HIV-1 Resistenzentwicklung nach Einsatz antiretroviraler Medikamentenregime zur Prävention der Mutter-Kind-Übertragung von HIV in Uganda und Tansania

Dissertation

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1 Einleitung

1.1 Die humanen Immundefizienzviren

Die humanen Immundefizienzviren (HIV) vom Typ-1 (HIV-1) und Typ-2 (HIV-2) wurden 1983 und 1986 [1,2] als verursachende Erreger der 1981 erstmalig beschriebenen [3] Immunschwächekrankheit AIDS (Acquired Immune Deficiency Syndrome) identifiziert.
Der Besitz einer Hülle und eines Einzel(+)-Strang RNA-Genoms, welches durch die viruseigenen Enzyme Reverse Transkriptase und Integrase in „provirale“ DNA umgeschrieben (Reverse Transkription) und in das Wirtsgenom integriert wird, führten zur Eingliederung von HIV in die Familie der Retroviren.
Die taxonomische Zuordnung in das Genus der Lentiviren (lat. lentus: langsam) beruhte auf dem langsam fortschreitenden, chronischen HIV-Infektionsverlauf. Nach einer akuten virämischen Phase, die ca. 7-10 Tage nach Infektion beginnt, pendeln sich die Viruslasten während der folgenden langen klinischen Latenzphase (Median bei HIV-1: 8-10 Jahre) auf stabile nachweisbare Werte, dem individuellen „set point“ ein. Die CD4+-T-Lymphozytenzahlen (kurz: CD4-Zellzahlen) nehmen jedoch langsam und kontinuierlich auf weniger als 200 Zellen pro Mikroliter (µl) ab, bis das Immunsystem letztlich die HIV-Vermehrung und opportunistische Infektionen nicht mehr zu kontrollieren vermag und zusammenbricht. Es kommt zur Ausbildung der AIDS-definierenden Erkrankungen und letztendlich zum Tod des Erkrankten [4]. Die „Centers for Disease Control and Prevention“ (CDC) teilen die HIV-Infektionsstadien seit 1993 [5] in die drei Kategorien A, B und C ein (A: asymptomatisch, B: HIV-assoziierte Infektionen, C: AIDS-definierende Erkrankungen). Bei Säuglingen verläuft die Krankheitsprogression zu AIDS sehr viel rascher. Ohne medizinische Intervention erreichen 20%-25% der vertikal infizierten Kinder bereits im ersten Lebensjahr und 40% der Kinder bis zum 5. Lebensjahr das AIDS-Stadium [6,7]. In den ressourcenlimitierten Ländern sterben 50% der infizierten Kinder bereits vor ihrem 2. Lebensjahr an AIDS-assoziierten Erkrankungen [8,9].
Eine Eradikation zellintegrierter Proviren und somit Heilung der Krankheit ist bislang nicht möglich.

1.1.1 Vorkommen und Verbreitung von HIV

HIV/AIDS hat sich seit den 80er Jahren zu einer Pandemie entwickelt, in deren Verlauf nach Angaben der Organisation Joint United Nations Program on HIV/AIDS (UNAIDS) bis 2009 weltweit bereits ca. 60 Millionen Menschen infiziert wurden und 25 Millionen Menschen starben [10]. Nach neuesten Schätzungen von UNAIDS waren Ende 2010

weltweit insgesamt 34 Millionen Menschen mit HIV infiziert, die Zahl der Neuinfektionen lag bei 2,7 Millionen in jenem Jahr. Im globalen Vergleich ist Subsahara-Afrika die von HIV/AIDS am stärksten betroffene Region. Im Jahr 2010 entfielen allein zwei Drittel der Menschen, die mit einer HIV-Infektion lebten und 70% der HIV-Neuinfektionen auf das südliche Afrika [11]. Von 1,9 Millionen Neuinfektionen in Subsahara-Afrika waren 350.000 Kinder unter 15 Jahren betroffen. 90% der weltweiten Neuinfektionen von Kindern finden in dieser Region statt [11]. Durch die disproportional hohe HIV-1 Prävalenz unter Frauen in Subsahara-Afrika (ca. 60% der HIV-1 Infektionen) [12] sind nahezu alle HIV-Infektionen bei Kindern die Folge der Übertragung von der Mutter auf das Kind [9,13].

1.1.2 Diversität von HIV

Von epidemischer Bedeutung sind ausschließlich die HIV-1 Stämme der Gruppe M („major"), die aufgrund phylogenetischer Kriterien weiter in die Subtypen A-D, F-H, J, K differenziert werden. Global sind die Subtyp C Infektionen am häufigsten (48%). In Amerika und Westeuropa ist der Subtyp B vorherrschend, während in Zentralafrika alle bekannten Subtypen vorkommen [14,15]. Der Subtyp scheint den Krankheitsverlauf [16], die Resistenzhäufigkeit [17-20] und Resistenzpfade bzw. -muster [21] zu beeinflussen. Die epidemisch nicht relevanten HIV-1 Stämme der Gruppen N ("new"), O ("outlier") und P ("pending the identification of further human cases") sowie die sich strukturell und biologisch unterscheidenden HIV-2 Stämme [22] sind fast ausschließlich auf Westafrika beschränkt [23-27]. HIV-2 Infektionen nehmen jedoch derzeit in Europa, Indien und den USA zu [28,29]. Koinfektionen mit verschiedenen Subtypen können zur Entstehung von rekombinanten Formen führen, die als CRF (*Circulating Recombinant Forms*) bezeichnet werden. Aktuell sind 51 solcher Mosaikviren beschrieben [30]. Die hohe Divergenz der Subtypen und Gruppen erschwert die Diagnostik von HIV erheblich.

1.1.3 Transmission von HIV

Eine HIV-1 Infektion erfolgt durch den direkten Kontakt mit infizierten Körperflüssigkeiten über Schleimhautverletzungen oder das Blut. Das Virus befindet sich in den infizierten CD4+-T-Lymphozyten, Monozyten, Makrophagen oder frei in Blut, Samenflüssigkeit, Vaginalsekret und Muttermilch der infizierten Person. Im Wesentlichen wird HIV-1 über ungeschützten sexuellen Kontakt sowie den gemeinsamen Gebrauch von Spritzenutensilien unter Drogenabhängigen übertragen. Dieser Infektionsweg stellt eine

„horizontale Transmission“ dar. Die Übertragung von HIV-1 von der Mutter auf das Kind wird als „vertikale Transmission“ bezeichnet. Ohne medizinische Intervention liegt das Risiko zwischen 25-48% [31]. Während der Schwangerschaft besteht ein Übertragungsrisiko von 5-10% (in-utero Transmission), zur Geburt von 10-20% (intrapartale Transmission) und in Abhängigkeit von der Stilldauer über die Muttermilch von 5-20% (postpartale Transmission) [31,32]. Das Risiko der vertikalen Transmission steigt mit zunehmender Viruslast der Schwangeren bzw. Mutter [33,34].

1.1.4 Maßnahmen zur Reduktion der vertikalen Transmission von HIV

In den westlichen Industrienationen kann das Risiko der vertikalen Übertragung bei Schwangeren mit bekanntem HIV-1 Status durch eine kombinierte Interventionsstrategie auf unter 2% reduziert werden [35-37]. Die Intervention besteht für die Schwangere ohne Therapieindikation aus der Einnahme eines zeitlich begrenzten antiretroviralen Medikamentenregimes zur Viruslastsenkung, einer elektiven Schnittentbindung [35,38], der antiretroviralen Prä- bzw. Postexpositonsprophylaxe für das Neugeborene und dem konsequenten Stillverzicht. Die Auswahl eines adäquaten Medikamentenregimes unter Berücksichtigung der HIV-Resistenzlage und die regelmäßige Kontrolle von CD4-Zellzahl und Viruslast führen im Regelfall zur erfolgreichen Senkung der Viruslast unter die Nachweisgrenze und somit zur Verhinderung der HIV-Übertragung. Ist die Viruslast der Schwangeren erfolgreich gesenkt, führt die Schnittentbindung zu keiner oder nur zu einer geringen zusätzlichen Reduktion der Transmissionsrate und stellt daher in Deutschland keinen festen Bestandteil der vertikalen Transmissionsprophylaxe mehr dar [37,39,40].

In ressourcenlimitierten Ländern bieten in die Schwangerschaftsvorsorge integrierte Maßnahmen zur Prävention der HIV-Mutter-Kind Übertragung (Prevention of mother-to-child transmission of HIV; PMTCT) schwangeren Frauen eine HIV-Beratung und -Testung an. HIV-positive Schwangere erhalten ein zeitlich begrenztes antiretrovirales Medikamentenregime zur Transmissionsprophylaxe oder bei Therapieindikation eine antiretrovirale Langzeittherapie. Elektive Schnittentbindung, Stillverzicht, regelmäßige Kontrolle von CD4-Zellzahl und Viruslast sowie HIV-Resistenzbestimmung sind in den infrastrukturell schwach ausgebildeten Regionen nur sehr eingeschränkt, meistens jedoch gar nicht durchführbar [32]. Auch ist der Zugang zu antiretroviralen Medikamenten in ressourcenschwachen Ländern weiterhin limitiert (Abbildung 1) [41].

1.2 Antiretrovirale Medikamente gegen HIV

Seit 1987 können HIV-Infizierte mit antiretroviralen Medikamenten behandelt werden. Ziel ist die Hemmung der Virusreplikation zur Absenkung der Viruslast unter die Nachweisgrenze von 40-50 Genomkopien/ml Plasma. Dies wird heutzutage in Form einer dauerhaften antiretroviralen Kombinationstherapie angestrebt. Zeitlich begrenzt werden antiretrovirale Medikamente auch routinemäßig zur Prävention der vertikalen HIV-Transmission eingesetzt.

Den Industrienationen stehen zur Behandlung inzwischen Medikamente aus fünf verschiedenen Wirkstoffklassen zu Verfügung:

1. Eintritts-Inhibitoren (Korezeptorantagonist und Fusionsinhibitor)
2. Nukleosidische bzw. Nukleotidische Reverse Transkriptase-Inhibitoren (NRTI)
3. Nicht-nukleosidische Reverse Transkriptase-Inhibitoren (NNRTI)
4. Integrase-Inhibitoren
5. Protease-Inhibitoren (PI).

Die Wirkstoffe hemmen unterschiedliche Schritte bzw. Proteine des viralen Replikationszyklus, der aus den Teilschritten Rezeptor- und Korezeptorbindung, Membranfusion, Reverse Transkription, Integration, Transkription und Translation, Assemblierung der viralen Komponenten und Virusknospung sowie -reifung besteht.
Die Eintritts-Inhibitoren verhindern bereits das Eindringen des Virus in die Zelle: Korezeptorantagonisten blockieren die CCR5-Korezeptoren der Zielzelle, während Fusionsinhibitoren durch Bindung an die transmembranen Untereinheiten (gp41) der insgesamt 72 Glykoproteinkomplexe in der Virushülle eine Fusion von Virushülle mit Zellmembran verhindern. Die Reverse Transkriptase-Inhibitoren (RTI) unterbrechen die Umschreibung viraler RNA in DNA. Zugelassene Integraseinhibitoren beeinträchtigen den Strangtransfer der viralen cDNA und dessen Integration ins Wirtsgenom. Proteaseinhibitoren (PI) konkurrieren mit den viralen Proteasen um die Substrate (*Gag*- und *Gag-Pol*-Vorläuferproteine), so dass die Prozessierung in funktionsfähige Proteine d.h. die „Virusreifung“ zum infektiösen Viruspartikel nicht stattfindet.
Da den RTI in dieser Arbeit eine größere Bedeutung zukommt, sollen auf diese genauer eingegangen werden: Aufgrund der unterschiedlichen Wirkungsmechanismen werden sie in NRTI und NNRTI unterteilt. Die NRTI sind Analoga der physiologischen DNA-Nukleoside/Nukleotide und konkurrieren mit diesen als alternative Substrate um den

Einbau in die DNA-Kette bei der Reversen Transkription. Da den NRTI die 3´-Hydroxylgruppe an der Desoxyribose fehlt, kann keine Phosphordiesterbindung zum darauffolgenden Nukleotid aufgebaut werden und es kommt zum Kettenabbruch bei der DNA-Synthese. Die NRTI sind Vorstufen der wirksamen Verbindungen und werden erst durch eine schrittweise intrazelluläre Phosphorylierung wirksam. Bei Nukleotidanaloga sind zwei, bei Nukleosidanaloga drei Phosphorylierungsschritte nötig. Die NNRTI binden direkt an der RT nahe der Substratbindungsstelle für Nukleoside. Dieser Komplex blockiert das aktive Zentrum der RT, so dass die Polymerisation deutlich verlangsamt wird.

1.2.1 Antiretrovirale Therapie

Eine antiretrovirale Therapie (ART) dient der Behandlung eines HIV-Infizierten zur Verbesserung seines eigenen Gesundheitszustandes. In der Regel besteht eine Therapie aus einer Kombination von mindestens drei verschiedenen Medikamenten zweier Wirkstoffklassen (**c**ombined **A**nti-**R**etroviral **T**herapy = cART), die meist lebenslang eingenommen werden müssen. Als erste Therapieoption sind in den Industrienationen üblicherweise zwei NRTI und ein PI oder NNRTI vorgesehen. Der Zeitpunkt für einen Therapiebeginn orientiert sich meist an der CD4-Zellzahl, die neben der Viruslast als prognostischer Marker des Infektionsstatus gilt. Dieser Wert variiert zwischen 350-500 CD4-Zellen/µl in den internationalen, US amerikanischen und deutschen Richtlinien [42-44]. Der Therapiebeginn bei Werten von 350-500 statt <350 CD4-Zellen/µl reduzierte in einer internationalen klinischen Studie sowohl die HIV-Transmissionsraten als auch die Krankheitsprogression der HIV-infizierten Personen [45]. Der Beginn einer Therapie bereits bei Absinken der CD4-Zellzahlen/µl unter die Grenze von 500 CD4-Zellen/µl oder ab HIV-positiver Testung wird daher international diskutiert [45-49].

1.2.2 Antiretrovirale Prophylaxeregime für die vertikale HIV-Transmission

Antiretrovirale Medikamentenregime werden nicht nur zur Behandlung von HIV-Infizierten, sondern auch zur Prävention der vertikalen HIV-Übertragung als so genannte „antiretrovirale Transmissionsprophylaxen“ eingesetzt. Hierbei erhalten HIV-positive Schwangere ohne Therapieindikation und ihre Neugeborenen ein temporäres antiretrovirales Medikamentenregime.

In Deutschland wird die initiale Prophylaxeindikation gemäß den Deutsch-Österreichischen-Leitlinien von 2011 [40] abhängig von der CD4-Zellzahl, Viruslast und

Resistenzlage am Anfang der Schwangerschaft gestellt und unter Beobachtung der Entwicklung dieser Parameter alle zwei Monate bei Bedarf korrigiert. Bei Werten von >350 CD4-Zellen/µl wird in Abhängigkeit von der Viruslast ab der 24. Schwangerschaftswoche (Viruslast >100.000 Kopien/ml) bzw. 28. Schwangerschaftswoche (Viruslast <100.000 Kopien/ml) mit einer Transmissionsprophylaxe begonnen, die aus zwei NRTI und wahlweise einem NNRTI oder PI besteht. Wenn keine Primärresistenzen gegen den NRTI Zidovudin (AZT) vorliegen, wird gegebenenfalls ausschließlich AZT verabreicht, da hier der Vorteil der geringeren Medikamentenbelastung besteht. Das Neugeborene erhält AZT postnatal für 2-4 Wochen [40].

Erstmalig wurde im Jahr 1994 eine Senkung der vertikalen HIV-Transmissionsrate um zwei Drittel aufgrund einer täglichen Einnahme von AZT durch die Mutter ab der 14. Schwangerschaftswoche und postnatal durch das Neugeborene in einer randomisierten, Placebo-kontrollierten klinischen Studie erzielt [50]. Weitere Studien mit AZT allein, mit AZT in Kombinationen mit Lamivudin (3TC) [51-55] oder mit dem NNRTI Nevirapin (NVP) als Einmaldosis für Mutter und Kind bei/nach Geburt [56] wurden in den Folgejahren durchgeführt. Aufgrund der Einfachheit, guten Verträglichkeit für Mutter und Kind und einer Transmissionsreduktion um die Hälfte (47%) bei geringem Kostenaufwand wurde die NVP-Einmaldosis in ressourcenlimitierten Ländern ab 2001 als bevorzugtes Medikamentenregime zur Reduktion der HIV-Übertragung von der WHO empfohlen [57]. Vor dem Hintergrund eines immer noch hohen Übertragungsrisikos von über 15% [56], einer schnellen Resistenzentstehung gegen NVP [58] mit den möglichen Folgen des Versagens anschließender antiretroviraler Therapien [59-61], änderte die WHO mehrfach die Empfehlungen zugunsten effektiverer Prophylaxeregime [62-66] (Tabelle 1). Die NVP-Einmaldosis wird jedoch weiterhin in Indien und in vielen Regionen Afrikas als Minimalstandardregime eingesetzt (Abbildung 1).

Tabelle 1: WHO PMTCT-Leitlinien von 2001, 2006 und 2010

WHO Leit-linien	Regime	In der Schwangerschaft	In den Wehen/ zur Geburt	Nach der Geburt
2001	**NVP-Einmaldosis**		NVP-Einmaldosis	Mutter: - Kind: NVP-Einmaldosis
2006	**Antiretrovirales Dreifachregime**	ab 28. SSW AZT	NVP-Einmaldosis AZT (alle 3 Stunden) 3TC (alle 12 Stunden)	Mutter: AZT für 7 Tage 3TC für 7 Tage Kind: NVP-Einmaldosis AZT für 7 Tage bis 4Wochen
2010	**Antiretrovirales Dreifachregime**	ab 14. SSW AZT	NVP-Einmaldosis AZT (alle 3 Stunden) 3TC (alle 12 Stunden)	Mutter: AZT für 7 Tage 3TC für 7 Tage Kind: NVP täglich bis zum Abstillen
		ab 14. SSW AZT+3TC+LPV/r o. AZT+3TC+ABC o. AZT+3TC+EVP o. TDF+3TC+LPV/r	AZT+3TC+LPV/r o. AZT+3TC+ABC o. AZT+3TC+EVP o. TDF+3TC+LPV/r	Mutter: AZT+3TC+LPV/r o. AZT+3TC+ABC o. AZT+3TC+EVP o. TDF+3TC+LPV/r Kind: -

SSW: Schwangerschaftswoche; NVP: Nevirapin; AZT: Zidovudin; 3TC: Lamivudin, LPV: Lopinavir, ABC: Abacavir, EVP: Etravirin, TDF: Tenofovir

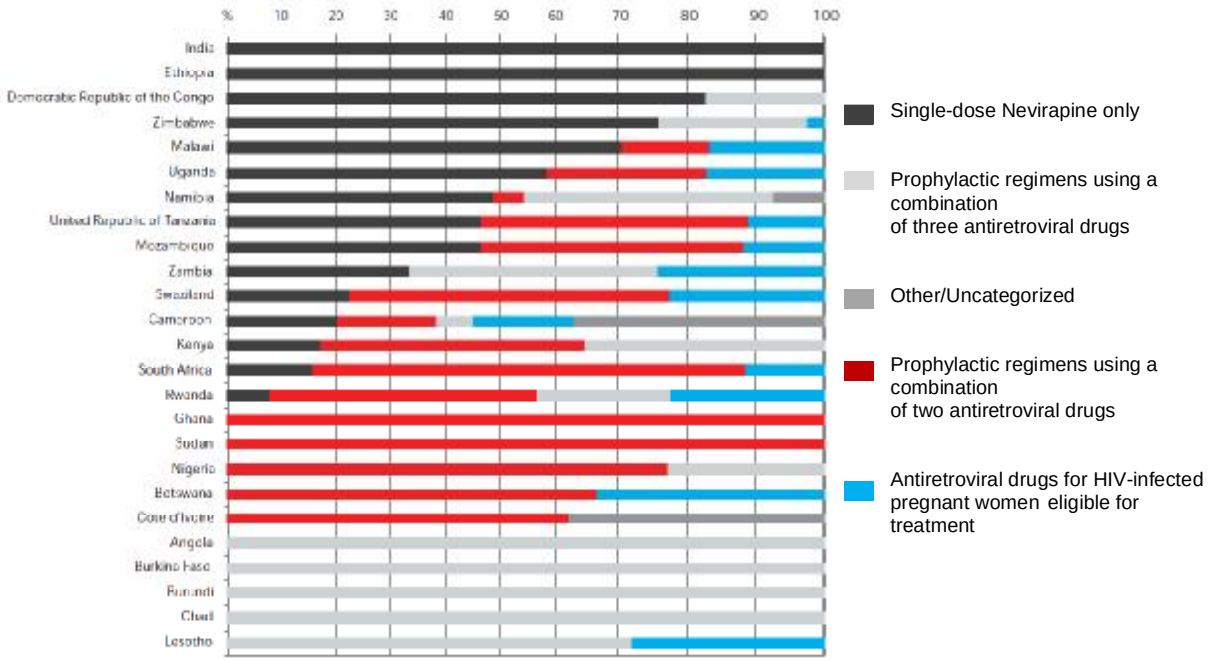

Abbildung 1: Antiretrovirale Prophylaxeregime der Mutter-Kind-Übertragung von HIV (UNAIDS 2010 Figure 3.11)

Die antiretroviralen Medikamente, die bis 2006 von der WHO zur Transmissionsprophylaxe vorgesehen waren (Tabelle 1), sind plazentagängige Reverse-Transkriptase-Inhibitoren [57,64]. AZT und 3TC gehören zur Wirkstoffgruppe der NRTI und NVP zu den NNRTI.

