)	400-mg tab[b] 600-mg tab[b]	2 tabs daily 1 tab twice daily	60 tabs 60 tabs	$1,230.20 $1,230.20
fosamprenavir (Lexiva)	700-mg tab	2 tabs twice daily 1 tab twice daily[b] 2 tabs once daily[b]	120 tabs 60 tabs 60 tabs	$1,812.68 $906.34 $906.34
indinavir (Crixivan)	400-mg cap	2 caps three times daily 2 caps twice daily[b]	180 caps 120 caps	$548.12 $365.41
nelfinavir (Viracept)	625-mg tab	2 tabs twice daily	120 tabs	$879.84
ritonavir (Norvir)	100-mg tab	1 tab once daily 1 tab twice daily 2 tabs twice daily	30 tabs 60 tabs 120 tabs	$308.60 $617.20 $1,234.40

Antiretroviral Drug Generic (Brand) Name	Strength	Dosing	Tabs/Capsules/ mLs per Month	AWP[a] (Monthly)
saquinavir (Invirase)	500-mg tab[b]	2 tabs twice daily	120 tabs	$1,088.84
tipranavir (Aptivus)	250-mg cap[b]	2 caps twice daily	120 caps	$1,335.14
Integrase Strand Transfer Inhibitor (INSTI)				
raltegravir (Isentress)	400-mg tab	1 tab twice daily	60 tabs	$1,171.30
Fusion Inhibitor				
enfuviritide (Fuzeon)	90-mg inj kit	1 inj twice daily	60 doses (1 kit)	$3,248.72
CCR5 Antagonist				
maraviroc (Selzentry)	150-mg tab 300-mg tab	1 tab twice daily 1 tab twice daily	60 tabs 60 tabs	$1,148.16 $1,148.16
Coformulated Combination Antiretroviral Drugs				
abacavir/lamivudine (Epzicom)	600/300-mg tab	1 tab daily	30 tabs	$1,118.90
tenofovir/emtricitabine (Truvada)	300/150-mg tab	1 tab daily	30 tabs	$1,391.45
zidovudine/lamivudine (generic) (Combivir)	300/150-mg tab 300/150-mg tab	1 tab twice daily 1 tab twice daily	60 tabs 60 tabs	$931.61 $1,035.12
abacavir/lamivudine/zidovudine (Trizivir)	600/150/300-mg tab	1 tab twice daily	60 tabs	$1,676.62
lopinavir/ritonavir (Kaletra)	200 mg/50-mg tab 400 mg/100 mg per 5-mL soln	2 tabs twice daily or 4 tabs once daily 5 mL twice daily	120 tabs 300 mL	$871.36 $871.34
rilpivirine/tenofovir/emtricitabine (Complera)	200/25/300 mg	1 tab daily	30 tabs	$2,195.83
efavirenz/tenofovir/emtricitabine (Atripla)	300/200/600 mg	1 tab daily	30 tabs	$2,080.97

[a] AWP = Average Wholesale Price in 2012 (source: First DataBank Blue Book AWP, accessed January 2012) Note that this price may not represent the pharmacy acquisition price or the price paid by consumers.

[b] Should be used in combination with ritonavir. Please refer to Appendix B, Table 3 for ritonavir doses.

Key to Abbreviations: AWP = average wholesale price; cap = capsule, DR = delayed release, EC = enteric coated, inj = injection, soln = solution, tab = tablet, XR = extended release

Table 1 B: 2012 recommendations of the International Antiviral Society-USA Panel for initial ART in adults (Source: Thompson, 2012[5])

Table 1. Recommended and Alternative Initial Antiretroviral Regimens, Including Strength of Recommendations and Quality of Evidence[a]

	Recommended Regimens	Alternative Regimens[b]	Comments
NNRTI plus NRTIs	Efavirenz/tenofovir/emtricitabine (AIa) Efavirenz plus abacavir/lamivudine[c,d] (AIa) in HLA-B*5701–negative patients with baseline plasma HIV-1 RNA <100 000 copies/mL	Nevirapine plus tenofovir/emtricitabine or abacavir/lamivudine (BIa) Rilpivirine/tenofovir/emtricitabine (or rilpivirine plus abacavir/lamivudine) (BIa)	Severe hepatotoxicity and rash with nevirapine are more common in initial therapy when CD4 cell count is >250/μL in women and >400/μL in men.
PI/r plus NRTIs[c]	Darunavir/r plus tenofovir/emtricitabine (AIa) Atazanavir/r plus tenofovir/emtricitabine (AIa) Atazanavir/r plus abacavir/lamivudine (AIa) in patients with plasma HIV-1 RNA <100 000 copies/mL	Darunavir/r plus abacavir/lamivudine (BIII) Lopinavir/r[d] plus tenofovir/emtricitabine (BIa) (or abacavir/lamivudine) (BIa)	Other alternative PIs include fosamprenavir/r and saquinavir/r but indications to use these options for initial treatment are rare.
InSTI plus NRTIs[c]	Raltegravir plus tenofovir/emtricitabine (AIa)	Raltegravir plus abacavir/lamivudine (BIIa) Elvitegravir/cobicistat/ tenofovir/emtricitabine[e](BIb)	Raltegravir is given twice daily; experience with elvitegravir/cobicistat/tenofovir/ emtricitabine[e] is limited to 48-week data.

Abbreviations: InSTI, integrase strand transfer inhibitor; NRTI, nucleos(t)ide reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor; /r, ritonavir-boosted.

[a] Ratings of the strength of the recommendations and quality of evidence are described in the eBox. Fixed-dose combinations are recommended when available and appropriate. Current fixed-dose combinations available are efavirenz/tenofovir/emtricitabine; tenofovir/emtricitabine; abacavir/lamivudine; rilpivirine/tenofovir/emtricitabine; lopinavir/ritonavir; zidovudine/lamivudine; and, if approved, elvitegravir/cobicistat/tenofovir/emtricitabine.

[b] Zidovudine/lamivudine is an alternative NRTI component of NNRTI-, PI/r-, and raltegravir-based regimens, but the toxicity profile of zidovudine reduces its utility.

[c] HLA-B*5701 screening is recommended before abacavir administration to reduce the risk of hypersensitivity reaction.

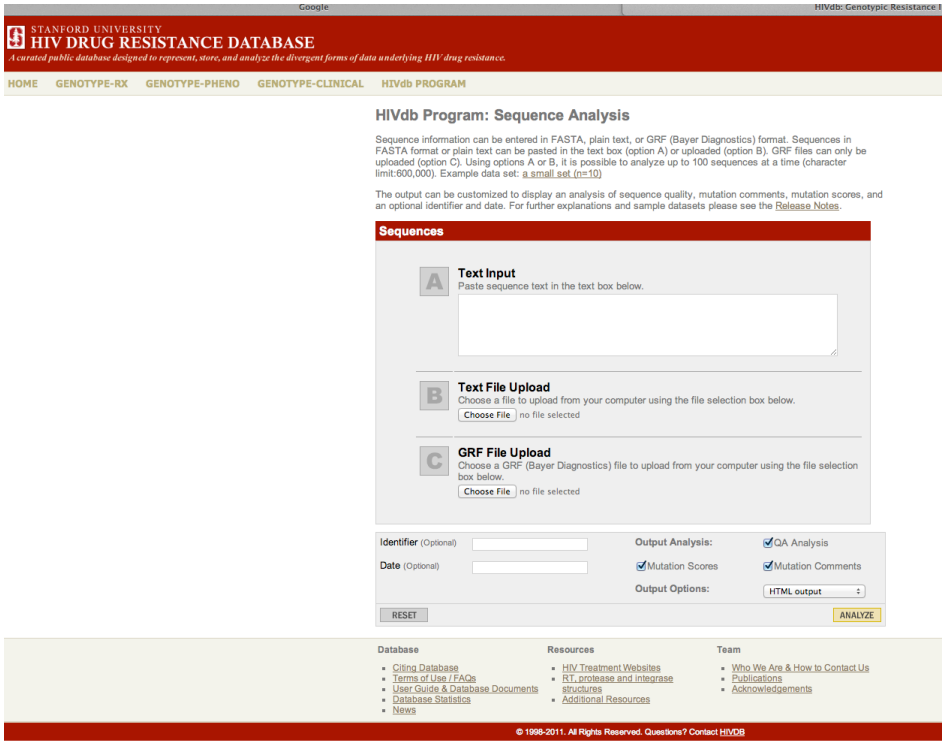
[d] Avoiding the use of abacavir or lopinavir/ritonavir might be considered for patients with or at high risk of cardiovascular disease.

[e] New Drug Application for this combined formulation has been filed with regulatory authorities. Approval decisions pending.

Even if a cure is still not possible, the lives of millions of people living with HIV/AIDS have been saved and improved significantly as a result of international collaboration in HIV basic and clinical research.[5-9] Against the backdrop of success and potential promise however[10], large numbers of eligible subjects are still without access to structured treatment programs and specialized medical care[11,12], especially those residing in remote areas and low-income countries.[13] Delayed diagnosis and treatment, as well as difficulties with regular follow-up visits and patient adherence to complex regimens have contributed to treatment failures and antiretroviral drug resistance increasing worldwide.[14,15 13,16]

With the development of each new antiretroviral drug, new studies emerged investigating the specific drug resistance profile generated by the respective new compound. The respective "signature drug resistance pattern" conferring resistance to a particular antiretroviral drug is usually identified by serial passage experiments, where wild-type HIV-1 isolates are grown in cell culture and in the presence of escalating doses of the antiviral substance in question.[17] The sequencing data derived from HIV outgrowing the drug pressure experiments are then compared to wild type virus as well as phenotypic *in vitro* analyses ("sensitivity assays").[18] The proof of concept is provided by drug exposure experiments with recombinant HIV harboring the same mutation(s), as well as patient data from clinical trials using the same drug. Extensive databases, such as the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu) have helped clinicians and researchers understand and interpret the significance of genotypic and phenotypic drug resistance information.[19]

Table 1 C. The Stanford HIV Drug Resistance Database (Sournce: http://hivdb.stanford.edu [20])



A number of studies have shown that making use of drug resistance information *prior to* initiating or switching ART may improve patient outcomes.[20] However, single drug experiments in laboratory isolates can only be approximations of what happens in real-life, especially since antiretroviral drugs are no longer used in isolation. An additional level of complexity has thus been introduced with the combination of 3-4 active antiretroviral compounds; in fact, the majority of patients harbor viruses that may be quite different from standard reference strains of HIV-1 subtype B.

New *in vitro* models are therefore needed to simulate and explore the effect of different antiretroviral combination therapies in actual patient isolates. The first key objective of this work was the development of a novel *in vitro* system for the analysis and head-to-head comparison of different drug combinations in multi-resistant clinical HIV isolates from salvage patients, i.e. patients who have already acquired resistance to most of the available drugs or routine antiretroviral regimens.

Objective 1: To develop an *in vitro* model simulating the development of drug resistance in HIV-1 clinical isolates during antiretroviral combination therapy.

MONITORING SUCCESS (AND FAILURE) OF ANTIVIRAL THERAPY

Ever since the "3-by-5 Initiative"[21] aiming to expand access to antiretroviral therapy to 3 million patients by the year of 2005, the importance of concerted efforts monitoring drug resistance and linking resistance data internationally, has gained recognition.[22-24] A number of studies have evaluated the cost-effectiveness of programs for the monitoring of virologic outcome parameters in resource-limited settings. [25-28]

In the early stages of the expanded access programs, ART was often introduced before the appropriate infrastructure for the systematic surveillance of drug resistance had been established. The long-term success of ART however, does not only depend on the availability of drugs, but also on the ability to monitor real-life effectiveness and safety. Treatment regimens need to be sustainable and accepted well among patients. In the long run, their ability to prevent or delay the emergence of drug resistance will be a key point.[29-31] Over time, sequencing technologies have become less expensive, yet highly sensitive alternative methods have been developed, such as the oligonucleotide ligation assay (OLA).[32,33] In addition, alternative modes of transport for blood samples have been suggested to provide simple modes of transportation across large distances without the need to maintain a cold-chain.[34,35]

A second key objective of this work was to translate drug resistance technologies to a resource-constrained setting and to evaluate the usefulness of the OLA from whole blood samples in comparison to consensus sequencing from dried blood spots in a real-life scenario. The research was conducted in the first pediatric treatment cohort in Lima, Peru, where school-aged children had finally gained access to antiviral therapy in 2002-5 through national programs supported by the Global Fund and the Peruvian Ministry of Health.[36]

Objective 2: To evaluate alternative methods for the monitoring of antiretroviral drug resistance in a developing country treatment cohort.

Meanwhile, a number of additional viral targets have become "treatable", including hepatitis viruses [37-42], *herpesviridae,*[43-45] and influenza[46,47], to name a few (Tables 1 D and E).[38,48,49] As a result of this success, the field of antiviral therapy has become increasingly complex. The pace of constantly evolving treatment strategies may be hard to keep up with for general practitioners, who may not yet be familiar with the concept of "treating viruses".[43,48,50,51] Meanwhile, antiviral drug resistance has been observed in a number of viral diseases, including seasonal and pandemic influenza A H1N1[52-54], hepatitis B[55,56] and C[57], herpes simplex-1 (HSV-1),[58,59] and cytomegaloviruses (CMV) disease.[60,55,56,58-65] The risk of developing drug resistance is particularly high in patients with irregular access to therapy and in immunocompromised individuals as well as high-risk groups including infants and children.[66-70]

Table 1 D: Overview of antiviral agents for herpesvirus infections (Source: Razonable et al., 2011[50])

TABLE 1. **Suggested Antiviral Drugs for the Treatment of Herpesvirus infections**[a,b,c]

Virus	Clinical disease	Drug name (route)	Recommended dosage	Comments
Herpes simplex viruses 1 and 2	Mucocutaneous disease	Acyclovir (IV)	5 mg/kg IV every 8 h	IV therapy is preferred for severe and disseminated disease Risk of crystalline nephropathy
		Acyclovir (oral)	400 mg orally 3 times daily 800 mg orally twice daily 200 mg orally 5 times daily	Localized disease and genital herpes
		Valacyclovir (oral)	1 g orally twice daily 500 mg orally twice daily	First episode of genital herpes Recurrent episodes of genital herpes
	HSV encephalitis	Acyclovir (IV)	10 mg/kg IV every 8 h	Risk of crystalline nephropathy
	Long-term suppression	Acyclovir (oral)	400 mg orally twice daily (400-800 mg 2 to 3 times daily for HIV-infected patients)	
		Valacyclovir (oral)	500 mg orally once daily 1 g orally once daily	Recurrence of <9 episodes per year Recurrence of >9 episodes per year
Varicella zoster virus	Varicella zoster	Acyclovir (IV)	10-12 mg/kg IV every 12 h	Recommended for immunocompromised hosts
		Acyclovir (oral)	600-800 mg orally 5 times daily 1 g orally every 6 h	Less preferred than valacyclovir because of poor bioavailability
		Valacyclovir (oral)	1 g orally 3 times daily	Preferred oral therapy for mild or localized disease
CMV	CMV disease in transplant recipients	Ganciclovir (IV)	5 mg/kg IV every 12 h	IV therapy is preferred for severe CMV disease, gastrointestinal disease, pneumonia, and encephalitis Transition to oral valganciclovir on clinical and virologic improvement Duration of therapy is guided by CMV surveillance using PCR or pp65 antigenemia Risk of myelosuppression
		Valganciclovir (oral)	900 mg orally twice daily	Indicated for CMV syndrome and mild to moderate CMV disease Duration of therapy is guided by CMV surveillance using PCR or pp65 antigenemia Risk of myelosuppression
		Foscarnet (IV)	Induction: 90 mg/kg every 12 h OR 60 mg/kg every 8 h Maintenance: 90-120 mg/kg every 24 h	Second-line therapy Indicated for ganciclovir-resistant CMV disease High risk of nephrotoxicity and electrolyte abnormalities Duration of therapy is guided by CMV surveillance using PCR or pp65 antigenemia
		Cidofovir (IV)	Induction: 5 mg/kg per dose once weekly for 2 doses Maintenance: 5 mg/kg every 2 wk	Alternative treatment of CMV disease; second-line agent Indicated for ganciclovir-resistant CMV disease High risk of nephrotoxicity; requires concomitant hydration and probenecid use Duration of therapy is guided by CMV surveillance using PCR or pp65 antigenemia
	Antiviral prophylaxis for CMV prevention in transplant recipients	Valganciclovir (oral)	900 mg orally once daily	Preferred drug for CMV prophylaxis Duration is generally for 3-6 mo; may be longer for lung transplant recipients Myelosuppression is major adverse effect
		Ganciclovir (IV)	5 mg/kg IV once daily	Preferred for intestinal transplant recipients or in clinical situations in which absorption is a concern
		Ganciclovir (oral)	1 g orally 3 times daily	Effective for CMV prevention but no longer a preferred drug because of its poor bioavailability; valganciclovir is preferred
		Valacyclovir (oral)	2 g orally 4 times daily	Shown to be effective mainly in kidney transplant recipients; not effective for other organ transplant recipients High doses increase risk of hallucinations and neurologic toxicities

(continued on next page)

TABLE 1. **Continued**[a,b,c]

Virus	Clinical disease	Drug name (route)	Recommended dosage	Comments
CMV (continued)	Preemptive therapy for asymptomatic CMV infection in transplant recipients	Valganciclovir (oral)	900 mg orally twice daily	CMV replication is detected by weekly CMV surveillance using PCR or pp65 antigenemia Preferred drug for the treatment of asymptomatic CMV infection in solid organ and hematopoietic stem cell transplant recipients
		Ganciclovir (IV)	5 mg/kg IV every 12 h	Less preferred than valganciclovir because of the logistics of IV administration Oral ganciclovir should not be used for treating active CMV infection
	CMV retinitis in HIV-infected patients	Valganciclovir (oral)	Induction: 900 mg orally twice daily for 14-21 d Maintenance: 900 mg orally once daily until immune reconstitution	For sight-threatening retinitis, use in combination with ganciclovir intraocular implant (see below)
		Ganciclovir (IV)	Induction: 5 mg/kg IV every 12 h for 14-21 d Maintenance: 5 mg/kg IV every 24 h until immune reconstitution	For sight-threatening retinitis, use in combination with ganciclovir intraocular implant (see below)
		Ganciclovir (intraocular implant)	One sustained-release intravitreal implant (4.5 mg/implant) every 6-8 mo	Replace every 6-8 mo until immune reconstitution Use in combination with systemic ganciclovir (or valganciclovir) because of the systemic nature of CMV disease
		Foscarnet (IV)	Induction: 90 mg/kg every 12 h OR 60 mg/kg every 8 h Maintenance: 90-120 mg/kg every 24 h	Second-line therapy Indicated for ganciclovir-resistant CMV disease High risk of nephrotoxicity and electrolyte abnormalities
		Cidofovir (IV)	Induction: 5 mg/kg per dose once weekly for 2 doses Maintenance: 5 mg/kg every 2 wk	Alternative treatment of CMV disease Indicated for ganciclovir-resistant CMV disease High risk of nephrotoxicity; requires concomitant hydration and probenecid use
	CMV disease other than retinitis in HIV-infected patients	Valganciclovir (oral)	Induction: 900 mg orally twice daily for 14-21 d Maintenance: 900 mg orally once daily until immune reconstitution	
		Ganciclovir (IV)	Induction: 5 mg/kg IV every 12 h for 14-21 d Maintenance: 5 mg/kg IV every 24 h until immune reconstitution	Preferred for severe disease (eg, pneumonitis, encephalitis) and for those with poor intestinal absorption
		Foscarnet (IV)	Induction: 90 mg/kg every 12 h OR 60 mg/kg every 8 h Maintenance: 90-120 mg/kg every 24 h	Second-line therapy Indicated for ganciclovir-resistant CMV disease High risk of nephrotoxicity and electrolyte abnormalities
		Cidofovir (IV)	Induction: 5 mg/kg per dose once weekly for 2 doses Maintenance: 5 mg/kg every 2 wk	Alternative treatment for CMV disease Indicated for ganciclovir-resistant CMV disease High risk of nephrotoxicity; requires concomitant hydration and probenecid use

[a] CMV = cytomegalovirus; HIV = human immunodeficiency virus; IV = intravenous; PCR = polymerase chain reaction.
[b] Doses are given for persons with normal renal function. Please consult individual drug's package insert for dose adjustments in persons with impaired renal function.
[c] No antiviral drugs have been approved for the treatment of Epstein-Barr virus or human herpesviruses 6, 7, and 8.

Table 1 E: Overview of antiviral agents for the treatment of Hepatitis B virus infections (Source: Razonable et al., 2011[50])

TABLE 2. **Antiviral Nucleos(t)ides for the Treatment of Chronic Hepatitis B**[a]

Drug name	Suggested dosage	Drug characteristics[b]	Toxicity[c]	Resistance
Adefovir	10 mg orally once daily	Acyclic nucleotide analogue of adenosine monophosphate	Nephrotoxicity Lactic acidosis Rebound hepatitis	rtN236T is most common
Emtricitabine	200 mg orally once daily	Nucleoside analogue of cytidine Very similar to lamivudine Used often in combination with tenofovir	Lactic acidosis Rebound hepatitis	rtM204V/I provides cross-resistance with lamivudine
Entecavir	0.5 mg orally once daily for treatment-naive patients 1 mg orally once daily for treatment-experienced patients and patients with decompensated liver disease	Nucleoside analogue of guanosine One of the most potent anti-HBV drugs	Well tolerated Lactic acidosis Rebound hepatitis	High barrier to resistance; requires 3 mutations for phenotype: rtM204V/I plus rtL180M plus rtT184S/A/I/L or rtS202G/C or rtM250L
Lamivudine	100 mg orally once daily	Nucleoside analogue of cytosine	Lactic acidosis Myopathy Rebound hepatitis	rtM204V/I is most frequent
Telbivudine	600 mg orally once daily	Synthetic thymidine nucleoside analogue No activity against HIV	Myopathy Peripheral neuropathy Lactic acidosis Rebound hepatitis	rtM204I is most frequent mutation; others include rtL80I/V, rtA181T, rtL180M, and rtL229W/V
Tenofovir	300 mg orally once daily	Acyclic nucleoside phosphonate diester analogue of adenosine monophosphate One of the most potent anti-HBV drugs	Nephrotoxicity Lactic acidosis Rebound hepatitis	Not well-defined; rtN236T is suggested but not yet confirmed

[a] HBV = hepatitis B virus; HIV = human immunodeficiency virus.
[b] All drugs inhibit hepatitis B replication by acting as a competitive substrate for the HBV DNA polymerase. All drugs except telbivudine have anti-HBV properties, and all patients with chronic hepatitis B who are considered for treatment should be screened for HIV.
[c] A common toxicity of the nucleos(t)ide analogues is lactic acidosis, with the potential to cause increases in serum alanine aminotransferase levels and hepatomegaly.

The recent influenza pandemic has led to more widespread use of antivirals in respiratory viral infections.[71] The classical antiviral drugs, amantadine and rimantadine, are targeting the entry of the virus into the host cell ("uncoating")[72], whereas the newer neuraminidase inhibitors oseltamivir and the inhaled drug zanamivir both inhibit the release of newly generated virus from the host cell.[49] Due to widespread resistance, amantadine and rimantadine are no longer recommended for the treatment of the influenza subtypes currently in circulation.[73]

While antibiotics have been overprescribed for decades,[74-76] the most vigorous discussions are targeting the use of the antiviral drug oseltamivir (Tamiflu™)[71,77-79] highlighting the fact that "antiviral therapy" may mean different things to different people.[80-88] A number of cost-effectiveness studies have been conducted addressing the issue.[89-94] Prescribing practices vary from year to year and from country to country reflecting a great deal of uncertainty.[79,95-97] Again, differences in levels of access to antiviral therapy are evident between developed and developing countries.[98,99] As in HIV/AIDS, the success of antiviral therapy of influenza is highly dependent on accurate diagnosis and timely treatment initiation, which is different from the usual practice in (suspected) bacterial respiratory infections, where antibiotic prescriptions may be delayed until the disease has progressed or worsened.[73]

An even greater challenge than finding consensus among health care providers on the most appropriate use of antivirals in respiratory infections may be the communication on antiviral therapy to the general public. A recent survey in the Netherlands revealed that 50% of the surveyed population held the belief that viral respiratory infections ought to be treated with antibiotics.[100] At the same time, antibiotic drug resistance is often perceived as a result of "the body becoming used to the drug".[101]

Utmost transparency and high quality information on the risks and benefits of "treating influenza" is a must, if the drug is to be used to the greatest possible benefit.[83,102] This includes the persistent implementation of evidence-based treatment guidelines, the monitoring of prescribing patterns in low- and high-risk groups, and the surveillance of drug efficacy, safety, and resistance in treatment cohorts.[46,97,103,104] Surveillance programs of neuraminidase inhibitor resistance revealed that resistance mutations in *pandemic* Influenza A H1N1 (2009) virus emerge predominantly at the neuraminidase position 275.[62,65,97,105-108] This mutation is already known as a neuraminidase inhibitor resistance mutation in *seasonal* influenza A H1N1 viruses.[109]

Resistant *pandemic* influenza A H1N1 (2009) viruses carrying the H275Y substitution do not seem to spread very efficiently however, and the majority of baseline isolates circulating in the general population appear to remain drug-sensitive.[53,110,111] This was also the case during the initial stages of drug resistance development in *seasonal* influenza.[112] Shortly before it was replaced by pandemic H1N1 however, the *seasonal* H1N1 virus had changed further by adopting compensatory mutations allowing the rapid spread of resistant H1N1 virus.[113-115] Of note, some investigators believe that neuraminidase resistance may be more common in children than in adults. This impression may however be slightly biased by the fact that oseltamivir was used in adults before it was subject to trials and licensed in children.