AZT (Azidothymidin, Zidovudin (ZDV), Handelsname Retrovir®)

AZT ist ein Thymidin-Nukleosidanalogon (NRTI) und wurde 1987 als erstes antiretrovirales Medikament zugelassen. Zu AZT gibt es die langjährigsten klinischen Erfahrungen, und es ist nach wie vor das einzig zugelassene antiretrovirale Medikament für den Einsatz in der Schwangerschaft [40,65]. Auf Grund der kurzen Halbwertszeit von 1-2 Stunden muss AZT zweimal täglich eingenommen werden [67].

3TC (Lamivudin, Dideoxy-Thiacytidin, Handelsname Epivir®)

3TC wurde im August 1996 als fünfter NRTI in Europa zugelassen. Es ist ein gut verträgliches Cytidinanalogon und daher als Kombinationspräparat mit AZT (zB. Combivir®) gut geeignet. 3TC hat eine Halbwertszeit von 5-7h [67] und ist für die tägliche Einmalgabe zugelassen.

NVP (Nevirapin , Handelsname Viramune®)

NVP war der erste zugelassene NNRTI (1997). Die sehr lange Halbwertszeit von 40-45h Stunden erlaubt eine einmalige Einnahme pro Tag. Dies und die gute Verträglichkeit [56,68] haben dazu beigetragen, dass NVP ein wichtiger Bestandteil von antiretroviralen Therapien und Prophylaxeregimen geworden ist. NVP ist auch als Sirup erhältlich und vereinfacht die Dosierung und Gabe an Kinder und Neugeborene.

1.3 HIV-Resistenzen gegen antiretovirale Medikamente

Wird die Virusreplikation trotz Medikamenteneinnahme nicht mehr gehemmt, kommt es zum Viruslastanstieg, man spricht vom „virologischen Versagen der antiretroviralen Therapie“. Häufigste Ursache hierfür sind Mutationen im Virusgenom, die zur Medikamentenresistenz führen. Dieser Zusammenhang wurde bereits kurz nach der Einführung von AZT beschrieben [69]. Mit Hilfe einer Vielzahl an antiretroviralen Substanzen und der genotypischen Resistenzbestimmung können in den Industrienationen auch dann noch wirksame Kombinationstherapien zusammengestellt werden, wenn Resistenzmutationen bereits vorliegen. Diese Möglichkeit fehlt bislang den Ländern der

ressourcenschwachen Endemiegebiete, der Zugang zu antiretroviralen Medikamenten unterschiedlicher Wirkstoffklassen ist meist noch stark eingeschränkt [70,71].

1.3.1 Entstehung von Resistenzen

Durch den hohen Turnover von HIV (10 Milliarden neuer Viruspartikel pro Tag) [72], gekoppelt mit der hohen Fehlerrate der RT (statistisch ca. eine Mutation pro neuem Viruspartikel) und durch die normalen Prozesse der Rekombination und Selektion sind die neu entstehenden Virusvarianten (Quasispezies) im HIV-Infizierten von großer Diversität.
Virusvarianten, die bei einem medikamentösen Selektionsdruck einen Replikationsvorteil haben, können sich zur dominanten Population entwickeln, d.h. zu „resistenten Virusvarianten“ gegenüber jenem Medikament [73,74]. Monotherapien, also die Einnahme nur eines Medikaments einer Wirkstoffklasse über längere Zeit begünstigen diese Art der Resistenzselektion.
Resistente Viren entstehen aber vor allem bei subtherapeutischen Wirkstoffspiegeln neu und haben in Gegenwart der Medikamente einen Selektionsvorteil gegenüber dem sensitiven Wildtyp. Ein unzureichender Wirkstoffspiegel kann sowohl Folge einer schwachen Resorption als auch einer erhöhten Umsatzrate im Patienten sein, oder als Resultat einer mangelnden Medikamentenadhärenz entstehen. Er liegt aber auch in Abhängigkeit von der Medikamenten-Halbwertszeit während der Abklingphase bei Therapieabbruch oder nach temporärer Medikamenteneinnahme vor. Hier werden bei noch geringem Wirkstoffspiegel im Blut, aber schon ansteigender Virusreplikation resistente Virusvarianten selektiert, es liegt ein so genannter „positiver Selektionsdruck“ vor [75-77].
Genügt zur Entstehung von Resistenz gegenüber einem oder sogar mehreren antiretroviralen Medikamenten der gleichen Wirkstoffklasse (Kreuzresistenz) bereits ein einziger Nukleotidaustausch im Virusgenom, spricht man von einer „geringen genetischen Resistenzbarriere“. Diese führt zur raschen Entstehung von Resistenzen.
In der jährlich aktualisierten HIV-Datenbank und der Mutationsliste der Internationalen AIDS-Gesellschaft, sind die aktuellen resistenzassoziierten Mutationen aufgeführt [78].

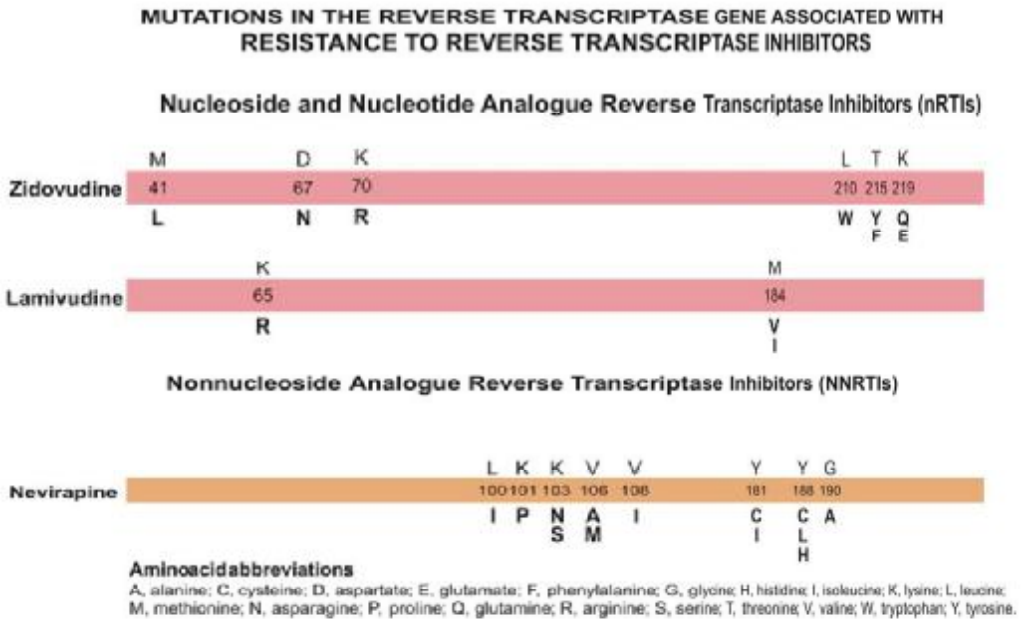

Abbildung 2: Resistenzmutationen, selektiert durch die Medikamente Zidovudin, Lamivudin und Nevirapin, geändert aus Johnson et al. 2011 [78]

AZT (Azidothymidin, Zidovudin (ZDV), Handelsname Retrovir®)

Zunächst unter Monotherpie mit AZT [69], aber auch häufig bei Therapie mit anderen NRTI beschrieben, entstehen die Thymidinanaloga-Resistenzmutationen (TAM). Es werden zwei Mutationspfade unterschieden: der TAM-1 Pfad mit den Aminosäureaustauschen M41L, L210W und T215Y und der TAM-2 Pfad mit D67N, K70R, T215F und K219Q/E [79-81]. Die K70R, die durch einen einzigen Nukleotidaustausch in der RT entsteht (AAA=>AGA), erscheint als erste Mutation bei der AZT-Resistenzentwicklung [81], sie tritt jedoch nur transient auf. Unter weiter anhaltendem Selektionsdruck von AZT erscheinen dann in Folge die TAM an Aminosäureposition 41 und 215. Für die Mutationen T215Y oder F ist ein 2-Basenaustausch notwendig (ACC zu TAC=>Y oder TTC=>F). Die genetische Barriere zur Resistenzentstehung ist also hoch [81,82]. In Abwesenheit des Selektionsdrucks revertiert die T215Y Mutation in Plasmaviren mit einem Nukleotid-Austausch in „Richtung“ Wildtyp zu den „Revertanten“ T215D/C/S [83,84]. Unter dem Selektionsdruck einer erneuten AZT-Einnahme zeigen sie daher eine raschere Entwicklung zur AZT-resistenten T215Y Mutation als der Wildtyp. Die genetische Resistenzbarriere ist bei den Revertanten demnach geringer als beim Wildtyp [85].

3TC (Lamivudin, Dideoxy-Thiacytidin, Handelsname Epivir®)

Für 3TC ist die genetische Barriere zur Resistenzentstehung gering. Bereits eine einzige Punktmutation (ATG=>GTG) in der Reversen Transkriptase führt zum Aminosäureaustausch M184V, der eine hochgradige Resistenz gegenüber 3TC verursacht [86]. Unter einer Monotherapie entsteht diese Mutation bereits nach wenigen Wochen [87]. Gleichzeitig führt die M184V zu einem starken Verlust der viralen Replikationskapazität [88,89] und wird in Abwesenheit des Selektionsdrucks schnell wieder durch den Wildtyp ersetzt [90].

Generell wird Resistenz gegenüber NRTI durch 2 verschiedene Mechanismen verursacht: die sterische Inhibition und die Pyrophosphorolyse. Mutationen der ersten Kategorie erlauben es dem RT-Enzym, strukturelle Unterschiede zwischen NRTIs und Nukleotiden zu erkennen und so den Einbau von NRTI zu verhindern. Bei der Resistenz durch die verstärkte Pyrophosphorolyse werden bereits eingebaute NRTI/Analogons aus der wachsenden DNA-Kette wieder freigesetzt.

NVP (Nevirapin , Handelsname Viramune®)

Resistenzen gegenüber NNRTI entstehen durch Mutationen in der Nähe des aktiven Zeitraums der RT, die eine Komplexbildung mit den NNRTI verhindern. Die genetische Barriere zur Resistenzentwicklung ist bei NVP sehr gering, bereits Punktmutationen führen zu Aminosäureaustauschen, die auch einzeln zur kompletten Resistenz gegenüber NVP und anderen Medikamenten der NNRTI-Klasse (Kreuzresistenz) führen. Häufigste Mutation nach Einnahme von NVP ist die K103N, gefolgt von Y181C/I und G190A in der Reversen Transkriptase [91,92]. Die NNRTI der zweiten Generation, also Efavirenz und Delavirdin, sind auch noch wirksam beim Vorliegen der K103N-Mutation.

1.3.2 Übertragung und Persistenz von Resistenzen

Resistente Virusvarianten werden auch übertragen. Die erste Transmission von AZT-resistenten Isolaten auf therapienaïve Personen wurde 1993 nur 5 Jahre nach Einführung von AZT beschrieben [93]. Die Prävalenz von resistenten Viren in frisch Infizierten variiert weltweit erheblich und ist in Ländern mit langjährigem Zugang zur antiretroviralen Therapie höher als in Ländern mit kürzerem und eingeschränktem Zugang. Nach einer Erhebung von 2009 (Analysezeitraum durchschnittlich 4 Jahre ab 2003) betrug die Prävalenz in den USA 14,3%, in Europa 7,7%, aber nur 5,3% in Afrika und 4,2% in

Asien [94]. Das SPREAD (Strategy to Control the Spread of HIV-1) Programm ermittelte sogar eine Prävalenz von 8,4% für 20 europäische Staaten und Israel (Analysezeitraum von 2002 bis 2005) [95]. Nach der Serokonverterstudie des Robert Koch-Instituts Berlin erfolgt die Übertragung resistenter HIV in Deutschland mit einer Häufigkeit von ca. 12% (Analysezeitraum von 1997 bis 2010) [96-98]. Ayouba *et al.* ermittelte 2009 eine noch geringe Prävalenz von HIV-Resistenzen (≤5%) bei therapienaiven neuinfizierten HIV-Patienten in Burkina Faso, Cote d'Ivoire, Senegal, Thailand, and Vietnam [99]. Übertragene Resistenzmutationen, insbesondere die K103N und T215-Reversionen, können jahrelang persistieren [100-103].

Mutationen in freien Plasmaviren revertieren in Abwesenheit der selektierenden Medikamente meistens zum Wildtyp, wenn sie die Aktivität der Protease und RT beeinträchtigen [104-106]. Sie können auch als archiviertes Provirus in der Wirtszelle persistieren („latente Reservoire“) [106-110] und zum Versagen einer anschließenden antiretroviralen Langzeittherapie beitragen [74,111-115].

1.3.3 Methoden der Resistenzbestimmung

Resistenz gegenüber antiretroviralen Medikamenten kann phänotypisch und/oder genotypisch untersucht werden. Bei der phänotypischen Resistenzbestimmung werden in der Regel rekombinante HIV-1, die eine Genomregion des Patientenisolates tragen, in Zellkultur auf die Empfindlichkeit gegenüber Medikamenten getestet. Hierbei werden auch Kreuzresistenzen, kompensatorische und neue Mutationen in ihrer Wirkung erfasst.

Die genotypische Resistenzbestimmung beruht auf der Detektion von resistenzassoziierten Mutationen in den Virussequenzen. Die Mutationen auf einem PCR-amplifizierten HIV-Genomabschnitt werden entweder über die Direktsequenzierung (Sanger Sequenzierung), durch Hybridisierungsverfahren mit mutanten Oligonukleotiden („LigAmp-“, Oligonukleotide Ligations- und Allel-spezifischen PCR Assays) [76,116,117] oder mit der ultra-sensitiven Sequenzierung nachgewiesen [114]. In der Routine hat sich die genotypische Resistenzbestimmung von Plasmaviren mittels Sanger Sequenzierung als Methode für das Therapie-Monitoring von HIV-Patienten durchgesetzt. Anhand der Populationssequenz des PCR-Amplifikats, die eine Konsensus-Sequenz der Quasispezies darstellt, können resistenzassoziierte Mutationen bzw. Mutationsmuster mit Hilfe von Webdiensten (Standford-USA; geno2pheno-D, GRADE-D; ANRS-F) über Vorhersage-

Algorithmen identifiziert und bewertet werden. Die Vorhersagetools basieren auf Regeln, die entweder auf aktuellen Literaturdaten d.h. wissensbasierten genotypischen Interpretationssystemen wie z. B. HIV-GRADE beruhen oder auf datenbasierten Interpretationssysteme aus paarweise durchgeführten Geno- und Phänotypisierungen, wie z.B. geno2pheno oder vircoType^{TM}. Hier wird dem individuellen genotypischen Resistenzmuster ein Phänotyp zugeordnet und das virologische Ansprechen „berechnet". Anhand populationssequenz-basierter Verfahren können resistente Varianten erst detektiert werden, wenn sie einen Anteil von mindestens 20% an der Gesamtviruspopulation ausmachen [118].

In der Forschung angewandte sensitivere Nachweismethoden konnten das Vorliegen von Virusvarianten in Proportionen unter 20% belegen [76,114,116,119-121]. Die Allel-spezifische PCR (*ASPCR*) ist eine Detektionsmethode für einzelne Resistenzmutationen und wurde zum Nachweis von HIV-1 Resistenzmutationen in Anteilen kleiner 1% in der Gesamtviruspopulation angewandt [76,120-125]. Die ultra-sensitive (*ultra-deep*) Sequenzierung (*UDS*) ist eine noch relativ neue Methode, die aufgrund der hohen Kosten erst in wenigen Laboren eingesetzt wird. Sie funktioniert nach dem Prinzip der Pyrosequenzierung an quasi-klonalen Sequenzen und wurde bislang zur Detektion der Resistenzmutationen in der RT, Protease und der Hüllglykoproteine mit Sensitivitäten von ca. 1% eingesetzt [114,126-130]. Die UDS gibt in einer einzigen Untersuchung Auskunft über alle vom Wildtyp abweichenden Mutationen der untersuchten HIV-Genome in der viralen Quasispezies. Beide Verfahren werden ausführlich in den Teilprojekten dieser Arbeit (Manuskript I und VII) beschrieben.

2 Zielsetzung

Zur Prävention der Mutter-Kind Übertragung von HIV-1 (prevention of mother-to-child transmission of HIV-1; PMTCT) in der weltweit meistbetroffenen Region Subsahara-Afrika werden antiretrovirale Medikamentenregime zur Prophylaxe der vertikalen HIV-Übertragung eingesetzt. Das Vorliegen subtherapeutischer Wirkstoffspiegel nach Beendigung eines temporären Medikamentenregimes bei langen oder unterschiedlichen Halbwertszeiten der Medikamente, geringe genetische Resistenzbarrieren und die Einnahme nur eines antiretroviralen Medikaments sind Ursachen für die Entstehung von Resistenzmutationen. Diese bergen die Gefahr eines späteren Therapieversagens für die HIV-positiven Mütter und deren vertikal infizierte Kinder.

Ziel dieser Arbeit war, die Entstehung und Persistenz von HIV-Resistenzen nach Einnahme einer NVP-Einmaldosis bzw. eines antiretroviralen Dreifachregimes mit AZT, 3TC und NVP zur Transmissionsprophylaxe zu untersuchen und die Anteile der resistenten Viruspopulationen zu quantifizieren. Die Analysen sollten in zwei Studienkollektiven aus Mutter-Kind-Kohorten durchgeführt werden, die im Rahmen von Untersuchungen des Instituts für Tropenmedizin und Internationale Gesundheit, Charité-Universitätsmedizin Berlin, in West-Uganda (Fort Portal District Hospital, Kabarole District) und Südwest-Tansania (Kyela Distrikt Hospital, Mbeya Region) aufgebaut worden waren. Die antiretrovirale Transmissionprophylaxe bestand zum Zeitpunkt der Untersuchung in Uganda aus einer Nevirapin (NVP)-Einmaldosis für Mutter und Kind um die Geburt und in Tansania aus einem Dreifachregime mit Zidovudin (AZT) ab der 28. Schwangerschaftswoche, einer NVP-Einmaldosis zu Beginn der Wehen sowie AZT und Lamivudin (3TC) zur Geburt und sieben weitere Tage für die Mutter und einer NVP-Einmaldosis mit AZT-Ergänzung für das Neugeborene.

Da die resistenten Virusvarianten nach einem kurzzeitigen medikamentösen Selektionsdruck oft nur in geringen Proportionen in der viralen Quasispezies vorliegen, können diese mit der routinemäßig eingesetzten Sanger Sequenzierung mit Nachweisgrenzen über 20% größtenteils nicht erfasst werden. Für diese Arbeit sollten daher hochsensitive Detektionsmethoden mit einer Nachweisgrenze <1% verwendet werden. Zunächst sollten Allel-spezifische PCR (ASPCR) Assays für die in West-Uganda prävalenten Subtypen A und D und für die beiden häufigsten NVP-selektierten Resistenzmutationen K103N und Y181C in der viralen Reversen Transriptase (RT) etabliert werden. Anschließend war die Etablierung von ASPCR Assays für die in Südwest-Tansania prävalenten Subtypen A, C und D und ausgewählte

AZT/3TC/NVP-selektierte Schlüsselmutationen (AZT: K70R, T215Y, T215F; 3TC: M184V; NVP: K103N, Y181C) der RT vorgesehen. Da die ASPCR jedoch auf die Detektion einzelner Punktmutationen limitiert ist, war für ausgewählte Proben eine zusätzliche Analyse mit der ultra-sensitiven Sequenzierung („ultra-deep sequencing"; UDS) vorgesehen, die Informationen über das Vorliegen aller medikamentenbedingten Resistenzmutationen in Anteilen von <1% in der viralen RT liefern sollte.

Anhand der Ergebnisse der Resistenzanalysen sollten für das NVP-Einmaldosis-Regime in Uganda Aussagen zum Entstehungszeitpunkt, Verlauf und zur Persistenz von resistenzassoziierten Mutationen in Plasmaviren (HIV-RNA) der Mütter und deren vertikal infizierten Kindern getroffen werden. Aufgrund des postnatalen Transmissionsrisikos durch das Stillen waren zusätzliche Nachweise von HIV-Resistenzmutationen in Viren der Brustmilch und der Vergleich der Resistenzmuster in mütterlichen Plasmaviren vorgesehen. Analog dazu sollte die Häufigkeit der Resistenzentstehung nach Einnahme des Dreifachregimes in Tansania und das Risiko der Entstehung multiresistenter Viruspopulationen in mütterlichem und kindlichem Blutplasma (HIV-RNA) ermittelt werden. Das Ausmaß der Archivierung von Resistenzen in Form integrierter Proviren (HIV-DNA) sollte durch die Analyse der maternalen peripheren mononukleären Blutzellen (PBMC) ermittelt werden. Der Verlauf der HIV-Viruslast der Mütter während des Untersuchungszeitraums und die Transmissionsraten trotz Einnahme der antiretroviralen Prophylaxeregime war als begleitender Analyseparameter vorgesehen.