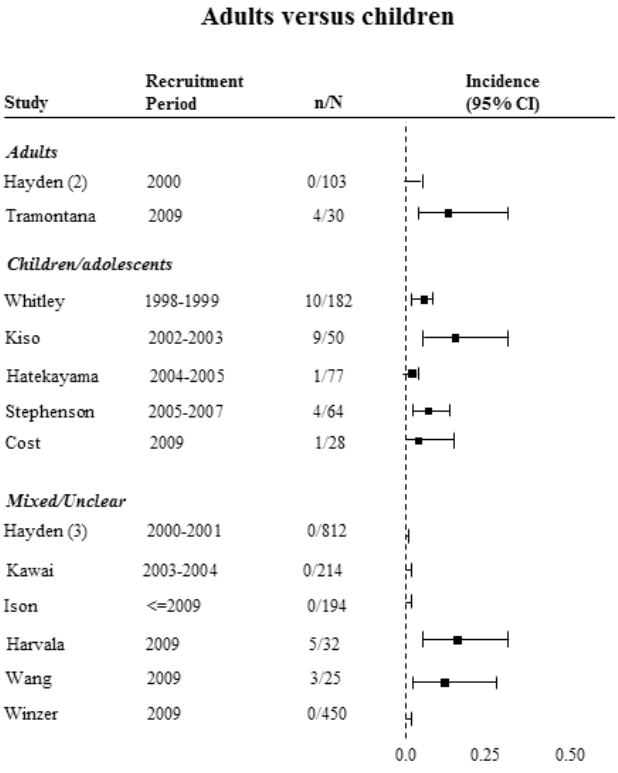


Figure 1 A: Forest plot of incidence of drug resistance in adult, pediatric and mixed trials (Source: Thorlund 2011[116])

In any case, neuraminidase resistance seems more common with oseltamivir treatment than with zanamivir treatment and more common in Influenza A H1N1 than in influenza H3N2 and influenza B (Figure 1 B)

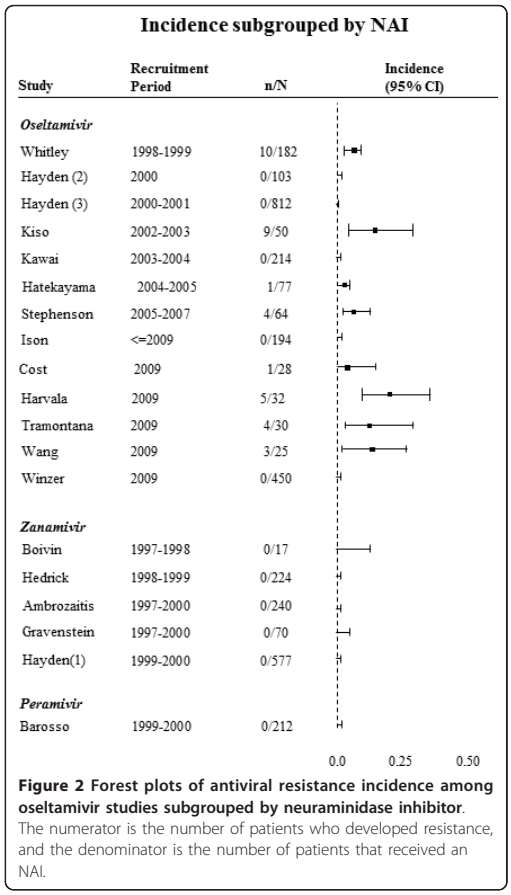


Figure 2 Forest plots of antiviral resistance incidence among oseltamivir studies subgrouped by neuraminidase inhibitor. The numerator is the number of patients who developed resistance, and the denominator is the number of patients that received an NAI.

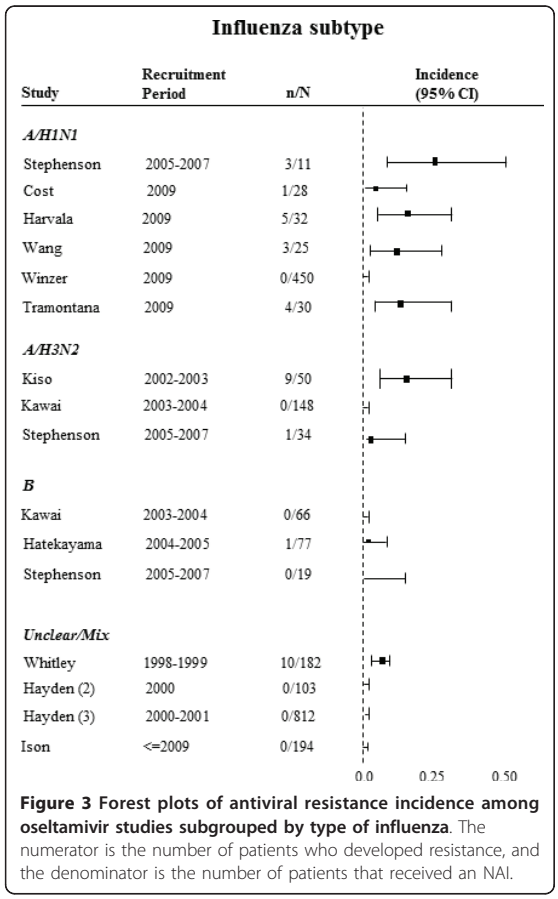


Figure 3 Forest plots of antiviral resistance incidence among oseltamivir studies subgrouped by type of influenza. The numerator is the number of patients who developed resistance, and the denominator is the number of patients that received an NAI.

Figures 1 B and C: Neuraminidase resistance against oseltamivir versus zanamivir (on the left) and in Influenza A H1N1 versus Influenza A H3N2 versus Influenza B (on the right; Source: Thorlund 2011[116])

The close monitoring of virus kinetics and drug resistance development is therefore an important aspect of treating pediatric influenza infection. The lessons learned from HIV and influenza passage experiments include the observation that viral drug resistance mutations tend to develop - and may even be experimentally induced - when antiviral drugs are consistently “under-dosed”, i.e. when concentrations are kept slightly below the range where 50% of viral growth would be inhibited (“IC50”). At and around the IC50, there is just enough evolutionary pressure to select for mutant virus while there is not enough drug to kill all of it.

Structured clinical studies of viral kinetics under therapy are cumbersome and rare, as they would require frequent visits and follow-up testing using costly viral load assays throughout the treatment period. Even fewer studies have addressed the issue of viral clearance and antiviral resistance development in high-risk patients. Immunocompromised individuals and infants for example, may harbor large viral loads at the beginning of the illness and thus take longer to clear the virus.[117-121] Despite the elevated risk of severe disease in this age group, antiviral therapy has only been licensed for children > 1 year of age, with the exception of an emergency use authorization (EUA) for infants during the 2009/10 influenza pandemic.[73,117,122-125]

The third key objective was therefore the analysis of virus kinetics and drug resistance development in infants and children infected with influenza A and B viruses undergoing oseltamivir therapy.

Objective 3: To assess the value of influenza subtyping and virus load testing in monitoring antiviral therapy and to establish predictors of drug resistance development against neuraminidase inhibitors.

2. STUDYING HIV DRUG RESISTANCE *IN VITRO*

The development of antiretroviral drugs is a direct result of the improved understanding of the HIV life cycle and potential viral drug targets following the discovery and initial description of HIV-1 as a „T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS)“ by Luc Montagnier and Françoise Barré-Sinoussi in 1983[126].

Within 25 years after the discovery, 25 antiretroviral drugs were licensed to treat infections with the human immunodeficiency virus.[127] These 25 different antiretroviral drugs belong to distinct drug classes that can be combined as they are interfering with different steps in the virus life cycle.[128,129]

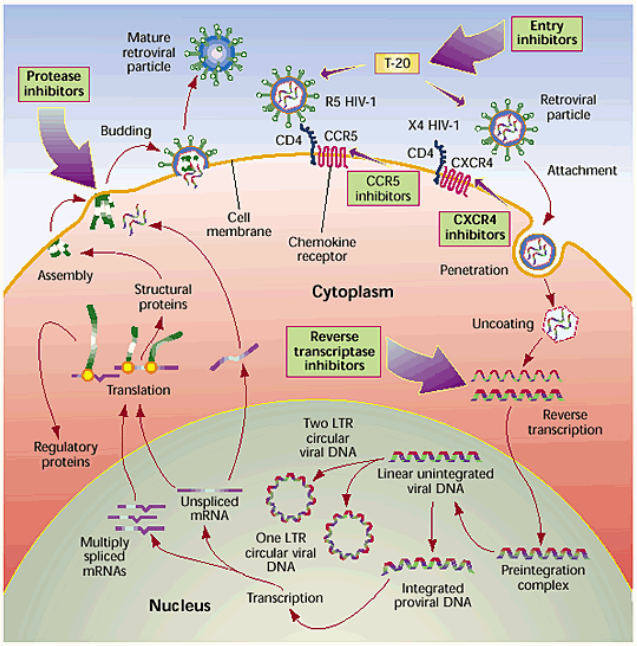


Figure 2 A: HIV-1 life cycle and antiretroviral drug targets (Source: Michael 1999[129])

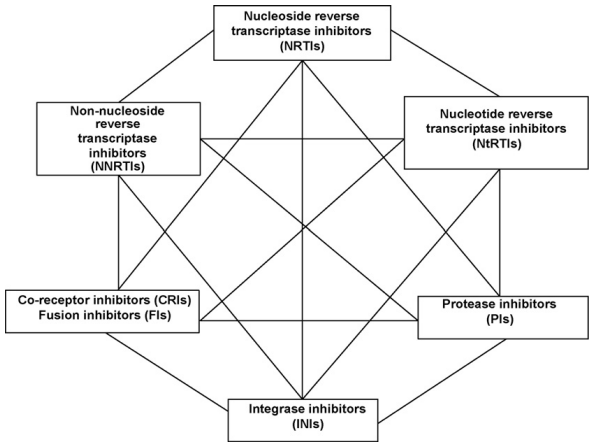


Figure 2 B: Antiretroviral drug classes and combinations (Source: DeClercq 2009)[127]

RNA viruses are known to replicate quickly and effectively while maintaining a maximum degree of flexibility and adaptability. This is also due to the high rate of spontaneous mutations with each replicative cycle. The replication pattern of RNA viruses has thus been described as "complex and dynamic mutant swarms", termed "viral quasispecies".[130] While mutation rates depend on the environment and the population size and composition, individual mutations may influence the phenotypic ability of the virus to adapt, to replicate and to adapt further.[130] Sometimes the need to adapt to a new environment occurs at the expense of the overall ability of the virus to replicate. Resistance mutations acquired under in the presence of an antiviral drug, for example, may yield a virus, which is less "fit", demonstrating a replicative disadvantage compared to wild type virus in the absence of drug pressure.[131]

Reverse transcriptase enzymes in HIV (i.e. "genetic copying mechanisms") lack proofreading functions, and mutant progeny are produced with every replicative cycle. Under evolutionary pressure, mutant variants with features providing a selective advantage may take little time to emerge. The HIV reverse transcriptase has thus been labeled a "low fidelity "enzyme.[132]

The influenza A virus RNA polymerase complex on the contrary, is deemed "a high fidelity enzyme", but "the multiple rounds of genome amplification per infective cycle are assumed to provide influenza A virus polymerase with ample opportunity to generate and amplify genomic founder mutations, and thus achieve optimal viral mutagenesis for its evolution".[133,134]

In either case, the error-prone reproductive mechanisms of HIV and influenza are blamed for the high rate of mutant viral progeny per infective cycle, including the potential for the production of drug-resistant quasi-species.[134,135] Many of the mutant quasi-species may not be viable, or harboring a replicative or "fitness" disadvantage. In the presence of antiviral drugs, the most resistant variants will eventually be selected for. It has been shown in HIV and other viral targets (such as Hepatitis C) that different antivirals may differ in their propensity to trigger the emergence of drug resistance mutations[17,57,136-139] This leaves hope for the design of "smart" treatment strategies, i.e. the development of specific drug combinations (and/or a particular sequence of drugs/regimens) to exert conflicting pressure on the virus thus delaying the emergence of drug resistant and highly transmissible "escape variants". [140-142]

Experience from several decades of antiretroviral therapy has shown that long-term success and sustainability depend on the stringent monitoring of clinical and virologic parameters and the availability of drug resistance testing when viral load rebound becomes evident. Periodic assessments of virus load and CD4 counts have become a mainstay in monitoring the success of antiretroviral therapy.[143] Increasing virus load (and as a result, declining CD4 cell counts) may indicate problems with adherence or with drug absorption, for example. Virologic failure, usually defined as repeat virus load measurements above levels of detection by RT-PCR, [144-146] is often a first indicator of drug resistance and the need to adjust or "switch" antiviral therapy (ART). A delay in switching therapy during emerging drug resistance has been shown to increase rates of failure during second-line therapy.[145] Furthermore, the periodic assessment of success versus failure of ART has been shown to be cost-effective [71].

Once antiretroviral drug resistance has developed, choices for subsequent second- or third line ART may become increasingly difficult. After many years of treatment with changing drug combinations, experienced patients may harbor virus that has accumulated sufficient resistance mutations to be resistant to all available standard regimens. In these cases, last-choice regimens (so-called "salvage therapy") may be attempted to contain the virus as best as possible.[147] Despite the rapid development of new and effective anti-retroviral medications over the past decades, treatment options in salvage patients are not unlimited. In patients with multi-drug resistant virus, it would be

desirable to make *ex vivo* predictions of virologic outcomes with any given regimen *before* the patient is exposed to a new drug or combination.[148-151]

CHOOSING STRATEGIC DRUG COMBINATIONS FOR HIV SALVAGE THERAPY

In the age of personalized medicine, it has become a major goal to develop predictors of drug safety and efficacy, leading to the discovery of new laboratory assays and biomarkers helping to tailor the most appropriate treatment regimen to the individual patient.[152,153]

The field of anti-infective therapy has been a positive example demonstrating the advantages of personalized medicine, for example when selecting the most appropriate antibiotic regimen for patients with specific bacterial infections, or the most promising antiretroviral combination for HIV-infected patients. Studies of the divergent evolution and transmission of drug resistance in HIV-infected couples have highlighted the importance of individualized therapy in HIV/AIDS.[154] A major challenge in the long-term success of ART is the emergence of drug resistance mutations over time. In children, who are facing a life-long need for successful ART, the need to switch treatment regimens should be delayed as much as possible, and there is little room for mistakes when choosing the next best regimen. The long-term success of a treatment regimen depends on the prevention of viral break-through. Every time a switch becomes necessary and new drugs or drug classes are introduced, the number of future options is reduced. In each patient, there is only one chance to pick the right regimen.[20,155]

Drug resistance testing is an efficient means of predicting the likelihood of virologic success versus failure in the individual patient. However, both genotypic and phenotypic tests are limited to predicting the success of an *individual* drug in a generic viral backbone.[20,156] The aim of the *in vitro* project presented in Study 1 is to simulate the effect of different combinations of antiretroviral drugs to determine the most sustainable drug combination in "real-life" patient isolates. The drug combinations used in the in vitro experiments were chosen to exert different levels of conflicting evolutionary pressure on the respective virus isolates.[142]

CHARACTERISTICS OF 3TC RESISTANT REVERSE TRANSCRIPTASE

A number of drug resistance mutations to all available antiretroviral drugs have been described to date. It is evident that antiretroviral drugs targeting a specific enzyme will induce drug resistance in the respective target region. Drug resistance mutations induced by protease inhibitors will be found in the protease gene, while resistance mutations induced by reverse transcriptase inhibitors (RTI) are to be found in the reverse transcriptase gene.[128] An updated overview of reverse transcriptase mutations can be found in Figure 2 C.

For the identification of drug resistance, usually one or more of the following methods and criteria have been applied:

(A) *in vitro* passage experiments (growing the virus in the presence of the drug)

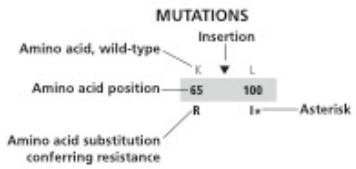
(B) validation of contribution to resistance by using site-directed mutagenesis (inducing the resistance mutation artificially and comparing to wild type virus)

(C) susceptibility testing of laboratory or clinical isolates (testing which concentration of drug a wild type versus resistant virus may be able to tolerate)

(D) nucleotide sequencing of viruses from patients in whom the drug is failing (determining the

sequence of a resistant virus, alignment with wild type virus to determine the difference)

(E) correlation studies between genotype at base-line and virologic response in patients exposed to the drug (following the emergence of drug resistance mutations *in vivo*). [128]



MUTATIONS IN THE REVERSE TRANSCRIPTASE GENE ASSOCIATED WITH RESISTANCE TO REVERSE TRANSCRIPTASE INHIBITORS

Nucleoside and Nucleotide Analogue Reverse Transcriptase Inhibitors (nRTIs)[a]

Multi-nRTI Resistance: 69 Insertion Complex[b] (affects all nRTIs currently approved by the US FDA)

	M	A	▼	K										L	T	K
	41	**62**		**69**	**70**									**210**	**215**	**219**
	L	**V**		**Insert**	**R**									**W**	**Y** F	**Q** E

Multi-nRTI Resistance: 151 Complex[c] (affects all nRTIs currently approved by the US FDA except tenofovir)

	A			V	F				F	Q						
	62			**75**	**77**				**116**	**151**						
	V			**I**	**L**				**Y**	**M**						

Multi-nRTI Resistance: Thymidine Analogue-Associated Mutations[d,e] (TAMs; affect all nRTIs currently approved by the US FDA)

	M			D	K									L	T	K
	41			**67**	**70**									**210**	**215**	**219**
	L			**N**	**R**									**W**	**Y** F	**Q** E

Abacavir[f,g]			K		L			Y		M						
			65		**74**			**115**		**184**						
			R		**V**			**F**		**V**						
Didanosine[g,h]			K		L											
			65		**74**											
			R		**V**											
Emtricitabine			K							M						
			65							**184**						
			R							**V** I						
Lamivudine			K							M						
			65							**184**						
			R							**V** I						
Stavudine[d,e,g,i,j,k]	M		K	D	K									L	T	K
	41		**65**	**67**	**70**									**210**	**215**	**219**
	L		**R**	**N**	**R**									**W**	**Y** F	**Q** E
Tenofovir[l]			K		K											
			65		**70**											
			R		**E**											
Zidovudine[d,e,j,k]	M			D	K									L	T	K
	41			**67**	**70**									**210**	**215**	**219**
	L			**N**	**R**									**W**	**Y** F	**Q** E

Nonnucleoside Analogue Reverse Transcriptase Inhibitors (NNRTIs)[a,m]

Efavirenz				L	K	K	V	V			Y	Y	G		P	
				100	**101**	**103**	**106**	**108**			**181**	**188**	**190**		**225**	
				I	**P**	**N** S	**M**	**I**			**C** I	**L**	**S** A		**H**	
Etravirine[n]	V	A	L	K		V		E		V	Y		G			M
	90	98	**100**	**101**		106		138		179	**181**		190			230
	I	G	**I***	E H **P***		I		A G K Q		D F T	**C* I* V***		S A			L
Nevirapine				L	K	K	V	V			Y	Y	G			
				100	**101**	**103**	**106**	**108**			**181**	**188**	**190**			
				I	**P**	**N** S	**A** M	**I**			**C** I	**C** L H	**A**			
Rilpivirine[o]					K				E		V	Y			H	F	M
					101				**138**		**179**	**181**			**221**	**227**	**230**
					E P				**A** G **K*** Q R		**L**	**C** I V			**Y**	**C**	**I** L

Figure 2 C: Overview of Reverse Transcriptase mutations (Source: Johnson 2011[128])

Individual drugs do not only differ in their ability to trigger specific drug resistance mutations in the target sequence, but resistant virus may also show altered enzymatic properties affecting the ability of the virus to adapt to new environmental stimuli.[31,128]

One of the most intensely studied mutant HIV enzymes is the lamivudine (3TC)-resistant reverse transcriptase (RT).[157] The most common drug resistance mutation selected under 3TC drug pressure conditions is the Methionine to Valine substitution at codon 184 ("mutation M184V") conferring high-level resistance to 3TC.[158,142,154] Cell-free enzymatic assays have revealed that 184V mutant RT displays a number of altered properties when compared to wild type RT, namely:

(A) Decreased ability to incorporate dNTP and to complete the elongation process.[159]

(B) Decreased RT processivity and "viral fitness".[160-163]

(C) Reduced number of viable progeny per infective cycle.[164,165]

(D) 3-49-fold increased fidelity of DNA-dependent and RNA-dependent polymerization.[163,166,167]

(E) Decreased production of quasispecies, and impairment of adaptation to environmental stimuli.[168,169,170]

It is obvious that most of the above mentioned features provide an evolutionary disadvantage ("fitness deficit") to M184V mutant compared to wild type virus - the only advantage being its ability to survive in the presence of the drug.[171] As soon as 3TC pressure is withdrawn (for example when the patient stops taking the medication), the selective pressure is reversed: wild-type HIV quasispecies *lacking* the (now superfluous) M184V mutation will display a selective advantage and be selected as the most "fit" to adapt to the new environment.[172,173] The loss of a previously acquired resistance mutation, such as the Methionine being re-selected at position 184 in M184V mutant virus, is termed "reversion". Rates of reversion in different virus populations seem to depend on the overall genetic backbone of the virus.[141,172-174]

It has been discussed whether or not it may be beneficial to preserve the M184V resistance mutation when switching to the next regimen after a 3TC-containing regimen has failed.[175] This would require continuing 3TC pressure during the subsequent ART regimen, even if counter-intuitive in the presence of high-level 3TC drug resistance.[173,176]

The passage experiments presented in Section 1 aim to explore the effect of continuing versus discontinuing 3TC pressure in this situation. The advantage of the *in vitro* approach is that all other parameters (drug levels and combinations) may be tightly controlled allowing head-to-head comparison of drug combinations in the same clinical isolate.

INTRODUCING NNRTI IN 3TC RESISTANT HIV

To test the behavior of M184V mutant virus in the context of intense drug pressure, the clinical isolates were derived from patients failing a 3TC-containing ART. The patients carried multi-drug resistant HIV, but had never been exposed to non-nucleotide reverse transcriptase inhibitors (NNRTI).

The drug chosen to exert maximum drug pressure in this context was nevirapine (NVP), an NNRTI known to trigger drug resistance rapidly *in vitro* as well as *in vivo.*[177-181] NVP drug pressure would be expected to trigger the acquisition/selection of NNRTI resistance mutations, a process that is hindered as long as the M184V mutation is still present, increasing fidelity thus decreasing the ability of the virus to adapt to a new environment.

The *in vitro* model was designed to explore the effect of different combinations of NVP, 3TC and ADV in four different clinical patient isolates.[182] The drugs were chosen for their potential to exert the opposite effect on the M184V mutation.[169,174,183] Adefovir (ADV) and its successor tenofovir, have been shown to select for reversion, with M184V mutant virus being overly sensitive ("hyper-susceptible") to both drugs.[174] In summary, both NVP and ADV were expected to act in favor of M184V reversion, while 3TC would be expected to act in favor of preserving the M184V mutation.

In vitro assays, such as the one presented in Study 1 may improve our understanding of the interplay between multiple antiretroviral drugs with respect to HIV replicative capacity/fidelity. "Real-life" effectiveness of antiviral drugs strongly depends on their use in the most appropriate setting. The concept of "locking the virus in" with competing drug pressures has been proposed to achieve the most sustainable drug regimen to combat HIV long-term.[142,31] *In vitro* experiments may help a) to explore basic mechanisms of interference and conflicting drug pressures, and b) take a first step towards the development of assays assisting in the selection of the most promising drug combination in the individual patient in the situation of salvage therapy (so-called "salvage patient").

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Article

Persistence *versus* Reversion of 3TC Resistance in HIV-1 Determine the Rate of Emergence of NVP Resistance

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Abstract: When HIV-1 is exposed to lamivudine (3TC) at inhibitory concentrations, resistant variants carrying the reverse transcriptase (RT) substitution M184V emerge rapidly. This substitution confers high-level 3TC resistance and increased RT fidelity. We established a novel *in vitro* system to study the effect of starting nevirapine (NVP) in 3TC-resistant/NNRTI-naïve clinical isolates, and the impact of maintaining *versus* dropping 3TC pressure in this setting. Because M184V mutant HIV-1 seems hypersusceptible to adefovir (ADV), we also tested the effect of ADV pressure on the same isolates. We draw four conclusions from our experiments simulating combination therapy *in vitro*. (1) The presence of low-dose (1 μM) 3TC prevented reversal to wild-type from an M184V mutant background. (2) Adding low-dose 3TC in the presence of NVP delayed the selection of NVP-associated mutations. (3) The presence of ADV, in addition to NVP, led to more rapid reversal to wild-type at position 184 than NVP alone. (4) ADV plus NVP selected for greater numbers of mutations than NVP alone. Inference about the "selection of mutation" is based on two statistical models, one at the viral level, more telling, and the other at the level of predominance of mutation within a population. Multidrug pressure experiments lend understanding to mechanisms of HIV resistance as they bear upon new treatment strategies.

Keywords: HIV-1; lamivudine; nevirapine; adefovir; resistance; selection; serial passage

1. Introduction

1.1. In vitro 'Combination Therapy'

Several antiretroviral agents have been licensed to treat infections with the human immunodeficiency virus Type 1 (HIV-1). Antiviral drugs that inhibit RT activity in wild-type HIV-1 select rapidly for drug-resistant variants. Current guidelines therefore recommend the use of several antiretroviral agents concomitantly rather than sequentially [1–3].

Two classes of drugs are active specifically against the reverse transcriptase enzyme of HIV-1. The nucleotide analogue RT inhibitors (NRTI) compete with the natural substrate and act as chain terminators in the RT catalytic site. The nonnucleoside analogue reverse transcriptase inhibitors (NNRTIs) are noncompetitive inhibitors that bind exclusively to a hydrophobic pocket in HIV type 1 RT [4]. Interactions between NRTI and NNRTI can be complex and difficult to assess experimentally.

We established a novel *in vitro* system to test the impact of NRTI pressure on the development of resistance to the NNRTI nevirapine (NVP) in highly NRTI-resistant but NNRTI-naïve isolates.

1.2. Lamivudine (3TC) Resistance—The M184V Substitution

When HIV-1 is exposed to the NRTI lamivudine (3TC) at inhibitory concentrations, resistant HIV variants carrying the RT substitution M184V (ATG→GTG) emerge rapidly [5–7]. The M184V substitution confers the highest level of resistance (up to 1000-fold) for any NRTI that has been described to date [8]. In cell-free RT assays the M184V mutant virus exhibits altered enzymatic properties. RT with the 184-Val substitution is less able to initiate reverse transcription, to incorporate dNTP, to perform chain elongation, and to undergo compensatory mutagenesis [9–13]. Decreased fitness and increased fidelity of 3TC-resistant virus limit the production of randomly mutant forms, many of which are not viable [14–16]. In M184V mutant enzyme, the process of polymerization becomes more accurate; fewer viral variants are produced; and adaptation to environmental stimuli is impaired [17–19].

1.3. Maintaining 3TC Pressure When NNRTI Are Introduced

To simulate an environment that promotes strong evolutionary pressure, we performed serial passages in escalating doses of the NNRTI nevirapine ([NVP]). Resistance against NVP develops rapidly *in vitro* and *in vivo* [20].

We hypothesize that if 3TC pressure is withdrawn and at the same time an NNRTI is introduced, then the M184V mutant strains will be at a competitive disadvantage to the more "fit" and "flexible" wild-type variants, which can adapt to the new drug more easily [19,21]. The concentration of 3TC used in our experiments is within the normal range of wild-type IC50. Since 3TC is known to exert selective pressure on M184V, we would expect inhibition of reversal to wild-type at position 184. In this study we address these questions. Can the anticipated 3TC effect be simulated in multidrug

pressure experiments? Does 3TC have the expected impact on reversal and mutation rates, even in the context of high-level 3TC resistance?