Abschließend sollten sowohl die beiden antiretroviralen Prophylaxeregime im Hinblick auf die Resistenzentwicklung als auch die beiden Nachweisverfahren ASPCR und UDS hinsichtlich Sensitivität, Durchführbarkeit und der Ergebnisse miteinander verglichen werden.

3 Ergebnisse

3.1 Liste aller Einzelarbeiten

Manuskript I

Detection and quantification of minor human immunodeficiency virus type 1 variants harboring K103N and Y181C resistance mutations in subtype A and D isolates by allele-specific real-time PCR

Hauser A, Mugenyi K, Kabasinguzi R, Bluethgen K, Kuecherer C, Harms G, Kunz A. Antimicrobial Agents and Chemotherapy 2009;53(7):2965-73.

Manuskript II

Emergence and persistence of minor drug-resistant HIV-1 variants in Ugandan women after nevirapine single-dose prophylaxis

Hauser A, Mugenyi K, Kabasinguzi R, Kuecherer C, Harms G, Kunz A.
PLoS ONE 2011;6(5):e20357.

Manuskript III

Dynamics of NVP-resistance development in HIV-1 of Ugandan mother-child pairs during 18 months after nevirapine single-dose exposure to reduce vertical transmission

Hauser A, Kunz A, Mugenyi K, Kabasinguzi R, Kuecherer C, Harms G.
(Manuskript in Vorbereitung)

Manuskript IV

Minor drug-resistant HIV type-1 variants in breast milk and plasma of HIV type-1-infected Ugandan women after nevirapine single-dose prophylaxis

Pilger D, Hauser A, Kuecherer C, Mugenyi K, Kabasinguzi R, Somogyi S, Harms G, Kunz A.
Antiviral Therapy 2011;16(1):109-13.

Manuskript V

Emergence of minor drug-resistant HIV-1 variants after triple antiretroviral prophylaxis for prevention of vertical HIV-1 transmission.

Hauser A, Sewangi J, Mbezi P, Dugange F, Lau I, Ziske J, Theuring S, Kuecherer C, Harms G, Kunz A.
PLoS ONE 2012;7(2):e32055.

Manuskript VI

Drug-resistant HIV-1 in cellular DNA of Tanzanian women following WHO-recommended antiretroviral prophylaxis for vertical transmission

Hauser A, Kuecherer C, Theuring S, Sewangi J, Mbezi P, Dugange F, Lau I, Ziske J, Harms G, Kunz A.

(Manuskript in Vorbereitung)

Manuskript VII

Minor drug-resistant HIV-1 variants in Tanzanian women after antiretroviral prophylaxis for vertical transmission detected by ultra-deep amplicon sequencing and allele-specific real-time PCR

Hauser A, Kunz A, Meixenberger K, Radonić A, Sewangi J, Mbezi P, Dugange F, Theuring S, Lau I, Ziske J, Harms G, Kuecherer C.

(Manuskript in Vorbereitung)

3.2 Eigenanteil an den Einzelarbeiten

Eigenanteil an den Einzelarbeiten in %

Manuskript	Konzeption und Etablierung der Methoden	Durchführung und Auswertung der Daten	Berichtabfassung
I	70	85	70
II	80	85	60
III	80	85	85
IV	80	85	10
V	95	100	85
VI	95	100	95
VII	60	85	95

3.3 Manuskript I

Detection and quantification of minor human immunodeficiency virus type 1 variants harboring K103N and Y181C resistance mutations in subtype A and D isolates by allele-specific real-time PCR

Abstract

Background: Nevirapine (single dose), commonly used to prevent the mother-to-child transmission of human immunodeficiency virus (HIV) in developing countries, frequently induces viral resistance. Even mutations which occur only in a minor population of the HIV quasispecies (<20%) are associated with subsequent treatment failure but cannot be detected by population-based sequencing.

Methods: We developed sensitive allele-specific real-time PCR (ASPCR) assays for two key resistance mutations of nevirapine. The assays were specifically designed to analyze HIV-1 subtype A and D isolates accounting for the majority of HIV infections in Uganda. Assays were evaluated using DNA standards and clinical samples of Ugandan women having preventively taken single-dose nevirapine.

Results: Lower detection limits of drug-resistant HIV type 1 (HIV-1) variants carrying reverse transcriptase mutations were 0.019% (K103N [AAC]), 0.013% (K103N [AAT]), and 0.29% (Y181C [TGT]), respectively. Accuracy and precision were high, with coefficients of variation (the standard ratio divided by the mean) of 0.02 to 0.15 for intra-assay variability and those of 0.07 to 0.15 (K103N) and 0.28 to 0.52 (Y181C) for inter-assay variability. ASPCR assays enabled the additional identification of 12 (20%) minor drug-resistant HIV variants in the 20 clinical Ugandan samples (3 mutation analyses per patient; 60 analyses in total) which were not detectable by populationbased sequencing. The individual patient cutoff derived from the clinical baseline sample was more appropriate than the standard-based cutoff from cloned DNA. The latter is a suitable alternative since the presence/absence of drug-resistant HIV-1 strains was concordantly identified in 92% (55/60) of the analyses.

Conclusions: These assays are useful to monitor the emergence and persistence of drug-resistant HIV-1 variants in subjects infected with HIV-1 subtypes A and D.

3.4 Manuskript II

Emergence and persistence of minor drug-resistant HIV-1 variants in Ugandan women after nevirapine single-dose prophylaxis

Abstract

Background: Nevirapine (NVP) single-dose is still a widely used antiretroviral prophylaxis for the prevention of vertical HIV-1 transmission in resource-limited settings. However, the main disadvantage of the Non-nucleoside Reverse Transcriptase Inhibitor (NNRTI) NVP is the rapid selection of NVP-resistant virus with negative implications for subsequent NNRTI-based long-term antiretroviral therapy (ART). Here, we analysed the emergence of drug-resistant HIV-1 including minor variants in the early phase after NVP single-dose prophylaxis and the rersistence of drugresistant virus over time.

Methods and Findings: NVP-resistant HIV-1 harbouring the K103N and/or Y181C resistance mutations in the HIV-1 reverse transcriptase gene was measured from 1 week up to 18 months after NVP single-dose prophylaxis in 29 Ugandan women using allele-specific PCR assays capable of detecting drug-resistant variants representing less than 1% of the whole viral

population. In total, drug-resistant HIV-1 was identified in 18/29 (62%) women; rates increased from 18% to 38% and 44% at week 1, 2, 6, respectively, and decreased to 18%, 25%, 13% and 4% at month 3, 6, 12 and 18, respectively. The proportion of NVP-resistant virus of the total viral population was significantly higher in women infected with subtype D (median 40.5%)

as compared to subtype A (median 1.3%; p = 0.032, Mann-Whitney U test). 33% of resistant virus was not detectable at week 2 but was for the first time measurable 6–12 weeks after NVP single-dose prophylaxis. Three (10%) women harboured resistant virus in proportions .10% still at month 6.

Conclusions: Current WHO guidelines recommend an additional postnatal intake of AZT and 3TC for one week to avoid NVP resistance formation. Our findings indicate that a 1-week medication might be too short to impede the emergence of NVP resistance in a substantial proportion of women. Furthermore, subsequent NNRTI-based ART should not be started earlier than 12 months after NVP single-dose prophylaxis.

3.5 Manuskript III

Dynamics of NVP-resistance development in HIV-1 of Ugandan mother-child pairs during 18 months after nevirapine single-dose exposure to reduce vertical transmission

Abstract

Background: A single-dose (SD) of nevirapine (NVP) is still applied in resource-limited settings to reduce mother-to-child transmission of HIV-1. NVP selects for resistant virus, which may negatively impact subsequent long-term therapy even if present as minor variants. The aim of our study was to compare emergence and persistance of resistant HIV-variants in HIV-positive Ugandan women and their vertically infected infants.

Methods: Eighty-six HIV-positive mothers and their children were followed up for 18 months after exposure to NVP-SD. Plasma samples of HIV-infected mother-child pairs, taken at delivery and at several points in time during the study period, were analysed for HIV-1 resistance mutations performing a highly sensitive allele-specific PCR for the most common NVP-selected resistance mutations K103N and Y181C.

Results: The HIV-transmission rate 6-8 weeks after birth was 9.3% (8/86), during the whole observation period totally 10/86 infants were infected. Follow up samples of 9/10 mother-child pairs were available for HIV-1 resistance testing. K103N and/or Y181C mutations were detected in plasmavirus of 7/9 (78%) mothers and 4/9 (44%) infants. Resistant virus emerged 2-8 weeks after NVP-exposure in 6/7 mothers and 3/4 infants and persisted for a maximum of 12 and 6 months, respectively. Resistant minor variants (<5%) were present in half of the women (4/7) and children (2/4). Vertical transmission of resistant virus could not be demonstrated.

Conclusions: In vertically infected children, NVP resistant HIV-variants did not emerge more frequently or persist longer than in their mothers. However, our findings suggest that the success of NNRTI-containing treatment might be limited for HIV-infected children if initiated within seven months after NVP-SD exposure.

3.6 Manuskript IV

Minor drug-resistant HIV type-1 variants in breast milk and plasma of HIV type-1-infected Ugandan women after nevirapine single-dose prophylaxis

Abstract

Background: Nevirapine single-dose (NVP-SD) reduces mother-to-child transmission of HIV type-1 (HIV-1), but frequently induces resistance mutations in the HIV-1 genome. Little is known about drug-resistant HIV-1 variants in the breast milk of women who have taken NVP-SD.

Methods: Blood and breast milk samples of 39 HIV-1-infected Ugandan women were taken 6-12 weeks after NVP-SD intake. Samples were analysed by population sequencing and allele-specific real-time PCR (AS-PCR) with detection limits for NVP-resistant HIV-1 variants (K103N and Y181C) of < 1% of the total viral population.

Results: AS-PCR results for both plasma and breast milk were obtained for 19 women who constituted the final study group (HIV-1 subtype frequencies were A1 n = 11, D n = 5, G n = 2 and C n = 1). A total of 7 (37%) and 10 (53%) women carried NVP-resistant virus in breast milk and plasma, respectively. Overall, 71% (5/7) women with NVP-resistant HIV-1 in breast milk displayed >1 drug-resistant variant. Resistance in breast milk was higher at week 6 (6/13 samples [46%]) compared with week 12 (1/6 samples [17%]). In total, 10 drug-resistant populations harbouring the K103N and/or Y181C mutation were detected in the 19 breast milk samples; 7 (70%) were caused by resistant minorities (< 5% of the total HIV-1 population). In the four women with drug-resistant virus in both plasma and breast milk, the mutation patterns differed between the two compartments.

Conclusions: Minor populations of drug-resistant HIV-1 were frequently found in breast milk of Ugandan women after exposure to NVP-SD. Further studies need to explore the role of minor drug-resistant variants in the postnatal transmission of (resistant) HIV-1.

3.7 Manuskript V

Emergence of minor drug-resistant HIV-1 variants after triple antiretroviral prophylaxis for prevention of vertical HIV-1 transmission

Abstract

Background: WHO-guidelines for prevention of mother-to-child transmission of HIV-1 in resource-limited settings recommend complex maternal antiretroviral prophylaxis comprising antenatal zidovudine (AZT), nevirapine single-dose (NVP-SD) at labor onset and AZT/lamivudine (3TC) during labor and one week postpartum. Data on resistance development selected by this regimen is not available. We therefore analyzed the emergence of minor drug-resistant HIV-1 variants in Tanzanian women following complex prophylaxis.

Method: 1395 pregnant women were tested for HIV-1 at Kyela District Hospital, Tanzania. 87/202 HIV-positive women started complex prophylaxis. Blood samples were collected before start of prophylaxis, at birth and 1-2, 4-6 and 12-16 weeks postpartum. Allele-specific real-time PCR assays specific for HIV-1 subtypes A, C and D were developed and applied on samples of mothers and their vertically infected infants to quantify key resistance mutations of AZT (K70R/T215Y/T215F), NVP (K103N/Y181C) and 3TC (M184V) at detection limits of <1%.

Results: 50/87 HIV-infected women having started complex prophylaxis were eligible for the study. All women took AZT with a median duration of 53 days (IQR 39-64); all women ingested NVP-SD, 86% took 3TC. HIV-1 resistance mutations were detected in 20/50 (40%) women, of which 70% displayed minority species. Variants with AZT-resistance mutations were found in 11/50 (22%), NVP-resistant variants in 9/50 (18%) and 3TC-resistant variants in 4/50 women (8%). Three women harbored resistant HIV-1 against more than one drug. 49/50 infants, including the seven vertically HIV-infected were breastfed, 3/7 infants exhibited drug-resistant virus.

Conclusion: Complex prophylaxis resulted in lower levels of NVP-selected resistance as compared to NVP-SD, but AZT-resistant HIV-1 emerged in a substantial proportion of women. Starting AZT in pregnancy week 14 instead of 28 as recommended by the current WHO-guidelines may further increase the frequency of AZT-resistance mutations. Given its impact on HIV-transmission rate and drug-resistance development, HAART for all HIV-positive pregnant women should be considered.

3.8 Manuskript VI

Drug-resistant HIV-1 in cellular DNA of Tanzanian women following WHO-recommended antiretroviral prophylaxis for vertical transmission

Abstract

Background: Previously, we reported the emergence of drug-resistant HIV-1 variants in plasma of Tanzanian women after antiretroviral triple prophylaxis for vertical transmission. However, since resistant strains could persist as provirus in maternal peripheral blood mononuclear cells (PBMCs) the aim of this study was to quantify drug-resistant HIV-1 strains in corresponding PBMC-DNA samples.

Methods: 50 HIV-1 infected, pregnant Tanzanian women having started the WHO 2006 standard recommended antiretroviral prophylaxis consisting of antenatal zidovudine (AZT), intrapartal nevirapine single-dose (NVP-SD) and intra/postpartal AZT/lamivudine (3TC) were tested for resistance of plasma virus. HIV-1 DNA samples (>100 copies) of corresponding 4-6 and 12-16 week buffy coat samples were available from 36 women for analysis of key resistance mutations to AZT (K70R, T215Y/F), NVP (K103N, Y181C) and 3TC (M184V) by highly sensitive allele-specific real-time PCR (ASPCR).

Results: HIV-1 drug-resistance was detected in PBMC-DNA of 10/36 women (28%). After a median antenatal AZT-intake of 54 days, HIV-1 DNA carrying AZT-resistance mutations were detected in 9/36 (25%) women (4 K70R, 5 T215Y/F +/-K70R), NVP-resistance in 3/36 (8%) and 3TC-resistance in 1/36 (3%) women. Similar proportions of resistant variants and resistance patterns were observed in plasma and PBMCs.

Conclusions: The proportion of drug-resistant HIV-1 including the highly resistant AZT mutation T215Y/F was as high in PBMCs as in plasma virus. According to our findings, antenatal intake of exclusively AZT enhances the risk for selection of AZT-resistant variants, potentially persisting as provirus and thus negatively impacting future maternal treatment options. Therefore, AZT monotherapy during pregnancy should be replaced by combination therapy.

3.9 Manuskript VII

Minor drug-resistant HIV-1 variants in Tanzanian women after antiretroviral prophylaxis for vertical transmission detected by ultra-deep amplicon sequencing and allele-specific real-time PCR

Abstract

Background: As part of a feasibility study carried out between 2008 and 2009 to implement a triple antiretroviral regimen for prevention of mother-to-child transmission of HIV-1 recommended in 2006 by the WHO, pregnant women were screened for the development of resistant strains of HIV-1. The study offered the opportunity to compare allele-specific real-time PCR (ASPCR) and amplicon-based ultra-deep sequencing (UDS) for the detection of minor resistant variants in the HIV-1 reverse transcriptase (RT).

Methods: Plasma samples from 13 Tanzanian women were analyzed by ASPCR for key resistance mutations in the viral RT selected by AZT, 3TC and NVP (K70R, K103N, Y181C, M184V, T215Y/F) and within the Roche 454 HIV RTPV3 Alpha Site Study researcher run using the Life Sciences´ GS FLX System for additional resistance mutations selected by the regimen at proportions as low as 1%.

Results: Regimen-selected resistance mutations were identified in HIV-1 of 9/13 women by UDS and of 10/13 women by ASPCR. The number of resistance mutations identified by UDS was three times higher than by ASPCR (33 vs 11). Furthermore, all but one of the key resistance mutations detected by UDS were also identified by ASPCR, whereas four ASPCR-detected mutations were missed by UDS. Amplicon generation from samples having viral loads below 20,000 copies/ml failed more frequently by UDS, resulting in lower sequence coverage.

Conclusion: Both methods can provide useful information about minor resistance mutations of HIV-1 and their impact on disease or treatment outcome. While the main limitation of ASPCR for genotype resistance testing is its restriction to single resistance mutations, UDS is limited by its requirement for high viral loads in order to achieve high coverage. The main advantage of ASPCR is the high sensitivity of detection whereas sequence information provided by UDS reveals the complete resistance patterns within the genomic region analyzed. UDS could therefore facilitate routine monitoring for the presence of minor variants in the HIV quasispecies.

4 Diskussion

Die zeitlich begrenzte Einnahme antiretroviraler Medikamentenregime zur Prävention der Mutter-Kind Übertragung von HIV birgt die Gefahr der Resistenzentstehung aufgrund der subtherapeutischen Wirkstoffspiegel während der Medikamenten-Ausklingphase. Besitzen diese Medikamente noch dazu eine lange Halbwertszeit wie z.B. Nevirapin (NVP), ist die genetische Barriere zur Resistenzentstehung zudem gering wie z.B. bei NVP und Lamivudin (3TC) und/oder wird das Medikament allein verabreicht (hier: NVP-Einmaldosis und Zidovudin (AZT) während der Schwangerschaft), ist das Risiko erhöht.

In den vorliegenden Einzelarbeiten wurde daher die Entstehung von Resistenzen bei HIV-positiven Müttern und ihren vertikal infizierten Kindern innerhalb zweier Mutter-Kind-Kohorten in West-Uganda bzw. Südwest-Tansania untersucht und beschrieben. Die eingenommenen antiretroviralen Prophylaxeregime bestanden während des Untersuchungszeitraums

1. im ugandischen Studienkollektiv aus einer NVP-Einmaldosis, basierend auf den WHO PMTCT Empfehlungen von 2001 und
2. im tansanischen Studienkollektiv aus einer zeitlich begrenzten Einnahme eines Dreifachregimes bestehend aus AZT, 3TC und NVP, den WHO PMTCT Leitlinien von 2006 folgend.

Zum Nachweis der Resistenzmutationen wurden zwei hochsensitive Allel-spezifische PCRs (ASPCR) für die jeweils in Uganda und Tansania prävalenten Subtypen etabliert (Manuskript I und V). Die Proben der HIV-positiven Mütter und der vertikal infizierten Kinder wurden hinsichtlich der durch die eingesetzten Medikamente am häufigsten selektierten Resistenzmutationen in der Reversen Transkriptase (RT) in Anteilen von weniger als 1% analysiert (Manuskript II-VI). Ausgewählte Proben wurden zusätzlich mit einer amplikonbasierten „ultra-deep sequencing“ (UDS) Methode auf das Vorliegen weiterer minoritärer Resistenzmutationen in der viralen RT untersucht (Manuskript VII).
In der nachfolgenden Diskussion werden die beiden hier angewandten sensitiven Nachweismethoden ASPCR und UDS mit ihren Vor- und Nachteilen einander gegenübergestellt. Anschließend folgt ein Vergleich der beiden antiretroviralen Prophylaxeregime hinsichtlich Entstehungshäufigkeit, Proportion und Persistenz der Resistenzmutationen. Die daraus resultierenden Folgen und Risiken für die HIV-positive Mutter und das vertikal infizierte Kind in den untersuchten wie auch aktuell empfohlenen antiretroviralen Prophylaxeregime werden abschließend diskutiert.

4.1 Vergleich von ASPCR und UDS

Sensitive Resistenz-Nachweismethoden erlauben eine präzise Detektion von geringsten Anteilen an resistenten Virusvarianten in der Gesamtviruspopulation. Die Sensitivität einer Detektionsmethode ist von deren Nachweisgrenze abhängig.

Die ASPCR-Assays konnten in der vorliegenden Arbeit mit Nachweisgrenzen unter 1% etabliert werden: für die in Uganda prävalenten HIV-Subtypen A und D wurden Nachweisgrenzen von 0,019%/ 0,013% und 0,29% für die K103N(AAC), K103N(AAT) und Y181C(TGT) Mutationen ermittelt (Manuskript I). Für die in Tansania prävalenten HIV-Subtypen A, C und D wurden Detektionslimits von 0,99%/ 0,04%/ 0,01%/ 0,35%/ 0,63%/ 0,33% und 0,42% für die K70R, K103N(AAC), K103N(AAT), Y181C, M184V, T215Y und T215F Mutationen erreicht (Manuskript V). Sie ergaben sich aus experimentellen Validierungen mit DNA-Standards, deren Sequenzen 100% komplementär zu den jeweiligen Primersequenzen der ASPCR-Assays waren und entsprechen denen aus publizierten Arbeiten [76,120-125]. Mit der angewandten UDS Methode wurde im 454 HIV Alpha Site Studienlauf II eine Nachweisgrenze von 1,3% ermittelt (Manuskript VII) [131]. Auch diese entspricht bereits beschriebenen UDS-Detektionslimits [114,126-130]. Sowohl mit der ASPCR als auch mit der UDS konnten resistente Virusvarianten demnach sehr sensitiv detektiert werden.