1.4. Adding Adefovir (ADV)

We studied the 3TC-induced "antimutator phenotype" [22] further in the presence of adefovir (ADV). M184V mutant HIV has been shown to be hypersusceptible to ADV (as well as its successor, tenofovir) *in vitro* [23]. When ADV is added in the absence of 3TC, does ADV select for wild-type virus at position 184? Does the reversal to wild-type virus at position 184 (M184V-reversal) facilitate the development of NVP resistance?

The studies reported here come against a backdrop of tension between diminishing viral load through the administration of drugs on the one hand, and constraining viral escape of resistant strains on the other.

Figure 1. Sequencing Data—Review of Mutations and Reversals in Different Drug Combinations: A complete review of RT sequence changes under the following drug conditions: *NVP_only*, *NVP+3TC*, *NVP+ADV*, *NVP+3TC+ADV*, *3TC+ADV*, *No_drug*. Individual isolates are displayed in different colors: #1 (green), #2 (dark blue), # 3 (light blue), #4 (pink), and #5 (orange). Sequence changes are listed with the passage number (PASS) and [NVP] where they were first observed. Any mutation away from wild-type is listed under 'MUT', reversal to wild-type under 'REV'. Shaded areas within 'REV' indicate M184V-reversal.

Sequencing Data-Review of Mutations and Reversals in Different Drug Combinations
(Figure 1)

NVP ONLY

PASS#	#1 MUT	#1 REV	#2 MUT	#2 REV	#3 MUT	#3 REV	#4 MUT	#4 REV	#5 MUT	#5 REV	NVP [μM]
1										W210L K211R	0.01
2			D218E				V108I	Y208H			0.02
3						S67N					0.04
4								Y215D		V184M	0.08
5					V106A						0.16
6	Y181C										0.32
7			V106A		V179I	V184M					0.64
8									Y181C		1.28
9				V184M			K101E	I35V			2.56
10											5.12
11					E224K						10.24
12	V106A	V184M									20.48
TIME	267 DAYS		327 DAYS		251 DAYS		343 DAYS		478 DAYS		TIME

NVP+3TC+ADV

PASS #	#1 MUT	#1 REV	#2 MUT	#2 REV	#4 MUT	#4 REV	#5 MUT	#5 REV	NVP [μM]
1						Y208H			0.01
2					L228Q				0.02
3									0.04
4				S67N					0.08
5	V108I				V106M				0.16
6			V106A						0.32
7									0.64
8			Y181C				V106A V108I		1.28
9	Y188C								2.56
10					F227L				5.12
11									10.24
12	E128Q						F227L		20.48
TIME	340 DAYS		309 DAYS		266 DAYS		318 DAYS		TIME

NVP+3TC

PASS #	#1 MUT	#1 REV	#2 MUT	#2 REV	#3 MUT	#3 REV	#4 MUT	#4 REV	#5 MUT	#5 REV	NVP [μM]
1								Y208H			0.01
2										W210L K211R	0.02
3											0.04
4											0.08
5	V106A										0.16
6					V106A	S67N	Y181C G190A				0.32
7			V106A	S67N					Y181C		0.64
8											1.28
9											2.56
10											5.12
11					Y181C						10.24
12			K103N		D218G						20.48
TIME	310 DAYS		327 DAYS		266 DAYS		343 DAYS		327 DAYS		TIME

ADV+3TC

PASSG	#1 MUT	#1 REV	#2 MUT	#2 REV	#4 MUT	#4 REV	#5 MUT	#5 REV	NVP [μM]
1						Y208H			0
2							H208Y		0
3									0
4									0
5									0
6									0
7									0
8									0
9									0
10									0
11			E122K						0
12									0
TIME	286 DAYS		215 DAYS		266 DAYS		258 DAYS		TIME

NVP+ADV

PASS #	#1 MUT	#1 REV	#2 MUT	#2 REV	#3 MUT	#3 REV	#4 MUT	#4 REV	#5 MUT	#5 REV	NVP [μM]
1											0.01
2											0.02
3			V106I	V184M	V106I	V184M		V184M			0.04
4											0.08
5							V106M	I35V	V108I		0.16
6	V106A										0.32
7							L228Q				0.64
8					G190A						1.28
9											2.56
10			V108I Y181C						Y188C	V184M	5.12
11											10.24
12	T69I	V184M			Y181C		F227L				20.48
TIME	258 DAYS		327 DAYS		251 DAYS		360 DAYS		509 DAYS		TIME

NO-DRUG CONTROL

PASSG	#1 MUT	#1 REV	#2 MUT	#2 REV	#4 MUT	#4 REV	#5 MUT	#5 REV	NVP [μM]
1									0
2									0
3						V184M			0
4				V184M				V184M	0
5									0
6									0
7									0
8									0
9									0
10						Y215C			0
11				S67N					0
12									0
TIME	157 DAYS		171 DAYS		186 DAYS		171 DAYS		TIME

1 V35T, M41L, V90I, E122K, D123E, D177E, M184V, G196E, I201V, E207Q, T215Y, K275Q, K277R, I293V, E297Q
2 K20R, D67S, T69N, K70R, V118I, D123S, I135T, M184V, I202V, E207Q, F214L, T215F, K219Q, A272P, E291D, E297T
3 K20R, D67S, T69N, K70R, V118I, D123S, I135T, M184V, I202V, E207Q, F214L, T215F, K219Q, A272P, E291D, E297T
4 V35I, M41L, D67N, R83K, E122K, E169D, M184V, E207Q, H208Y, L210W, R211K, T215Y, I293V
5 M41L, E122K, D123E, I135T, M184V, E207Q, L210W, R211K, T215Y, A272P, P294T

2. Results and Discussion

Figures 1–3 summarize explicit baseline mutations and experimental mutations by isolate and drug combination. They also summarize counts of mutations together with incidence of V184M-reversal by isolate and passage number.

2.1. Mutations and Reversals in Different Drug Combinations

Reverse transcriptase (RT) mutational patterns and selected mutations/reversals of the individual isolates #1-5 at baseline and throughout twelve serial passages (P1-P12) are shown in Figure 1, below. All newly selected mutations and reversals persisted throughout P12. No mutations were observed in the *No_drug* control setup.

2.1.1. Introducing First-time NNRTI in NRTI-Resistant/NNRTI-naïve Clinical Isolates

Baseline isolates #1-5 (see legends) exhibit RT resistance patterns that commonly are observed in salvage therapy, all having changes at positions 184 and 215. It is noteworthy that of 55 baseline mutations, none is known to be in the NNRTI binding pocket, which includes positions 101, 103, 106, 108, 179, 181, 188, 190, 224, 227, and 228.

All isolates exposed to escalating doses of NVP showed a gradual appearance of one to three mutations, a total of 42 mutations. Of them, 38 were known to be in the NNRTI binding pocket, the remainder are considered noncanonical mutations or polymorphism. All RT mutations were tracked, including those not known to be associated with drug resistance. Please note the remarkably small p-values for the null hypothesis that these 38 mutations were "equidistributed" (that is to say, exchangeable) among possible codons.

2.1.2. Significance of NNRTI Binding Pocket Mutations

For the 19 isolates for which treatment included escalating doses of NVP, there were 38 NNRTI binding pocket mutations. Of these, 13 were at codon 106, seven at 181, and five at 108. The p-values for the findings that the "most popular" codon of 11 had at least 13 mutations, alternatively that the "second most popular" had at least 11, under the common null hypothesis that codons are "equidistributed" (exchangeable) were computed thus.

Isolates are taken to be independent, codons within isolates chosen at random without replacement from among the 11. Reading from isolate #1 through #5, successively from *NVP only* through *NVP+3TC+ADV*, the respective numbers of NNRTI binding pocket mutations were seen to be 2,1,3,2,1,1,2,1,2,1,1,3,2,3,1,2,2,3,3. Therefore, the number of ways NNRTI binding pocket codons could be chosen is

$$\binom{11}{2}\binom{11}{1}\binom{11}{3}\binom{11}{2}\cdots\binom{11}{3}\binom{11}{3},$$ a product of 19 numbers.

We made this choice at random 50,000 times, each time noting the codon chosen the largest, respectively next largest, number of times, thereby obtaining the joint sampling distribution of these two random quantities. Of the 50,000 trials, the "most popular" codon seen was seen only 12 times,

and that occurred for only four trials. The "next most popular" was seen seven times (2056 trials), eight times (129 trials), and nine times (seven trials). It follows that the respective estimated p-values are 1/50,001 (which is about 0.00002) and (2057+129+7)/50,001 (which is about 0.044) when the null hypotheses are as given. The first hypothesis seems untenable, and possibly not the second, either.

2.1.3. Maintaining *versus* Withdrawing 3TC Pressure

Maintaining 3TC Pressure prevented M184V reversal in all instances. Whenever 3TC was withdrawn, we selected for M184V-reversal, except for #4 in *NVP_only* (reversal at 215, 208, and 35 instead) and #1 in *No_drug.*

2.1.4. The Impact of Chance Effects

Isolates #2 and #3 were derived from the same baseline isolate. During lower passage numbers, these two isolates generated similar patterns. The impact of chance effects ('stochasticity') on the evolution of these two separate populations became more obvious at later passages. As expected, the two isolates #2 and #3 did not develop identically throughout combination passage experiments, but more similarly than isolates derived from different baseline patient isolates. In all isolates tested, preexisting sequence differences at baseline and viral variants below levels of detection may have contributed to the observed differences in mutational patterns. Since all baseline isolates underwent the same treatments, comparisons can be drawn across treatment groups.

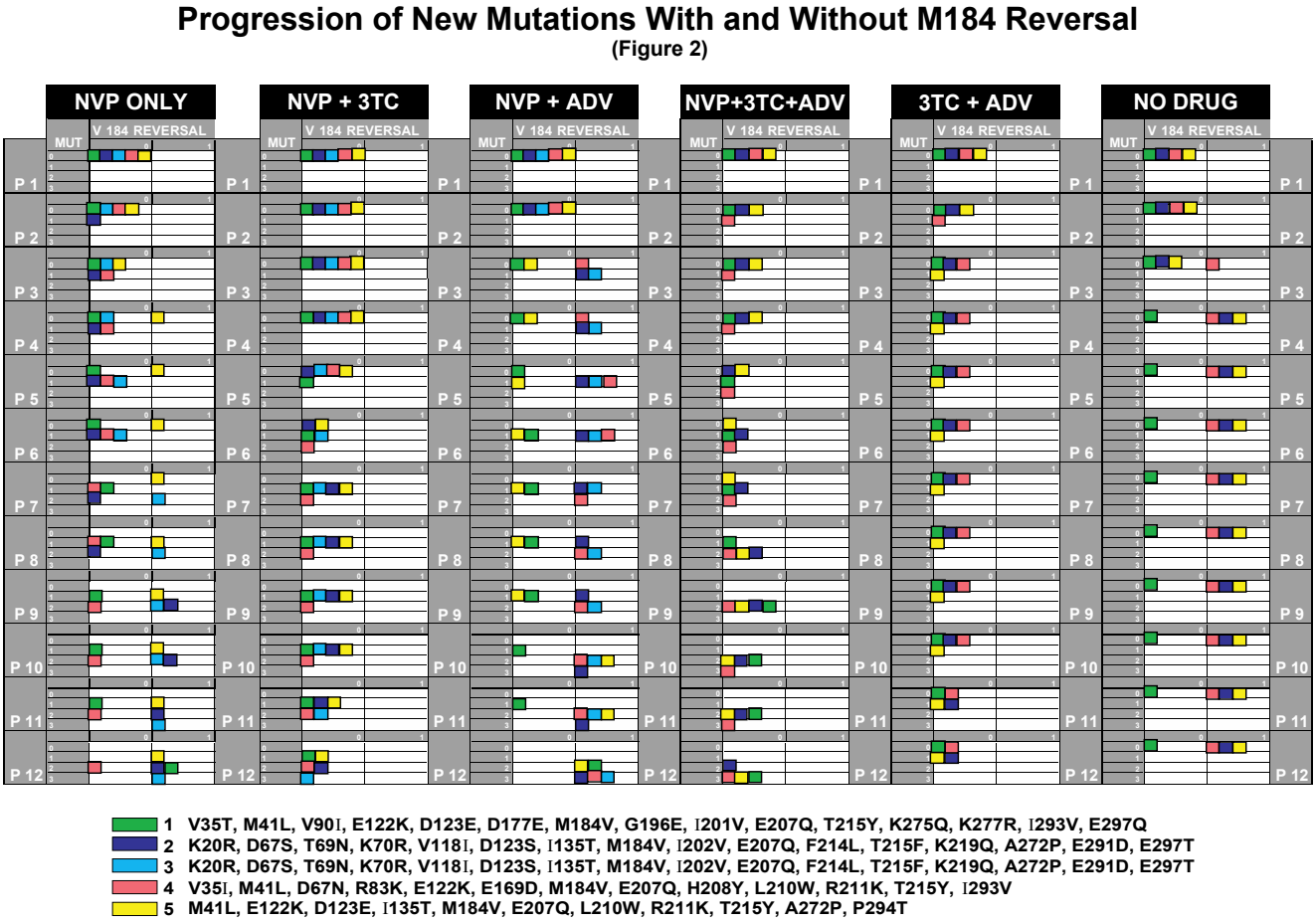
It is evident that in this experimental setting, selective forces due to increasing evolutionary pressure override the impact of genetic differences at baseline**.** We note that when a mutation was selected at a particular codon for a particular triple, the mutation persisted in subsequent isolates. To be conservative, for "significance" we require a difference in numbers of detected mutations when isolates are compared for a given passage. In the particle model, we also test for whichever treatment has the smaller number of mutations, since the number of particles remains stable with subsequent passage.

2.2. Progression of Mutations with and without M184V Reversal

For improved visualization of HIV evolution and dynamics during serial passage experiments, we summarized *in vitro* responses to different drug combinations in an innovative fashion using a Serial Passage Integrated Display ("Cube Model") with 2-by-4 tables based on reversal/no reversal and the number of newly selected mutations per clinical isolate ("cube").

Figure 2 illustrates how different drug combinations direct the movement of cubes into preferred directions: downward (new mutations), to the right (reversal), or diagonal (both).

Figure 2. Progression of Mutations With and Without M184V-reversal. All mutations and M184V-reversals for every passage and isolate are summarized. Isolates #1-5 (represented by colored cubes) started with *a priori* no mutations 'MUT' and no M184V-reversal (0MUT/0REV). At P1-P12, each cube could either remain in its position or move vertically to 1, 2, or 3 mutations and/or horizontally from 0 to 1 M184V-reversal. 'Movements' of cubes under defined drug pressures can be followed along P1-P12 or compared across equivalent passages.



2.2.1. NVP_only

NVP_only simulates the effect of NVP monotherapy and serves as the basic experiment for the comparison with the other NVP escalation experiments. When all isolates in *NVP_only* are viewed together, we see the selection of wild-type at position 184 (M184V-reversal) in most (4/5) cases, though only one through the first six passages. There are 1, 2, or 3 newly selected mutations at least by P12, mostly known NVP resistance mutations (see Figure 1).

2.2.2. NVP+3TC

With the addition of 3TC (***NVP+3TC***) we observe several changes in comparison to *NVP_only*. M184V-reversal was prevented by the addition of low-level 3TC in all cases despite an exponential increase in NVP doses of up to >2000-fold. In the presence of 3TC, no RT changes could be found when NVP was escalated from P1 through P4 (1 to 8-fold [NVP] and from P7 to P10 (64 to 510-fold

NVP). Under extreme pressure (P12, 2048-fold NVP) we observe total numbers of 2x1, 2x2 and 1x3 mutations, but no M184V-reversal. Testing the correlation in the viral particle model, there is a significant difference in the two regimes at the 5% level according to in the suspected direction for isolate #2 at P2 through P5 and for isolate #4 at P4. There are significant differences in the opposite direction for isolate #1 at P5 and isolate #4 at P6.

2.2.3. NVP+ADV

The addition of ADV to *NVP_only (**NVP+ADV**)* selects rapidly for M184V-reversal (P3). At P12 all isolates have reverted. Moreover, we see a higher total number of NNRTI mutations in comparison to both *NVP_only* and *NVP+3TC*. When *NVP* alone is compared to *NVP+ADV* by the viral particle model, there are significant differences in the anticipated direction for isolate #3 at P3 and for isolate #5 at P5 and P6. There is a significant difference in the opposite direction for isolate #4 at P3.

2.2.4. NVP+3TC+ADV

NVP+3TC+ADV can be viewed as the 3TC with *ADV+NVP*, as *NVP_only* plus *3TC+ADV,* or as *ADV* with *NVP+3TC;* 3TC again prevented M184V-reversal in all cases. However, the addition of ADV to *NVP+3TC* increased the number of NVP mutations selected at lower passage numbers. The high degree of heterogeneity in the presence of ADV was independent of M184V-reversal, which was prevented by 3TC. Comparing *NVP+3TC* with *NVP+3TC+ADV* in the viral particle model, the only significant differences in mutation (other than reversals) are for isolate #4, P2 through P4. They are all in the suspected direction: more mutations appeared when *ADV* was part of the treatment regime.

2.2.5. 3TC+ADV

3TC+ADV serves as a control experiment; [3TC] and [ADV] were maintained at the same level from P1 through P12. The low number of total mutations suggests that the degree of evolutionary pressure was not comparable with the NVP-escalation experiments; ([ADV] at 2 μM) as an active drug exerted evolutionary pressure and generated mutations. Interestingly, E122K (#2/P11) and H208Y (#5/P3) would not be considered resistance mutations to ADV [24].

2.2.6. No_Drug

No_drug simulates a treatment interruption and demonstrates that upon 3TC withdrawal, M184V tends to revert, even if the environment is stable. By P4, 3/4 isolates had reverted.

2.2.7. Testing the 3TC-Effect

The null hypothesis that 3TC does not lead to altered 184 reversal has p- value 5.982×10-6 , at least if 3TC is specified in advance of the computation. Figure 2 will convince a reader that the attained significance level for any reasonable model should be very small since there was no reversal with 3TC.

Further inference in this section is devoted to testing the null hypotheses that numbers of mutations result in identical sampling distributions no matter which of two combinations of drugs was

administered. In order that tests are conservative, we computed p-values for two-sided alternatives; that is, in principle either combination of drugs could have resulted in a sampling distribution of mutations stochastically smaller or larger than that of the other.

We employed exact distributions of a Mann-Whitney (equivalently Wilcoxon rank-sum) statistics in computing attained significance [25], respecting that resulting 2×4 tables [with rows representing treatment and columns numbers of mutations (0, 1, 2, or 3)] have many tied observations. Our statistics are, in fact, permutation statistics. When we say "significant" in the table that follows, we mean that the (two-sided) p-value was <0.05. Alert readers will see that we have made no attempt to correct for multiple testing and have not employed false discovery rates [26]. Evidence for our claims is transparent from cursory examination of Figures 1 and 2; we feel that the conservative p-values we supply are sufficient to make our points. (Table 1).

(A) *NVP_only* has significantly more mutations than *3TC+ADV* at passages 8 through 10 and 12.

(B) *NVP+3TC* has significantly fewer mutations than *NVP+3TC+ADV* at passages 9 and 10.

(C) *NVP+3TC* has significantly more mutations than *3TC+ADV* at passages 7 through 10.

(D) *NVP+ADV* has significantly more mutations than *3TC+ADV* at passages 6 through 12.

These are the most extreme comparisons with respective p-values 0 .048, 0.040, 0.032, 0.032, 0.024, 0.032, and 0.008.

(E) *NVP+3TC+ADV* has significantly more mutations than *3TC+ADV* at passages 9 through 12.

Table 1. Comparison across passage numbers and drug combinations; testing the null hypotheses that numbers of mutations result in identical sampling distributions no matter which of two combinations of drugs was administered. P-values (<0.05 in **bold**) for comparisons (A) to (E); passage numbers 6–12.

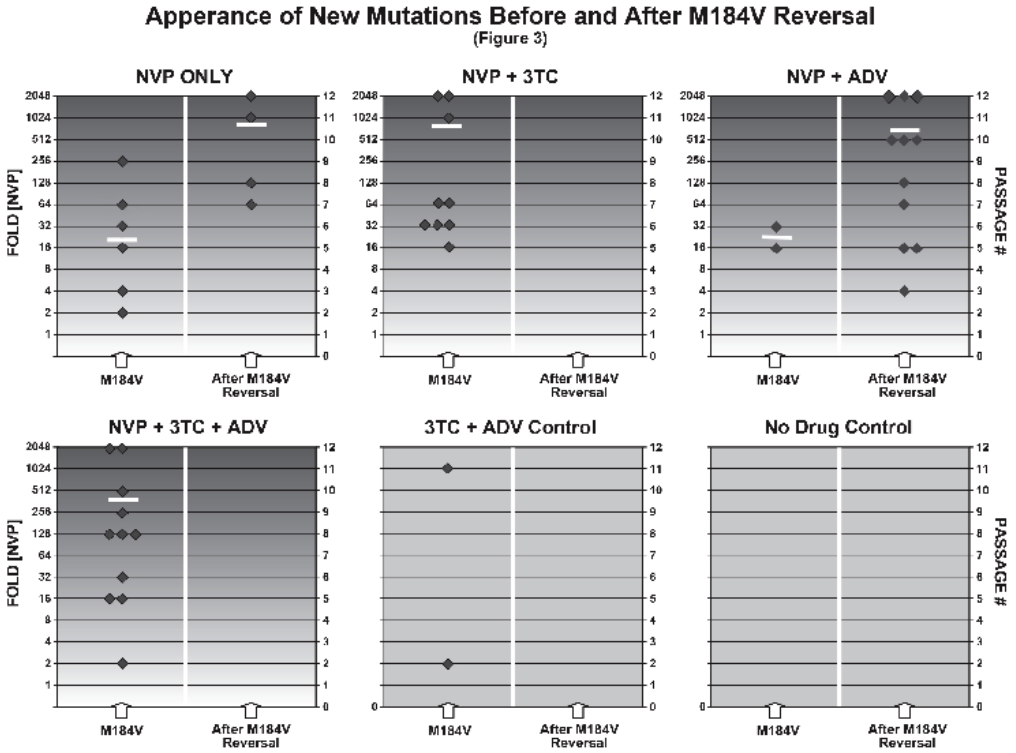
Comparison	**Passage Number**						
	6	**7**	**8**	**9**	**10**	**11**	**12**
A) *NVP_only versus 3TC+ADV*	0.5238	0.1429	**0.0317**	**0.0476**	**0.0476**	0.0794	**0.0317**
B) *NVP+3TC versus NVP+3TC+ADV*	0.9762	1.000	0.2063	**0.0476**	**0.0397**	0.1190	0.1429
C) *NVP+3TC versus 3TC+ADV*	0.4444	**0.0397**	**0.0397**	**0.0397**	**0.0397**	0.1587	0.0794
D) *NVP+ADV versus 3TC+ADV*	**0.0476**	**0.0397**	**0.0317**	**0.0317**	**0.0238**	**0.0317**	**0.0079**
E) *NVP+3TC+ADV versus 3TC+ADV*	0.3714	0.3714	0.0571	**0.0286**	**0.0286**	**0.0286**	**0.0286**

2.3. Appearance of New Mutations before and after 184 Reversal

Figure 3 summarizes the selection of mutations prior to and after M184V-reversal, respectively. Though numerical averages may be of limited descriptive value for random quantities that change by doubling, for completeness these numerical averages are displayed as horizontal white bars.

NVP_only shows a mixed picture. Mutations were selected at P2, P3, P5, and P6 without prior or simultaneous reversal at position 184 (*M184V*; left column). The first new mutation with reversal (*After M184V Reversal*; right column) became prevalent at P7. More mutations after reversal appeared at P8, P11, and P12, though additional mutations were selected at P7 and P9 without reversal.

Figure 3. Appearance of new mutations before and after 184 Reversal: All mutations generated (diamonds) per drug setting, before (*M184V*, left column) and after M184V-reversal (*After 184 Reversal*, right column) are summarized. We display the appearance of each selected mutation in relation to the respective passage number and NVP concentration on the y-axis. *FOLD [NVP]* represents the concentration at which a mutation first became prevalent. *FOLD [NVP]* $=2^{P}$ (with P = passage number). As reported in Figure 1, all observed mutations persisted up to P12. During drug escalation, the event of M184V-reversal is required for a mutation to appear in the right column. Diamonds in either column can therefore only be compared between different drug settings. Horizontal bars in the logarithmic *FOLD [NVP]* scale indicate the average [NVP] for mutations to appear.



With ***NVP+3TC*** there was no M184V-reversal. With *3TC+NVP* we selected for a total number of 9 mutations, which was the lowest among all NVP escalation experiments. Importantly, the first mutation that appeared with *NVP+3TC* was selected under substantially higher NVP concentrations (P5; 16-fold), by contrast with what was seen with *NVP_only* (P2; 2-fold) or in any other setting.

With ***NVP+ADV***, mutations appeared after M184V-reversal, the first one at very low NVP concentration (P3; 4-fold). The average [NVP] was lower than in *NVP_only* for both *M184V* (left column, 24-fold *versus* 62-fold) and *After M184V Reversal* (right column: 682-fold *versus* 816-fold).

In the ***NVP+3TC+ADV*** experiments, 3TC precluded M184V-reversal. The total number of selected mutations (11) was lower than in *NVP+ADV* (13) and higher than in *NVP+3TC* (9). It must be noted, however, that *NVP+3TC+ADV* was done with only four isolates (*versus* five in the other NVP-settings). The first mutation appeared at P2 (2-fold [NVP]). However, the average concentration needed to generate mutations was high (483-fold in *NVP+3TC+ADV*), but lower than without ADV (595-fold in *NVP+3TC*).

2.4. Summary of Results

We draw four conclusions from our experiments simulating combination therapy *in vitro*.

1. The presence of low-dose (1μM) 3TC prevented reversal to wild-type from an M184V mutant background.
2. Adding low-dose 3TC in the presence of NVP delayed the selection of NVP-associated mutations.
3. The presence of ADV, in addition to NVP, led to more rapid reversal to wild-type at position 184 than NVP alone.
4. ADV plus NVP selected for greater numbers of mutations than NVP alone.

Inference about the "selection of mutation" is based on two statistical models, one at the viral level, more telling, and the other at the level of predominance of mutation within a population.

2.5. Discussion

It is evident from Figures 1 through 3 that during each passage, there is tension between diminishing viral load through the administration of drugs and constraining viral escape through the selection of mutant forms. Our contributions begin with the establishment of an *in vitro* system to study the impact of continued 3TC pressure on the selection of both M184V-reversal and resistance to NVP.

Several clinical trials demonstrated that the use of genotypic resistance data is associated with improved virologic and clinical outcome in salvage therapy and can be cost-effective [27–29]. When sequence data are available to direct the choice of a new regimen and a known resistance mutation is found, it seems plausible that the respective drug has lost antiviral activity and should be discontinued. Specific combinations of antiretroviral agents can exert conflicting genetic pressures [30]. A novel strategy that remains to be established is the continued use of certain individual compounds with the goal to preserve "suppressor mutations" impairing the evolution of resistance to other compounds [4,15,22,31].

Some authors have suggested continuing 3TC therapy even in the context of high-level 3TC resistance [18,19,32,33]. The strategy is to preserve the resistance mutation M184V, which has been linked to an HIV-1 reverse transcriptase with altered biochemical properties. Previous *in vitro* studies have shown that M184V may not delay the emergence of some protease inhibitors (PI) mutations [34], but of some PI and NNRTI [12]. In our study we test for NRTI-NNRTI interactions allowing for structural or functional constraints within the RT enzyme to interfere with the acquisition of new mutations.

Jonckheere *et al.* compared HIV wild-type to M184V mutant virus with three different stable doses of NVP in the absence of 3TC pressure [35]. Breakthrough of NVP-resistant virus was generally observed one passage later with M184V mutant than with wild-type virus. This study is in agreement with our results, but did not address the effect of concomitant 3TC pressure on diverse clinical isolates. Diallo and Balzarini *et al.* tested the combination 3TC with NNRTI (NVP and efavirenz, respectively) [12,36]. Again, viral breakthrough was delayed significantly when wild-type and 184V recombinant HIV were exposed to 3TC plus NNRTI. The above experiments by Jonckheere, Balzarini

and Diallo *et al.* used clonal HIV-1 IIIB laboratory isolates passaged in tumor cell lines, which would be considered the standard method when examining the effect of individual mutations on the emergence of drug resistance mutations. In our experiments we confirmed that this additive 3TC-NNRTI effect is preserved even in the context of "real-life" patient isolates carrying the M184V "naturally", *i.e.*, after *in vivo* exposure to 3TC. This is even more remarkable as our method used diverse clinical isolates, which may have contained minority variants below the 20% detection level with population-based sequencing [37]. Future developments of this method should employ second generation sequencing methodologies to examine the role of minority variants in the mix, or to compare artificial mixtures of minority variants outcompeting each other under drug pressure.

It remains to be noted that in our assay, we used pooled donor PBMC rather than immortalized laboratory cell lines, aiming to simulate the real-life scenario as closely as possible. While pooled PBMC may pose a potential caveat due to the variability in the composition of PBMC over time, the pooled PBMC culture technique has been developed and established at the Stanford Center for AIDS Research (CFAR) in the early 1990s [38–42] and has since then become part of standard quantitative PBMC culture protocols in the NIAID Virology Manual for HIV Laboratories [43]. Quan *et al.* observed an additive effect of 3TC+NVP in enzymatic assays when measuring the amount of full-length RT product in M184V mutant virus. The authors suggested that 2.5–20 μM 3TC might exhibit a modest antiviral effect in M184V mutant virus despite high-level resistance [32]. Our data are in agreement with this hypothesis, but for 3TC-concentrations as low as 1 μM.

Our results underline the importance of drug combination testing in patient isolates that are resistant at baseline. In 38 of 42 isolates exposed to NVP we selected for mutations that are located in the NNRTI binding pocket. We note that of them, 13 were at site 106, seven at 181, and five at 108. It seems plausible that structural constraints favor NNRTI mutations in positions 103-108 over changes in positions 181 or 188 as long as M184V predominates. The structure of M184I-HIV-1 has been solved and published [8], NNRTI have been co-crystallized with wild-type as well as Y181C and Y188L mutant RT [44]. For a better understanding of NRTI-NNRTI interactions complex three-dimensional models of dual/triple resistant virus will be required.

In our study we investigate the overall effect of maintaining *versus* withdrawing 3TC pressure in clinical isolates at the first-time use of NNRTI. It is well known that NNRTI mutations are generated quickly *de novo*, even in wild-type laboratory strains. Clinical studies have shown that NNRTI- naïve patients may harbor HIV-1 viral variants with reduced NVP-susceptibility [45,46]. In our study, all isolates were fully susceptible to NVP and ADV at baseline (data not shown). All isolates were exposed to identical experimental conditions, allowing comparisons across the different drugs present in the growth media. In this context it is not surprising that the two isolates (#2 and #3) of common genetic lineage behaved similarly. As we expected, patterns of resistance evolved differently, but always in concordance with one basic hypothesis. That is, no matter the genetic background, 3TC precluded M184V-reversal and impaired the selection of mutations in the NNRTI binding pocket. Thus, continuing 3TC and adding at least two more active agents to the regimen should delay the initial selection of NNRTI resistance.

The observation that *ADV+NVP* select for greater numbers of RT mutations than *NVP_only* can be explained by the selective pressure of both drugs, *ADV+NVP*, being greater than that of NVP alone, but sufficiently low to allow viral replication and selection. The pressures imposed by ADV would be

expected to select changes conferring advantages to replication in the presence of ADV, such as 184 reversion. M184V reversion was promoted by ADV pressure only in the absence of 3TC pressure, contrary to reports of TDV+3TC serial passage experiments in SIV [47]. Interestingly, the mutations observed with ADV in our experiments were, with one exception (T69I), all positioned within the NNRTI binding pocket as opposed to the NRTI binding pocket. Thus, the majority of mutations selected with *NVP+ADV* and *NVP+ADV+3TC* were mutations known to confer NVP, not ADV, resistance. *ADV+3TC* alone selected for random changes at positions 208 and 122. Another NRTI, zidovudine (ZDV) has previously been reported to increase mutation rates [48,49].

The rapid outgrowth of 184Met virus in the absence of 3TC in our experiments indicates that fitness disadvantages are compensated for, as soon as drug pressure is released. We screened for replicative fitness in P10-12 supernatants and found that TCID50 values, when measured several times in the absence of drug, were extremely low. Surprisingly, the same isolates showed dramatically improved growth in the presence ADV, NVP and 3TC, independent of the dose range applied (data not shown). Dose-dependent enhancement of viral growth by NNRTI has been reported [44]. The observed phenomenon of dose-independent, but drug-dependent growth enhancement in some of our isolates will be a subject for further investigation [50].

It has been suggested that the observed benefits of 3TC in combination therapy, even after 3TC-resistance arises, may be attributed to the net-effect of decreased adaptability and a deficit in viral fitness [5,12,51]. The simulation of combination therapy *in vitro* is a new method that provides an important link between *in vitro* assays and *in vivo* studies in animal models and human subjects. Our data support a chain of evidence derived from biochemical assays and single-drug experiments in laboratory isolates, as we report. We approximate the actual clinical scenario further by using multiple drugs simultaneously in clinical isolates with diverse genetic backgrounds.

The genetic background has been determined by consensus genotyping as the current method of choice when switching drug regimens. As indicated in the mathematical models used, this includes only the view of majority variants composing >80% of the virus population [37]. In this assay, each virus population was allotted the time required to outgrow drug pressures with each passage before the next passage was started. Future studies using different mathematical models will be addressing growth kinetics during single passages. Allele-specific assays may help determine the role of minority variants in the evolution of drug resistance against combinations of antiviral drugs.

The next level of complexity would be reached in a clinical trial that ought to account for additional parameters, such as patient compliance, virus-host interactions, and the distribution of viral populations within body compartments [31]. Several studies tested the decay of M184V during salvage therapy as well as treatment interruptions [51–54]. Resistant variants with impaired fitness disappeared within weeks after discontinuation of highly active antiretroviral therapy (HAART), accompanied by rapid viral load rebound. Only well-designed prospective clinical trials can assess the *in vivo* risk/benefit ratio and justify a prolonged, possibly once-daily use of 3TC in 3TC-resistant patients, not only in the context of strategic treatment interruptions, but also when a new regimen is started [31,55–57].

3. Experimental Section

3.1. Test Compounds

Lamivudine (3TC) was kindly provided by GlaxoSmithKline (Research Triangle Park, NC), ADV by Gilead (Foster City, CA). NVP was obtained from Boehringer Ingelheim (Ridgefield, CT).

3.2. HIV Strains

Six clinical isolates #1 through #5, were cell-free supernatants expanded by cocultivation with donor PBMC (NIAID Virology Manual for HIV Laboratories). They were stored at −70 °C. These frozen stocks were derived anonymously from individuals who had received long-term antiretroviral therapy but who had never been exposed to NNRTIs. The primary samples were sequenced up to RT amino acid position 300. A complete list of initial RT mutations (as compared to the Los Alamos consensus HIV-1B) can be found in the legends of Figures 1 and 2.

3.3. Cells and Cell Culture

Pooled HIV-negative donor PBMC (Stanford Blood Bank) were isolated by centrifugation on Ficoll-Paque and were cultured in RPMI medium containing 15% heat-inactivated fetal calf serum, IL-2, PenStrep and L-Glu. Before use, these cells were stimulated for 2–3 days with phytohemagglutinin (Sigma, St. Luis, MO) and washed [40–42].

3.4. Passage Experiments

Isolates # 1–5 were set up in five different drug combinations: *NVP_only*, *NVP+3TC*, *NVP+ADV*, *NVP+3TC+ADV*, *3TC+ADV* and *No_drug*, #3 and #2 were aliquots from the same baseline sample, but were run as independent experiments in *NVP_only*, *NVP+3TC*, and *NVP+ADV*. With each passage, the concentration of NVP was doubled. The NVP starting dose was 0.01 μM, around the IC 50 of the NNRTI-naïve baseline isolates. 3TC [1 μM] and ADV [2 μM] were added and maintained at levels around the IC50 of the respective baseline isolates.

Newly passaged cultures were set up as follows: 100 μl supernatant, incubated with 5 Mio PBMCs in 1ml media without drug. After 2 hours of incubation at 37 °C/5% CO2, the culture was dissolved in 10ml media with the respective drug combinations. The cultures were transferred to 25-mL flasks and again incubated at 37 °C/5% CO2.

Viral growth was monitored once weekly using a p24 antigen assay on supernatants (Abbott Laboratories, Chicago, IL). At p24 ELISA values $<3 \times 10^4$ pg/mL, cultures were split: 2.5 Mio PBMCs were replaced by new donor PBMC in media containing the respective drugs in the same molar concentration. At $\geq 3 \times 10^4$ pg/mL, the cultures were passaged after a 2-hour incubation time. With every passage [NVP] was doubled. The amount of supernatant to infect new cells was adapted according to p24 values obtained before passage. All experiments were carried to the 12th passage (P12), *i.e.*, 2048-fold [NVP], well below cytotoxic levels. Average time to P12 was 293 days (range 157–509 days). With every passage, supernatant was harvested and stored in aliquots at −70 °C.

3.5. ABI Sequencing

Population-based sequencing [37] was done at baseline as well as from the supernatant obtained with every passage. As was described previously [58], purified proviral RNA (Quiagen Viral RNA Extraction Kits, Chatsworth, CA) from cultured cell pellets was amplified by nested PCR. A Superscript-One-Step RT-PCR reagent (Life Technologies, Gaithersburg, MD) was used to obtain DNA segments for sequencing. First-round nested PCR primers were RT-21 [59] and MAW-26 [58] and second-round primers were PRO-1 [60] and RT-20 [59], respectively. Second-round products were sequenced using a dRhodamine labeled terminator kit (PE Applied Biosystems, Warrensburg, UK) and the previously described [61] primers RT-a, RT-b (forward), RT-y and HXBR2-89 (reverse). Sequencing was performed using ABI Model 377 equipment and software.

Sequences were aligned, proofread, and edited in a blinded fashion. Sequencing data were compared to the corresponding baseline isolates and to the consensus B sequence from the Los Alamos HIV Sequence Database, as well as to data obtained from earlier passages in the same experiment. Any mutation away from consensus B-sequence was defined as mutation. Any mutation back towards consensus HIV-1B was defined as a reversal, even if it was not "all the way back" to consensus.

3.6. Statistical Methods

For a given triple (drug combination, isolate, passage), the individual viral particle is the sampling unit for our first model. Its assumptions are that for each of (approximately) $3x10^4$ viral particles and at each of the 300 RT key codons, the amino acid at that codon is either as it was at baseline or has a mutated value. Based on published estimates, the sensitivity of population based sequencing is at least 80% [37]. We assume that for a fixed codon the probability of mutation is constant across particles [62,63]. For a fixed codon *c*, what might vary for two particles *i* and *i'* is the correlation between the indicator functions of the amino acid values at the codon for the two particles. Recall that the indicator function of an event is 1 if the event occurs and 0 if not. Therefore, the indicator for the c^{th} codon of the i^{th} particle is 1 if that codon has baseline value. Otherwise it is 0. Symbolically, this is $\rho(i,i') = Corr(I_i(c),I_{i'}(c))$.

We ask this question: "What would be the maximum value of this correlation, averaged over pairs of particles, so that two isolates differ at the 5% level of significance when one isolate has baseline values at all codons and the other a single mutation away from baseline?"

The mathematical details and discussion of this novel statistical model are summarized in *3.6.1. Viral Particle Model* and *3.6.2. Population Models*, below:

3.6.1. Viral Particle Model

One approach to testing differences at two fixed passages between two isolates, neither descended from the other, was by what we have termed the *viral particle model*. The basis for comparison at a fixed codon is a two-sample t-like statistic that is the difference of two fractions divided by an estimate of the standard deviation of that difference. We denote the two isolates by *a* and *b*.

For *a*, say, the indicator of particle *i* having its baseline value at codon *c* is $I_i(c)$, which has value 1 if *c* is wild type, and otherwise is 0. There is an analogous indicator for *b*. We take the numbers of viral

particles to be 30,000. We speak of the correlation ρ between two indicators. For two codons i and i' we write this correlation $\rho(i,i')$. What matters is actually the value of $\rho(i,i')$ averaged over pairs (i,i') of codons. From the constraint that the variance of a sum cannot be negative, it follows easily that the average $\rho(i,i')$ cannot be less than -3.33×10^{-5}. In fact we expect that ρ, which we cannot know exactly, is positive and small. Because two particles within the same isolate and passage may have replicated inside the same cell, and also because of the physical proximity of any two particles within a flask can vary, we do not assume *a priori* that $\rho = 0$.

For a comparison of differences, the numerator of the t-like statistic is

$$\hat{p}_a - \hat{p}_b, \quad \text{where } \hat{p}_a = (1/30,000) \sum_{i=1}^{30,000} I_i(c)$$

and $\hat{p}_b$ is defined by analogy.

From a well-known computation with sums of random variables that assume only values 0 and 1 it follows that the variance of $\hat{p}_a$,

$$V(\hat{p}_a) = (p_a(1 - p_a)/30,000) + ((29,999/30,000)\rho\, p_a(1 - p_a)), \qquad (A.1)$$

where $p_a = E(I_i(c)) = Prob(I_i(c) = 1)$.

Note that this probability is assumed here not to depend on i. (Of course, computations that follow in this appendix, and that are required elsewhere in the paper, show this assumption to be false, decisively. However, the net effect of our assumptions is to make the p-values of our test extremely conservative.) We estimate $V(\hat{p}_a)$ by replacing p_a on the right hand side of (A.1) by $\hat{p}_a$.

$V(\hat{p}_b)$ is estimated analogously. Because $\hat{p}_a$ and $\hat{p}_b$ are clearly independent, our t-like statistic is now seen to be

$$t_c = \frac{\hat{p}_a - \hat{p}_b}{\sqrt{\hat{V}(\hat{p}_a) + \hat{V}(\hat{p}_b)}}$$

The missing ingredient in t_c is ρ, which we admittedly have no way of knowing exactly. But for that, we could approximate p-value for testing the null hypothesis "no difference between given isolates and passages at codon c" by $Prob(|Z| > t)$, where Z is a standard Gaussian random variable; and t the observed value of t_c. We could then test the null hypothesis "no difference at any codon" by $300\,Prob(|Z| > t)$. The latter computation uses the simple Bonferroni bound. In fact what we wish to do with t_c is to find and use the largest value of ρ for which the cited 80%–20% difference at some codon for fixed isolates and passages is significant at the 5% level for the null hypothesis as given. First, we solve $Prob(|Z| > t) = 0.05/300$ for t, arriving at $t = 3.7482$.

Then, set $\hat{p}_a = 0.8$, $\hat{p}_b = 0.2$, and $t_c = 3.7482$; and solve for ρ. The resulting ρ is 0.08.

3.6.2. Population Models

Two further models are based on the notion that for each (drug, passage) combination, isolates are sampling units. Thus, the sample size is five for *NVP_only*, *NVP+3TC*, and *NVP+ADV*, and for *NVP+3TC+ADV*, *3TC+ADV*, and *No_drug*, for a total sample size of 27 for each cited pair.

Numbers of mutations across isolates within a particular passage for a "counting process" such as ours might be taken to be what is conventional in such applications, a Poisson process. The Poisson model arises when there are many chances for "success" but few "successes", and in addition trials are independent. These assumptions might apply when we take mutations themselves as sampling units. The presence of mutation or mutations within an isolate and drug combination can be assumed independent across passages; they are certainly independent across isolates. A sum of independent Poisson random variables has a Poisson distribution no matter the respective parameters of the summands. Conversely, if a sum of independent random variables has a Poisson distribution, then according to D. Raikov [64], each summand has a Poisson distribution. Therefore, we can test the null hypothesis that the Poisson model applies to numbers of mutations within a drug combination by looking at Passage 12 to see if the distribution of numbers of mutations across isolates is Poisson. We begin with the usual approach to assessing the Poisson model: via the "Poisson dispersion test" [63]. (In a Poisson model the mean and variance are equal as numbers.) The test statistic is proportional to the ratio of sample variance to sample mean. If the Poisson model for mutations were correct for a fixed treatment (or drug combination), but there was a change by isolate in the Poisson parameter with 184 reversal, then there would be evidence for over-dispersed data and thus evidence against a strict Poisson model. Other aspects of the experiment could lead to over-dispersed or under-dispersed numbers of mutations. (Over[under]-dispersion in a model means that the variance is greater [less] than what the model would constrain it to be.) There are some 0s in the sample variances when isolates are pooled within treatments. The ratio of sample variance to sample mean disregards information in the sample mean when the sample variance is 0. This observation and a Taylor series argument not given here led us to use as a test statistic the difference of sample mean and sample variance rather than the usual ratio. On the null hypothesis that the data are Poisson, the difference should be 0 to within noise. Because there are at most five isolates per drug combination, we could not rely on asymptotic distributions computed under the null hypothesis. Instead, we used the parametric bootstrap distribution [62] of the test statistic under the null hypothesis. This amounts to sampling independently from a Poisson distribution with parameter (mean and variance) the average number of mutations observed at the twelfth passage. The resulting distribution is the reference distribution for the cited difference when the null hypothesis is true. We took 1,000 bootstrap samples per drug combination. This approach enabled us to compute p-values for the null hypothesis separately for alternatives of over-dispersion and under-dispersion relative to the Poisson.

When the number of mutations across isolates within a particular passage is hypothesized to have a Poisson distribution and tested as specified, then the p-value for "over-dispersion" is never less than 0.85. However, for the model with "under-dispersion", the respective p-values are 0.137 for *NVP only*, 0.147 for *NVP+3TC*, 0.058 for *NVP+ADV, 0*.061 for *NVP+3TC+ADV*, and 0.370 for *3TC+ADV*. There were no mutations but V184M reversal (and two other reversals) for the No drug regimen. Clearly, none of the five p- values is less than 0.05. However, when we combine them by Fisher's

technique [65] of summing minus twice the natural logarithms of the p-values and comparing the sum with a chi-square distribution with 10 degrees of freedom, the overall p-value for the null hypothesis of "under-dispersion" comes to 0.03. For this reason we did not use the Poisson model for numbers of mutations. Instead, our test statistic was nonparametric. Given two candidate drug regimes, it was simply the difference between cumulative numbers of mutations, pooled across isolates. The significance of this difference at each passage was assessed by a permutation test [62].

In summary, the first of the two involves a Poisson assumption that is standard for data like ours but that was discarded after careful study.

The second of the two models involves a nonparametric statistic. That is, given two candidate drug regimens, it is simply the difference between cumulative numbers of mutations, pooled across isolates. The significance of this difference at each passage was assessed by a permutation test [62]. That the passages of the isolates are independent conditional on their origin is all that matters for the validity of this second test. Sampling distributions of reversal by isolate ought to be closer for the second and third isolates than for any other pair when they are exposed to the same challenges. With the second of the cited two approaches here, we can assign separate p-values for the null hypothesis of "no difference in mutation rate" *versus* each of the two alternatives where one treatment produces a greater mutation rate than the other.

The issue of whether 3TC precludes V184M reversal can be approached by means of a 2-by-2 table and Fisher's exact test [63]. One of rows or columns corresponds to "treatment with 3TC or not" and the other to "reversal" or not. There are 27 isolates (Figure 2).

4. Conclusions

Using a novel *in vitro* assay and statistical model, we explored useful strategies of combining antiretroviral drugs with potentially divergent effects on the RT substitution M184V, exerting high-level resistance to 3TC.

We noted a pronounced "antimutator effect" when continuing low-dose 3TC while introducing a first-time NNRTI (NVP) in NRTI resistant/NNRTI naïve clinical isolates. Even in the context of high-level resistance, maintaining 3TC pressure prevented reversal M184V in all instances while delaying the emergence of NNRTI resistance. The opposite effect was exerted by ADV; M184V mutant HIV-1 has previously been shown to be hypersusceptible to ADV (as well as its successor, tenofovir) [23].

For improved visualization of HIV evolution and dynamics during serial passage experiments, we summarized *in vitro* responses to different drug combinations in an innovative fashion using a Serial Passage Integrated Display ("Cube Model", Figure 2) with 2-by-4 tables based on reversal/no reversal and the number of newly selected mutations per clinical isolate ("cube").

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Conflict of Interest

The authors declare no conflict of interest.

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3. MANAGING HIV DRUG RESISTANCE *IN VIVO*

REAL-WORLD EFFECTIVENESS OF ANTIRETROVIRAL THERAPY

A number of factors determine the success of a given drug regimen, including the combination of drugs, the timing and sequencing of ART, as well as virologic subtype, resistance and compensatory mutations. An additional level of complexity is reached when antiretroviral therapy is administered in real-life. A number of host factors will influence the effectiveness of a given regimen, including:

(A) Intra-individual differences in drug absorption and metabolism;[184]

(B) Differences in drug concentrations in different body tissues and compartments;[185-187]

(C) Drug interactions affecting the pharmacokinetics of antiretroviral drugs;[188-190]

(D) Patient adherence to the regimen;[191,192]

(E) Distribution of HIV quasi-species: typically, proviral HIV-1 RNA is circulating in plasma whereas integrated viral DNA is archived in the lymphocyte compartment;[20]

(F) Levels of immune suppression at the time of treatment initiation.[193-195]

The impact of these additional factors needs to be examined *in vivo*. While clinical trials provide an idealized environment to test the potential effect of a drug regimen under controlled conditions, the "real-world" effectiveness of a treatment intervention will best be studied in observational studies and cross-sectional analyses of treatment cohorts. Individualized care however requires flexibility and consistent allocation of resources, as well as access to highly specialized medical care and laboratory expertise.[196,197]

OVERCOMING CHALLENGES IN LOW-RESOURCE SETTINGS

WHO statistics indicate that great progress has been made in improving access to antiretroviral therapy in low- and middle income countries.[198] Evaluations of the real-world effectiveness of antiretroviral therapy should account for the additional challenge of implementing treatment programs in low-resource settings.[196]

When antiretroviral therapy was first introduced, many countries chose to establish standard first- and second-line regimens allowing the purchase of antiretroviral medications *en gros* to save cost. The establishment of stringent eligibility criteria and fixed treatment regimens also helped to ensure that scarce resources were allocated to those most in need when only few HIV specialists were available to take care of adults and children living with HIV/AIDS.

The urge to provide access to ART as soon as possible was immediate. With the help of the Global Fund, WHO and a number of successful public private partnerships, enormous effort was undertaken to expand access to therapy worldwide.[199-201] During the "3-to-5 Initiative", the majority of funding was allocated to the distribution of drugs; less attention was paid to the establishment of sustainable infrastructure to monitor resistance in the newly treated individuals, since it required substantial investments in laboratory facilities and expertise.[197,202-207]

The study presented in Study 2 aims to investigate the emergence of drug resistance in a cohort of children at a large pediatric hospital in Lima, Peru, gaining first-time access to structured ART. When

the program was initiated, drug resistance testing was not generally available. To save cost, the monitoring of viral load (semi-annually) and CD4 counts (every 3 months) was conducted on the same day for all patients at the Instituto Nacional del Salud (INS; Peruvian National Institutes of Health). When a major portion of this first pediatric treatment cohort began to show evidence of viral failure however, the need to test fur drug resistance emerged along with the quest for the most appropriate second-line therapy.[208] With cost being a major constraint, the cross-sectional analysis was designed to evaluate the feasibility and usefulness of different potentially cost-saving alternatives for the monitoring of drug resistance in low-resource settings:

(A) A cross-cohort design was selected since it is more affordable as it allows running batched samples simultaneously.[209]

(B) The oligonucleotide ligation assay (OLA) was evaluated as an alternative method to detect key drug resistance mutations without the need for costly sequencing staff and equipment.[32,33]

(C) The OLA from DNA was compared with the OLA from RNA with respect to sensitivity and its ability to predict treatment virologic success versus failure.[210]

(D) Samples for RNA consensus genotyping were transported as dried blood spots on filter cards obviating the need for expensive blood tubes and shipping requirements.[211-213]

CROSS-SECTIONAL ANALYSIS OF RNA VERSUS DNA RESISTANCE

The OLA was developed as a highly sensitive method to detect previously known or expected drug resistance mutations. The laboratory protocol and oligonucleotide kits are available upon request via the US National Institutes of Health. The method requires the appropriate equipment and trained laboratory staff to perform DNA/RNA extractions, to generate cDNA, and to perform PCR, ligation and ELISA assays. The laboratory needs to be adequate to accommodate the handling of infectious samples without cross-contamination. The final step in the protocol involves a relatively costly multi-color detection system to discriminate between mutant, wild type and mixed populations in the sample.[32,210,214]

Previous studies have shown promising results using the OLA method, including the analysis of samples from developing countries.[32,33,210] While most studies had focused on either DNA or RNA-OLA, it was still unclear which one of the two methods may be superior in the setting of population-based cross-sectional analyses[143,214,215]

DISPLAYING TREATMENT OUTCOMES ACROSS COHORTS

When entire treatment cohorts are evaluated on a fixed time schedule, rather than based on individual patient outcome, different analysis methods may be required. One of the aims of the analysis presented in Study 2 was to develop innovative ways of displaying overall-treatment outcomes across cohorts and real-world effectiveness of a given regimen over time.