Bei der Analyse von Patientenmaterial kam es in beiden Verfahren zu methodenbedingten Einschränkungen, deren Lösungsansätze hier kurz vorgestellt werden sollen:

1) Da für sensitive Assay-Nachweisgrenzen eine entsprechende Anzahl von viralen RNA-Kopien analysiert werden musste (z.B. mindestens 1000 untersuchte Viruskopien für den Nachweis von 0,1% Mutantenkopien), wurde die ASPCR-Nachweisgrenze zusätzlich mit RNA-Standards experimentell ermittelt. RNA-Standards wurden aus Virusvermehrungen von rekombinanten Mutanten- und Wildtyp-Viren (T215Y und T215T; exemplarisch für alle Mutationen) in Zellkulturen gewonnen und für deren Mischungen (0,1% bis 10% Mutante in Wildtyp) der kleinste nachweisbare Anteil in den verschiedenen Verdünnungen (10^3, 10^4 und 10^5 Kopien/ml Plasma) ermittelt. Anhand einer Regressionsgeraden aus den jeweiligen Nachweisgrenzen ließen sich Mutantenanteile identifizieren und diejenigen ausschließen, die unterhalb des cut-offs einer niedriglastigen Probe ($<1x10^4$ Kopien/ml Plasma) lagen (Manuskript V, Supplementary Materials and Methods).

2) Aufgrund der hohen Variabilität der HIV-Sequenzen kommt es bei der ASPCR zu den bekannten Limitierungen der PCR- basierten HIV-Diagnostik: Virussequenzen, die nicht 100% komplementär zu den Primersequenzen sind, führen zu mehr oder weniger deutlichen Verschiebungen der Cycle-Theshold (CT)-Werte in der real-time PCR und somit zu falschen Ergebnissen. Für dieses inhärente Problem wurden zwei Kompensationsmöglichkeiten entwickelt:

a) Subtyp-spezifische HIV-Sequenzunterschiede in den afrikanischen Proben konnten mit Hilfe einer begrenzten Anzahl von „wobble"- und/oder Inosin-Basen (subtyp-generische Basen) in den Primersequenzen ausgeglichen werden. Diese Vorgehensweise wurde auch von anderen Arbeitsgruppen beschrieben [117]. Die Primer der Subtyp A-, C- und D-spezifischen ASPCR-Assays enthielten daher entsprechend mehr generische Basen als die Subtyp A- und D-spezifischen ASPCR-Assays (Manuskript I und V).

b) Individuelle Sequenzunterschiede (Polymorphismen), die spezifisch für die virale Quasispezies des Infizierten waren, wurden durch die Verwendung einer Ausgangsprobe (vor Medikamenteneinnahme) zur Berechnung der CT-Werte-Änderung eines jeden Patienten erfolgreich kompensiert (individueller Wildtyp). Die Vorteile dieser Herangehensweise wurden bereits ausführlich in Manuskript I diskutiert. Stand diese Ausgangsprobe nicht zur Verfügung, wurde auf die Verwendung eines Wildtyp-Standards mit einer Assay-Spezifität von 93,5% ausgewichen (Manuskript I).

3) Bei der UDS wurde in der vorliegenden Arbeit die empfohlene Anzahl von 50 Mutantensequenzen innerhalb von 5000 Gesamtsequenzen für die valide Detektion von Minoritäten unter 1% [132] für keine AZT/3TC/NVP-resistenzassoziierten Positionen erreicht. Das Vorliegen einer Mutation wurde daher als valide erachtet, wenn 10 und mehr Mutantensequenzen innerhalb von mindestens 800 Gesamtsequenzen für diese Position vorlagen. Diese Definition ermöglichte die Bewertung von Resistenzmutationen bis zu 0,3% und ließ somit den Vergleich der Anzahl von Nachweisereignissen bei UDS und ASPCR zu (Manuskript VII). Da es sich bei der amplikonbasierten UDS um eine neue Methode zur HIV-Resistenzbestimmung handelt, ist die Definition des Detektionslimits noch nicht abschließend festgelegt. Auch in der Literatur sind die Angaben zu den Nachweisgrenzen dementsprechend sehr unterschiedlich (1%-0,05%) [127,133].

Mit der ASPCR werden Mutationen aufgrund der Hybridisierung von spezifischen Primern während einer PCR detektiert. Sie erlaubt so den sensitiven Nachweis einzelner Punktmutationen. Da für jede resistenzassoziierte Mutation (Codon) ein eigenständiger

Assay etabliert werden musste, wurden in der vorliegenden Arbeit einzelne medikamentenselektierte Schlüsselmutationen in der RT als Marker ausgewählt (AZT: K70R, T215Y, T215F; NVP: K103N, Y181C; 3TC: M184V), die zur Beschreibung der Entstehungshäufigkeit von Resistenzen in den jeweiligen Studienkollektiven dienten (Manuskript I-V). Mit Hilfe der UDS hingegen ließen sich in einem einzigen Assay alle potentiellen Resistenzmutationen im gesamten untersuchten Sequenzabschnitt der RT nachweisen. Die Ergebnisse lieferten ein vollständiges RT-Resistenzprofil der Virusvarianten einer Probe (Manuskript VII). Dadurch konnten mit der UDS insgesamt dreimal mehr Resistenzmutationen detektiert werden, von denen zwei Drittel an Positionen auftraten, die mit den ASPCR-Assays nicht untersucht wurden (z.B. K65R für 3TC, G190A und F227L für NVP, D67G und M41L für AZT u.a.). Die daraus resultierenden Medikamentenresistenzen für die jeweils untersuchten Proben waren dennoch nicht signifikant verschieden: beide Methoden zeigten vergleichbare Entstehungshäufigkeiten von AZT- und 3TC- Resistenzen in den 13 mütterlichen Plasmaproben (ASPCR: 38% und 8%, UDS: 31% und 8%, Fisher's exact test beide p=1,00). Die Entstehung von NVP- und dualresistenten Viruspopulationen wurde mit der ASPCR im Vergleich zur UDS tendenziell, wenn auch noch nicht signifikant unterschätzt (62% versus 31% und 31% versus 0%, Fisher's exact test p=0,24 und 0,10) (Manuskript V und VII). Der Vergleich anhand eines größeren Probenpanels ist erforderlich.

Sowohl die ASPCR als auch die UDS sind RT-PCR-basierte Nachweismethoden und setzten als ersten Schritt die Generierung eines spezifischen PCR-Produktes voraus. Die ASPCR wurde in der vorliegenden Arbeit als 2-stufige („nested“) real-time PCR etabliert. Das „äußere“ PCR-Produkt mit einer Länge von 644 Basenpaaren ließ sich aus Proben mit Viruslasten von nur 650 bzw. 1000 Viruskopien/ml Plasma und geringem Ausgangsvolumen (nur 150µl Plasma der Neugeborenen) zuverlässig amplifizieren und anhand eines Konzentrationsstandards quantifizieren (Viruslastmessung). Mit den spezifischen Produkten der „äußeren“ PCR konnten anschließend sowohl die verschiedenen Assays („innere“ PCRs) zur Quantifizierung der zu testenden Resistenzmutationen als auch die Sanger-Sequenzierung zur Ermittlung einer Populationssequenz durchgeführt werden (Manuskript I und V).
Das Roche UDS Protokoll erforderte neben einem Mindest-Plasmavolumen (500µl) auch eine empfohlene Mindest-Viruslast von 20.000 Kopien/ml. Tatsächlich war in dieser Arbeit die Generierung der 6 Ausgangsamplikons (RTP 1-6) nur für die Proben

erfolgreich, die Viruslasten größer als 20.000 Kopien/ml aufwiesen. Die Gründe hierfür wurden ausführlich in Manuskript VII diskutiert. Die ASPCR ist somit ca. 30mal sensitiver in der Amplifikation des Ausgangsproduktes als die UDS.

Beide beschriebenen Methoden ermöglichten einen sensitiven Nachweis von Resistenzmutationen, um retrospektiv die Risiken der Resistenzentstehung nach einem antiretroviralen Prophylaxeregime zu erkennen. Die Ergebnisse im Hinblick auf die Häufigkeit der Medikamentenresistenz waren nicht signifikant verschieden. Da die in dieser Arbeit zu analysierenden Proben (n= ca. 600) häufig von geringen Volumina und/oder geringer Viruslast waren, erwies sich für diese Studie die sensitivere ASPCR als bevorzugte Methode. Obwohl sie auf die Analyse einzelner Punktmutationen beschränkt ist, stellte sie ein probates Verfahren zur Abschätzung der Entstehungshäufigkeit von Resistenzen in den jeweiligen Studienkollektiven dar. Die UDS wird sich dennoch voraussichtlich in Zukunft als Methode zur Detektion minoritärer Virusvarianten für Proben mit höheren Viruslasten durchsetzen, da sie ein vollständiges Bild der Resistenzlage liefert. Ein Bedarf in der Individualdiagnostik besteht jedoch erst, wenn die klinische Relevanz und der Einfluss der Minoritäten eindeutig geklärt ist.

4.2 Vergleich der HIV-Resistenzentstehung nach Einsatz zweier antiretroviraler Medikamentenregime zur Prävention der HIV-Transmission

4.2.1 HIV-Resistenzentstehung bei Müttern

Die NVP-Einmaldosis führte im ugandischen Studienkollektiv innerhalb von drei Monaten bei 62% der Mütter zur Entstehung von resistenten Plasmaviren mit den NVP-selektierten Mutationen K103N und/oder Y181C (Manuskript II). Nach Einnahme eines Dreifachregimes bestehend aus AZT, 3TC und NVP kam es im gleichen Zeitraum bei 40% der Mütter des tansanischen Studienkollektivs zur Selektion der HIV-Resistenzmutationen K70R und T215Y/F (AZT), K103N und Y181C (NVP) sowie M184V (3TC) (Manuskript V). Beide Medikamentenregime sind demnach mit einem hohen Risiko der Resistenzentstehung verbunden.

Bei NVP ist die genetische Barriere zur Resistenzentstehung gering. Zudem führt die lange Halbwertszeit von NVP (ca. 45 Stunden) zu einem lang anhaltenden suboptimalen Wirkstoffspiegel nach dem Absetzen des Medikaments. Beide Eigenschaften begünstigen die schnelle Selektion von NVP-Resistenzen. Bereits nach einer NVP-Einmaldosis zur Transmissionsprophylaxe wurde die Resistenzentstehung zahlreich beschrieben [58]. Mit der Einführung der postpartalen AZT/3TC-Ergänzung im 2006 empfohlenen Dreifachregime war eine Reduktion der NVP-Resistenzen vorgesehen [64]. Tatsächlich war in den hier untersuchten Studienkollektiven eine Abnahme in der Resistenzentstehung zu erkennen: die Entstehungshäufigkeit der Resistenzen sank von 62% nach der NVP-Einmaldosis (Manuskript II) auf 18% nach der postpartalen AZT/3TC-Ergänzung des Dreifachregimes (Manuskript V). Diese Beobachtung wurde unterstützt von der Tatsache, dass die Frau, welche die AZT/3TC-Ergänzung nicht erhalten hatte, die höchsten Anteile NVP-resistenter Virusvarianten aufwies. Trotz postpartaler Medikamentenkombination entstanden noch immer bei 18% der Frauen NVP-resistente Viren. Aufgrund der langen Halbwertszeit liegen NVP-Wirkstoffspiegel im mütterlichen Blut über einen längeren Zeitraum vor [134] und führen auch noch nach Wochen zur Resistenzselektion. In beiden Studienkollektiven waren zu Woche 6 die höchsten Anteile an Frauen mit NVP-resistenten Viruspopulationen zu finden. Aufgrund der kurzen Halbwertszeiten von AZT und 3TC (1-2 bzw. 5-7 Stunden) erzielen diese lediglich über die 7-tägige Einnahmedauer replikationshemmende Wirkstoffspiegel. Schon eine auf vier Wochen verlängerte postpartale Einnahme der kombinierten Wirkstoffe reduzierte die Häufigkeit NVP-resistenter Virusvarianten deutlich effizienter auf 1,8% [135,136].

Die präpartale AZT-Einnahme führte zu einer signifikanten Abnahme der Viruslast zur Geburt, nicht aber zu einer Senkung unter die Nachweisgrenze. Virusreplikationen fanden offenbar unter dem Selektionsdruck von AZT weiterhin statt und resultierten in einer signifikant häufigeren Entstehung von AZT-Resistenzmutationen bei langer (>10 Wochen) als bei kurzer AZT-Einnahmedauer (<10 Wochen, Manuskript V). Eine Einnahme über durchschnittlich 7,5 Wochen während der Schwangerschaft und für sieben Tage postpartum führte bei knapp einem Viertel der tansanischen Mütter (22%) zur Selektion von AZT-Resistenzmutationen in Plasmaviren. Bei der Mehrheit dieser Frauen (14%) handelte es sich um die transient auftretende K70R Mutation. Diese ist lediglich mit einer geringen phänotypischen Resistenz gegen AZT assoziiert [123], doch ihr frühes Auftreten gilt als eindeutiger Indikator für die Entstehung weiterer AZT-Resistenzmutationen. Trotz hoher genetischer Resistenzbarriere entstanden bei 8% der Frauen Viruspopulationen mit T215Y/F-Mutationen, die mit einer hochgradigen AZT-Resistenz assoziiert sind [137].
Eine weitere häufige Ursache der Resistenzentstehung ist die mangelnde Medikamentenadhärenz, zu der es insbesondere bei komplexen Medikamenteneinnahme-Schemata kommen kann [138]. Die Frauen, die für Resistenztestung ausgewählt wurden, wiesen jedoch aufgrund der Auswahlkriterien (mind. drei Follow-up Proben) generell eine hohe Bereitschaft zur korrekten Teilnahme auf. Die Adhärenz war bei ihnen hoch (97,5% IQR 86-100; Berechnung nach Kirsten *et al.* 2011 [138]) und unterschied sich nicht signifikant in den Gruppen der Frauen mit und ohne AZT-resistente Virusvarianten (T-Test: p= 0,85). Die Auswirkung einer nicht- adhärenten Medikamenteneinnahme kann daher anhand dieses untersuchten Studienkollektivs nicht beurteilt werden.
Für 3TC ist ebenfalls die genetische Barriere zur Entstehung der Resistenzmutation M184V sehr gering. Während der einwöchigen postpartalen AZT/3TC-Ergänzung entstanden zwar bei 8% der Mütter Plasmaviren mit dieser Mutation, sie lagen jedoch ausschließlich in sehr geringen Anteilen unter 1% vor (Manuskript V). Diesen M184V Minoritäten wurde in der Literatur bislang noch keine klinische Bedeutung zugeordnet.

Während durch eine NVP-Einmaldosis monoresistente Virusvarianten selektiert werden, (Manuskript II, III und IV), entstehen als Resultat des antiretroviralen Dreifachregimes auch dual- oder multiresistente Viruspopulationen (AZT/3TC, NVP/3TC, AZT/NVP, AZT/3TC/NVP; Manuskript V-VII). Der effektiven Reduktion der NVP-Resistenzen steht die nachteilige Entstehung von AZT- und Dualresistenzen entgegen.

4.2.2 Mögliche Auswirkungen der HIV-Resistenzentstehung für den Erfolg anschließender antiretroviraler Therapien

Ohne medikamentösen Selektionsdruck wird die Mehrzahl der Resistenzen rasch wieder vom Wildtyp ersetzt [104-106]. Bei einem Viertel der Frauen des ugandischen Studienkollektives konnten jedoch auch noch 6-18 Monate nach der NVP-Einmaldosis resistente Virusvarianten im Plasma mit der ASPCR detektiert werden (Manuskript II).

Im tansanischen Studienkollektiv wiesen noch 21% (6/29) der Mütter resistente Virusvarianten in den 3-Monats-Plasmaproben auf. Da Proben von späteren Zeitpunkten nicht vorhanden waren, konnte eine Persistenz über diesen Zeitraum hinaus nicht ermittelt werden. Es können lediglich Prognosen anhand von Literaturangaben gestellt werden:

4% der Frauen wiesen im Untersuchungszeitraum Plasmaviren mit der AZT-selektierten Mutation T215Y auf. In Abwesenheit des Selektionsdrucks revertiert diese gewöhnlich statt zum Wildtyp zu den „Revertanten“ T215D/C/S [83,84], die über Jahre im Plasma persistieren können [100]. Unter erneutem Selektionsdruck von AZT mutieren diese aufgrund der niedrigeren genetischen Barriere rasch wieder zur T215Y [85].

Bei 28% der Frauen des tansanischen Studienkollektives wurden resistente Virusvarianten in den PBMCs der Buffycoat-Proben identifiziert. Unter diesen lagen bei 25% der Frauen AZT-selektierte Resistenzen (11% K70R, 8% T217Y/F), bei 8% NVP-Resistenzen und bei 3% 3TC-Resistenzen in der HIV-DNA vor (Manuskript VI). Die resistenztragende Virus-DNA wird während des Replikationszyklus in das Wirtszellgenom integriert und kann dort langfristig als Provirus persistieren. Belec *et al.* [139] identifizierte T215Y/F-Mutationen noch 20 Monate nach dem Absetzten von AZT in proviraler DNA; NVP-Resistenzmutationen wurden noch 10,5 Monate nach NVP-Exposition in replikationskompetenten Proviren nachgewiesen [140]. Es wird vermutet, dass diese unter erneutem selektiven Druck und Re-Aktivierung der Virusreplikation rasch zur dominierenden Viruspopulation im Plasma ansteigen können [141-143].

Mit einer Archivierung und Re-Aktivierung von proviralen NVP-Resistenzen ist u.U. die folgende Beobachtung zu erklären: drei Monate nach der NVP-Einmaldosis lag im Plasmavirus einer Mutter (Nr.9, Manuskript II) die Y181C-Mutation zu Anteilen von ca. 10% vor, diese sank in den Monat 6 und 12 Proben zu undetektierbaren Anteilen ab (100% Wildtyp) und wurde durch den Beginn einer NVP-haltigen Therapie in Monat 6 zu Anteilen von 55% in der Monat 18 Probe hochselektiert. Buffycoat-Proben dieser Mutter zur Identifikation von Resistenzen in proviraler DNA lagen leider nicht vor.

Insgesamt wiesen die Ergebnisse der vorliegenden Arbeit sowohl nach NVP-Einmaldosis als auch nach Einnahme des Dreifachregimes auf eine Persistenz von resistenten Virusvarianten über mehrere Monate hin. Das Vorliegen von NVP-resistenten Viren zu Therapiebeginn wurde mehrfach mit einem virologischen Versagen von NVP- oder NNRTI-haltigen Therapien in Verbindung gebracht [112,115,144-146]. Da Resistenztestungen vor Therapiebeginn in Subsahara-Afrika nicht zur Verfügung stehen, wird ein Therapiebeginn generell erst 6-12 Monate nach NVP-Exposition empfohlen [61]. Die erste Therapieoption besteht aus der Einnahme von 2 NRTI und einem NNRTI. Versagt diese, ist die Gabe eines Proteaseinhibitors (PI) statt NNRTIs als zweite Therapieoption vorgesehen [65]. NVP-Einmaldosis exponierte Frauen mit virologischen Versagen einer NNRTI-haltigen Therapie aufgrund der NVP-Resistenzen erhalten die Chance eines Therapieerfolges mit einer PI-haltigen Medikamentenkombination. Für Frauen mit AZT- und AZT/NVP-resistenten Viruspopulationen aus vorheriger Dreifachprophylaxe (Manuskript V und VI) sind die Therapieoptionen deutlich stärker eingeschränkt, da auch die Medikamente der NRTI-Klasse (Kreuzresistenz gegen Didanosin und Stavudin) verminderte Wirkung zeigen könnten [147,148].

In den beiden untersuchten Studienkollektiven lagen ASPCR-detektierte Resistenzmutationen bei 50% bzw. bei 70% der Mütter (NVP-Einmaldosis/ Dreifachregime) als minoritäre Virusvarianten in Anteilen unter 5% in der Gesamtviruspopulation vor. Die klinische Bedeutung der Minoritäten für den Verlauf einer Therapie ist noch nicht abschließend geklärt. Eine Reihe von Studien zeigte einen Zusammenhang zwischen deren Vorliegen und dem Versagen von NNRTI-haltigen [74,112,115,144-146], PI-haltigen [107,149,150] und AZT-haltigen Therapien [151]. Es liegen aber auch solche Studien vor, die keinen Zusammenhang beobachteten [152]. Die klinische Relevanz ist vermutlich auch bei Minoritäten abhängig von der Art der Resistenzmutation, der resultierenden viralen Fitness und von der jeweiligen Folgetherapie [153]. Während NNRTI-selektierte Punktmutationen bereits mit einer hohen Resistenz gegenüber allen NNRTI assoziiert sein können, entstehen Resistenzen gegenüber PI und AZT aus der Akkumulation von verschiedenen Mutationen [154]. Es ließ sich bislang kein Grenzwert oder „kritischer Anteil“ an resistenten Virusvarianten erkennen, zumal Minoritäten innerhalb von Wochen durch rasche Selektion zu Majoritäten in der viralen Quasispezies heranwachsen können [74].