When cohorts are assessed periodically rather than sporadically, laboratories are able to run batched samples. The expensive equipment and special expertise required to run these assays usually lead to the establishment of centralized laboratory infrastructure. This may lead to the need to ship samples across large distances. In many tropical countries, where the shipment of frozen infective samples provides an additional challenge, alternative modes of transportation have been sought, such as the use of filter cards to transport dried blood spots for viral load assessments.[216-218]

The periodic measurement of CD4 count remains standard of care in antiretroviral therapy, with the exception of few developing country studies proposing the absolute lymphocyte count as an alternative.[219,28,220] When clinical parameters are monitored along with CD4 counts, these parameters in combination may reveal useful to measure the real-life impact of ART.[221-223] It has been suggested that with delayed access to ART, clinical disease may be advanced and CD4 counts may be low, increasing the likelihood of virologic failure.[224] In Study 2 we aimed to explore new ways of comparing treatment outcomes within the same cohort over time, as well as from one cohort to another.

ACCESS AND ADHERENCE TO THERAPY

In addition to regular assessments of CD4 counts and viral loads, resistance testing should always be accompanied by standardized adherence measures to detect suboptimal or inconsistent exposures to antiretroviral drugs and the development of drug resistance early-on. Up-to-date knowledge about the prevalence of drug resistance mutations in treatment cohorts may also be used to guide efforts limiting the transmission of drug-resistant virus within a population.[215]
In Study 2, we used a standard adherence questionnaire developed by the Pediatric AIDS Clinical Trials Group (in Spanish and English language) for use in pediatric clinical trials. Once virologic failure has occurred, the measurement of drug resistance should help to detect "true cases of" treatment failure and discriminate patients who have acquired drug resistance from those who have not been using the medication at all. The reasons for failure should thus always be addressed with adherence questionnaires, MEMS caps or telephone reminders.[225] Adherence instruments however will never be able to replace drug resistance testing.

There has been extensive discussion about the ideal time point to initiate ART, a difficult decision especially in children facing life-long therapy. Recent studies suggest that chances to achieve long-term virus suppression may be diminished in patients starting ART late (especially in resource-limited settings where ART may begin long after patients have become eligible). This recent evidence lead to the adjustment of ART guidelines to accommodate earlier initiation of ART before any significant decrease in immunologic parameters has occurred.[226,66,227,228] Standardized methodologies are needed allowing the head-to-head comparison of treatment outcomes across populations over time to assist public health agencies and stakeholders in selecting not only the best regimen, but also the ideal timing of ART.[142] Measuring the real-world effectiveness/ impact of treatment programs[229] may also provide powerful tools to demonstrate the positive impact and cost-effectiveness of successful and timely antiviral therapy while antagonizing the recent decrease in HIV/AIDS funding ("donor fatigue").[230]

MANUSCRIPT (2)

"ANTIVIRAL RESISTANCE AND PREDICTORS OF VIROLOGIC FAILURE IN THE FIRST COHORT OF HIV-INFECTED CHILDREN GAINING ACCESS TO STRUCTURED ANTIRETROVIRAL THERAPY IN LIMA, PERU: A CROSS-SECTIONAL ANALYSIS"

Title Page

Antiviral Resistance and Predictors of Virologic Failure in the first Cohort of HIV-Infected Children Gaining Access to Structured Antiretroviral Therapy in Lima, Peru: A Cross-Sectional Analysis

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Abstract

Background

The impact of extended use of ART in developing countries has been enormous. To improve management and outcomes among pediatric patients, a thorough understanding of factors influencing therapeutic success is required. The current study aims to investigate the value and feasibility of cross-sectional drug resistance monitoring using DNA and RNA oligonucleotide ligation assays (OLA) in the first cohort of children gaining access to structured ART in Peru.

Methods

Between 2002-5, 46 eligible children started the standard regimen of AZT, 3TC and NFV (median age 5.6 years (range: 0.7-14y), median VL $1.7 \cdot 10^5$ RNA/mL (range: $2.1 \cdot 10^3$ – $1.2 \cdot 10^6$), median CD4-count 232 cells/μL (range: 1-1591). Of these, 20 patients were classified as CDC clinical category C and 31/46 as CDC immune category 3. At the time of cross-sectional analysis in 2005, adherence questionnaires were administered. DNA OLAs and RNA OLAs were performed from frozen PBMC and plasma, RNA genotyping from dried blood spots.

Results

During the first year of ART, 44% of children experienced virologic failure, with an additional 9% failing by the end of the second year. Virologic failure was significantly associated with the number of resistance mutations detected by DNA-OLA ($p < 0.001$) during cross-sectional analysis, but also with low immunologic CDC-scores at baseline ($p < 0.001$). Children previously exposed to unsupervised short-term ART showed significantly higher numbers of resistance mutations by DNA-OLA ($p = 0.01$). Detection of M184V (3TC resistance) by RNA-OLA and DNA-OLA demonstrated a sensitivity of 0.93 and 0.86 and specificity of 0.67 and 0.7, respectively, for identification of virologic failure. Mutations

N88D and L90M (NFV resistance) detected by DNA-OLA were correlated with virologic failure, whereas mutations at RT position 215 (AZT resistance) were not associated with virologic failure.

Conclusions

Previous unsupervised ART and severe immune suppression at baseline negatively impaired subsequent treatment success, whereas prevention of mother-to-child transmission with AZT +/- NVP did not. DNA-OLA from frozen PBMC provided a highly specific tool to detect archived drug resistance. RNA consensus genotyping from dried blood spots and RNA-OLA from plasma consistently detected drug resistance mutations, but merely in association with virologic failure.

Background

Antiretroviral therapy (ART) has, for the past years, increased the hope for survival of millions of people living with the human immunodeficiency virus (HIV) worldwide, adults as well as children. A clear survival advantage was achieved for HIV-infected patients with a dramatic decrease in new AIDS cases.[1] Immune reconstitution ensues when viral replication can be suppressed successfully over time.[2]

Once a first-line regimen has failed however, the reasons for such failure may be complex, including malnutrition and co-morbidities leading to poor absorption of medications. Lack of economic resources and education may further complicate the already difficult adherence to complex medication schedules.[3-11] Some patients may have been pre-exposed to intermittent or erratic courses of antiretrovirals through aid programs, private activities and contacts abroad. HIV-infected children may have also been infected with a resistant maternal virus through mother-to-child transmission (MTCT).[12, 13] In resource-limited settings where medications for standard first-line ART medications are often purchased *en bloc* and large groups of patients are started on ART simultaneously, cross-sectional drug resistance testing may be particularly useful.

This study aims to test the value and feasibility of cross-sectional resistance testing and innovative tools to display disease progression or clinical/immunological improvement in the first cohort of children starting ART in Peru. With Global Fund support, structured ART first became available in August 2002 to a select group of HIV-infected children at the Instituto Nacional de Salud del Niño (INSN) in Lima, based on the criteria established by the Guideline for the Management of the HIV-Infected Child by the Peruvian Ministry of Health (MINSA).[14-17]

In contrast to a neonatal cohort starting ART several years later, the majority of patients in this first cohort at the INSN were school-age, had already progressed to AIDS when starting ART and were born before the broad introduction of prevention of mother-to-child transmission (pMTCT) programs in Peru.[18] Therefore, most patients were considered ART-naive prior to starting the Peruvian standard first-line regimen, consisting of zidovudine (ZDV, 100 mg/m^2 every 12 hours) with lamivudine (3TC, 4 mg/Kg. every 12 hours) and nelfinavir (NFV, 25 mg/Kg. every 8 hours).[17]

At the time of introduction of ART in Peru, access to drug resistance testing was still limited. To save cost, alternative testing methodologies and transportation modalities were sought, such as the Oligonucleotide Ligation Assay (OLA)[19-21] and filter cards for the transportation of blood samples as dried spots.[22-26]

The aims of the study were:

1. To determine the prevalence of antiretroviral drug resistance in children with virologic failure versus no virologic failure.
2. To evaluate the sensitivity of the DNA-OLA from frozen peripheral blood mononuclear cells (PBMC) as compared to the OLA from virion RNA (plasma) and RNA consensus sequencing from dried blood spots.
3. To determine factors associated with virologic failure and drug resistance development.
4. To design a simple and integrative display of clinical/immunological progression of HIV disease after ART initiation

Methods

Patient population and study procedures

Participants underwent standard medical procedures and routine HIV medical care at the Infectious Diseases Service at the INSN according to the MINSA Guideline for the Management of the HIV-Infected Children, CD4+counts were determined every 3 months, and viral load every 6 months at the Peruvian National Institutes of Health (Instituto Nacional del Salud, INS).[16] Antiretroviral therapy for eligible patients was provided free of charge by the MINSA. Eligibility criteria for ART provided by the Peruvian Ministry of Health included: Established perinatal HIV infection* and age < 18 months, or age >18 months and CDC immune category 2 or 3. Exceptions were planned for asymptomatic patients with a rapid decline in CD4+ or viral load >100,000cp/ml (or >10,000-20,000 in those > 30 months). [16]

IRB approval was obtained at participating institutions in the US and Peru.

All eligible subjects undergoing ART according to the MINSA program who agreed to participate and whose parents/guardians had signed the informed consent, were included in the cross-sectional analysis. Basic clinical and virologic parameters until the date of testing were recorded from routine medical records and laboratory reports (viral load and CD4 testing data). Additional parameters were obtained such as CDC stage[27], opportunistic and other infections, medication and dosing information and adverse events attributable to ART. A previously published standardized adherence questionnaire (*PACTG P1042S*) was used at the time of cross-sectional analysis to systematically measure adherence based on information provided by parents and caregivers. [28, 29].

At the time of the first regular follow-up, blood sampling was performed at the INS after entry into the study. 5 ml of citrated blood were collected from study participants for resistance

* confirmed by DNA-PCR/viral load at 6 months, or by ELISA at/after 18 months or AIDS-defining diagnosis

testing. In addition, two Guthrie filter cards were collected with 4 capillary blood spots (finger prick) of 50 uL each.

Virologic Testing

Ficoll-Hypaque centrifugation and separation of the citrated blood was performed at the PRISMA laboratory in Lima. Plasma and PBMC were immediately stored separately at -20C and shipped on dry ice to the Tulane and LSU PACTU laboratory for RNA and DNA extraction. Viral loads in plasma were quantified by real-time RT-PCR as described.[30]

The OLA was conducted according to the NIH protocol for mutations at HIV-1B protease positions D30N, I50V, V82A, V82S, V82T, I84V, N88D, and L90M as well as reverse transcriptase positions K103N, Y181C, K65R, T215F, T215Y, M184V, and Q151M.[21, 31] Dried Blood Spots (DBS) collected on Guthrie cards were stored at room temperature to be shipped to the Stanford Center for AIDS Research for consensus RNA sequencing.[32]

Definition of Virologic Failure

For the purposes of the study, virologic failure was defined by two or more HIV RNA measurements above the detection limit (400cp/ml) 4 – 6 months after the initiation of ART therapy.

Sample Size Calculation

We assessed the population size N needed for assessing differences in resistance development between patients failing ART and those successfully treated.

We assumed that 50% of patients would eventually fail ART $P(\text{failure}) = 0.5$ and that those failing ART would with 90% probability develop drug resistance $P(\text{res.}|\text{failure}) = 0.9$.

Conversely, successfully treated patients may with 10% probability develop resistance $P(\text{res}|\text{success}) = 0.1$. We can therefore compute the expected number of patients with failure

and resistance $a = P(\text{res.}|\text{failure}) \cdot P(\text{failure}) \cdot N$, with failure and no resistance $b = \left(1 - P(\text{res.}|\text{failure})\right) \cdot P(\text{failure}) \cdot N$, with no failure and resistance $c = P(\text{res.}|\text{success}) \cdot \left(1 - P(\text{failure})\right) \cdot N$ and with no failure and no resistance $d = \left(1 - P(\text{res.}|\text{sucess})\right) \cdot \left(1 - P(\text{failure})\right) \cdot N$. According to Fisher's exact statistics $p = \dfrac{\binom{a+b}{a}\binom{c+d}{c}}{\binom{N}{a+c}}$ for the underlying contingency table, we could show significance at the 5% level ($p \leq 0.05$) for a sample size of $N = 12$. For values $P(\text{res.}|\text{failure}) = 0.8$, $P(\text{res.}|\text{failure}) = 0.7$ and $P(\text{res.}|\text{failure}) = \left(1 - P(\text{res.}|\text{sucess})\right)$ population sizes of $N = 12$ and $N = 22$ would be required.

Rates of clinical/immunological progression

The rate of clinical and immunological progression r_C and r_I respectively (average change of CDC score per year throughout the study population) were computed with the following formula

$\begin{pmatrix} r_I \\ r_C \end{pmatrix} = \begin{pmatrix} \sum_{m_I} F_{m,I} \cdot m_I \\ \sum_{m_C} F_{m,C} \cdot m_C \end{pmatrix}$, where m_I and m_C denote the magnitude (number of scores) of change observed and $F_{m,I}$ and $F_{m,C}$ the fraction that has changes by that magnitude within a certain time interval. For our evaluations, we computed the rates of immunological and clinical progression between enrolment and year 1, year 1 and year 2 and year 2 and the time thereafter.

Results

Demographics:

A total number of 46 children were enrolled between September 2002 and March 2005. Median age at enrolment was 5.6 years (range: 0.7-14y). The median virus load at enrolment was $1.7 \cdot 10^5$ RNA/mL (range: $2.1 \cdot 10^3$ – $1.2 \cdot 10^6$) and the median CD4-count was 232 cells/μL (range: 1-1591). Notably, five children had CD4 counts below 10 cells/μL. The median weight at enrolment was 18kg (range: 5.5-45). Notably, 43/46 (93%) had a negative z-score compared to the reference child weight[33] in their corresponding age groups, indicating malnutrition in this cohort. The median z-score was -2 (range: -4 to 0). The clinical categories at baseline and at each follow-up visit were classified according to the CDC 1994 Revised Classification System for HIV Infection in Children.[27] Seven children were recorded as clinical category N (not symptomatic), 4 children fell into clinical category A (mildly symptomatic), 15 were in category B (moderately symptomatic) and 20 were in category C (severely symptomatic). Notably, eight children (17%) were co-infected with active tuberculosis at enrolment. Children were also staged in terms of immune categories, according to the 1994 CDC classification system.[27] Four children were in category 1, 11 were in category 2, and 31 fell into category 3. Basic demographic characteristics are displayed in Table 1.

>> Table 1 <<

Vertical HIV transmission was the mode of infection for all but two children, who were infected by blood transfusion. Seven mothers had received antiretroviral prophylaxis with AZT +/- NVP for the prevention of mother-to-child transmission (pMTCT). Three children had been exposed to postnatal AZT for pMTCT (P019T, P020T, P028T) and four children had been exposed to unsupervised ART prior to enrolment: two children (P057T, P067T) received 3TC+AZT prior to enrolment. One child (P067T) continued NFV+3TC+AZT

without any gap, while P053T and P016T had received NFV+3TC+AZT prior to initiation of the program. One child P016T continued with only a few weeks interruption whereas for P053T there was a gap of one year between his prior ART medication and ART medication provided through this program.

Viral Dynamics & Virologic Failure Rates

Viral load measurements for all children are displayed in Figure 1A. Virologic failure was defined by two or more measurements demonstrating > 400 copies/ml RNA after 16 weeks of treatment (see filled squares in Figure 1A). The cumulative probability of virologic failure is shown in Figure 1B.

>>> Figure 1 <<<

As can be seen, 44% of children experienced virologic failure during the first year of ART, half of the children failed before the end of the second year of ART. By the end of the study 56 ± 15% had experienced virologic failure.

Both patients that were infected by blood transfusion (2/2) and all children with previous ART exposure (4/4) eventually experienced viral failure. None of the 7 children whose mothers had received pMTCT prophylaxis with AZT +/- NVP (0/7) and none of the children who had received post-natal AZT prophylaxis for pMTCT (0/3) experienced virologic failure.

Children who were younger at entry were slightly more likely to fail ART ($p = 0.06$ by Wilcoxon rank sum test). Virologic failure was significantly associated with the immunologic CDC-score at baseline (i.e. when starting structured ART; $p < 0.001$), with severely immune-suppressed patients being most likely to fail ART.

In contrast, the CDC clinical category at baseline was not predictive of virologic failure during subsequent ART. Children who had reported missing >50% of doses (according to the adherence questionnaire administered) were also more likely to experience virologic failure ($p = 0.05$).

>>> Figure 1 <<<

Rates of Immunologic & Clinical Progression

Neither immunologic CDC classification, nor clinical CDC classification at baseline were correlated with the age of the children (albeit the time between infection and start of therapy, $p = 0.39$ and $p = 0.83$)

Study participants were classified in terms of CDC clinical and immune categories at enrolment, during year 1, year 2 and after year 2, as shown in Figure 2A-D.

For the purpose of this analysis, CDC categories were applied in a novel way, assigning new CDC categories at each assessment time point ignoring previous CDC scores.

It can be seen that the majority of study participants are clustered in the lower right corner in Figure 2A (intensity of shading & percentages shown in the respective fields), which represents immunologic suppression (high immunologic CDC scores) and numerous opportunistic infections (immunologic scores 'B' & 'C'). After the onset of treatment, during year 1 (Figure 2B) the study participants are distributed almost equally throughout the space defined by the clinical- and immunologic classifiers. During year 2 after treatment initiation, most of the study participants showed evidence of immunologic recovery and an overall decrease in the rate of clinical signs of HIV/AIDS such as opportunistic diseases (increasing percentage are found in the upper left corner in Figure 2C). After year two, a higher percentage of subjects are represented in the upper left corner of Figure 2D, while at the same time there is a slight regression to the right, indicating an overall clinical deterioration.

>>> Figure 2 <<<

The rate of clinical/immunologic progression per year is shown in Figure 3B-D for the first year after enrolment (panel B), the second year after enrolment (panel C) and for the time thereafter (panel D). It can be seen that treatment had a very positive effect on both

immunologic and clinical parameters during the first year after initiation of ART as well as during the subsequent year (the blue arrow pointing towards the upper-left in Figure 3B and C). The rate of improvement was -0.4 immunologic stages and -0.77 clinical stages in the first year after treatment initiation and -0.65 immunologic and -0.61 clinical stages from year 1 to year 2.

Immunologic improvement was minimal during year three (-0.1 stages), whereas the clinical status of the study participants worsened slightly by 0.16 stages on average (the blue arrow pointing towards the upper-right in Figure 3D).

The immunologic CDC-scores at the time of final assessment were significantly correlated with virologic failure ($p<0.01$), with patients failing therapy having higher scores (i.e. being more severely compromised immunologically), while the final clinical CDC-scores were not linked.

>>> Figure 3 <<<

In summary, immunologic improvement became evident soon after initiation of ART and could be maintained in this cohort of first-line ART recipients, whereas the clinical improvement (with respect to CDC scores) seemed to lag behind, possibly due to the fairly advanced disease states at baseline.

Drug resistance testing

On average, the drug resistance testing was performed at 2.4 years after the initiation of structured ART. Prior to cross-sectional analysis of the treatment cohort, drug resistance information was not available to direct the choice of treatment regimens. In ART-failing patients, the vast majority of drug resistance tests (96%) were performed at time points after virologic failure.

Samples for RNA consensus sequencing were transported as dried blood spots on Guthrie cards. RNA amplification for consensus genotyping was possible in 14/46 samples (including 3 samples with a viral load slightly below 400 cp/ml), in 4 instances only the protease gene (PR) could be sequenced.

Overall, 70% of HIV-1 RNA sequences were derived from individuals eventually failing ART. In the remaining cases, RNA could be amplified from two patients whose viral load had just dropped below 400cp/ml, one had repeat measurements slightly below threshold.

Samples for DNA and RNA OLA testing were transported frozen as plasma and PBMC samples after Ficoll-Hypaque centrifugation and separation. Of these, RNA-OLA testing was performed successfully in 20/46 (43%), in one case only the protease mutations could be tested by RNA-OLA. As expected, the majority of samples yielding RNA-OLA results (80%) were derived from patients with detectable viral load. DNA-OLA testing however was successful in almost all patient samples (45/46, 98%), of which 47% showed no evidence of virologic failure at the time of testing. Hence, DNA-OLA from frozen PBMC provided a sensitive tool for the cross-sectional assessment of archived drug resistance in a patient cohort, whereas RNA consensus genotyping from dot blots as well as RNA-OLA from plasma virions yielded results mainly in those individuals with established virologic failure (over-representing those with viral loads above the 400cp/ml threshold).

Drug Resistance Mutations

The M184V reverse transcriptase mutation was detected in 80% of the sequenced RNA samples and tested positive in 74% and 47% by RNA-OLA and DNA-OLA, whereas thymidine associated mutations (TAMs: M41L, D67N, K70R, L210W, T215F/Y, K219Q/E [34]) were detected in 50% of sequenced viral RNA. Using RNA-OLA and DNA-OLA, the T215Y and T215F mutations tested positive in 47% and 42%, respectively.

The protease mutation D30N was detected in 43% of RNA genotyping samples and in 0% and 2% of available RNA- and DNA-OLA samples. The N88D and L90M protease mutations were detected in 36% and 21% of genotyping samples, in 25 % and 20% of RNA-OLA samples, and in 42% and 44% of DNA-OLAs, respectively.

Children who were previously exposed to short-term antivirals showed significantly higher numbers of resistance mutations detected by DNA-OLA ($p = 0.01$), but not by RNA-OLA ($p = 0.26$) or genotyping ($p = 0.18$) at the time of cross-sectional analysis. Virologic failure was strongly associated with the number of resistance mutations detected by DNA-OLA ($p < 0.001$).

The detection of the M184V reverse transcriptase mutation (indicating 3TC resistance) by any of the three methods (genotyping, RNA-OLA or DNA-OLA) was significantly more frequent in patients with virologic failure ($p = 0.07$[1], $p < 0.05$[2] and $p < 0.001$[3]). Also, the mutations N88D and L90M (NFV resistance) were more frequently detected in DNA-OLA in patients with virologic failure ($p < 0.001$ and $p < 0.05$, respectively). The protease mutation D30N was not detected more commonly in patients with virologic failure in any of the assays used, neither were TAMs selected differentially in failing vs. non-failing patients.

Detection of the M184V, N88D and L90M substitutions by RNA OLA was highly sensitive for predicting virologic failure (sensitivity: 0.93, 1.0 and 1.0). The ability to perform RNA OLA, together with positive detection of mutations M184V, N88D and L90M may suggest virologic failure in this cohort of patients.

The detection of the same mutations (M184V, N88D and L90M) by DNA-OLA yielded a slightly lower sensitivity of 0.86, 0.9 and 0.75 for predicting virologic failure, but the assay could be performed in almost all patient samples (regardless of virologic success or failure) indicating that virologic failure may indeed be attributed to resistance development at these

[1] Fisher's exact test
[2] χ^2 test

three residues (these specific mutations appear significantly more frequently in failing patients, see Table 2).

>>> Table 2 <<<

Relative sensitivities and specificities of the DNA- and RNA-OLA

We evaluated the DNA-OLA and RNA-OLA relative to each other: The DNA-OLA yielded a sensitivity of 59% relative to the RNA-OLA. Its relative specificity was 96%. Reversely, the sensitivity of the RNA-OLA relative to the DNA-OLA was 86%, whereas its specificity was 88%. (Table 3)

>>> Table 3 <<<

Discussion

Two aspects were quite unique in this patient cohort: a) all patients received the same first-line antiretroviral regimen and b) patients, on average, were in advanced stages of HIV/AIDS when starting their first antiretroviral regimen.[42] When antiretroviral therapy was first introduced in Peru, uniform criteria were established by the MINSA to ensure the allocation of resources and medication to those most in need. This first cohort of patients at the largest children's hospital in Peru suddenly became eligible for therapy at a time when many had already progressed to disease stages beyond the eligibility threshold.

The effect of delayed access to ART in this first cohort becomes evident in comparison to a recent study observing the transmission of resistant virus in a much younger cohort of neonates and children with timely access to pMTCT and ART in Peru, revealing a predominance of NNRTI mutations while mutations conferring high-level resistance to ARV were still found to be rare.[18] This observation is unlikely an effect of age. Even though our cohort started treatment after the disease had progressed significantly, age by itself was not associated with an advanced clinical stage at enrollment. To the contrary, young age (thus earlier treatment initiation) seemed to favor virologic failure. This may also be due to a survivor effect, i.e. slower progression in those patients who survived the first years after MTCT.

The rate of virologic failure was high in this first pediatric cohort gaining access to ART in Peru in 2002/3, with ~44% showing virologic failure after the first year of ART, ~53% after two years. The majority of children were in poor health, as evidenced by malnutrition (93% were below the reference weight in their age-class[33]) and a high prevalence of opportunistic infections with 43% showing AIDS-defining conditions and 17% co-infection with active tuberculosis. Immunologically, 67% of the children had already reached the immunologic

CDC category 3 (corresponding to an adult CD4 levels of < 200 cells/μL) prior to gaining access to structured ART regimens.

Immunologic classification at baseline was very predictive for virologic failure. In agreement with studies in industrialized countries[35, 36], this may indicate that the percentage of CD4 cells (i.e. the immunologic category) could be used to guide treatment initiation in this population. The immunologic classification may be more valuable for the decision of ART initiation than relying on DNA-PCR results alone.[37]

Despite relatively high rates of virologic failure in this cohort, both immunological and clinical conditions improved during ART, in particular throughout the first and second years of treatment. Thereafter little additional improvement was achieved. Overall, from the time of initiation of ART up until the time of the cohort assessment, 57% had shown marked improvement with respect to their clinical status (as measured by CDC category/visit), whereas 35% had not changed, and only 8% showed progression of disease. With respect to the immunologic CDC-scores, 76% had improved, 22% had experienced no change, and 2% showed a decrease in CD4 counts.

For improved visualization of the overall development of the cohort during ART, we summarized the clinical and immunological response to therapy in an innovative fashion using a Clinical Course Integrated Display with 3-by-4 tables based on the revised CDC clinical and immunological categories.[27] Here, we applied the CDC scores as a flexible tool to examine the cohort on a yearly basis, allowing for CDC scores to improve or deteriorate, according to the CD4 counts and reported clinical symptoms. Using this simple system in cross-sectional analyses and surveillance programs, rates of disease progression (Figure 3) may be computed for different cohorts allowing the comparison of treatment strategies in

terms of their clinical and immunologic effects in a given population. This system may be applicable to similar cohort studies in developed and developing countries, especially in conjunction with cross-sectional analyses of antiretroviral drug resistance.