4.2.3 Mögliche Auswirkungen der HIV-Resistenzentstehung für die vertikale Transmission von HIV

Die Einführung der präpartalen AZT-Einnahme im Dreifachregime hatte eine effektivere Senkung des in-utero und intrapartalen Transmissionsrisiko durch die prophylaktische Wirkung des plazentagängigen Medikaments im Kind selbst [155], und durch eine Viruslastsenkung bei der Mutter zum Ziel [65]. Tatsächlich konnte die durchschnittliche Viruslast im tansanischen Studienkollektiv während der Schwangerschaft insgesamt signifikant gesenkt werden, doch war die Viruslast zum Geburtstermin bei Frauen mit AZT-resistenten Plasmaviren in den Geburtsproben bis zu zehnmal höher als bei Frauen ohne AZT-resistente Virusvarianten zur Geburt (Manuskript V). Auch wenn der Zusammenhang von hoher Viruslast bzw. AZT-resistenten Plasmaviren zur Geburt und höheren Transmissionsraten im tansanischen Studienkollektiv nicht belegt werden konnte (Manuskript V), so ist doch zahlreich belegt, dass das Risiko der vertikalen Transmission mit zunehmender Viruslast der Schwangeren bzw. Mutter steigt [33,34]. Schlussfolgerungen zur Auswirkung von Resistenzen auf die vertikale HIV-Transmission sind in dieser Arbeit aufgrund der geringen Fallzahlen nicht möglich.
Das Vorliegen von NVP-Resistenzen zum Zeitpunkt einer folgenden Schwangerschaft wurde nicht mit einem erhöhten Transmissionsrisiko in Verbindung gebracht [156].

Insgesamt waren die HIV-Transmissionsraten ca. 6 Wochen nach Geburt in beiden Studienkollektiven mit 9,3% nach der NVP-Einmaldosis und 14,3% nach Einnahme des Dreifachregimes (Fisher's exact test: $p=0.41$) sehr hoch (Manuskript III+V). Der postpartale Transmissionsweg ist hier wahrscheinlich, da alle infizierten Kinder in Uganda und Tansania gestillt wurden.

Im ugandischen Studienkollektiv wurden bei 37% der Mütter meist minoritäre NVP-resistente Virusvarianten in Brustmilch 6 oder 12 Wochen nach NVP-Einmaldosis detektiert. Die Entstehung von resistenten Virusvarianten in Brustmilch nach Dreifachprophylaxe wurde bislang nicht untersucht, ein Vergleich für die beiden antiretroviralen Regime ist daher nicht möglich. Die Übertragung resistenter Viren von der Mutter auf das Kind wurde schon früh beschrieben [157] und stellt daher ein reales Risiko für das Kind dar. Sie konnte aber in keinem der beiden Studienkollektive nachgewiesen werden.

4.2.4 HIV-Resistenzentstehung bei vertikal infizierten Kindern

Das Auftreten von Resistenzen konnte auch bei den HIV-infizierten Kindern nach Einnahme des Prophylaxeregimes dokumentiert werden. Das Risiko für eine Resistenzentstehung in den ersten drei Lebensmonaten war in beiden Studienkollektiven mit 4/9 Kindern (Uganda) und 3/7 Kindern (Tansania) ähnlich hoch (Manuskript III+V). Während es sich bei den ugandischen Kindern ausschließlich um NVP-resistente Virusvarianten handelte, wurden bei den tansanischen Kindern sowohl NVP- als auch AZT-resistente Viren identifiziert, die bei einem Kind sogar als dualresistente Viruspopulation vorlagen. Während bei den ugandischen Kindern diejenigen die höchsten Anteile an resistenten Virusvarianten trugen, die in-utero infiziert wurden (Manuskript III), ließ sich in dem einzigen in-utero infizierten Kind aus Tansania keine Resistenzentstehung nachweisen. Die höchsten NVP-Resistenzanteile wurden hier bei einem Kind detektiert, das kein postnatales AZT in Ergänzung zu NVP erhalten hatte. Die postnatale AZT-Einnahme würde demnach die Entstehung von NVP-Resistenzen auch bei Kindern reduzieren, eine generelle Aussage ist auf Grund der geringen Fallzahl (n=1) nicht möglich.

Bei den ugandischen Kindern wurde eine Persistenz bis zu 6 Monaten beobachtet und entspricht damit den Beobachtungen von Persaud *et al.* [158], der resistente HIV-Varianten in 26% der Kinder (n=19) noch sechs Monate nach NVP-Einmaldosis nachweisen konnte. Über die Persistenz der HIV-Resistenzen bei den tansanischen Kindern über den Untersuchungszeitraum von drei Monaten hinaus konnte aufgrund fehlender Proben keine Aussage getroffen werden (Manuskript V).

4.3 Ausblick: HIV-Resistenzentstehung nach Einsatz aktueller antiretroviraler Medikamentenregime zur Prävention der HIV-Transmission und mögliche Auswirkungen

2010 wurden die Leitlinien der WHO erneut überarbeitet: die Vorverlegung des Medikamenten-Einnahmebeginns von der 28. auf die 14. Schwangerschaftswoche sieht eine Reduktion der präpartalen HIV-Übertragung vor. Da das Stillen in ressourcenlimitierten Ländern trotz HIV-Transmissionsrisiko aufgrund hoher Sterberaten nicht-gestillter Babys durch Diarrhoe, Pneumonie und Unterernährung weiterhin favorisiert wird [159], wurde das Regime auf die Zeit des Stillens ausgeweitet. Die Anhebung der CD4-Zellzahl von 200 auf 350 Zellen/µl führt hier zu einem früheren

Beginn der Langzeittherapien im Infektionsverlauf. Eine der empfohlenen Prophylaxeoptionen entspricht dem Medikamenten-Einnahmeschema von 2006 mit dem Unterschied, dass die präpartale AZT-Anwendung der Mutter in einer 6- statt 3-monatigen Einnahme resultiert und die kindliche NVP-Dosis um die Stillphase ausgedehnt wird. Alternativ wird die Kombination von drei Medikamenten (cART) für die Mutter von der Schwangerschaft bis zum Abstillen empfohlen (Tabelle 1).

Aufgrund der vorliegenden Ergebnisse besteht der dringende Verdacht, dass eine 6-monatige präpartale AZT-Einnahme der Mütter die beschriebenen Risiken der HIV-Resistenzselektion erhöht (Manuskript V). Auch die tägliche NVP-Dosis zur postnatalen Transmissionsprophylaxe für die gestillten Kinder birgt für bereits vertikal infizierte Kinder ein hohes Risiko der Resistenzentstehung. Fogel *et al.* [160] identifizierte noch 6 und 12 Monate nach 14-tägiger NVP- oder NVP/AZT-Prophylaxe bei 80-85% bzw. 60-75% der infizierten Kinder der PEPI-Malawi-Studie NVP-resistente Virusvarianten. Eine tägliche NVP-Dosis zur postnatalen Transmissionsprophylaxe führt bei vertikal infizierten Kindern offenbar zu subtherapeutischen Wirkstoffspiegeln mit resistenzselektierenden Folgen [158]. Da im Jahr 2009 in den ressourcenlimitierten Ländern überhaupt nur 15% der Kinder in den ersten Lebensmonaten auf eine HIV-Infektion getestet wurden [161], bleibt die Infektion der Kinder oft lange unerkannt. Der Benefit der verlängerten NVP-Einnahme zur Reduktion der postnatalen HIV-Transmission steht somit dem Risiko der HIV-Resistenzentstehung bei unerkannt infizierten Kindern gegenüber.
Die Empfehlung einer cART zur Transmissionsprophylaxe (Tabelle 1) basiert auf Ergebnissen der „Kesho Bora study“ ("A better future", Swahili) [162]. Hier konnte die HIV-Infektion bei Kindern um 43% bis zum 1. Lebensjahr und die Transmission durch das Stillen ohne schwerwiegende Nebenwirkungen um 54% gesenkt werden [163,164]. Eine cART vermag bei adhärenter Medikamenteneinnahme das Risiko der HIV-Resistenzentstehung auf ein Minimum zu reduzieren [165]. Da cART jedoch die kostenintensivere Variante ist, wird bereits in einigen Ländern Subsahara Afrikas - so auch in Tansania - das Prophylaxeregime mit 6-monatiger AZT-Einnahme in der Schwangerschaft als Standardintervention in die PMTCT Versorgung etabliert (mündliche Kongressinformationen, Tansania PMTCT-Leitlinien 2011). Unabhängig von solchen Empfehlungen ist die Realität in den meisten Gegenden Subsahara Afrikas eine andere: in den ländlichen Gegenden ist oftmals nur die NVP-Einmaldosis als „Minimalstandard“ verfügbar (Abbildung 1) [166].

Die Verbreitung und Transmission resistenter Viruspopulationen gewinnt in Afrika zunehmend an Bedeutung. Eine Studie aus Mwanza, Tanzania ermittelte für therapienaive neuinfizierte Patienten eine dramatisch hohe Prävalenz an HIV-Resistenzen von 19,1% [167]. Gerade für Regionen mit fehlenden zweiten Therapieoptionen sind zirkulierende Resistenzen ein absehbares Problem.

Die Ergebnisse dieser Arbeit liefern eindeutige Hinweise darauf, dass die Einnahme der beiden hier beschriebenen antiretroviralen Medikamentenregime mit einem hohen Risiko der Resistenzentstehung für HIV-infizierte Mütter und Kinder verbunden ist. Mit einer Einschränkung anschließender Therapieerfolge muss aufgrund der Persistenz der Resistenzen gerechnet werden, wenn die Therapie innerhalb eines Jahres nach Geburt begonnen wird. Während eine NVP-Einmaldosis die Therapieoption mit Medikamenten der NNRTI-Klasse limitieren kann, ist nach einem antiretroviralen Dreifachregime mit präpartaler AZT-Einnahme die Therapieoption mit Medikamenten der NRTI- und NNRTI-Klasse unter Umständen deutlich stärker eingeschränkt. Solange Medikamente der RTI-Klasse einzeln verabreicht werden (hier: AZT in der Schwangerschaft und NVP-Einmaldosis), bleibt auch die gravierende Resistenzproblematik bestehen. Unter dem hier beschriebenen Aspekt der Resistenzentwicklung, aber auch zur effektiveren Reduktion der vertikalen Transmission, ist eine cART für HIV-positive Schwangere und Mütter mindestens für die Dauer der Schwangerschaft und Stillzeit eine wünschenswerte Alternative. Da die individuelle Resistenzlage vor Therapiebeginn in ressourcenlimitierten Ländern nicht abgeklärt werden kann, ist der retrospektive Nachweis der Resistenzhäufigkeit zur generellen Risikoabschätzung essentiell. Sensitive Nachweismethoden sind dabei hilfreich.

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6 Zusammenfassung

Subsahara-Afrika ist die weltweit am stärksten betroffene Region von HIV/AIDS. Im Jahr 2010 entfielen ca. 68% aller HIV-Infektionen und 90% der HIV-Neuinfektionen bei Kindern (<15 Jahre) auf diese Region. Nahezu alle Infektionen von Kindern sind die Folge einer HIV-Übertragung von der Mutter auf das Kind (vertikale Transmission). Zur Prävention der Mutter-Kind-Übertragung von HIV werden in Subsahara-Afrika seit 2001 antiretrovirale Regime eingesetzt, die auf Empfehlungen der WHO basieren. Eine Nevirapin (NVP) - Einmaldosis für die HIV-positive Schwangere und das Neugeborene zur Geburt stellten für viele Jahre die antiretrovirale Standardprophylaxe in den meisten Ländern Subsahara-Afrikas dar. Zur effektiveren Reduktion der HIV-Transmission aktualisierte die WHO 2006 ihre Empfehlungen zugunsten eines antiretroviralen Dreifachregimes. Dieses bestand für die Mutter aus Zidovudin (AZT) ab der 28. Schwangerschaftswoche, einer NVP-Einmaldosis zur Entbindung sowie AZT und Lamivudin (3TC) für sieben weitere Tage und sah für das Neugeborene eine NVP-Einmaldosis zur Geburt mit siebentägiger postnataler AZT-Ergänzung vor. Die zeitlich begrenzte Einnahme solcher Regime birgt jedoch die Gefahr der Resistenzentstehung insbesondere dann, wenn lange Halbwertszeiten der Medikamente zu suboptimalen Wirkstoffspiegeln im Blut führen. Sowohl dies als auch der Einsatz nur eines Medikaments führen zu einem Selektionsdruck auf die replizierende Viruspopulation und resultieren, in Verbindung mit einer geringen genetischen Resistenzbarriere, in einer sehr raschen Entstehung resistenter Virusvarianten.
Resistenzen können wiederum zukünftige Behandlungsoptionen für die HIV-positiven Mütter und deren vertikal infizierte Kinder einschränken. Die Kenntnis um das Ausmaß der Resistenzentstehung nach antiretroviralen Prophylaxeregimes ist daher in Ländern ohne Zugang zur genotypischen Resistenzbestimmung von großer Bedeutung für den Erfolg einer später notwendigen antiretroviralen Langzeittherapie.

Da die Resistenzen oft nur in geringen Anteilen in der viralen Quasispezies des HIV-Infizierten vorkommen (minoritäre Varianten), werden sie mit der routinemäßig durchgeführten genotypischen Resistenzbestimmung (Sanger-Sequenzierung der Populationssequenz; Detektionslimit ca. 20%) methodenbedingt nicht erfasst. Daten zur Entstehung von minoritären Resistenzen in Anteilen <20% nach NVP-Einmaldosis liegen nur begrenzt vor und fehlen für das antiretrovirale Prophylaxeregime mit AZT, 3TC und NVP nach 2006 WHO-Empfehlungen. In den vorliegenden Einzelarbeiten wurden daher

resistente Viruspopulationen inklusive minoritärer Varianten in Plasma- und/oder Brustmilchproben von 39 HIV-positiven Müttern und deren vertikal infizierte Kinder nach einer NVP-Einmaldosis (Studienkollektiv: West-Uganda 2003-2005) bzw. in Plasma- und/oder Buffycoat-Proben von 50 Mutter-Kind-Paaren nach Dreifachregime (Studienkollektiv: Südwest-Tansania 2008-2009) untersucht. Hierzu wurden hochsensitive Allel-spezifische PCR (ASPCR) Assays für die in Uganda und Tansania prävalenten HIV-Subtypen A und D bzw. A, C und D und die regimebedingt am häufigsten zu erwartenden Resistenzmutationen in der Reversen Transkriptase (RT) etabliert. Zusätzlich wurden 13 ASPCR-voranalysierte Proben mit einer amplikonbasierten ultra-sensitiven Sequenzierungsmethode (UDS) auf das Vorliegen weiterer minoritärer Resistenzmutationen in der viralen RT getestet. Ziele der Promotionsarbeit waren die Eignung der beiden Methoden zum sensitiven Resistenznachweis und auch die beiden antiretroviralen Medikamentenregime unter dem Aspekt der Resistenzentwicklung zu bewerten.

Beide Methoden, ASPCR und UDS, ermöglichten den Nachweis von resistenten HIV-Varianten in Anteilen kleiner als 1%. In der Amplikon-Generierung als der wesentliche initiale Schritt beider Methoden war die ASPCR 30mal sensitiver als die UDS (650 versus 20.000 Kopien/ml). Die ASPCR war daher die bevorzugte Methode zur sensitiven Resistenztestung von Proben der Studienkollektive mit niedrigen Viruslasten.

Ein Vergleich der ASPCR-Ergebnisse zeigte, dass sowohl die NVP-Einmaldosis als auch das Dreifachregime mit AZT, 3TC und NVP häufig zur Entstehung resistenter Virusvarianten führten (62% bzw. 40%). Bei jeweils der Hälfte der Frauen lagen diese als minoritäre Virusvarianten in Anteilen <5% im Plasma vor. Während nach Einnahme des Dreifachregimes die Entstehungshäufigkeit von NVP-Resistenzen geringer war als nach einer NVP-Einmaldosis allein (62% versus 18%), entstand jedoch bereits bei einer durchschnittlichen pränatalen AZT-Einnahme über 7,5 Wochen ein erheblicher Anteil an AZT-resistenten Virusvarianten (22%). Die HIV-Übertragungsraten waren in beiden Studienkollektiven vergleichbar und mit 9,3% (NVP-Einmaldosis) und 14,3% (Dreifachregime) sehr hoch. Bei jeweils knapp der Hälfte der vertikal infizierten Kinder wurden ebenfalls resistente HIV-Varianten identifiziert; eine vertikale Transmission resistenter HIV konnte jedoch nicht nachgewiesen werden. Das Vorliegen von resistenten Viren im mütterlichen Plasma noch 18 Monate nach der NVP-Einmaldosis und in proviraler DNA mütterlicher Blutzellen nach Einnahme des Dreifachregimes weist auf die Persistenz von Resistenzmutationen über mehrere Monate hin. Diese könnten den Erfolg

von Therapien, die innerhalb des ersten Jahres nach Transmissionsprophylaxe begonnen werden, einschränken. Während die Entstehung monoresistenter Viren nach NPV-Einmaldosis die Medikamentenoptionen für NNRTI-haltige Therapien limitiert, könnten AZT/NVP-selektierte Resistenzmutationen nach einem Dreifachregime anschließende Therapieoptionen durch den Ausschluss von mehreren Medikamenten der NNRTI- und NRTI-Klasse drastisch einschränken. Zur effektiveren Reduktion der vertikalen Transmission und der Resistenzentstehung sollten daher schwangere HIV-positive Frauen antiretrovirale Medikamente nur als Kombinationstherapie (cART) mindestens für die Dauer der Schwangerschaft und Stillzeit erhalten.

7 Abstract

Sub-Saharan Africa represents the most heavily HIV-affected region worldwide. In 2010 this region accounted for approximately 68% of all HIV-infections and 90% of new HIV infections in children (<15 years). Almost all HIV infections in children are the result of mother-to-child transmission (vertical transmission). Antiretroviral regimens based on WHO recommendations have been implemented in Sub-Saharan Africa since 2001 to prevent the mother-to-child transmission of HIV. A single-dose (SD) of nevirapine (NVP) for the HIV-positive pregnant women and their newborn children at birth has been the standard regimen in most Sub-Sahara African countries for many years. In order to more effectively reduce HIV transmission, the WHO recommendations were updated in 2006 in favor of a triple antiretroviral regimen. This regimen consists, for the mother, of zidovudine (AZT) starting at the 28th week of pregnancy, NVP-SD during labor and AZT plus lamivudine (3TC) at birth and for seven days thereafter. The child receives NVP-SD at birth and AZT for seven days. However, the temporary nature of such regimes poses the risk of resistance development, particularly if the long half-lives of drugs results in suboptimal levels in the blood. Both this and the use of only a single drug lead to a selective pressure on the replicating virus population and, in combination with a low genetic barrier to development of drug resistance, result in the rapid emergence of resistant viral variants. Resistance may negatively impact future treatment options for HIV-positive mothers and their vertically infected children. Knowledge of the scope of resistance development after antiretroviral prophylaxis in countries without access to genotypic resistance testing is therefore of great importance for the success of a subsequent long-term therapy.

Resistance mutations are frequently present in the viral quasispecies of HIV-infected patients at only low proportions (minor variants) and are not detectible by routine genotypic resistance testing (Sanger sequencing of population sequence, detection limit approximately 20%). Data is limited regarding the emergence of resistance in proportions under 20% after NVP-SD and is lacking for the antiretroviral regimen using AZT, 3TC and NVP recommended by the WHO in 2006. To address this, the sub-studies presented here investigated in detail the emergence of resistant virus variants, including minor variants, in the plasma and/or breast milk of 39 HIV-positive mothers and their vertically infected children after exposure to the NVP-SD (study cohort: Western Uganda 2003-2005) or in the plasma and/or buffy coats of 50 mother-child pairs after triple antiretroviral

prophylaxis (study cohort: Southwest Tanzania, 2008-2009). A highly sensitive allele-specific PCR (ASPCR) specific for the most prevalent subtypes A and D in Uganda or A, C and D in Tanzania and for the most common regimen-selected resistance mutations in the reverse transcriptase (RT) was established for the analysis. In addition, 13 samples pre-analysed by ASPCR were tested in parallel with an amplicon-based ultra-deep sequencing method (UDS) for the presence of further minor resistance mutations within the viral RT. The aim of this dissertation was to assess the suitability of the two sensitive detection methods and of the antiretroviral prophylaxis regimen in terms of resistance development.

Both methods, ASPCR and UDS, enabled the detection of resistant HIV-variants at proportions under 1%. ASPCR was found to be 30 times more sensitive than the UDS (650 versus 20,000 copies/ml) in amplicon generation, an essential initial step for both methods and was therefore the preferred method for the sensitive resistance testing of study samples with low viral loads.