Previous exposure to (often incomplete) ART was significantly associated with virologic failure, indicating that short courses of unsupervised ART prior to the initiation of coordinated long-term treatment programs may be counterproductive and lead to the development of drug resistance. Exposure of the newborn to post-natal pMTCT with AZT on the other hand, did not increase the likelihood for subsequent virologic failure neither did maternal exposure to pMTCT with AZT +/- NVP. Archived drug resistance mutations that have been acquired during previous exposure to antiretrovirals and that are still present in the PBMC compartment may be detected by DNA OLA.

The DNA OLA may be particularly useful for the purposes of population-based surveillance in low resource settings and in countries where genotyping tests may not be readily available. The DNA-OLA was very indicative for the presence of resistance (high specificity, low false pos. rate), but less indicative for the absence of resistance (low sensitivity, high false neg. rate) in comparison to the DNA OLA. To the contrary, the RNA-OLA was more useful for predicting the absence rather than the presence of resistance. Therefore, DNA-OLA can be used to rule-in resistance, whereas RNA-OLA may be used to rule-out resistance.

The detection of the resistance mutations M184V, N88D and L90M by DNA-OLA was highly predictive of virologic failure in this cohort treated with lamivudine-zidovudine-nelfinavir as first-line therapy. The analysis of archived HIV-DNA resistance in PBMC provided useful results in most patients, even if virologic failure was not (yet) evident. The DNA-OLA may detect resistance mutations that have been acquired during previous exposure

to erratic short-term ART, still present in the lymphocyte compartment. This may occur in low-resource settings before antivirals become universally available, when patients and their families are restricted to temporary access to limited, often insufficient amounts of antiviral medications. Turnover rates within the lymphocyte compartment may however be too low for the early detection of antiretroviral drug resistance during therapy (i.e. in time before viral failure becomes apparent).

A possible strategy for the improvement of ART in resource-poor settings (where genotyping is often not available) could be to use the DNA-OLA as a baseline screening tool before starting therapy. This could be combined with the use of RNA-OLA in those patients experiencing virologic failure. Notably, a positive RNA OLA at positions M184V, N88D or L80M was highly sensitive for virologic failure (sensitivity: 0.93, 1.0 and 1.0 respectively). Therefore, drug resistance monitoring at key residues using RNA OLA in patients experiencing virologic failure may be particularly useful as an economical indicator of drug resistance and could suggest a treatment change.

Success rates could likely be improved even further if treatment was initiated at higher CD4 counts, in line with recent revisions of the treatment guidelines in industrialized countries (initiation of treatment at an adult CD4 count of 350 cell/μL). [35, 36] This is in agreement with recent reports from other cohorts in Latin America. A recent cross-sectional analysis and evaluation of clinical outcomes of ART in Latin America showed that nearly half of the patients were so-called “late testers/presenters”. Evaluations of outcomes with ART in Latin American children revealed a higher incidence of opportunistic infections when compared to US cohorts (such as PACTG 129C).[35, 36]

While consensus RNA genotyping (if available) will likely remain the mainstay of individualized resistance testing during ongoing antiretroviral therapy, the applicability of the OLA in population-based surveillance remains to be fully assessed in larger cohorts, including the cost-effectiveness and the personnel and training required for either method. At the time of the study, genotyping was not available. In recent years, capacities for monitoring drug resistance have been expanded at the Peruvian INS including sequencing facilities and an e-health driven, web-based laboratory information system.[38, 39] The national ART program was expanded in 2004 to include larger parts of the population living with HIV/AIDS, including infants in earlier stages of HIV infection.[39-41]

Our data emphasize the need for timely antiretroviral treatment initiation and early HIV testing to contribute to this aim.[5, 12, 42, 43] For children undergoing therapy, regular follow-up visits with viral load and resistance testing and concrete measures to monitor and improve adherence (using PDA's, cellphone reminder and other e-health features) may be a key to success of ART in Latin America and beyond. [44-51]

Conclusions

1. HIV drug resistance was the major factor contributing to virologic failure of antiretroviral therapy in this cohort of children with delayed access to structured ART in Lima, Peru.

2. In most instances, virologic failure occurred early in the course of treatment and commonly after previous exposure to unsupervised ART, but not in relation to pMTCT.

3. The DNA OLA method detected antiretroviral resistance at key positions independently of virologic failure in the form of integrated DNA (in PBMC), whereas the RNA OLA detected antiviral resistance in viral RNA (in plasma) only after virologic failure. Antiviral resistance was more readily detected by OLA than by RNA consensus genotyping (from dried blood spots).

4. DNA-OLA could be used before treatment initiation to assess archived drug resistance and with standard regimens, in particular when previous exposure to ARV is anticipated. RNA-OLA could be used to guide treatment switches in patients experiencing virologic failure.

List of abbreviations

INS= Instituto Nacional del Salud (Peruvian National Institutes of Health)

IESN= Instituto Especializado de Salud del Niño

PRISMA = Asociación Benéfica Proyectos en Informática, Salud, Medicina y Agricultura

MINSA= Ministerio de Salud del Peru

PACTG=Pediatric AIDS Clinical Trials Group

ART= Antiretroviral Therapy

MTCT= mother-to-child transmission

pMTCT= prevention of mother-to-child transmission

ZDV = Zidovudine

3TC= Lamivudine (LMV)

NFV= Nelfinavir

NRTI = Nucleoside-analogue Reverse Transcriptase Inhibitors

NNRTI= Non-nucleoside-analogue Reverse Transcriptase Inhibitors

PI= Protease Inhibitor

OLA= Oligonucleotide Ligation Assay

PCR= Polymerase Chain Reaction

RNA= Ribonucleic Acid

DNA= Desoxyribonucleic Acid

WHO = World Health Organization

HIV = Human immunodeficiency virus

AIDS = Acquired Immunodeficiency Syndrome

Competing interests

None

Authors' contributions

Study concept and design: BAR, RAO, RVD, DKK. Acquisition of data: BAR, GSC, MEC, LK. Laboratory Analyses: BAR, PC; AMA, JER, DKK. Analysis and interpretation of data: MVK, BAR. Drafting of the manuscript: BAR, MVK. Critical revision of the manuscript for

intellectual content: DKK, RAO, RVD, AMA, GSC, PC. Statistical analysis: MVK. All authors read and approved the manuscript.

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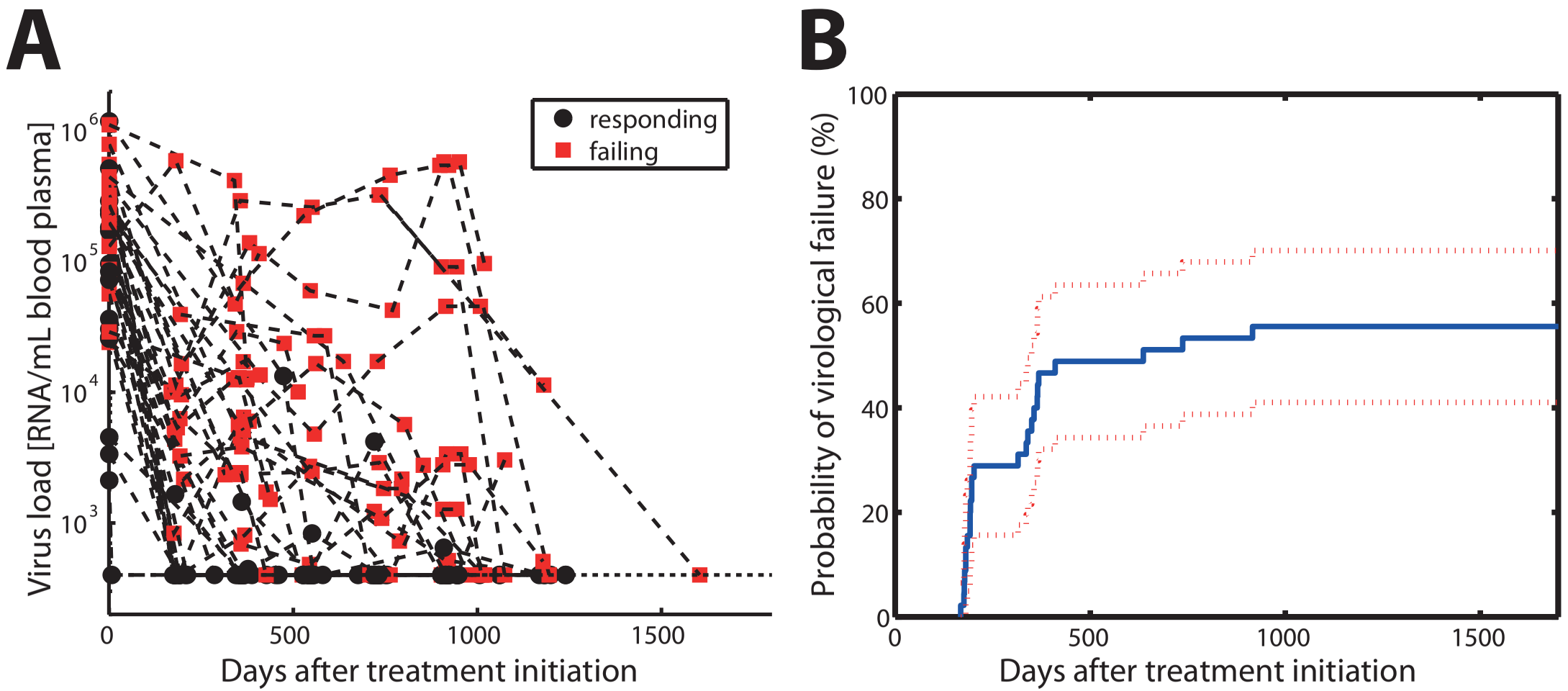
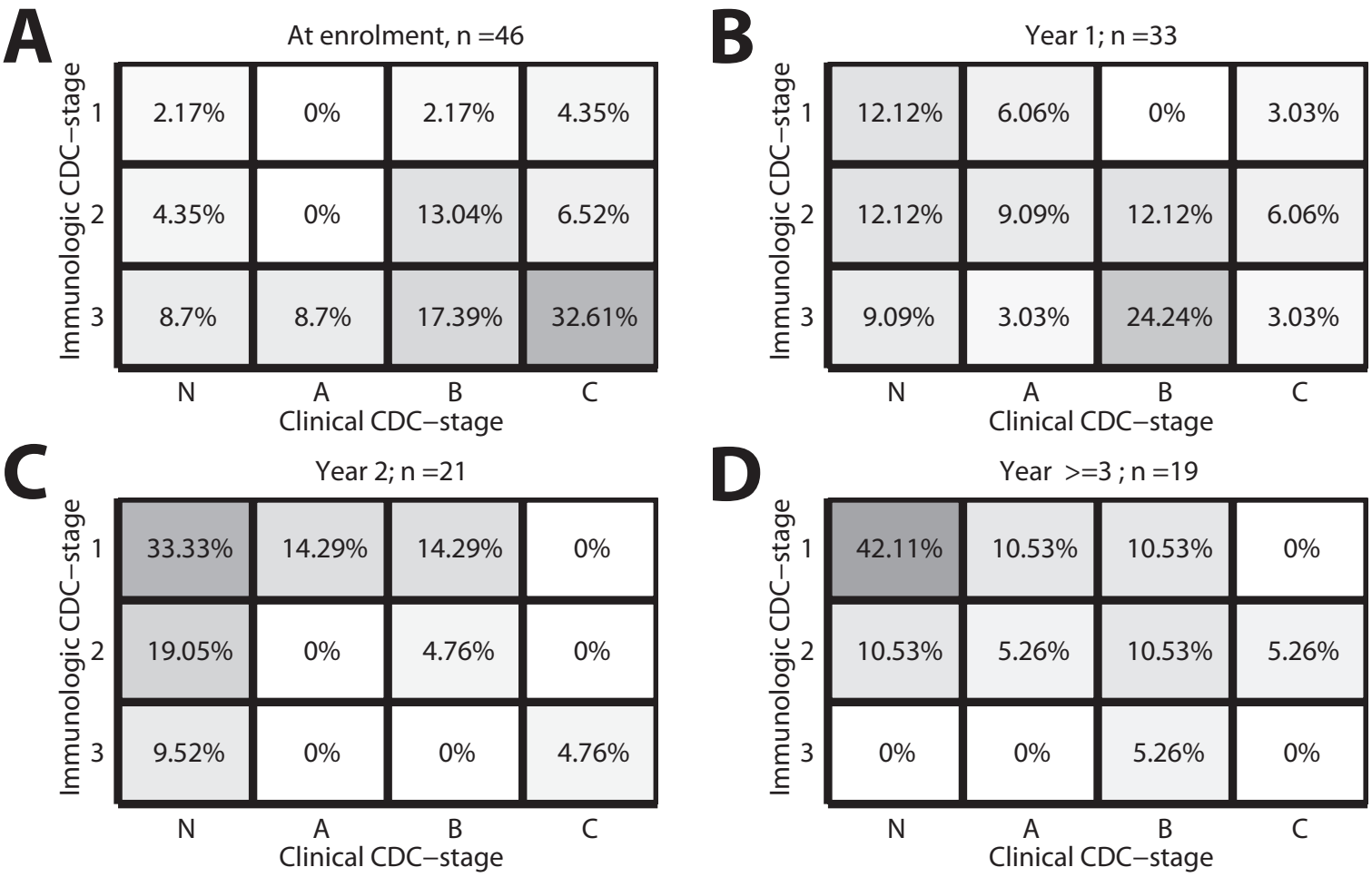
Figure Legends

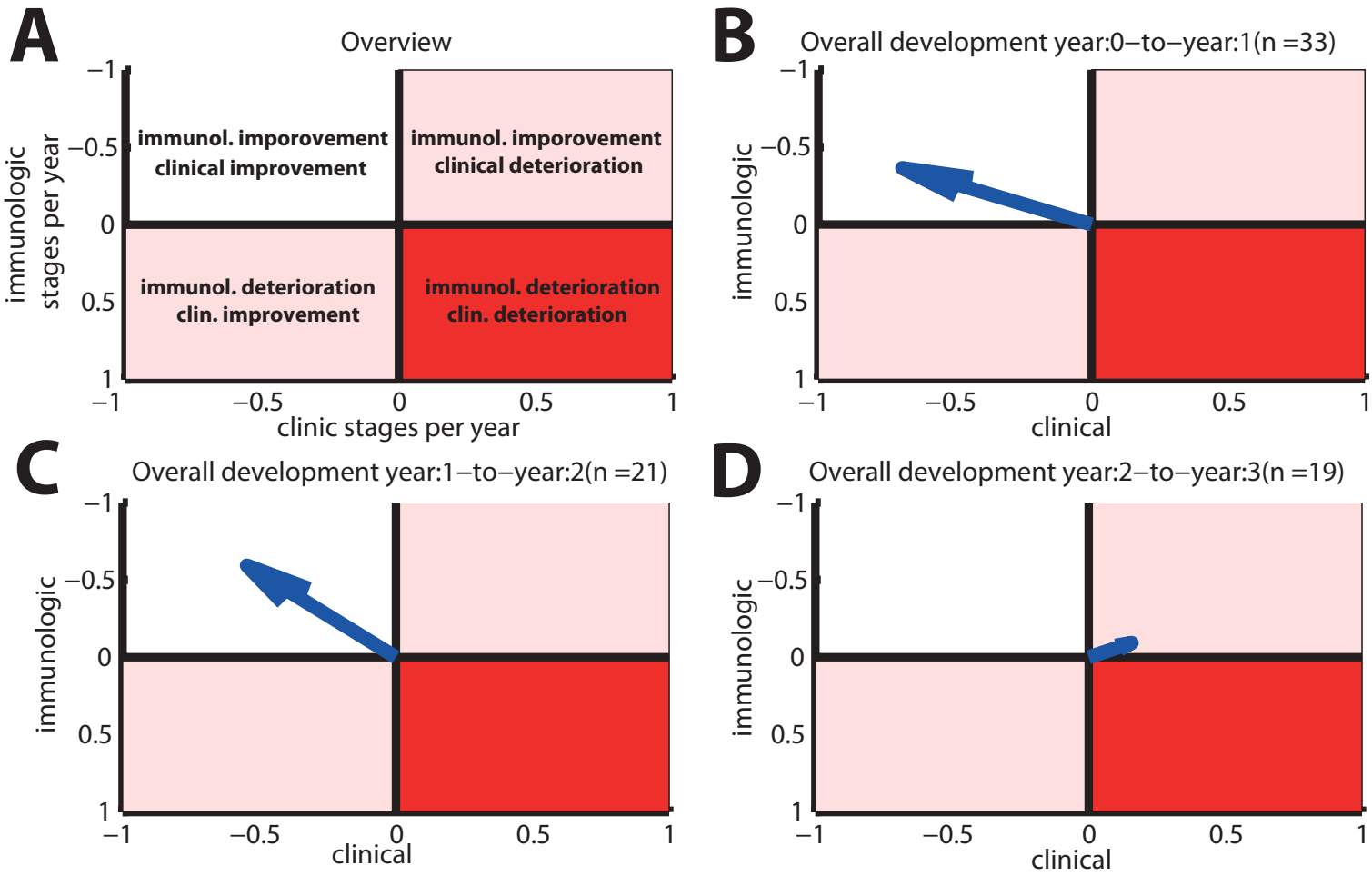
Figure 1: A: Viral load dynamics in children after treatment initiation. **B:** Cumulative probability of virologic failure after treatment initiation.

Figure 2: Immunologic and clinical classification of study participants at treatment initiation, through years 1-2 and ≥ 3 years after initiation of ART. The numbers in the distinct fields and the intensity of the shading represent the percentage of individuals falling within the respective CDC classification. **A:** Classification at enrolment. **B:** Classification during year 1 after treatment initiation. **C:** Classification during year 2 after treatment initiation and **D:** Classification after year 2.

Figure 3: Average rates of progression with respect to clinical and immune classifiers. **A:** The upper-left area indicates an overall improvement in terms of clinical and immune classifiers, whereas the upper-right area indicates immunological improvement but clinical deterioration. The lower-left area indicates immunological deterioration but clinical improvement, and the lower right area indicates deterioration with respect to both immunologic and clinical classifiers. **B:** The blue arrow indicates the overall rate of progression in the first year after treatment initiation (i.e. both clinical and immunologic parameters are improving). **C:** Overall rate of progression during the second year. **D:** Overall progression during the third year.


A
B
responding
failing
Virus load [RNA/mL blood plasma]
10^6
10^5
10^4
10^3
0
500
1000
1500
Days after treatment initiation
Probability of virological failure (%)
100
80
60
40
20
0
0
500
1000
1500
Days after treatment initiation


A
At enrolment, n =46
Immunologic CDC−stage
1
2
3
2.17%
0%
2.17%
4.35%
4.35%
0%
13.04%
6.52%
8.7%
8.7%
17.39%
32.61%
N
A
B
C
Clinical CDC−stage
B
Year 1; n =33
Immunologic CDC−stage
1
2
3
12.12%
6.06%
0%
3.03%
12.12%
9.09%
12.12%
6.06%
9.09%
3.03%
24.24%
3.03%
N
A
B
C
Clinical CDC−stage
C
Year 2; n =21
Immunologic CDC−stage
1
2
3
33.33%
14.29%
14.29%
0%
19.05%
0%
4.76%
0%
9.52%
0%
0%
4.76%
N
A
B
C
Clinical CDC−stage
D
Year >=3 ; n =19
Immunologic CDC−stage
1
2
3
42.11%
10.53%
10.53%
0%
10.53%
5.26%
10.53%
5.26%
0%
0%
5.26%
0%
N
A
B
C
Clinical CDC−stage


A
Overview
immunol. imporovement
clinical improvement
immunol. imporovement
clinical deterioration
immunol. deterioration
clin. improvement
immunol. deterioration
clin. deterioration
immunologic
stages per year
clinic stages per year
B
Overall development year:0–to–year:1(n =33)
immunologic
clinical
C
Overall development year:1–to–year:2(n =21)
immunologic
clinical
D
Overall development year:2–to–year:3(n =19)
immunologic
clinical

Tables

Table 1: Basic characteristics of study participants

	All n = 46	With subseq. virol. failure n = 26	Without subseq. virol. failure n = 20
Gender (male n)	27	16	11
Age (years)	5.6 (0.2;14)	5.0 (0.67; 13.9)	6.5 (0.7; 13.8)
Weight below WHO child reference (n)[33]	43	24	19
Weight median z-score (range)	-2.0 (-4; 0)	-2.5 (-4; 0)	-1 (-4; 1)
Baseline virus load (RNA/mL)	1.7e5 (2.1e3;1.2e6)	2.1e5 (2.4e4; 1.1e6)	8.4e5 (2.1e3; 1.2e6)
CD4 count (cells/µL)	232 (1; 1519)	154 (1; 1591)	381 (2; 870)
Tubercoulosis coinfection (n)	8	3	5
Clinical CDC stage			
N (not symptomatic)	7	5	2
A (mildly symptomatic)	4	3	1
B (moderately symptomatic)	15	7	8
C (severely symptomatic)	20	11	9
imm. CDC stage			
1	4	1	3
2	11	1	10
3	31	24	7

Table 2: Frequency of mutations detected in different assays.

	M184V	**TAM**	**n**	**Virol. Fail.**	**D30N**	**N88D**	**L90M**	**n**	**Virol. Fail.**
RNA Genotyping	**80%***	50%	10	70%	43%	36%	21%	14	70%
RNA-OLA	**74%****	47%[1]	19	84%	0%	25%	20%	20	80%
DNA-OLA	**47%*****	42%[1]	45	53%	2%	**42%*****	**44%****	45	53%

*associated with virologic failure ($p < 0.1$),

**strongly associated with virologic failure ($p < 0.05$),

*** very strong association with virologic failure ($p < 0.001$)

Table 3: Detection of resistance mutation with DNA-OLA vs. RNA-OLA.

	DNA+	DNA-	Sum
RNA+	36	25	61
RNA-	6	278	184
Sum	42	203	

The field ‘DNA+/RNA+’ denotes the number of resistance mutations positively detected by both DNA-OLA and RNA-OLA, whereas the field ‘DNA-/RNA+’ denotes the number of resistance mutations where the DNA-OLA yielded a negative result and the RNA-OLA yielded a positive result.

[1] only T215F and T215Y

4. PREDICTING INFLUENZA DRUG RESISTANCE *IN VIVO*

TRANSLATING FROM HIV TO INFLUENZA ANTIVIRAL THERAPY

While the periodic testing of virus kinetics and drug resistance has become common sense in the treatment of HIV infection, the same is not the case in influenza antiviral therapy. Compared to life-long HIV therapy, the treatment of influenza infection is considered a short-term affair. Influenza virus loads and drug resistance mutations are not monitored routinely outside clinical trials or sentinel surveillance programs. [73,231]

Several important differences between HIV-1 and influenza therapy need to be acknowledged. While current HIV therapy aims to interfere with the viral replication cycle at several targets simultaneously (see Figure 4, above), the antiviral agents that are currently recommended for the treatment of influenza (oseltamivir and zanamivir) are effective only at one step in the influenza life cycle, i.e. during the exit and release of infectious viral particles (Figure 7).[232] This means that monotherapy is currently the standard of care in the antiviral treatment of influenza infection.[73]

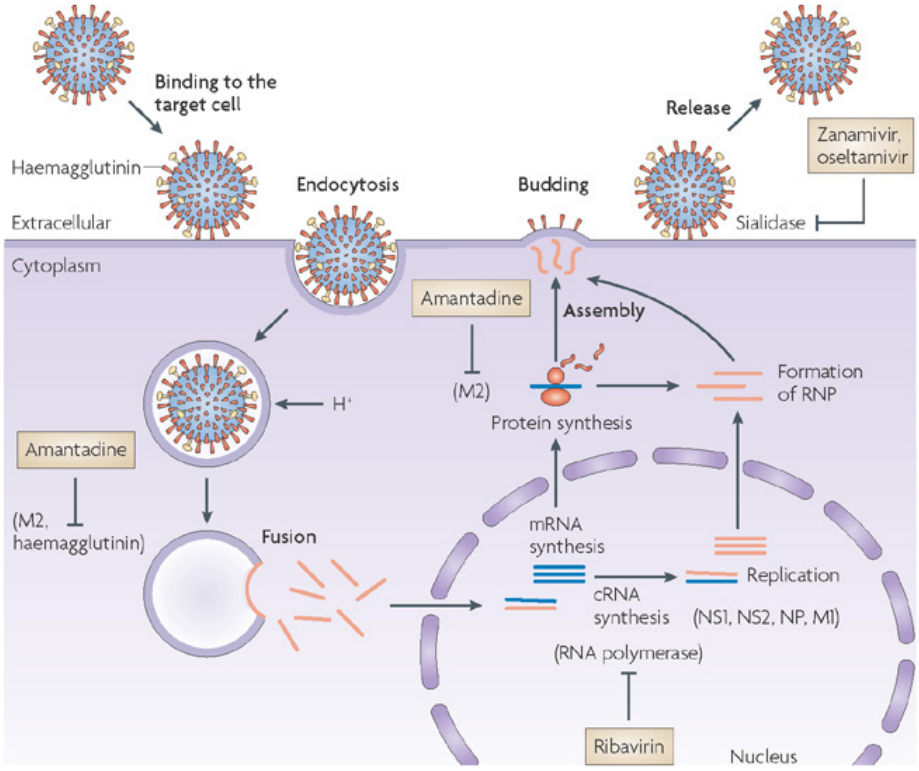


Figure 4 A: Influenza life cycle and potential drug targets (Source: von Itzstein, 2007[232])

As indicated in Figures 4 A-C, the knowledge of the exact mechanisms and clinical significance of drug resistance development in influenza, as well as the prevalence and location of important compensatory mutations in different influenza subtypes, is currently limited. To improve the

knowledge of the disease burden with influenza disease and the "real-world" effectiveness of antiviral therapy, a broad range of patients need to be studied in different age groups, including subjects with underlying conditions and other risk factors, who are not usually part of phase 1-3 clinical trials. [233-235] To this end, a pediatric quality management program was started in November of 2009 at the Charité University Department of Pediatrics in Berlin, Germany, in collaboration with the National Reference Centre for Influenza at the adjacent Robert Koch Institute (RKI), also in Berlin, Germany. In an attempt to minimize selection bias and inter-rater variability, all patients fulfilling pre-defined Influenza-Like Illness (ILI) criteria are screened prospectively, tested (using nasopharyngeal swabs/secretions) and monitored clinically until discharge from the hospital.

During the 2010/11 influenza season, patients who tested positively for influenza and whose physician decided to treat with neuraminidase inhibitors, were followed closely with clinical assessments as well as viral load and neuraminidase resistance testing. In collaboration with Klinikum Worms, additional patients treated in a semi-urban environment were included in the analysis.