A comparison of ASPCR results demonstrated that the NVP-SD as well as the triple regimen with AZT, 3TC and NVP frequently selected for resistant viral variants (62% versus 40% respectively). Resistant plasma virus variants were present as minorities at proportions under 5% in half of the women from both study cohorts. While the development of NVP-resistance was reduced after triple prophylaxis compared to the NVP-SD alone (from 62% to 18%), a high proportion of additional AZT-resistance mutations emerged after a median 7.5 weeks of prepartal AZT treatment (22%). The transmission rates were comparable and very high in both study cohorts (9.3% for NVP-SD and 14.3% for the triple regimen). Both regimens selected for resistant virus in almost half of the vertically infected children, although vertical transmission of resistant virus could not be demonstrated. The presence of resistance mutations in maternal plasma-virus for 18 months after NVP-SD and in the proviral DNA of the maternal host cells after treatment with the triple regimen demonstrates the persistence of resistant mutations that might impact the success of a “first line” therapy if initiated within the first year after antiretroviral transmission prophylaxis. While the presence of mono-resistant viruses after NVP-SD may limit the options of first-line therapies using NNRTIs, the AZT/NVP-resistance mutation selected by the triple regimen could drastically reduce subsequent treatment options by excluding several NNRTIs and NRTIs. Therefore, to reduce vertical transmission and the development of resistance, a combination of antiretroviral drugs (cART) should be offered to all pregnant HIV-infected women at least for the duration of pregnancy and breast-feeding.

8 Liste der veröffentlichten Arbeiten

- **Hauser A**, Mugenyi K, Kabasinguzi R, Bluethgen K, Kuecherer C, Harms G, Kunz A. Detection and quantification of minor human immunodeficiency virus type 1 variants harboring K103N and Y181C resistance mutations in subtype A and D isolates by allele-specific real-time PCR.
  Antimicrobial Agents and Chemotherapy 2009;53(7):2965-73.

- **Hauser A**, Mugenyi K, Kabasinguzi R, Kuecherer C, Harms G, Kunz A.
  Emergence and persistence of minor drug-resistant HIV-1 variants in Ugandan women after nevirapine single-dose prophylaxis.
  PLoS ONE 2011;6(5):e20357.

- Pilger D, **Hauser A**, Kuecherer C, Mugenyi K, Kabasinguzi R, Somogyi S, Harms G, Kunz A.
  Minor drug-resistant HIV type-1 variants in breast milk and plasma of HIV type-1-infected Ugandan women after nevirapine single-dose prophylaxis.
  Antiviral Therapy 2011;16(1):109-13;
  http://dx.doi.org/10.3851/IMP1698.

- **Hauser A**, Sewangi J, Mbezi P, Dugange F, Lau I, Ziske J, Theuring S, Kuecherer C, Harms G, Kunz A.
  Emergence of minor drug-resistant HIV-1 variants after triple antiretroviral prophylaxis for prevention of vertical HIV-1 transmission.
  PLoS ONE 2012;7(2):e32055.

9 Anhang

9.1 Danksagung

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9.2 Lebenslauf

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

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Detection and Quantification of Minor Human Immunodeficiency Virus Type 1 Variants Harboring K103N and Y181C Resistance Mutations in Subtype A and D Isolates by Allele-Specific Real-Time PCR▽

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Nevirapine (single dose), commonly used to prevent the mother-to-child transmission of human immunodeficiency virus (HIV) in developing countries, frequently induces viral resistance. Even mutations which occur only in a minor population of the HIV quasispecies (<20%) are associated with subsequent treatment failure but cannot be detected by population-based sequencing. We developed sensitive allele-specific real-time PCR (ASPCR) assays for two key resistance mutations of nevirapine. The assays were specifically designed to analyze HIV-1 subtype A and D isolates accounting for the majority of HIV infections in Uganda. Assays were evaluated using DNA standards and clinical samples of Ugandan women having preventively taken single-dose nevirapine. Lower detection limits of drug-resistant HIV type 1 (HIV-1) variants carrying reverse transcriptase mutations were 0.019% (K103N [AAC]), 0.013% (K103N [AAT]), and 0.29% (Y181C [TGT]), respectively. Accuracy and precision were high, with coefficients of variation (the standard ratio divided by the mean) of 0.02 to 0.15 for intra-assay variability and those of 0.07 to 0.15 (K103N) and 0.28 to 0.52 (Y181C) for inter-assay variability. ASPCR assays enabled the additional identification of 12 (20%) minor drug-resistant HIV variants in the 20 clinical Ugandan samples (3 mutation analyses per patient; 60 analyses in total) which were not detectable by population-based sequencing. The individual patient cutoff derived from the clinical baseline sample was more appropriate than the standard-based cutoff from cloned DNA. The latter is a suitable alternative since the presence/absence of drug-resistant HIV-1 strains was concordantly identified in 92% (55/60) of the analyses. These assays are useful to monitor the emergence and persistence of drug-resistant HIV-1 variants in subjects infected with HIV-1 subtypes A and D.

Thirty-three million adults and children are living with human immunodeficiency virus (HIV) worldwide, most of them in sub-Saharan Africa (36). Antiretroviral prophylaxis for the prevention of mother-to-child transmission (PMTCT) of HIV as well as antiretroviral long-term treatment (ART) are used to reduce the burden of the HIV/AIDS epidemic (7). The nonnucleoside reverse transcriptase inhibitor (NNRTI) nevirapine (NVP) is widely applied as an antiretroviral prophylaxis for the prevention of vertical transmission of HIV and as a backbone of ART in many developing countries (16, 35). However, the low genetic barrier of NVP leading to resistance by a single nucleotide mutation (25) predisposes for the emergence of drug-resistant HIV variants. Even a single dose of NVP is associated with the frequent selection of NVP/NNRTI-resistant HIV strains (2, 10–14, 18, 29). Accordingly, the response to an NVP-containing ART was shown to be compromised in women following single-dose prophylaxis with NVP (21, 23). Most drug-resistant HIV populations are present only at low levels which cannot be detected by standard population-based sequencing with a detection limit of ≥20%. However, minor drug-resistant variants can become the dominant population, thereby leading to treatment failure (4, 20, 22, 27, 34). Allele-specific real-time PCR (ASPCR) assays were shown to detect drug-resistant populations at levels of <1% (17) and have previously been developed for the detection of NNRTI-associated resistance mutations in the *pol* gene of HIV type 1 (HIV-1) subtypes B and C (6, 18–19, 24, 26, 28, 31, 33). ASPCR assays in general are highly susceptible to the impaired binding of primers due to polymorphisms in the primer-binding regions (30). Due to the high variability of HIV-1 sequences, subtype-generic ASPCR assays are hardly available. So far, no ASPCR assay has been established for the analysis of the most frequent NVP-associated drug-resistant mutations in HIV-1 subtype A and D strains. These subtypes are common in East African and Central African countries and account for more than 95% of all Ugandan HIV infections (15, 32).

We developed and evaluated highly sensitive ASPCR assays enabling the detection and quantification of the two most common NVP-associated resistance mutations, K103N and Y181C, in the *pol* open reading frame of subtypes A and D. In addition, we tested the blood specimens of 20 HIV-infected pregnant Ugandan women who had preventively been treated with single-dose NVP to evaluate the ASPCR assays by using clinical samples.

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TABLE 1. Oligonucleotide sequences of outer RT-PCR analysis, ASPCR assays, and allele-specific PCR primers

Assay and primer name	Sequence (5′ to 3′)	Nucleotide position (HXB2)
RT-PCR		
OUT FOR1	AAACAATGGCCRTTGACAGAAGA	2613–2635
OUT REV1	GGATGGAGTTCATAHCCCATCCA	3234–3256
K103N ASPCR		
Forward primer FOR2	GGCCTGAAAATCCATAYAATACTCC	2701–2725
Generic DNA reverse primer REV4	CCCACATCYAGTACTGTTACTGATTT	2859–2884
AAC-specific reverse primer REV6	CCCACATCYAGTACTGTTACTGATTGG	2858–2884
AAT-specific reverse primer REV7	CCCACATCYAGTACTGTTACTGATTCA	2858–2884
Y181C ASPCR		
Forward primer FOR3	AAATCAGTAACAGTACTRGATGTGGG	2859–2884
Generic DNA reverse primer REV9	ATCCTACATACAARTCATCCATGTATTGA	3092–3120
TGT-specific reverse primer REV11	ATCCTACATACAARTCATCCATGTATTGCC	3091–3120

MATERIALS AND METHODS

Construction of standards. The detection limit of HIV RNA in plasma samples was determined by using serial dilutions of HIV-negative human plasma spiked with a defined amount of HIV-1 NL4.3 virus (1).

To quantify drug-resistant HIV-1 variants carrying the Y181C mutation in the reverse transcriptase (RT), DNA standards were generated by cloning amplified *pol* fragments (nucleotides [nt] 2001 to 3454 of HXB2; accession no. K03455) (37) from patients with mutant and wild-type RT into the plasmid pBluescript II SK(+) (pBSSK+, pBlueskript; Stratagene GMBH, Heidelberg, Germany). K103N DNA standard mixtures were obtained by assembling clonal plasmid DNA carrying either the mutant codon AAC or AAT at position 103 of the RT with pNL4.3 carrying the wild-type sequence (AAA). Standard curves were established with defined ratios of wild type (pNL4.3) and mutant plasmid controls (0%, 0.01%, 0.1%, 1%, 10%, and 100% of the proportion of mutant variants) to quantify the amount of drug-resistant variants. The total amount of input DNA was the same for all standard mixtures used (10^7 copies/ml). All standards were handled the same way as the samples of patients and thus were also subjected to the outer PCR analysis.

Clinical samples. Two samples each were collected from 20 Ugandan women who had participated in a PMTCT of HIV program in western Uganda; ethical approval by the Uganda National Council for Science and Technology was granted. The drug-naïve women ingested a single dose of 200 mg of NVP, and none of the women received ART during the observation period. The first blood sample was collected directly after the intake of NVP and was thus supposed to contain only wild-type HIV-1 (5). The second blood sample was taken 1 to 6 weeks after the NVP intake.

Primers. Primers (Table 1) were designed to be subtype A and D specific using the reference alignment of the Ugandan HIV-1 M group sequences accessible via the Los Alamos HIV sequence database available at http://www.hiv.lanl.gov/components/hiv-db/combined_search_s_tree/search.html. The primer-binding sites of the standards including the wild-type control HIV-1 NL4.3 virus (subtype B) (1) were 100% complementary to the primers used in the outer RT-PCR and the inner ASPCR assays.

The mutant-specific primers exhibited an additional mismatch on the penultimate (second to the terminal) base of the antisense primer to diminish unspecific priming and to increase the specificity for the target mutation (8).

RT-PCR and outer real-time PCR. Virus was pelleted from 450 μl of plasma by centrifugation for 90 min at 14,000 rpm and 4°C. RNA was extracted using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) and eluted in 60 μl of buffer provided with the kit. Aliquots of 10 μl were stored at −70°C.

A total of 9 μl of RNA (68-μl plasma equivalents) was reverse transcribed; after denaturation of the RNA at 65°C for 5 min, the RT mix with 100 U SuperScript II RT, 1× RT buffer, 5 mM dithiothreitol, 500 μM deoxynucleoside triphosphate (all reagents from Invitrogen, Karlsruhe, Germany), 40 U RNasin (Promega, Mannheim, Germany), and 60 μM of random hexamers (Roche, Mannheim, Germany) was added to a final volume of 20 μl. The reaction mixture was incubated for 1 h at 42°C and stopped by heating for 5 min at 95°C.

The outer real-time PCR was performed with the LightCycler 480 system and the 480 SYBR green I master kit (Roche, Mannheim, Germany). The PCR amplifies a 644-bp *pol* fragment comprising codons 103 and 181 of the RT (nt 2613 to 3256 on HXB2; accession no. K03455) (Fig. 1). The 20-μl reaction mixture contained 4 μl of cDNA and 0.6 μM each of forward primer OUT FOR1 and reverse primer OUT REV1 (Table 1). PCR amplifications were started with a 10-min denaturation step at 94°C, followed by 50 amplification cycles (15 s at 94°C, 20 s at 62°C, and 1 min at 72°C) and a final extension step at 72°C for 7 min. Melting point analysis of the PCR products from 60°C to 95°C was carried out to ensure the specificity of the amplified products, and only those samples melting at the expected temperature of 78°C were further analyzed by ASPCR.

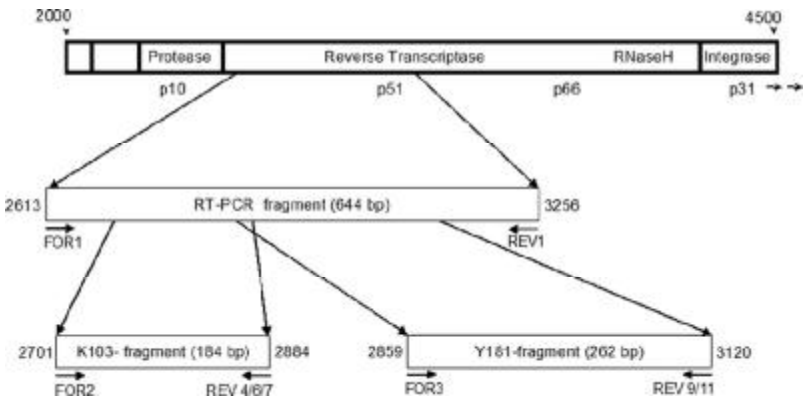

FIG. 1. HIV-1 *pol* gene and amplified PCR fragments of RT-PCR and ASPCR assays. Nucleotide positions correspond to the nucleotide sequence of HXB2; accession no. K03455.

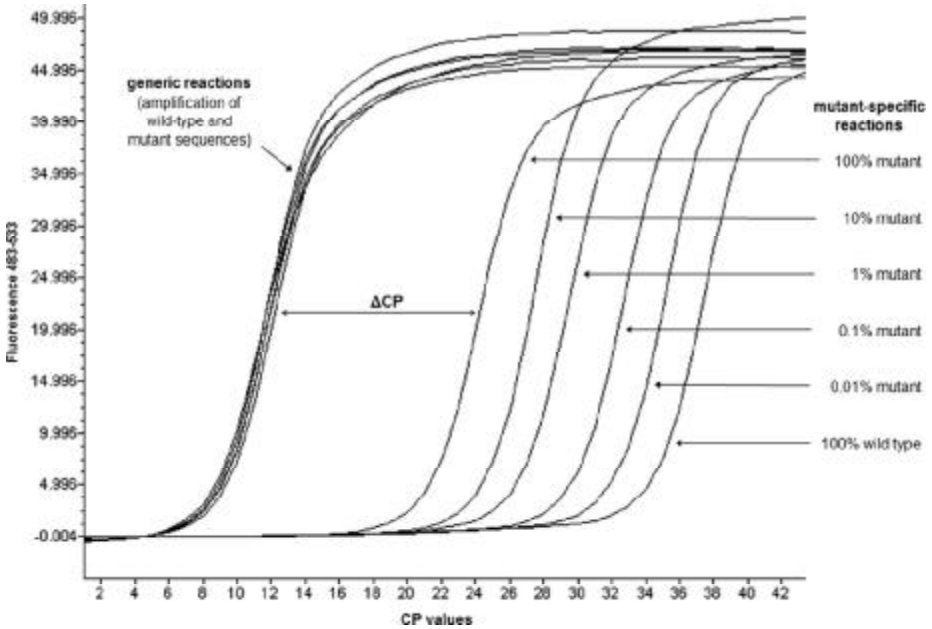

FIG. 2. Example of amplification curves of cloned DNA standards with different frequencies of mutant alleles in the real-time ASPCR assay.

Quantification of mutant populations by using ASPCR. The outer RT-PCR was diluted 1:1,000 in 5 mM Tris-HCl buffer, pH 8. Approximately 10^8 to 10^9 copies/reaction (quantitated by real-time PCR using the outer PCR) were used as the cDNA input for the ASPCR. For detection and quantification of mutant populations, two separate ASPCR reactions per mutation were performed, one to quantify generically all viral molecules and one to quantify specifically the mutant molecules only.

The K103N assay was composed of three separate reactions per sample, resulting in a 184-bp fragment (nt 2701 to 2884 on HXB2; accession no. K03455) (Fig. 1). The forward primer FOR2 (Table 1) was combined either with the reverse primer REV4 (amplification of the wild-type and mutant sequences), REV6 (mutant-specific sequence AAC), or REV7 (mutant-specific sequence AAT). The forward primer FOR3 (Table 1) was combined with the generic reverse primer REV9 to amplify all viral copies or with the mutant-specific primer REV11 (262-bp fragment; nt 2859 to 3120 on HXB2; accession no. K03455) (Fig. 1) to detect the Y181C mutant (codon TGT).

Generic and mutant-specific amplifications were performed in parallel under identical conditions: 2 μl of the outer PCR product (diluted 1:1,000) and primers (each at 0.5 mM), using the 480 SYBR green I master kit (Roche, Mannheim, Germany) according to the manufacturer's instructions in a 20-μl final volume. All ASPCR reactions were started with an initial denaturation step (95°C for 5 min). The ASPCR for K103N was followed by 45 cycles (95°C for 10 s, 62°C for 10 s, and 72°C for 10 s). For the Y181C ASPCR, 45 amplification cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s were performed. Amplification specificities of PCR products were checked by thermal denaturation analysis. All ASPCR assays were run in duplicate, and the arithmetic mean was used for further analysis. Each run was performed with a complete set of mutant and wild-type DNA controls (see "Construction of standards").

Establishment of cutoff for the detection of mutant variants. The threshold cycle/crossing point (CP) of real-time PCR analysis indicates the cycle number at which the fluorescence emission exceeds the background fluorescence. In the generic reaction, all viral templates were amplified. Therefore, the CP of the generic reaction is always the same, regardless of the presence of mutant template (Fig. 2). However, the higher the amount of mutant template in a given sample, the lower the CP of the mutant-specific reaction (Fig. 2). Thus, the higher the proportion of a mutant population in a given sample, the lower the difference between the CP of the mutant-specific reaction and that of the generic reaction (ΔCP) (Fig. 2). The cutoff indicating the presence of mutant populations was defined as the mean ΔCP (eight independent runs) of the 100% wild-type control minus three standard deviations (SD). For each sample, the ΔCP was calculated, and any ΔCP below the cutoff indicated the presence of a mutant population. The term "standard-related cutoff" is used throughout the text if this definition was applied.

An additional cutoff calculation was used for the clinical samples of Ugandan women. Since the baseline samples were obtained from women within a few hours after the intake of NVP prophylaxis, these samples were supposed to contain wild-type HIV-1 variants only. This assumption is reasonable as K103N-containing minor HIV variants were found in only 2% of drug-naïve Ugandan women (5). Therefore, we used the mean ΔCP (minus 3 SD) of the baseline sample of each woman as an individual cutoff. Furthermore, the presence of a mutant HIV-1 population was considered only if the ΔCP value of the follow-up sample was at least two cycles lower than the ΔCP value of the baseline sample. For this definition, the term "patient-specific cutoff" is used throughout the text.

Quantification of mutant variants. Standard curves were calculated by the defined mixtures of wild-type and mutant DNA controls. The ΔCP of mutant DNA controls was compared to the ΔCP of the 100% wild-type DNA control (ΔΔCP equals the ΔCP of 100% wild type minus the ΔCP of the sample). A standard curve of the ΔΔCP values of the defined mixtures of wild-type and mutant DNA controls was established for each of the three mutations (K103N [AAC], K103N [AAT], Y181C [TGT]) and was used to quantify the proportions of mutant HIV-1 populations in samples from the mothers treated with NVP.

Evaluation of the precision of the ASPCR. The precision and accuracy of the assays were evaluated by testing the DNA controls with a nominal proportion of mutant allele ranging from 0.01% to 100% four times in the same run (intra-assay variability) and in eight independently performed runs (interassay variability). The SD and coefficient of variation (the SD divided by the mean) as a relative measure of data dispersion were calculated to determine precision and variability. Accuracy was evaluated by comparing the nominal input of the mutant allele with the calculated amount.

Population-based sequencing. Population-based sequencing was performed using the Viroseq HIV-1 genotyping system version 2.0 (Abbott, Wiesbaden, Germany) and the 3130xl Genetic Analyzer automated sequencer (Applied Biosystems, Darmstadt, Germany).

Determination of HIV-1 subtypes. HIV-1 subtyping of the *pol* sequence was performed using the Rega subtyping tool, which determines the HIV-1 subtype of a query sequence using phylogenetic analysis and bootscanning methods (http://dbpartners.stanford.edu/RegaSubtyping/) (9). Only subtype classification based on bootstrap values of >70% in the tree topology were taken into account.

RESULTS

RT-PCR. The minimum input of HIV RNA required to amplify reproducibly was 1,000 copies per RT-PCR. All 20 clinical samples were successfully amplified and quantified in the outer RT-PCR (median viral load, 12,935 copies/ml; interquartile range, 5,219 to 49,287 copies/ml; range, 1,700 to 1,000,000 copies/ml).

Cutoff for the detection of mutant HIV variants using cloned DNA standards. The standard curves calculated by the ΔΔCP values of the DNA plasmid control mixtures with different mutant allele frequencies are shown in Fig. 3. The lower limit of detection of minor drug-resistant variants was 0.019% for K103N (AAC), 0.013% for K103N (AAT), and 0.29% for Y181C. The 100% HIV wild-type controls were correctly identified in all runs (100% specificity). The sensitivity for 0.1% to 100% mutant controls (K103N) and 1% to 100% mutant controls (Y181C) was 100%. The ΔΔCP values were highly correlated ($r > 0.99$) to the proportion of mutant DNA (Fig. 3).

Precision and accuracy of the ASPCR using cloned DNA standards. Intra-assay and interassay variability is shown in Table 2. The coefficients of variation (CVs) of intra-assay variability were 0.02 to 0.15 for all samples with proportions of mutant DNA ranging from 0.1 to 100%. The CVs of interassay variability for samples with proportions of mutant DNA ranging from 0.1 to 100% were 0.07 to 0.15 (codon 103) and 0.28 to 0.52 (codon 181). The lower the input of mutant DNA, the higher the CV, and controls harboring 0.01% mutant allele resulted in interassay CVs of 0.3 to 0.74.

All calculated values for mutant populations represented 50% to 180% of the nominal input in the range of 0.1% to 100% mutant frequency.

Analysis of clinical samples. The first sample of the 20 Ugandan women was taken within a few hours (median, 6 h; interquartile range, 4 to 8 h; range, 2 to 25 h) after the intake of 200 mg NVP. All but one (subtype K) of the HIV subtypes were subtype A1 ($n = 10$), D ($n = 7$), or A1D ($n = 2$) (Table 3).

Eighteen samples (90%) in the AAC-K103N assay, 18 samples (90%) in the AAT-K103N assay, and 17 samples (85%) in the Y181C assay were identified to contain 100% wild-type virus (Table 3). For these samples, the median CP value of the generic reactions was 9 (range, 7 to 11). Seven drug-resistant HIV populations were identified in clinical samples of five women (patient no. 16 to 20) (Table 3). K103 mutants with 0.3% AAC plus 6.2% AAT and 1.6% AAC plus 1.1% AAT mutant frequencies and Y181C mutants with frequencies of 0.3%, 0.6%, and 1.2%, respectively, were detected. In three of these five women, an additional single nucleotide polymorphism close to the 3′ end of the reverse primer-binding site (patient no. 16, 17, and 18) (Table 3) was identified in the RT sequence. The CP values of the generic reaction were high for these three samples (17–20). No primer mismatch was revealed in the two other samples carrying the HIV Y181C mutation at a frequency of 0.6% and 0.3% (patient no. 19 and 20) (Table 3). Based on population-based sequencing in all samples, only wild-type sequences at both codons 103 and 181 of the RT could be identified.

The follow-up samples were collected 1 to 6 weeks after the intake of NVP single-dose prophylaxis. The 60 ASPCR results of the 20 samples (three mutation analyses per sample) applying the patient-specific cutoff and the standard-related cutoff as well as the results of population-based sequencing were compared (Table 4).

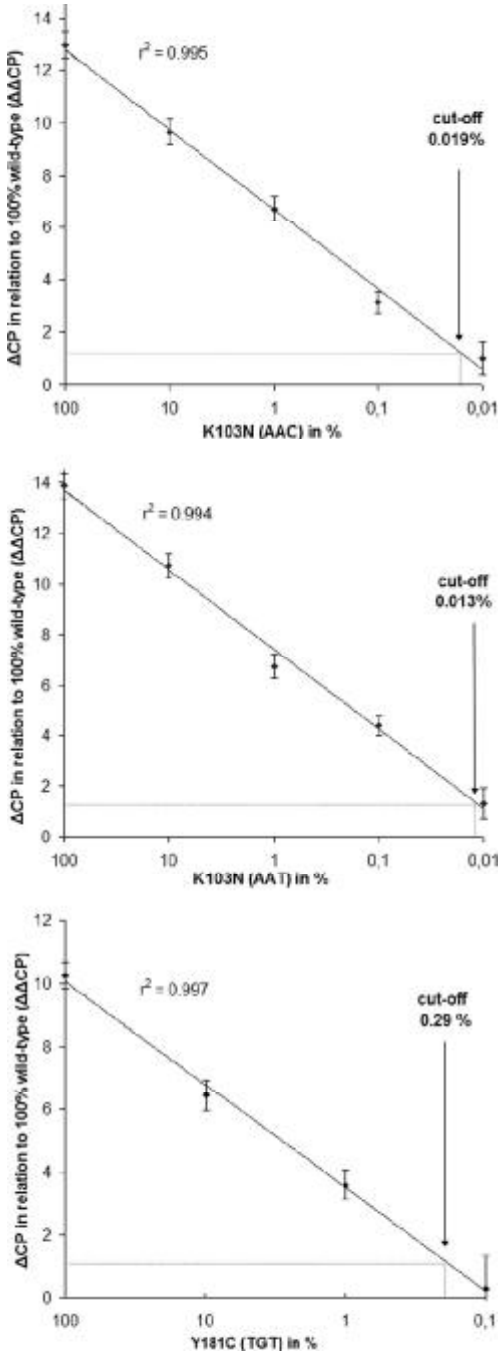

FIG. 3. Sensitivity of the K103N (AAC), K103N (AAT), and the Y181C (TGT) ASPCR assays. Data points represent the mean of eight independent experiments, and error bars indicate the SD.

Whenever both the patient-specific cutoff method and the standard-related cutoff method of the ASPCR assays indicated 100% wild-type HIV-1, the population-based sequencing con-

TABLE 2. Intra-assay and interassay variability of ASPCR assays to detect NVP-resistant HIV-1 variants[a]

% Nominal mutant allele	Intra-assay variability (mean % mutant allele ± SD)			Interassay variability (mean % mutant allele ± SD)		
	AAC (K103N)	AAT (K103N)	TGT (Y181C)	AAC (K103N)	AAT (K103N)	TGT (Y181C)
100	118.6 ± 2.4	97.6 ± 4.8	100.2 ± 6.6	116.4 ± 12.8	114.0 ± 14.4	135.5 ± 37.5
10	10.3 ± 0.41	11.1 ± 0.94	7.5 ± 0.48	9.4 ± 0.81	11.4 ± 1.28	9.7 ± 3.11
1	1.09 ± 0.05	0.53 ± 0.02	0.90 ± 0.11	1.01 ± 0.07	0.62 ± 0.07	1.23 ± 0.37
0.1	0.097 ± 0.01	0.076 ± 0.01	0.135 ± 0.02	0.068 ± 0.01	0.105 ± 0.02	0.182 ± 0.09
0.01	0.019 ± 0.005	0.009 ± 0.001	0.091 ± 0.011	0.014 ± 0.004	0.013 ± 0.009	0.131 ± 0.053
0	0.009 ± 0.003	0.006 ± 0.002	0.059 ± 0.003	0.007 ± 0.002	0.005 ± 0.002	0.091 ± 0.035

[a] Means were calculated from four replicates measured in one run (intra-assay variability) and from eight independent experiments (interassay variability).

cordantly showed only wild-type virus (n = 35; group A) (Table 4).

The presence of the K103N or Y181C mutation as identified by population-based sequencing was always confirmed in the ASPCR assays (n = 8; group B) (Table 4).

Both cutoffs, the standard-related cutoff as well as the patient-specific cutoff of the ASPCR assays, provided evidence for the presence of low-level minority mutations in 20% of the analyses (n = 12) which were not detectable by population-based sequencing (group C) (Table 4).

In 5 out of the 60 (8.3%) analyses, the qualitative results differed depending on the patient-specific and standard-related cutoff applied (group D) (Table 4). All drug-resistant variants were present at levels ≤1.1% in these five patients; population-based sequencing detected wild-type HIV only and a mismatch in each of the primer-binding sites of the reverse primer in all five patients.

Quantification results of standard-related and patient-specific cutoff differed more than 10-fold in four samples. In one sample, application of the standard-related cutoff yielded a much lower proportion of the HIV variant with the Y181C mutation (1.3% versus 30%; patient no. 14; group B) (Table 4), while population sequencing detected the mutant variant in the presence of the wild type. In contrast, the standard-related cutoff method resulted in much higher proportions of mutant populations in three samples (patient no. 16, K103N [AAC], 2.4% versus 0.05%; patient no. 17, K103N [AAC], >100% versus 3.3% K103N; and patient no. 18, Y181C, 15% versus 1.1%) which were all identified as 100% wild-type HIV-1 by population-based sequencing. Polymorphisms in the primer-binding region of the reverse primer were detected in the HIV genome of the four patients; three out of the four samples exhibited extremely high CP values (14–20) in the generic reactions.

TABLE 3. Results of ASPCR assays of baseline samples of 20 HIV-infected Ugandan women[a]

Patient no.	Subtype	% K103N (AAC)	% K103N (AAT)	% Y181C (TGT)
1	A1	No mut	No mut	No mut
2	A1	No mut	No mut	No mut
3	D	No mut	No mut	No mut
4	A1	No mut	No mut	No mut
5	D	No mut	No mut	No mut
6	A1/D	No mut	No mut	No mut
7	A1	No mut	No mut	No mut
8	D	No mut	No mut	No mut
9	A1/D	No mut	No mut	No mut
10	A1	No mut	No mut	No mut
11	A1	No mut	No mut	No mut
12	D	No mut	No mut	No mut
13	A1	No mut	No mut	No mut
14	D	No mut	No mut	No mut
15	D	No mut	No mut	No mut
16	A1	0.3	6.2	No mut
17	A1	1.6	1.1	No mut
18	K	No mut	No mut	1.2
19	A1	No mut	No mut	0.6
20	D	No mut	No mut	0.3

[a] No mut, no drug-resistant HIV-1 population detected.

DISCUSSION

Due to its low genetic barrier, NVP selects rapidly for resistance. Minor drug-resistant variants are of special interest since they may pave the way for treatment failure (4, 20, 22, 27, 34). Here, three ASPCR assays to detect minor HIV-1 variants exhibiting K103N and Y181C resistance mutations in subtype A and D isolates were successfully developed and evaluated.

Performance of ASPCR assays using cloned DNA standards. K103N and Y181C variants were detected at very low levels as follows: 0.019% (K103N [AAC]), 0.013% (K103N [AAT]), and 0.29% (Y181C). For K103N, the precision achieved was high with low mean interassay CVs (<0.15) across a broad range of 0.1% to 100% mutant frequency. Mean interassay CVs were higher (>0.28) for the Y181C mutation, thus not matching the typical criteria for quantitative assays. In this context, it is important to note that the main aim of resistance testing is the qualitative assessment of drug-resistant variants. As for the determination of HIV viral load, we suggest that a true increase/decrease of drug-resistant variants should be considered only at a change of at least 0.5 to 1 log. The accuracy of the three ASPCR assays was high. The assays overestimated slightly the actual proportion at very high frequencies of mutant alleles (100%).

Application to clinical samples. Potential polymorphisms in primer-binding sites of clinical samples compared to cloned DNA standards with primer-binding sites that are 100% complementary to the primer sequence necessitate the evaluation of clinical samples. We therefore analyzed clinical samples of 20 women who participated in a PMTCT program in Uganda.

The first blood samples were taken within a few hours after the intake of single-dose NVP from previously antiretroviral drug-naïve women. As expected, most samples harbored wild-type HIV only (Table 3), while minor drug-resistant HIV populations (0.3% to 6.2%) were identified in 7/60 (12%) analyses

TABLE 4. Results of the ASPCR assays and the population-based sequencing of plasma samples collected from 20 HIV-infected Ugandan women 1 to 6 weeks after the intake of single-dose NVP[a]

Group	K103N (AAC)				K103N (AAT)				Y181C (TGT)			
	Patient no.	ASPCR assay result		Population-based sequencing result	Patient no.	ASPCR assay result		Population-based sequencing result	Patient no.	ASPCR assay result		Population-based sequencing result
		Patient-specific cutoff	Standard-related cutoff			Patient-specific cutoff	Standard-related cutoff			Patient-specific cutoff	Standard-related cutoff	
A	1	wt	wt	wt	1	wt	wt	wt	1	wt	wt	wt
	3	wt	wt	wt	3	wt	wt	wt	3	wt	wt	wt
	4	wt	wt	wt	4	wt	wt	wt	4	wt	wt	wt
	6	wt	wt	wt	6	wt	wt	wt	6	wt	wt	wt
	8	wt	wt	wt	7	wt	wt	wt	8	wt	wt	wt
	10	wt	wt	wt	8	wt	wt	wt	10	wt	wt	wt
	11	wt	wt	wt	10	wt	wt	wt	12	wt	wt	wt
	12	wt	wt	wt	11	wt	wt	wt	13	wt	wt	wt
	13	wt	wt	wt	12	wt	wt	wt	15	wt	wt	wt
	15	wt	wt	wt	15	wt	wt	wt	16	wt	wt	wt
	19	wt	wt	wt	16	wt	wt	wt	17	wt	wt	wt
					19	wt	wt	wt	20	wt	wt	wt
B	2	Mixture (28)	Mixture (20)	Mixture	9	Mixture (35)	Mixture (15)	Mixture	5	Mixture (11)	Mixture (38)	Mixture
	7	Mixture (21)	Mixture (6.2)	Mixture					9	Mut (>100)	Mixture (25)	Mixture
	9	Mixture (26)	Mixture (5.9)	Mixture					14	Mixture (30)	Mixture (1.3)	Mixture
	14	Mixture (44)	Mixture (60)	Mixture								
C	5	Mixture (11)	Mixture (4.1)	wt	2	Mixture (1.9)	Mixture (2.2)	wt	2	Mixture (15)	Mixture (9.5)	wt
	16	Mixture (0.05)	Mixture (2.4)	wt	5	Mixture (13)	Mixture (3.3)	wt	18	Mixture (1.1)	Mixture (15)	wt
	17	Mixture (3.3)	Mut (>100)	wt	13	Mixture (0.03)	Mixture (0.03)	wt				
	18	Mixture (1.6)	Mixture (0.9)	wt	18	Mixture (0.6)	Mixture (0.4)	wt				
	20	Mixture (0.2)	Mixture (0.06)	wt	20	Mixture (0.3)	Mixture (0.03)	wt				
D					14	wt	Mixture (0.04)	wt	7	Mixture (0.4)	wt	wt
					17	wt	Mixture (0.9)	wt	11	Mixture (1.1)	wt	wt
									19	wt	Mixture (0.4)	wt

[a] For ASPCR assays, the results for both cutoff methods (patient-specific and standard-related) are shown and quantifiable drug-resistant HIV populations are indicated in percentages. According to the results of the ASPCR assays and the population-based sequencing four groups were defined as follows: A, concordant detection of wild-type HIV by all assays; B, concordant detection of mutant populations by all assays; C, detection of mutant populations by ASPCR assays with both cutoff methods but not by population-based sequencing; and D, divergent results of the ASPCR assays between the two cutoff methods. wt, detection of wild-type HIV only; Mut, detection of mutant HIV only; Mixture, detection of wild-type and mutant HIV (percent mutant HIV).

and in 5/20 (25%) women, respectively. Population-based sequencing revealed no NVP-associated mutations but an additional polymorphism within the last four nucleotides of the reverse primer-binding site in the ASPCR assay in three of these five women although not in two of the women (Y181C, 0.3% and 0.6%). This mismatch on the 3′ end of the primer-binding site had much more of an impact on the binding specificity of the generic primer compared to that of the mutant-specific primer (designed with a mismatch already at the penultimate position). Therefore, the presence of natural polymorphisms resulted in a much higher CP of the generic primer. Consequently, the difference between the CP values of generic and mutant-specific primers (ΔCP) became lower, thus simulating the presence of mutant populations. However, this misinterpretation can simply be avoided by checking the CP values of the different PCRs; if the CP value using the generic primer is much higher than usual but the CP value with the mutant-specific primer is within the usual range, there is much evidence for the presence of polymorphisms. We therefore assume that at least the three of the five analyses exhibiting high CP values in the generic reaction most likely represent false positive results. However, it cannot be excluded that low-level mutant populations were indeed present in these samples from drug-naïve mothers. Primary drug resistance mutations in the HIV genome were shown to occur in some Ugandan women without any previous drug intake (5).

Altogether, these results reflect the higher variability of clinical samples compared to cloned DNA standards. To overcome this problem and to avoid false positive results, the increase of the cutoff for detection of the mutant population up to 1% could be a rational alternative for the analysis of clinical samples. In addition, samples exhibiting an abnormally high CP value in the ASPCR with the generic primer should be excluded from analysis since they cannot be reliably analyzed.

The 60 ASPCR results from follow-up samples matched strongly with the results obtained by population-based sequencing (Table 4). However, the ASPCR assays were able to detect low-level drug-resistant HIV populations in 20% of the analyses. The quantification results of these additionally detected drug-resistant variants were 0.03% to 15% (patient-specific cutoff) and thus below the detection limit of the population-based sequencing.

To compensate for individual sequence variability in the primer-binding site, it is advantageous to have an individual reference sample prior to the intake of antiretroviral drugs. This baseline sample can be used for the determination of a patient-specific cutoff. However, most often such a sample is not available. In this case, a standard cutoff derived from cloned DNA standards has to be applied to all patient samples. To investigate the usefulness of a standard cutoff, we compared the qualitative and quantitative results of the patient-specific cutoff with the standard-related cutoff. The qualitative results of the 60 analyses were highly concordant: the presence (20 analyses) or absence (35 analyses) of drug-resistant HIV populations was indicated by both cutoffs in 92% of the analyses (55 analyses), while the presence of drug-resistant HIV populations was indicated in 8% of the analyses (5 analyses) by one cutoff only (Table 4). Assuming that the results obtained by applying the patient-specific cutoff are true, the standard-related cutoff falsely indicated the presence of low-level mutant populations in three patients and failed to detect it in two patients. However, in all five patients, the detected proportions of mutant populations were very low (0.04% to 1.1%).

The quantification results of the 20 analyses indicating the presence of drug-resistant HIV variants differed by more than 10-fold between the two cutoff methods in only four (20%) analyses. In three of them, the standard-related cutoff seemed to overestimate the frequency of mutant populations (2.4% versus 0.05%, 15% versus 1.1%, and >100% versus 3.3%) since population-based sequencing revealed exclusively wild-type HIV in all three patients. All three samples exhibited a mismatch located within the last three bases of the 3′ end of the generic primer, indicated by an elevated CP value of the generic primer. As already discussed, elevated CP values may not only simulate the presence of mutant HIV variants but can also lead to an overestimation of mutant proportions.

Despite these limitations, we consider the use of a standard-based cutoff in the absence of a baseline sample to be a very suitable alternative for generating reliable results in most cases.

Conclusions. Polymorphisms in the primer-binding site are a general problem for ASPCR assays and can be the reason for underestimation or overestimation of mutant populations by using these techniques (6, 30). Indeed, we found that polymorphisms within the primer-binding site affected the efficiency of amplification of wild-type and mutant alleles.

There are four options to handle these difficulties. First, patient-specific primers designed according to the results of population-based sequencing could be used. However, this option is not suitable for routine use. Second, if a baseline sample prior to antiretroviral drug intake (assumed to be 100% wild type) was stored, a patient-specific cutoff could be determined. Third, special attention has to be paid to samples exhibiting a high CP value in the PCR with the generic primer. We have shown here that abnormally high CPs tend to overestimate or even falsely indicate the presence of mutant populations. Such samples should be omitted from ASPCR analysis. Fourth, increasing the cutoff indicating the presence of mutant populations up to 1% could increase the specificity of the assays but would also lower the sensitivity for low-level mutant populations. In this context, the choice between a falsely detected minor drug-resistant population and the failure to detect existing minor drug-resistant populations has to be made. So far, the clinical implications of low-level mutant populations at a <1% frequency are not really understood. Recently, it was shown (3) that K103N and Y181C mutants below 41% or 33%, respectively, were not detected by a phenotypic assay. This seems to be reassuring, but the main risk of minor drug-resistant HIV variants is their archiving with possible outgrowth under future drug pressure (4, 20, 22, 27, 34). However, an exact threshold for minor drug-resistant variants at which treatment regimens should be changed is not established so far. Since at present no clinician would change a successful ART due to the presence of drug-resistant variants at very low levels only, we believe that it is acceptable to increase the cutoff of ASPCR assays in routine use up to a detection level of 1%.

We have shown that our developed ASPCR assays reliably detected NVP-associated drug resistance mutations in HIV-1 subtype A and D genomes with much higher sensitivity than population-based sequencing. For analysis of subtypes other

than A and D, primers and PCR conditions have to be designed very carefully due to the inherent vulnerability of ASPCR assays to polymorphisms. These sensitive methods can be used to detect and quantify NNRTI-resistant minor variants in samples of mothers and children after NVP prophylaxis within a PMTCT intervention. However, the assays can also be applied to samples from patients under long-term ART to monitor the emergence of HIV with NNRTI-associated resistance mutations. Resistance formation is also important from a public health perspective. The transmission of drug-resistant virus should be monitored in each and every country. In resource-limited settings, this is often not feasible. ASPCR assays are cheaper and the performance less time consuming than population-based sequencing. The assays developed here focus on the key NNRTI-associated resistance mutations and could be used to analyze blood and other secretions (e.g., vaginal secretions and semen) to monitor the spread of resistant virus in populations infected with HIV-1 subtypes A and D.