ASSESSING BASELINE VIRUS LOAD AND SUBTYPE

The predictive value of self-reported symptoms[236] or physician's clinical diagnoses of "influenza" are low.[237] Depending on the clinical environment and the respective physicians in charge, the suspicion of "the flu" is often highly subjective and may be improved by the systematic use of screening criteria or case definitions.[238] Nevertheless, other viral infections are often misdiagnosed as "influenza".[239]

A basic step in improving the practice of influenza antiviral therapy is the timely establishment of the influenza diagnosis making use of rapid turnaround times for PCR diagnostics at RKI, supported by the use of point-of-care testing.[240,241] With the availability of RT-PCR diagnostics to all patients in the QM program, it became possible to estimate viral loads based on CT (cycle threshold) values.[242-244]

MONITORING VIRUS KINETICS AND DRUG RESISTANCE DURING THERAPY

The aim of repeat virologic testing was to establish virus load kinetics and to determine the "typical slope" of virus load decline during influenza antiviral therapy. To this end, the RT-PCR testing was repeated at least once during therapy. With the average course of oseltamivir treatment lasting five days,[71,73] the second obligational time point was determined to be day five of treatment. Additional follow-up testing during and after the antiviral treatment were optional. The viral load information was useful to the treating physician, as the need to maintain infection control measures depends on the duration of viral shedding.[243,245]

Little is known as to how long viral shedding 'should' last in different age groups[246-249] and whether virus load could potentially be linked to disease severity.[250] It seems intuitive however that clearance should occur sooner with antiviral treatment than without,[251] in adults sooner than children, and that additional host factors may contribute to the viral clearance rates.[252-254] It may further be assumed that the time to achieve non-detectable virus load will depend on the level of viral load at baseline, i.e. prior to treatment initiation,[242] and that there may be differences among influenza subtypes.[109]

PREDICTING DRUG RESISTANCE DEVELOPMENT

The analysis in Study 3 tests the hypothesis that impending development of neuraminidase inhibitor resistance may delay the clearance of influenza virus during antiviral therapy.[118,120]

The predominant oseltamivir drug resistance mutation is the Histidine to Tyrosine mutation in neuraminidase position 275 ("mutation H275Y"). The spread of oseltamivir-resistant pandemic influenza A H1N1 has thus far been limited due to the fitness disadvantage conferred by the H275Y mutation, but previous experience with seasonal influenza A H1N1 virus has shown that this handicap may ultimately be overcome resulting in rapidly spreading drug-resistant variants. [54,112] The monitoring of viral loads may thus be crucial when treating influenza, not only to detect issues with adherence/absorption but also to detect new mutational patterns and drug resistant variants early-on.[255]

If clearance rates under therapy were stable and /or comparable for the respective influenza virus subtype, the slope of viral clearance could indicate success versus failure of antiviral therapy. If the "slope" of viral clearance under therapy were to "lag behind" expectations for the respective virus subtype for example, this might be an early biomarker for emerging drug resistance and/or difficulties with drug absorption. The analogies to the monitoring of HIV therapy and the *in vitro* experiments described in Study 1 would be obvious.

MANUSCRIPT (3)

"VIRUS LOAD KINETICS AND RESISTANCE DEVELOPMENT DURING OSELTAMIVIR TREATMENT IN INFANTS AND CHILDREN INFECTED WITH INFLUENZA A (H1N1) 2009 AND INFLUENZA B VIRUSES."

Virus Load Kinetics and Resistance Development During Oseltamivir Treatment in Infants and Children Infected With Influenza A(H1N1) 2009 and Influenza B Viruses

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Background: Infants and small children are the most effective transmitters of influenza, while bearing a high risk of hospitalization and adverse disease outcomes. This study aims to investigate virus load kinetics and resistance development during oseltamivir therapy in infants and children infected with influenza A(H1N1) 2009 and influenza B viruses.

Methods: Virus load in nasopharyngeal samples and phenotypic/genotypic neuraminidase inhibitor resistance were determined at baseline, at day 5 and in additional follow-up samples, if available. Patient-specific viral clearance indices $CL_V(i)$ were determined along with estimates of the time required to achieve nondetectable virus load.

Results: No evidence of baseline oseltamivir resistance was detected in 36 patients infected with influenza A(H1N1) 2009 (n = 27) or influenza B (Victoria, Yamagata; n = 9) before oseltamivir therapy. On average, viral loads were lower for influenza type B (median = $5.9 \cdot 10^3$/mL) than for drug-resistant (median = $2.6 \cdot 10^6$/mL) and sensitive A(H1N1) 2009 (median = $4.8 \cdot 10^4$/mL), $P = 0.04$ and $P = 0.09$, respectively. Time required to achieve nondetectable virus load was significantly longer in drug-resistant A(H1N1) 2009 (median 15.4 days) compared with drug-sensitive A(H1N1) 2009 ($P = 0.003$; median 7.7 days) and drug-sensitive influenza B ($P = 0.001$; median 5 days). No evidence of viral rebound was observed once viral clearance was achieved.

Conclusions: Our data indicate that influenza subtyping in combination with baseline viral load measurements might help to optimize the duration of antiviral therapy in the individual child. Lower than expected virologic response rates in patients without malabsorption or compliance issues may suggest resistance development.

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This work was part of a quality management program at the Charité Department of Pediatrics in collaboration with the National Reference Centre for Influenza at the Robert Koch Institute, as well as an ongoing study (Neuraminidase Study) at Klinikum Worms. Treatment was provided either off-label or via Roche Protocol Nr. WP22849. B.R. and H.S. are principal investigators in the influenza surveillance programs with Robert Koch Institute as described above, as well as in Roche Study Protocol Nr. WP22849, Clinicaltrials.gov Identifier NCT00988325 (B.R. and H.S.) and Protocol Nr. NV25182, Clinicaltrials.gov Identifier NCT01286142 (B.R.); both institutions (Charité and Klinikum Worms) have received research funding from Roche. No personal funding has been received by B.R. M.v.K. received research funding through the DFG-research center MATHEON. The authors have no other funding or conflicts of interest to disclose.

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The risk of complications from seasonal and pandemic influenza is high in children, in particular in infants and small children, who carry the highest per capita risk of hospitalization.[1–4] Antiviral treatment with oseltamivir (Tamiflu, Roche Laboratories Inc, Nutley, NJ) has so far been approved only for adults and children older than 1 year of age,[5] with the exception of an emergency use authorization during the influenza A(H1N1) 2009 pandemic.[6] Retrospective studies in the United States and Germany suggest that oseltamivir may be tolerated well in infants[7,8] following dosing recommendations by the US Centers for Disease Control and Prevention, the Infectious Diseases Society of America and the German Society for Pediatric Infectious Diseases.[5,9–12]

Little is known about the virologic impact of oseltamivir therapy in infants, where in the absence of timely and effective therapy, serious disease outcomes may occur.[5,9] Infants and small children are known to display elevated viral loads at disease onset and an increased risk of prolonged viral shedding[5,13–17] with significant implications on infection control.[18] In the absence of therapy, viral loads tend to remain high during the first 3 days of illness, especially in children with severe disease or underlying conditions.[17,19]

The optimal duration of treatment with neuraminidase inhibitors is difficult to determine in the individual child. The Centers for Disease Control and Prevention guidelines state that treatment regimens beyond 5 days may be required in severely ill or immunocompromised individuals with a risk of ongoing viral replication.[5] Similarly, the World Health Organization (WHO) guidelines recommend that "where the clinical course remains severe or progressive, despite five or more days of antiviral treatment, monitoring of virus replication and shedding, and antiviral drug susceptibility testing is desirable. Antiviral treatment should be maintained without a break until virus infection is resolved or there is satisfactory clinical improvement."[10]

The aim of this study is to investigate neuraminidase resistance development,[20–22] virus load kinetics and basic clinical characteristics in 2 hospital-based cohorts of infants and children undergoing oseltamivir therapy after being infected with either influenza A(H1N1) 2009 or influenza B. The goal is to assess correlates of drug response toward optimizing duration of treatment in this sensitive age group.

METHODS

Patient Population

During the 2010 to 2011 influenza season, the pediatric departments at Charité University Hospital (Charité Influenza-Like

Disease = ChILD Cohort)[23] and Worms City Hospital (Klinikum Worms)[8] monitored viral load and resistance development in infants and children undergoing oseltamivir treatment for influenza infection in collaboration with the German National Reference Centre at Robert Koch Institute after approval by the respective Institutional Review Boards.

Patients with laboratory-confirmed influenza started treatment with oseltamivir phosphate within the first 2 days after disease onset, in accordance with German (DGPI) and WHO-EURO recommendations.[12,24] The average duration of therapy was 5 days, but could be extended when there was clinical evidence of ongoing viral replication (as defined according to the WHO: "persistent or recurrent high fever and other symptoms beyond three days without signs of resolution").[12]

Baseline clinical factors and nasopharyngeal swabs for real-time polymerase chain reaction (RT-PCR) were obtained immediately before the first dose, on day 5 of treatment and during additional follow-up visits, if applicable. Temperature and basic clinical factors (rhinitis, cough, vomiting and diarrhea) were also recorded at the time of the swabs.[25]

Virology

Nasopharyngeal swabs were obtained by a specifically trained study team and delivered to the laboratory immediately, optimizing both sample quality and sensitivity of subsequent RNA extraction and viral culture.[26,27] Both centers kept samples refrigerated until sending to Robert Koch Institute overnight. Virologic testing was conducted at the German National Reference Centre for Influenza.

Polymerase Chain Reaction Detection

Nasopharyngeal swabs were washed out in 2.0 mL cell culture medium. RNA was extracted from 300 μL using the MagAttract Viral RNA 48 Kit (Qiagen, Hilden, Germany) and eluted in 80 μL elution buffer. Synthesis of complementary DNA was performed using 25 μL of RNA and 200 U M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, California) in a total reaction volume of 40 μL. RT-PCR was performed in 25 μL reaction mix using 3 μL of complementary DNA, primer and probes targeting the M gene for universal detection of influenza A viruses as well as primer and probes targeting the HA and NA genes for further subtyping of influenza A viruses including the specific detection of A(H1N1) 2009 viruses as recently described.[28] Identification and differentiation of influenza B viruses into the B/Victoria/2/87 lineage (B Victoria) and the B/Yamagata/16/88 lineage (B Yamagata) were done according to Biere et al.[29] Determination of the viral load was performed by comparative analysis of the RT-PCR threshold (threshold cycle) values obtained for each influenza-positive patient sample with serially diluted plasmid standards. All reactions were performed using the Light Cycler 480 real-time PCR system (Roche Deutschland Holding GmbH, Germany).

Antiviral Resistance

Genotypic resistance to neuraminidase inhibitors was analyzed as recently described.[21] In brief, a 229 bp fragment was amplified by PCR using a primer pair with a biotinylated sense primer followed by pyrosequencing for detection of resistance-associated NA-N1substitutions H274Y and N294S. Genotypic resistance analysis of influenza B viruses was performed by cycle sequencing (primer sequences on request). Phenotypic resistance analysis was performed for viruses that could be isolated from patient specimens. The 50% inhibitory concentration (IC_{50}) for oseltamivir and zanamivir was determined in a fluorometric enzyme assay with 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (Sigma-Aldrich, St. Louis, MO) used as substrate.[30] Oseltamivir resistance of influenza A(H1N1) 2009 viruses was characterized by NA-H274Y mutation and/or IC_{50}-values >100 nM (100-fold compared with sensitive viruses).[21] Type B influenza viruses with >10-fold increased IC_{50}-values were defined as resistant to neuraminidase inhibitors (>300 nM).

Calculation of Virus Load Kinetics

For the purpose of viral clearance estimates, all patients were considered, where at least one baseline viral load and one day 5 viral load measurement were available (n = 36).

Patient-specific viral clearance indices $CL_V(i)$ [1/day] were estimated by the optimization routine *lsqcurvefit* in MATLAB version 7.10 (MathWorks Inc., Natick, MA), using a weighted least-squares criterion to minimize the residual error:

$$\underset{V_0(i),CL_V(i)}{\arg\min} \sum_t \left| \frac{\hat{V}(i,t) - V(i,t)}{V(i,t)} \right|^2 \quad (1)$$

where $\hat{V}(i,t)$ and $V(i,t)$ denote the estimated and the experimental viral load in nasopharyngeal secretions of patient i at time t after treatment initiation, respectively and $V_0(i)$ denotes the patient-specific virus load at treatment initiation. $\hat{V}(i,t)$ was assumed to exhibit a first-order decay according to:

$$\hat{V}(i,t) = V_0(i) \cdot e^{-t \cdot CL_v(i)} \quad (2)$$

Estimating the Time Required to Achieve Undetectable Viral Load

We defined the time required to achieve undetectable viral load as the time to the first viral load measurement below levels of detection by RT-PCR (approximately 10 copies/ reaction). Based on our virus clearance estimates (above), we assessed the time when virus load would drop below limits of detection $t_{\varnothing}(i)$ for each patient i. We therefore set $\hat{V}(i,t_{\varnothing})=10$ (copies/reaction) in the equation above and solved for

$$t = t_{\phi}(i) = \ln\left(\frac{V_0(i)}{10}\right) \cdot \frac{1}{CL_V(i)} \quad (3)$$

Clinical Predictors of Viral Load

We assessed the correlation of virus load measurements in nasopharyngeal secretions with clinical parameters (maximum temperature, and symptoms of rhinitis, cough, diarrhea, vomiting) in terms of linear and rank correlation.

RESULTS

Patient Characteristics

Eligible Patients

At Charité, 9 inpatients with laboratory-confirmed influenza during the 2010 to 2011 season were not treated with oseltamivir (age > 2 years and no risk factors) whereas the remaining 22 were treated, and all were followed up with at least one baseline and one day 5 sample. Five Worms patients were excluded from the analysis when either the baseline sample was taken after the initiation of oseltamivir treatment[4] or the day 5 sample was missed.[1] All remaining 14 Worms patients were included in the analysis.

The overall number of eligible patients (ie, patients with at least 2 viral load measurements before and during therapy) in both cohorts was n = 36. Of these, 27 (75%) were infected with influenza A(H1N1) 2009, 8 (22%) with influenza B (Victoria) and 1 (3%)

with influenza B (Yamagata). All baseline isolates were sensitive to oseltamivir. In the group of A(H1N1) 2009 infected patients, 7 of 27 (26%) developed oseltamivir resistance. All viruses remained sensitive to zanamivir.

Patient characteristics for all eligible patients from both cohorts, Charité University Hospital, Berlin (CH; n = 22) and Worms City Hospital (WO; n = 14) are displayed in Table, Supplemental Digital Content 1, http://links.lww.com/INF/B220. At Charité, preexisting conditions were noted in 3 of 22 (14%) patients, and 19 of 22 (86%) patients were below the age of 2. At Klinikum Worms, preexisting conditions were noted in 4 of 14 (28%) patients whereas all 14 (100%) were below the age of 2. Patients who experienced a decrease in virus load during treatment were defined as treatment "responders." Patients who showed a virus load increase or plateau while on therapy, were termed "non-responders."

Responders

In total, n = 32/36 (89%) patients responded to oseltamivir treatment by a decrease in virus load upon treatment initiation; 24 (75%) oseltamivir responders were infected with influenza A(H1N1) 2009 and 8 (25%) were infected with influenza type B (Victoria). Of patients who had additional follow-up samples taken after nondetectable viral loads had been achieved (n = 21), viral load remained nondetectable in all cases, including 2 of 2 patients who had developed oseltamivir resistance.

Nonresponders

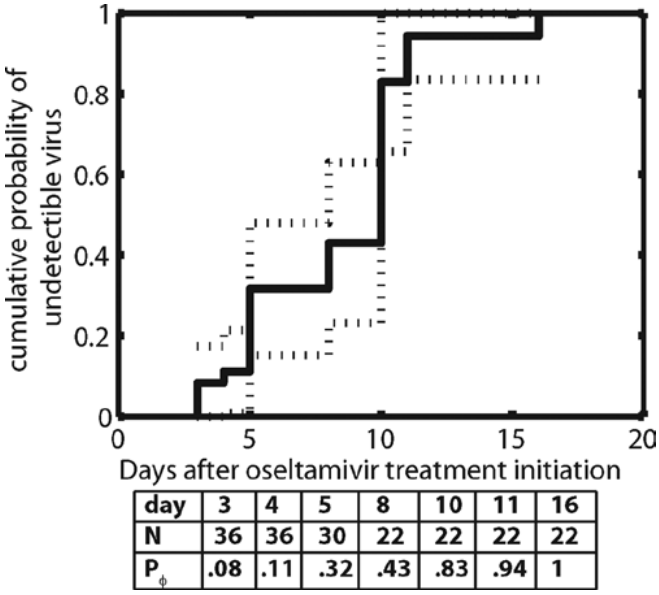
Of the 4 nonresponders (ie, patients who did not show a decrease in viral load during therapy: WO13, WO2, CH11, CH20), 3 patients (WO13, WO2 and CH20) presented with severe diarrhea and/or vomiting before initiation of therapy, which may suggest malabsorption. One of them (WO13) subsequently developed oseltamivir resistance. This patient (WO13), who also had a cardiac malformation and pneumonia, was also the only patient who showed a coinfection with respiratory syncytial virus. One patient with influenza A(H1N1) 2009 (WO2, no underlying conditions) failed to respond without development of resistance as did 1 patient with Yamagata Line Influenza type B (CH20, cardiac malformation). Patient CH11 was without underlying conditions but diagnosed at the very onset of symptoms with very low baseline viral loads (162 copies/mL), possibly before reaching a peak viral load.

Kaplan-Meier Estimate of Cumulative Probability to Achieve Undetectable Virus

The cumulative probability to achieve undetectable virus load in nasopharyngeal secretions of all patients in both cohorts is shown in Figure 1. All patients in the Charité (ChILD) cohort (n = 22) eventually reached undetectable virus load. None of the Worms (NA Study) patients (n = 14) had reached undetectable virus load at their final visit on day 5 of therapy. However, patients at the site in Worms had significantly higher initial viral loads compared with the patients from Berlin (P = 0.0001). Overall, our data indicate that 32% of patients had achieved undetectable virus loads by day 5, and the majority (94%) achieved undetectable virus load by day 11. One patient who achieved undetectable virus load only by day 16 (CH10), had developed drug-resistant influenza A(H1N1) 2009 during the course of treatment.

Virus Kinetics During Oseltamivir Treatment

For all 36 eligible patients (Fig. 2A–C), we estimated individual viral clearance $CL_v(i)$ according to Equation 1. Viral clearance estimates were not different for patients coming from the 2 distinct hospitals participating in this study (Klinikum Worms versus Charité, Berlin), P = 0.55. We found that patients infected with drug-sensitive A(H1N1) 2009 were most effective in eliminating the virus (median CL_v = 1.36/day, range = [−0.18; 2.26]), followed by patients infected with drug-sensitive Influenza B (median CL_v = 0.88/day, range = [0.37; 2.49]). Patients infected with oseltamivir-resistant A(H1N1) 2009 cleared the virus least effectively (median CL_v = 0.75/day, range = [−0.13; 1.28]); see Figure 3A and Table, Supplemental Digital Content 2, http://links.lww.com/INF/B221.



day	3	4	5	8	10	11	16
N	36	36	30	22	22	22	22
P_ϕ	.08	.11	.32	.43	.83	.94	1

FIGURE 1. Kaplan-Meier estimate of the cumulative probability to achieve undetectable virus load. The solid lines indicate the empirical estimate of the cumulative probability to achieve undetectable virus in nasopharyngeal secretions. The dashed lines are the confidence bounds, which were computed using the Greenwood formula.

Virus clearance was significantly lower in A(H1N1) 2009 infected patients, who developed oseltamivir resistance compared with drug-sensitive A(H1N1) 2009 infected patients (P = 0.03, Wilcoxon rank-sum test). Virus clearance in patients infected with drug-sensitive Influenza B and drug-sensitive A(H1N1) 2009 was not significantly different (P = 0.59). There was also no significant difference in viral clearance between patients infected with drug-sensitive Influenza B virus and drug-resistant A(H1N1) 2009 at the 5% level (P = 0.35).

Estimating the Time Required to Achieve Undetectable Virus Load

We estimated the time required to achieve nondetectable virus load by RT-PCR $t_\varnothing(i)$ (using Equation 3). In 2 of the nonresponders (WO2 and WO13), we observed a slight increase in virus load at the second/final measurement on day 5; therefore, we cannot infer the time when these patients would have eventually reached nondetectable virus load. The estimated time required to achieve nondetectable viral load for the remaining patients is depicted in Figure 3B.

Influenza A(H1N1) 2009 infected patients developing oseltamivir resistance appear to require the longest time to achieve nondetectable virus load (median = 15.4 days, range = [10; 25.2]) in

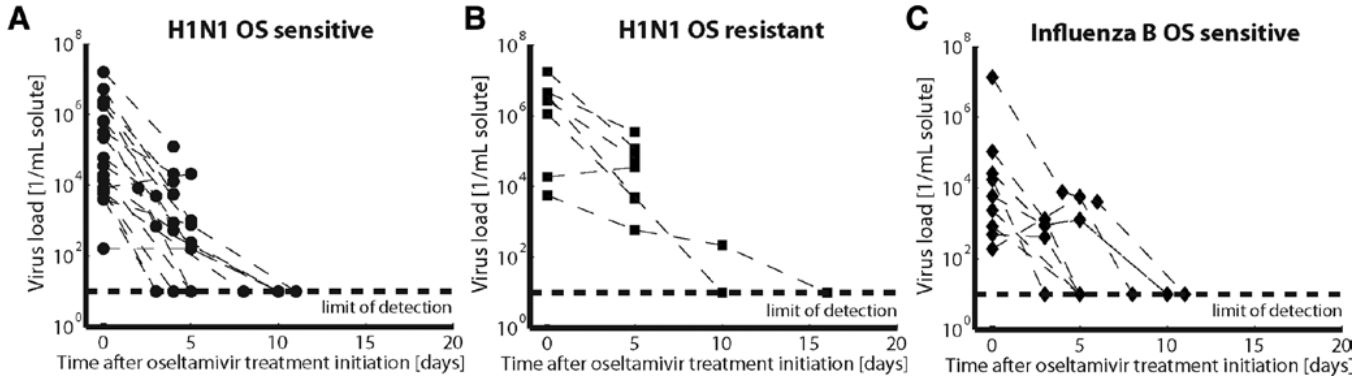


FIGURE 2. Viral dynamics after treatment initiation. A, Viral kinetics in patients infected with drug-sensitive influenza A(H1N1) 2009 (filled circles). B, Viral kinetics in patients infected with drug-resistant influenza A(H1N1) 2009 (filled squares). C, Viral kinetics in patients infected with drug-sensitive influenza B (filled diamonds).

comparison to drug-sensitive influenza A(H1N1) 2009 (median = 7.7 days, range = [3; 15.2]) and influenza B (median = 5 days, range = [3; 11.5]).

Time required to achieve nondetectable virus load was significantly longer in drug-resistant A(H1N1) 2009 compared with drug-sensitive A(H1N1) 2009 ($P = 0.003$) and drug-sensitive influenza B ($P = 0.001$). A pooled estimate of the median time required to achieve nondetectable viral loads in nasopharyngeal secretions yielded a value of 8 days.

Note that our estimate of the time required to achieve nondetectable viral load takes into account both the individual viral load before treatment initiation $V_0(i)$ and the estimated virus clearance rate $CL_v(i)$. On average, influenza type B viral loads (median = $5.9 \cdot 10^3$/mL) were lower than those of drug-resistant (median = $2.6 \cdot 10^6$/mL), and sensitive A(H1N1) 2009 viruses (median = $4.8 \cdot 10^4$/mL), $P = 0.04$ and $P = 0.09$, respectively.

Clinical Predictors of Viral Load

The correlation between viral load and fever is depicted in Figure 4A. On the basis of all available data (fever/virus load at presentation and during follow-up), we found a weak positive (albeit not linear) correlation indicated by Spearman rank correlation coefficient ($R_S = 0.49$). This correlation was significantly different from zero ($P < 0.001$), indicating that high fever indicates large viral load. The precise (mathematical) relation of these 2 parameters is however not known. Therefore, it is not possible to derive crude estimates of individual viral loads based on temperature measurements alone.

We also assessed whether the number of key symptoms present (rhinitis, cough, diarrhea, vomiting) would indicate larger viral loads (Fig. 4B). In fact, we found that the severity of illness (defined by 0–4 key symptoms) correlates with viral load $R_S = 0.44$. This correlation was significantly different from zero

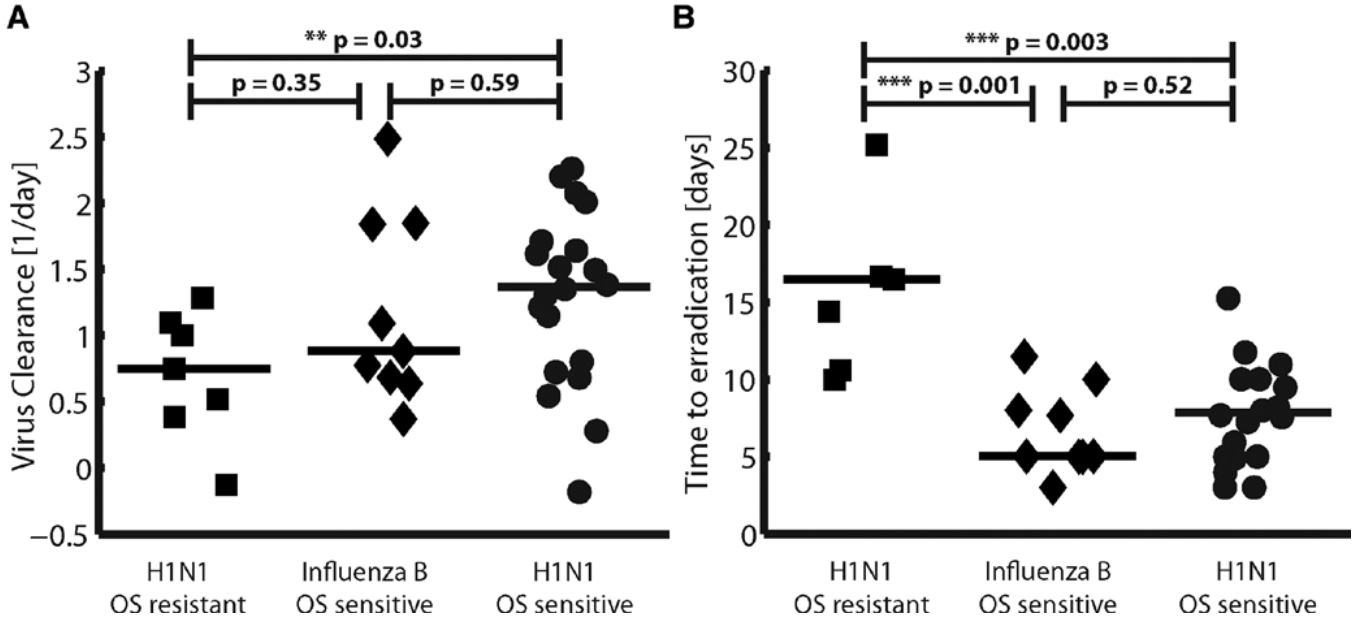


FIGURE 3. Estimated viral clearance $CL_v(i)$ and time required to achieve nondetectable viral load in nasopharyngeal secretions. A, Estimated viral clearance. B, Estimated time to achieve nondetectable virus load after initiation of oseltamivir treatment. Estimates in patients infected with drug-resistant A(H1N1) 2009, drug-sensitive influenza B and drug-sensitive A(H1N1) 2009 viruses are depicted by filled squares, filled diamonds and filled circles. OS indicates oseltamivir.

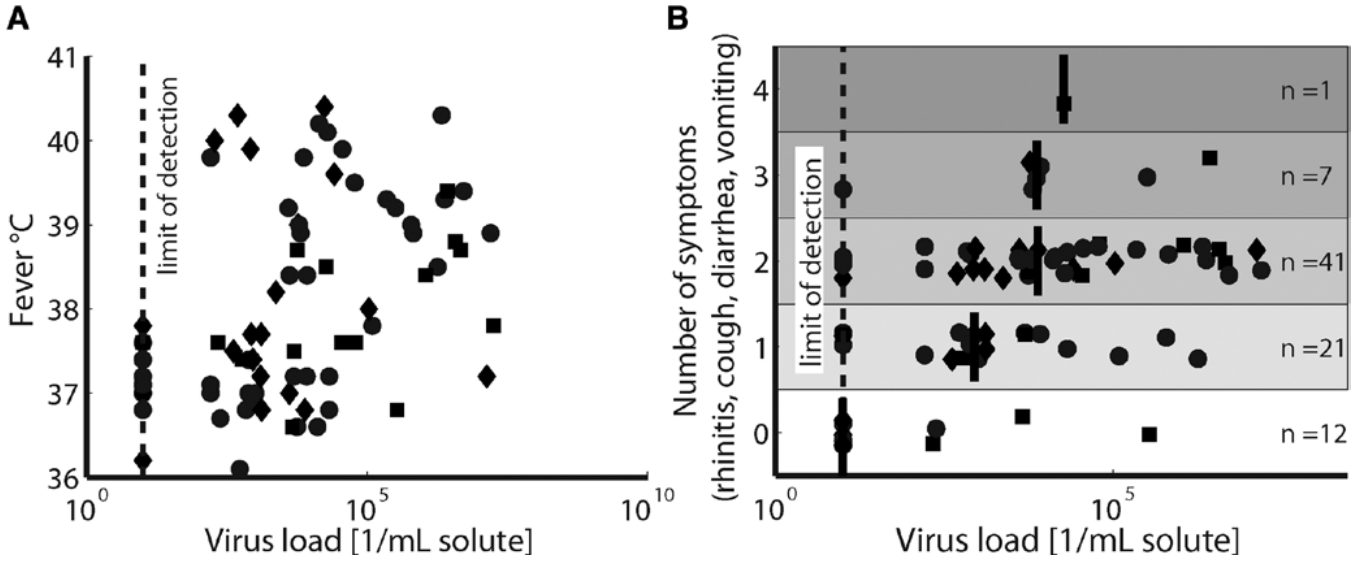


FIGURE 4. Observed correlation between virus load and fever and between the number of symptoms and virus load in nasopharyngeal secretions. A, Correlation between virus load and fever in all patients. B, Correlation between the number of symptoms observed and the measured virus load. The solid vertical bars denote the respective median virus load for each category. The number of data points available for each category is denoted at the right side of the illustration. Data from patients infected with influenza B are displayed by filled diamonds, whereas data from patients infected with drug-sensitive or drug-resistant A(H1N1) 2009 influenza viruses are depicted by filled squares and circles, respectively.

($P < 0.001$), indicating a positive association between the number of key symptoms and viral load in our study. However, further research is warranted to derive more sophisticated relations between clinical endpoints and virology.

DISCUSSION

Our data allow a first comparison of resistance development, clinical response rates and viral kinetics in infants and children with pandemic influenza A(H1N1) 2009 and seasonal influenza B, the most prevalent strains during the 2010 to 2011 flu season in Europe.[31]

No evidence of baseline oseltamivir resistance was detected in 36 pediatric patients infected with influenza A and B before initiation of antiviral therapy. These findings are consistent with recent WHO data showing no extended circulation of neuraminidase inhibitor-resistant pandemic influenza A(H1N1) 2009 viruses in the general population.[22] Enhanced surveillance in the Netherlands and in Germany of A(H1N1) 2009 viruses revealed only 1 case of 1100, and 2 cases of 1580, respectively, of resistant viruses not linked to oseltamivir therapy.[21,32]

The occasional appearance of neuraminidase-resistant pandemic influenza A(H1N1) 2009 during oseltamivir therapy was observed in our study as well as by other investigators[33] but resistance development appeared to be a self-limited phenomenon: once viral loads dropped below the limits of detection (10 copies/reaction), no evidence of viral rebound was detected in any of the n = 20 patients followed up to day 30 after initiation of therapy. This may explain why preexisting/baseline oseltamivir resistance is still very rare in untreated individuals infected with pandemic influenza A(H1N1) 2009.[34,35]

The development of neuraminidase inhibitor resistance in influenza B viruses[34,36] has so far been observed only sporadically and was not observed in our study (neither genotypically nor phenotypically).

In our study, elevated baseline viral loads correspond to an extended time required for viral loads to drop below levels of detection. In isolated cases where compliance or absorption issues may have prevented adequate response to treatment and/or where oseltamivir resistance developed, additional time was required to achieve complete viral clearance. Clearance rates of susceptible influenza A(H1N1) 2009 and B in patients responding to therapy were very similar, with an average clearance $\overline{CL_v}$ [1/day] of 1.29 and 1.18, respectively (see Table, Supplemental Digital Content 2, http://links.lww.com/B221). Viral clearance was significantly slower in 7 cases of patients infected with influenza A(H1N1) 2009 developing oseltamivir resistance ($\overline{CL_v}$ [1/day] = 0.7) indicating residual replication.

Among clinical factors tested, the maximum temperature showed a weak (albeit not linear) correlation with viral load measurements. The implications are that high fever may be related to large viral burden, that is, the virology is the reason for high fever in our sample of patients. The precise (mathematical) relation of these 2 parameters is, however, not known and warrants further investigation in larger trials.

Our naive analysis in Figure 4B showed that the number of symptoms (rhinitis, cough, diarrhea, vomiting were analyzed) is related to the viral burden. Further research using larger patient samples may identify combinations of symptoms that may provide informative clinical classifiers. This could be achieved by, for example, fitting artificial neural networks and validating them on large data sets that were not used for model fitting. Research pointing in this direction will be left for the future.

The decision whether to continue or discontinue oseltamivir treatment on day 5 is often difficult to make based on clinical factors alone, especially in infants and small children. Patients with hyperreactive airways may display continued pulmonary obstruction and mucus secretion even after viral clearance has been achieved. Comparisons of febrile versus afebrile patients in Chinese surveillance studies (using a threshold of 37.3°C) showed similar durations of

influenza PCR positivity regardless of baseline temperatures. Univariate analysis of the risk of pandemic A(H1N1) 2009 virus shedding beyond 5 days, however, showed significant differences based on maximum temperatures.[13]

This study has several limitations. First, the size of 36 treated infants and children is moderate, and untreated controls are absent for ethical reasons. However, this is one of the first prospective analyses of infants undergoing oseltamivir treatment in a tightly controlled setting closely monitored by infectious disease specialists. The virologic follow-up was intense due to quality management programs in collaboration with the National Reference Centre for Influenza.

Second, the clinical information collected in this study was restricted to maximum temperatures as well as 2 basic symptoms of uncomplicated influenza (rhinitis and cough), and 2 factors of drug absorption and tolerance (vomiting and diarrhea). Our data suggest that in isolated cases with severe vomiting and diarrhea before initiation of therapy, malabsorption may have led to a limited response to antiviral therapy. Although oseltamivir appears to be tolerated well in this age group[7,8] future studies will provide additional safety and clinical data as well as detailed information on pharmacokinetics and pharmacodynamics in infants.

Third, the number of data points available per patient to estimate viral clearance rates $CL_v(i)$ was limited, especially in patients where only 1 follow-up sample was available. In these instances, clearance indices represent conservative estimates as viral loads may have dropped below limits of detection even before the respective follow-up swab confirming viral elimination was obtained. We therefore tested whether a limited number of data points may correlate with lower $CL_v(i)$ parameter estimates, but could not find any significant correlation ($R_S = -0.23$, $P = 0.9$), indicating that the number of data points collected did not bias our estimates and therefore did not play a major role in our study.

The reported study also has several strengths. Our data resulting from close virologic follow-up in infants and children suggest that the assessment of baseline viral load measurements (in addition to influenza subtyping) may provide useful information to estimate the optimal duration of oseltamivir treatment in the individual patient. A less than expected decrease in viral load during therapy would then point toward resistance development, compliance issues or malabsorption. Clinical parameters in comparison seem to be poor indicators of response to antiviral therapy.

Large-scale studies should focus on clinical characteristics of nonresponders versus responders, as well as patients exhibiting susceptible versus resistant virus.[37] Furthermore, the prevalence of pandemic influenza A(H1N1) 2009 should be monitored closely throughout future influenza seasons and extended to different geographic areas with a focus on pediatric patients as the most effective transmitters of influenza.[38,39]

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5. CONCLUSION AND DISCUSSION

The three studies reported in this thesis have generated three strategies to improve the success and sustainability of antiviral therapy in HIV and influenza infection.

STRATEGY #1: USING *IN VITRO* MODELS TO DELAY DRUG RESISTANCE

When treating infections with the human immunodeficiency virus, there is only one chance per patient to select the most promising drug combination at a time. In vitro models may assist the physician in making the best choice.

The first strategy proposes drug combination passage experiments as a means to identify general principles guiding the interaction between the HIV and antiretroviral drugs. The *in vitro* model may be developed further to establish a personalized medicine approach to treating HIV infection. The assay could be modified into a "pre-exposure *in vitro* system" involving parallel short-term exposures of patient isolates to several drug combinations simultaneously. This technology could be used to predict chances of virologic success versus failure *before* ART is switched, particularly in salvage therapy settings involving multi-resistant virus and limited drug choices.

STRATEGY #2: IDENTIFYING PATIENTS AT RISK OF FAILURE

The second strategy aims to predict success versus failure across treatment cohorts. The strategy is exemplified in the cross-sectional analysis of a developing country pediatric cohort receiving one standard first-line ART regimen. While the majority of patients may benefit from standard ART, there will always be subsets of patients who may have been pre-exposed to erratic short-term antiviral therapy, thus harboring archived drug resistance. These patients would be bound to fail the standard regimen. It might be beneficial and even cost-effective to use an individualized approach in this subset of patients.

When a cohort is tested with the *DNA OLA* prior to initiation of ART, it should be possible to predict which patients may benefit most from the standard regimen - and which individuals might require a different approach. Once ART is underway, regular cross-cohort assessments should make use of the *RNA-OLA* as the most sensitive method to detect newly emerging drug resistant variants in time to change ART before viral load rebounds. Regular - for example annual - cross-cohort drug resistance evaluations could be tied in with periodic assessments of CD4 counts and viral load, as these are commonly implemented in developing countries. To compare clinical and immunological response to therapy across cohorts, treatment periods and/or programs, stakeholders could make use of the Clinical Course Integrated Display based on the revised CDC clinical and immunological categories, as proposed in Study 2.

STRATEGY #3: DETECTING DRUG RESISTANCE AHEAD OF TIME

The third strategy is an attempt to translate the lessons learned from antiretroviral therapy to influenza antiviral therapy. Here, we present a cross-cohort analysis of infants and small children with laboratory confirmed influenza A and B infection undergoing antiviral therapy. The key question is not the choice of drug regimen, but rather the decisions of "how long to treat" and "when to look

for drug resistance mutations". When antiviral therapy is administered, intra-individual differences (based on immunologic and host factors) are becoming less relevant, and the slope of virus load decline seems to be determined mostly by the virus subtype (at least with pandemic influenza A H1N1 versus influenza B). Depending on the baseline virus load, the clearance rate can be predicted, as the expected time to achieve non-detectable virus load. Patients who do not clear the virus in time may be the ones benefiting from either prolonged therapy or elevated drug dosing. Drug resistance testing should target these patients, to detect resistant variants early-on and to switch to alternative drugs, if possible.

Future studies will need to demonstrate the feasibility and applicability of the proposed strategies in large numbers and diverse settings. The *targeted* use of sensitive virologic assays along with the right choice and timing of antiviral drugs may prove to be cost-effective while improving patient outcomes in both developed and developing countries.

6. OUTLOOK

In addition to new strategies, the studies have led to a number of subsequent follow-up projects addressing the limitations of the pilot projects while making use of the lessons learnt.

One of the aspects that have not yet been addressed in the *in vitro* experiments in Study 1 is the role of minority variants in the mix and their impact on rates of emergence of drug resistance and the selection of mutant virus. The research of RNA viral evolution has become a fast-paced field where innovative and interdisciplinary methods are developed by virologists, biochemists, bioinformaticians and mathematicians seeking an improved knowledge of the complex interplay between selection, recombination and the study of gene clusters and coevolving gene sites.[256]

New mathematical models are being developed and tested in collaboration with colleagues at the Free University in Berlin to deepen our understanding of the role of resistance and compensatory mutations, viral fitness and the "stochasticity effect" (ability to increase genetic diversity) of different antiviral drugs and combinations. Analyses are underway, starting with the "no-drug experiments" and investigating the observed differences when more drugs are added into the mix. Interestingly, the fitness effects that can be modeled based on growth characteristics during the *in vitro* passage experiments are in concordance with published data in the HIV Drug Resistance database [257]

As indicated in Studies 1 and 2, a major drawback of consensus genotyping is the limited sensitivity of population-based sequencing methodologies, usually not exceeding the 80%-level.[18] Recently developed second-generation sequencing technologies and allele-specific assays could be employed in future models to deepen our understanding of evolutionary dynamics in clinical patient isolates. Initial deep sequencing techniques achieved increased sensitivity, but also introduced additional bias during the initial PCR amplification step, especially in patient samples with low template numbers; newer deep sequencing technologies are being developed using dual tags (one code unique to sequence and a primer ID) to increase sensitivities to >99% without introducing artefactual diversity. [258] Competitive fitness experiments with different mixtures of wild type and mutant virus – possibly even in the presence of different drugs - as well as clonal experiments and enzyme kinetics could help establish the impact of different resistance and compensatory mutations further. Phenotypic sensitivity assays could be performed at each passage to test the predictive value of IC_{50} to different drugs in comparison to the outcome of combination passage experiments.

If "combination IC_{50}" data looked promising, rapid drug exposure assays could be attempted and developed into a new tool to test, predict and compare the effectiveness of different drug combinations *ex vivo* before administering a new regimen to patients in real-life. It would be hoped

that individualized drug combination assays might help in the choice of the most promising next ART regimen in the individual patient. This would be a further step towards personalized HIV/AIDS medicine. As mentioned previously, the sustainability of an ART regimen is of particular importance in children living with HIV/AIDS. Unless a cure can be found, children will be facing life-long therapy. Drug combination assays may therefore be of particular interest to the pediatric HIV specialist.

Evidently, sustainability of ART in the individual patients is the key prerequisite for sustainability of ART programs in treatment cohorts. Study 2 demonstrates the usefulness of the highly sensitive OLA method to examine the prevalence of key drug resistance mutations not only in the individual child patient (i.e. virus populations in the plasma and lymphocyte compartment), but also across treatment cohorts as a whole. The routine use of the OLA is now being explored at the Instituto Nacional del Salud (INS) in Lima. As a positive side-effect of our research collaboration with the team at Stanford University and the Peruvian NIH (INS), the consensus sequencing capacity has been fully established at the time of the second anniversary of universal access to ART in Peru in 2006, benefiting both adults and children with HIV/AIDS in Peru. The universal treatment of HIV/AIDS was introduced in 2004, the brochure by the Peruvian MINSA celebrating the first 2 years of ART in Peru underlines the success of the program.

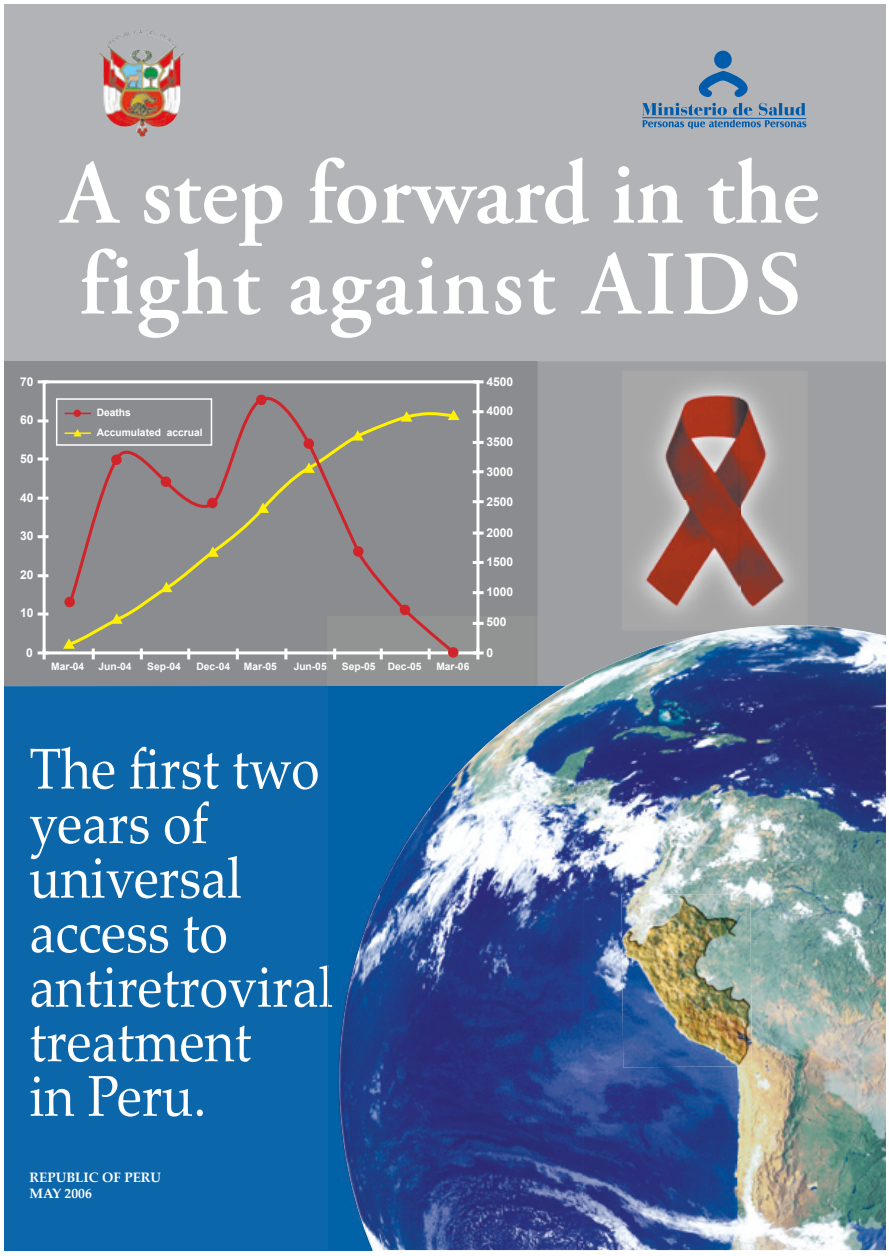

Figure 6 A: Cover of the brochure issued by the Peruvian Ministry of Health (MINSA) celebrating the 2nd anniversary of universal access to antiretroviral therapy in Peru[259]

The graph on page 47 of this brochure[259] proves the effectiveness of pMTCT in Peru. It is hoped that exposed infants who have been unfortunate enough to be infected prenatally may be treated soon enough to prevent substantial damage to the immune system. The ongoing epidemic of HIV in young adults will lead to an increase in numbers of exposed infants and sustained efforts will be necessary to control the pediatric epidemic in Peru.[259]

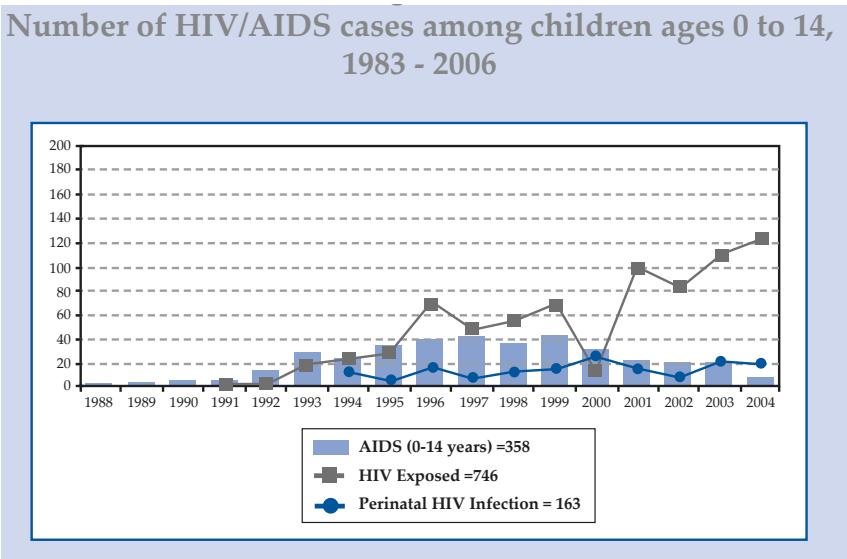

Figure 6 B: Number of HIV-exposed children in Peru, 1983-2006 (Source: MINSA 2006[259])

In the same brochure, decentralization and sustainability are emphasized as a major challenge for the future success of the universal access program in Peru. In many developing country settings, very few health care providers and HIV specialists may be available to take care of large populations. New communication and telemedicine tools as well as information technologies including SMS reminders and messaging may help to monitor and improve drug safety as well as patient compliance in remote settings.[260,261] Drug resistance testing and the use of alternative methodologies for the transportation of patient samples across large distances will remain priorities. The RNA yield from dried blood spots sent on Guthrie cards to the Stanford Center for AIDS Research (CFAR) in Study 2 did not prove as successful as published elsewhere [262], with RNA consensus sequencing being successful in only 30 % of the samples. It is hoped that the transportation of dried blood spots on filter cards specifically designed to bind DNA for subsequent testing with the DNA-OLA (originally developed for malaria trials) may be more successful.[32,263] Precautions must be taken to avoid cross-contamination when obtaining dried blood spots.[264] The display of patient outcomes across cohorts, as developed in Study 2, will provide useful to stakeholders observing the disease status in different population strata over time. This innovative display of disease progression across cohorts in Study 2 was inspired by the "cube" model in Study 1, displaying the progression of HIV-1 during drug pressure experiments.

The focus of ongoing and future projects at Charité results mostly from Study 3, in the attempt to translate lessons learnt from HIV antiretroviral therapy to influenza antiviral therapy. Study 3 as well as current projects and analyses are part of a quality management (QM) program at the Charité Department of Pediatrics in collaboration with the National Reference Center for Influenza at the Robert Koch Institute.[265] The program was instituted during the 2009 influenza pandemic for the systematic surveillance of infants and children with influenza-like illness (ILI)[266] presenting to the emergency department and pediatric and neonatal inpatient units at Charité.[240,241,255,267] The QM program (now in its third year) allows the unbiased real-time assessment of disease expressions in "true" influenza infection versus ILI due to other respiratory viruses and/or bacterial pathogens.[268]

The virus load kinetics of influenza A H1N1 (2009) and B viruses during the 2010/11 influenza pandemic in Germany will now be compared to kinetics in treated and untreated children infected with influenza A H3N2 during the 2008/9 and 2011/12 influenza seasons. Recent investigations at the National Reference Centre for Influenza at the Robert Koch Institute indicate that rare neuraminidase mutations may have developed in H3N2-infected infants undergoing oseltamivir treatment in 2012

(data not shown) leading to 4000-fold resistance to oseltamivir and partial resistance to zanamivir.[269-271] Fitness assays and competition experiments comparing mutant and wild-type H3N2 virus are underway.

Several clinical trials are being conducted testing the safety, tolerability, pharmacokinetics and pharmacodynamics of oseltamivir in sensitive pediatric subpopulations, including neonates and infants as well as children infected with influenza while undergoing chemotherapy or bone marrow transplantation.[267] Similar to HIV antiretroviral therapy, there seems to be a thin line between "too much and too little drug", i.e. drug safety on one hand - and resistance development on the other.

An effort will be made to develop innovative methodologies to standardize and compare resistance development and viral shedding in influenza cohorts throughout several seasons. Second-generation sequencing methodologies are being employed to investigate the impact of influenza minority quasispecies in the context of pandemic and seasonal influenza surveillance. For the large-scale surveillance of common resistance mutations, such as the H275Y neuraminidase mutation in Influenza A H1N1 viruses, the OLA may provide a useful and cost-effective alternative to sequencing technologies. The concept of studying virus kinetics during oseltamivir therapy has been taken to the next level in a recent project exploring the value of rapid immunoassays in the surveillance of treated and untreated children during confirmed influenza infection. Additional analyses will be investigating the potential link between influenza virus load and disease severity.[244,248,272,273]

In Study 3, only few clinical parameters were examined (namely vomiting, diarrhea, cough and rhinitis as well as body temperature). This was due to the limited information available from the collaborating site in Worms. The QM program at Charité will be able to provide a detailed picture of real-time clinical presentations throughout the entire course of illness up until discharge from the ED or hospital.[240,241] The detailed clinical picture allows the analysis of individual host factors and their role with respect to virus kinetics and disease expression during influenza infection. It is assumed that intra-individual differences in viral clearance rates may be "blunted" during therapy, but more pronounced in untreated patients.[255] A variety of immunological factors will be explored in the context of the EU project BioVacSafe (www.biovacsafe.eu) looking for biomarkers of vaccines Immunosafety while examining cytokine panels, autoantibodies and genetic host factors in "natural infection" versus immunization. The ultimate goal should be the development of safe and effective vaccines, which are the only means to effectively obviate the need for antiviral therapy in both adults and children

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