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Emergence and Persistence of Minor Drug-Resistant HIV-1 Variants in Ugandan Women after Nevirapine Single-Dose Prophylaxis

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Abstract

Background: Nevirapine (NVP) single-dose is still a widely used antiretroviral prophylaxis for the prevention of vertical HIV-1 transmission in resource-limited settings. However, the main disadvantage of the Non-nucleoside Reverse Transcriptase Inhibitor (NNRTI) NVP is the rapid selection of NVP-resistant virus with negative implications for subsequent NNRTI-based long-term antiretroviral therapy (ART). Here, we analysed the emergence of drug-resistant HIV-1 including minor variants in the early phase after NVP single-dose prophylaxis and the persistence of drug-resistant virus over time.

Methods and Findings: NVP-resistant HIV-1 harbouring the K103N and/or Y181C resistance mutations in the HIV-1 reverse transcriptase gene was measured from 1 week up to 18 months after NVP single-dose prophylaxis in 29 Ugandan women using allele-specific PCR assays capable of detecting drug-resistant variants representing less than 1% of the whole viral population. In total, drug-resistant HIV-1 was identified in 18/29 (62%) women; rates increased from 18% to 38% and 44% at week 1, 2, 6, respectively, and decreased to 18%, 25%, 13% and 4% at month 3, 6, 12 and 18, respectively. The proportion of NVP-resistant virus of the total viral population was significantly higher in women infected with subtype D (median 40.5%) as compared to subtype A (median 1.3%; $p = 0.032$, Mann-Whitney U test). 33% of resistant virus was not detectable at week 2 but was for the first time measurable 6–12 weeks after NVP single-dose prophylaxis. Three (10%) women harboured resistant virus in proportions >10% still at month 6.

Conclusions: Current WHO guidelines recommend an additional postnatal intake of AZT and 3TC for one week to avoid NVP resistance formation. Our findings indicate that a 1-week medication might be too short to impede the emergence of NVP resistance in a substantial proportion of women. Furthermore, subsequent NNRTI-based ART should not be started earlier than 12 months after NVP single-dose prophylaxis.

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Introduction

Mother-to-child transmission of HIV-1 in developing countries is still a major concern. Although more effective prophylaxis regimens like the combination of 3 different antiviral drugs are recommended by current WHO guidelines [1], nevirapine single-dose (NVP-SD) is still a frequently used option in resource-constrained settings due to its simplicity.

The major drawback, however, of using the Non-nucleoside Reverse Transcriptase Inhibitor (NNRTI) NVP is the frequent emergence of NVP-resistant HIV-1 variants even after single-dose intake as a result of NVP's low genetic barrier [2-7]. There is evidence that treatment failure of a subsequent NVP- or other NNRTI-based antiretroviral therapy (ART) is more likely for women with prior NVP exposure which has been connected to drug resistance [8-10]. Drug-associated resistance mutations in the HIV-1 genome fade over time [2,3,5,11] but even drug-resistant HIV-1 variants representing only a minor population of the total viral population are important since they can also predispose to treatment failure [12–14]. Limited data is available about the time of emergence of minor drug-resistant HIV-1 in the early phase (1 to 2 weeks) after NVP-SD prophylaxis.

Here, we analysed the emergence and persistence of NVP-resistant HIV-1 in Ugandan women mainly infected with subtype A and D. Samples were taken at tight schedule from 1 week up to 18 months after NVP-SD intake. Highly sensitive allele-specific PCR (ASPCR) assays were applied to quantify drug-resistant HIV-1 at proportions as low as 1%.

Methods

Ethics Statement

All study participants were HIV-1 positive pregnant women enrolled in a prevention of mother-to-child (PMTCT) programme at Fort Portal District Hospital (Kabarole District, western Uganda) if they had given written informed consent. The study was approved by the National ethical committee of Uganda (National Council of Science and Technology) and by the ethical committee of the Charité - Universitätsmedizin Berlin in Germany.

All women had taken NVP-SD (200 mg) at the onset of labour following the HIVNET012 protocol [15]. Blood samples were taken at delivery (baseline), 1, 2 and 6 weeks and 3, 6, 12 and 18 months after NVP-SD intake. Women were included into the final analysis if they had never taken antiretrovirals before NVP-SD prophylaxis and if at least 4 samples after NVP-SD intake were available. Additionally, the baseline sample from delivery (supposed to contain HIV-1 wild type only) had to be amplifiable in order to establish an individual cut-off in the ASPCR for detection of NVP-resistant virus; quantification of drug-resistant HIV-1 variants carrying the K103N and/or the Y181C mutation in the *pol* gene were done by ASPCR assays as previously described [16]. The detection limits for the 3 mutations as estimated from plasmid DNA controls were 0.019% K103N (AAC), 0.013% K103N (AAT) and 0.29% Y181C (TGT) in the presence of wild-type HIV-1 [16].

Population-based sequencing which is a much less sensitive method (detection limit for drug-resistant HIV-1 is approximately 20%) was conducted on all samples exhibiting NVP-resistant HIV-1 and on 22% randomly chosen samples without evidence of drug-resistant virus as determined by ASPCR. Population-based sequencing was performed using the automated sequencer 3130xl Genetic Analyzer (Applied Biosystems, Darmstadt, Germany) and the Viroseq HIV-1 Genotyping System version 2.0 (Abbott, Wiesbaden, Germany). For HIV-1 subtype analyses the REGA HIV-1 subtyping tool was applied [17].

Statistical analysis was performed using the program SPSS, version 17.0 (SPSS Inc, Chicago, IL, USA). The proportions of K103N (codon AAC) and K103N (codon AAT) mutants were summed to obtain the total proportion of virus harboring the K103N mutation. Fisher's exact test (two-tailed) was used to assess significant associations among categorical variables like the resistance frequencies between different HIV-1 subtypes. The non-parametric Mann-Whitney U test was used to compare the maximum proportions of NVP-resistant virus between women infected with different subtypes. For this purpose, the maximum proportion of K103N *or* Y181C ever observed during the observation period of an individual was used as the presence of both mutations on the same genome cannot be excluded; this approach underestimates the total proportion of NVP-resistant virus in case the 2 mutations are located on different HIV-1 genomes.

Results

29 women fulfilled the inclusion criteria and constituted the final study group. The median baseline data were age 25 years (IQR 22–29), parity 2 children (IQR 1.5–4.5), viral load 15050 copies/mL (IQR 6220–46725) and CD4 count 546 cells/mm3 (IQR 452–730). In total, 158 blood samples after NVP-SD intake were available and amplifiable (mean: 5.5 samples per woman). One mother started ART (d4T, 3TC and NVP) 6 months after NVP-SD and thus during the observation period. 52% (n = 15) were infected with HIV-1 subtype A1, 38% (n = 11) with subtype D and 1 woman each with subtype C, subtype G and an HIV-1 isolate which was not assignable according to the REGA HIV-1 subtyping tool.

Altogether, 18/29 (62%) women developed NVP resistant virus during the observation period (Table 1): 7/15 (47%) women with subtype A1, 8/11 (73%) women with subtype D and the 3 women infected with subtype C, subtype G and the unclassifiable isolate. The frequency of resistance was not significantly different between subtype A and subtype D (p = 0.25, Fisher's exact test).

9/18 (50%) women with resistance formation exhibited the K103N *and* the Y181C mutation in their viral population. In 8 of these women, both mutations were simultaneously present when resistance formation was detected for the first time; in 1 woman (Table 1, no. 10) the development of Y181C preceded the emergence of K103N. In the remaining 9/18 women with resistance formation the resistant virus carried either the K103N (n = 7) *or* the Y181C (n = 2) mutation during the observation period.

In 3 of the 18 women with resistant virus during the observation period amplification of the week 2 samples failed. In 10 of the remaining 15 women (67%), resistant variants were for the first time detectable 1–2 weeks after NVP-SD. In 5/15 (33%) women the resistance emerged later and was visible for the first time at week 6 or in one woman (Table 1, no. 10 infected with subtype G) at month 3, respectively.

Resistance formation took place in a time-dependent manner with an increase in frequency up to week 6 followed by fading of mutations. In detail, resistant virus was detectable in 2/11 (18%) week 1 samples, in 9/24 (38%) week 2 samples, in 11/25 (44%) week 6 samples, in 4/22 (18%) month 3 samples, in 7/28 (25%) month 6 samples, in 3/24 (13%) month 12 samples and in 1/24 (4%) month 18 samples.

The maximum proportion of NVP-resistant HIV-1 of the total viral population was higher in subtype D than in subtype A samples (median 40.5% for subtype D versus 1.3% for subtype A, Mann-Whitney U test: p = 0.032).

In 50% (9/18) of the women having developed drug-resistant HIV-1 variants, the relative proportion of the resistant population did not exceed 5% during the whole study period. This applied to 5/7 (71%) women infected with HIV-1 subtype A1 and to 2/8 (25%) women infected with subtype D (p = 0.13, Fisher's exact test).

At month 6, drug-resistant HIV-1 variants were still detectable in 25% of the women; NVP-resistant virus at proportions higher than 10% were detected in 3 women (10%). In 2 of these women (Table 1, no. 24 and no. 27), the resistant HIV-1 population faded away and was no longer detectable at month 12. In 1 woman (Table 1, no. 19), the drug-resistant HIV-1 variant (100% Y181C) which had emerged early at week 1 persisted throughout the whole observation period and was still present at month 12 (18-month sample was missing).

In another woman (Table 1, no. 9), the resistant virus population being 10% (Y181C) at month 3 was not detectable at month 6 and month 15 but re-emerged and constituted the majority of the HIV-1 population at month 18; this woman was infected with subtype C and had started NVP-containing ART 6 months after NVP-SD intake.

Population-based sequencing was conducted on all samples of the 18 women exhibiting drug-resistant virus during the study (n = 37) and on 27 (22%) randomly chosen samples without indication of drug-resistant virus by ASPCR. The results of ASPCR and population-based sequencing matched very well; all samples without detectable drug-resistant HIV-1 or with drug-

Table 1. Nevirapine-resistant HIV-1 variants in plasma samples of 29 Ugandan women taken 1 week up to 18 months after nevirapine (NVP) single-dose prophylaxis as analysed by allele-specific PCR (ASPCR).

			proportion of NVP-resistant HIV-1 in % by ASPCR										proportion of NVP-resistant HIV-1 in % by ASPCR						
no	**sub-type**	**mutation**	**w1**	**w2**	**w6**	**m3**	**m6**	**m12**	**m18**	**no**	**sub-type**	**mutation**	**w1**	**w2**	**w6**	**m3**	**m6**	**m12**	**m18**
1	A1	K103N	wt	wt	wt	wt	wt	wt	wt	**16**	nt	K103N	n/a	**2.2**	**1.7**	wt	**0.1**	wt	n/a
		Y181C	wt	wt	wt	wt	wt	wt	wt			Y181C	n/a	**1.1**	wt	wt	wt	wt	n/a
2	A1	K103N	n/a	wt	wt	n/a	n/a	n/a	n/a	**17**	D	K103N	wt	wt	wt	wt	wt	wt	wt
		Y181C	n/a	wt	**0.5**	n/a	n/a	n/a	n/a			Y181C	wt	wt	wt	wt	wt	wt	wt
3	A1	K103N	n/a	**30**	**16**	wt	**0.5**	wt	n/a	**18**	A1	K103N	n/a	wt	wt	n/a	wt	wt	wt
		Y181C	n/a	**15**	wt	wt	wt	wt	n/a			Y181C	n/a	wt	wt	n/a	wt	wt	wt
4	D	K103N	wt	**9.4**	**7.0**	wt	wt	wt	wt	**19**	D	K103N	n/a	**3.3**	**61**	**1.4**	wt	wt	n/a
		Y181C	wt	**45**	**0.6**	wt	wt	wt	wt			Y181C	n/a	**100**	**100**	**100**	**100**	**100**	n/a
5	A1	K103N	n/a	n/a	wt	wt	wt	wt	wt	**20**	A1	K103N	wt	wt	n/a	wt	wt	wt	wt
		Y181C	n/a	n/a	wt	wt	wt	wt	wt			Y181C	wt	wt	n/a	wt	wt	wt	wt
6	A1	K103N	n/a	wt	n/a	n/a	wt	n/a	wt	**21**	A1	K103N	wt	wt	wt	wt	wt	wt	wt
		Y181C	n/a	wt	n/a	n/a	wt	n/a	wt			Y181C	wt	wt	wt	wt	wt	wt	wt
7	D	K103N	wt	wt	**1.4**	wt	wt	wt	wt	**22**	A1	K103N	n/a	n/a	wt	wt	wt	n/a	wt
		Y181C	wt	wt	wt	wt	wt	wt	wt			Y181C	n/a	n/a	**1.3**	wt	**0.9**	n/a	wt
8	D	K103N	**25**	**0.9**	wt	wt	wt	wt	wt	**23**	A1	K103N	n/a	wt	wt	n/a	wt	wt	wt
		Y181C	**11**	**8.4**	wt	wt	wt	wt	wt			Y181C	n/a	wt	wt	n/a	wt	wt	wt
9§	C	K103N	n/a	**0.4**	n/a	wt	wt	*** 0.05**	wt	**24**	D	K103N	n/a	wt	**50**	n/a	**100**	wt	wt
		Y181C	n/a	**16**	n/a	**9.8**	wt	* wt	**55**			Y181C	n/a	wt	wt	n/a	wt	wt	wt
10	G	K103N	n/a	wt	wt	wt	**0.1**	n/a	wt	**25**	A1	K103N	wt	**3.3**	wt	n/a	wt	wt	wt
		Y181C	n/a	wt	wt	**0.5**	wt	n/a	wt			Y181C	wt	wt	wt	n/a	wt	wt	wt
11	A1	K103N	n/a	**0.1**	wt	n/a	wt	**0.1**	wt	**26**	A1	K103N	**0.03**	wt	wt	wt	wt	wt	wt
		Y181C	n/a	wt	wt	n/a	wt	wt	wt			Y181C	wt	wt	wt	wt	wt	wt	wt
12	D	K103N	wt	n/a	**59**	wt	wt	n/a	wt	**27**	D	K103N	n/a	**11**	n/a	**36**	**11**	wt	wt
		Y181C	wt	n/a	wt	wt	wt	n/a	wt			Y181C	n/a	**30**	n/a	wt	wt	wt	wt
13	D	K103N	n/a	n/a	wt	wt	wt	wt	n/a	**28**	D	K103N	n/a	wt	**0.1**	wt	wt	wt	wt
		Y181C	n/a	n/a	wt	wt	wt	wt	n/a			Y181C	n/a	wt	wt	wt	wt	wt	wt
14	A1	K103N	wt	n/a	**21**	wt	wt	wt	wt	**29**	D	K103N	n/a	wt	wt	wt	wt	wt	wt
		Y181C	wt	n/a	**0.4**	wt	wt	wt	wt			Y181C	n/a	wt	wt	wt	wt	wt	wt
15	A1	K103N	n/a	wt	wt	wt	wt	wt	wt										
		Y181C	n/a	wt	wt	wt	wt	wt	wt										

w: week; m: month
wt: wild-type HIV-1
n/a: not applicable (missing sample or sample that failed amplification)
§: start of antiretroviral long-term treatment at month 6 (d4T + 3TC + NVP)
*: sample collected at month 15
nt: not typable according to REGA-tool
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resistant variants at proportions lower than 5% in the ASPCR were classified to contain HIV-1 wild type only by population sequencing. In all samples harbouring NVP-resistant virus at proportions higher than 20% in the ASPCR assays, population-based sequencing confirmed the presence of drug-resistant virus. Whenever population-based sequencing detected HIV-1 variants carrying the K103N and/or Y181C mutation, the ASPCR assays indicated the presence of these mutations as well.

Discussion

NVP-SD is widely used for prevention of mother-to-child transmission of HIV-1 in resource-constrained settings but it frequently induces resistance mutations in the HIV-1 genome [2–7]. Here, we measured NVP-resistant HIV-1 at proportions as low as 1% in 29 Ugandan women from 1 week up to 18 months after NVP-SD prophylaxis. The aim of this study was to define the time of emergence of NVP-resistant virus including minority variants during the early phase after NVP-SD intake (1–2 weeks) and the persistence of NVP-resistant HIV-1.

62% of the women developed resistant virus during the observation period. The rate of resistant virus increased from week 1 (18%) over week 2 (38%) to week 6 (44%) before it continuously declined to 25% (month 6), 13% (month 12) and 4% (month 18). Most likely, NVP concentrations at week 1 which were shown to exceed the IC50 of NVP more than tenfold in most

women [18] were sufficiently high to prevent a breakthrough of resistant virus at this early time.

Higher rates of NVP-associated resistance mutations in HIV-1 subtype D strains as opposed to subtype A strains have been observed in other studies [2]. In our study, the emergence of NVP-resistance during the observation time (73% in subtype D versus 47% in subtype A) and the frequency of drug-resistant variants exceeding 5% of the total viral population (75% in subtype D versus 29% in subtype A) was more common in women infected with subtype D than with subtype A; these differences were not statistically significant, presumably due to the small sample size (Fisher's exact test: $p = 0.25$ and $p = 0.13$, respectively). However, the maximum proportions of the NVP-resistant HIV-1 population in subtype D samples (median 40.5%) significantly exceeded those in subtype A (median 1.3%) samples ($p = 0.032$, Mann-Whitney U test).

50% of the women with resistance formation never harboured NVP-resistant variants at proportions higher than 5%. However, even these minor variants are of great importance as they were shown to increase the risk of subsequent treatment failure using NNRTI-containing ART [12–14].

An interval of 6 months between NVP prophylaxis and NNRTI-based ART seemed to be sufficient for unrestricted treatment response in a trial conducted in Botswana [10]. However, in our study, NNRTI-resistant virus was present in 25% of the women at month 6. Furthermore, 3 women (10%, all subtype D) exhibited proportions of resistant HIV-1 higher than 10% (K103N: 11%, K103N: 100%, Y181C: 100%) at month 6. Starting ART in these women 6 months after NVP-SD would probably lead to the outgrowth of resistant virus, thus preparing the ground for treatment failure. Therefore, the proposed 6-month interval between NVP prophylaxis and start of NNRTIs including ART may not be long enough for all women. Consistently, Stringer et al. [8] recommended recently not to start an NNRTI-containing drug regimen within the first 12 months after NVP-SD intake as they found an increased risk of treatment failure in Zambian, Kenyan and Thai women up to 1 year after NVP-SD.

It poses a fundamental problem that the same drug is used as single-dose for prophylaxis and as part of subsequent ART. Unfortunately, neither extended combination prophylaxis regimens nor NNRTI-sparing ART using a protease inhibitor are currently realistic options in many resource-limited settings.

Of note, in the only woman who started ART during the observation period (Table 1, no. 9, start of NVP-containing treatment at month 6), resistant virus harbouring the Y181C mutation re-emerged under ART. In this woman infected with HIV-1-subtype C, drug-resistant virus was detectable early after NVP-SD intake (Y181C: 16% at week 2), disappeared and was still undetectable 9 months after the initiation of ART. However, 12 months after starting ART the majority of the viral population carried the Y181C mutation. Most likely, the resistant virus was selected, archived under NVP-SD and reappeared under the selection pressure of NVP-containing ART. Recently, it was shown that NVP-resistant HIV-1 arising after NVP-SD intake can indeed be archived as stably integrated provirus within the latent reservoir of resting CD4 cells [19].

Resistant viruses emerged in most women about 2 weeks after intake of NVP-SD. In one third of the women, however, drug-resistant virus was not detectable 2 weeks after NVP-SD intake but emerged later. Current WHO PMTCT guidelines recommend the additional postnatal intake of AZT and 3TC to reduce NVP resistance formation [1]. According to our results a 1-week course will not be sufficient to avoid the development of NVP-resistant virus in a considerable proportion of women. Accordingly, other studies have shown that 7–10% of women still exhibited NVP-resistant virus 6 weeks after NVP-SD intake despite a postnatal 1-week course of AZT and 3TC [20,21]. This indicates that a 7-day postpartum course of AZT/3TC can diminish but not eliminate the selection of NVP resistance mutations. It is therefore conceivable that an extension of the postnatal drug intake could further diminish the emergence of NVP resistant virus. In fact, Lallemant et al. applied a 1-month postpartum course of AZT plus didanosine and almost completely prevented the selection of NVP-resistant HIV-1 (0% resistant virus using population sequencing and 1.8% resistant virus using a highly sensitive assay) [22]. In this context it is important to note that none of the dual short-course antiretroviral prophylaxis regimens fully suppresses viral replication and all share the disadvantage of not preventing postnatal transmission via breastfeeding. Recently, the Kesho Bora study and other trials have proven that maternal highly active ART during pregnancy and breastfeeding efficiently reduces vertical transmission as well as the emergence of drug-resistant virus [23–26] thus maximizing future treatment options. These findings are reflected in the latest WHO PMTCT guidelines which recommend as one option highly active ART for all HIV positive pregnant women irrespective of their CD4 cell count [1]. On the other hand, possible negative implications of highly active ART like higher rates of preterm delivery, lower birth weight and cardiac effects in infants have to be considered and counterbalanced [27–29]. It is crucial to define the best option for prevention of mother-to-child transmission in order to reduce the burden of HIV/AIDS in these most severely affected regions.

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Author Contributions

Conceived and designed the experiments: AH CK GH AK. Performed the experiments: AH. Analyzed the data: AH AK. Contributed reagents/materials/analysis tools: KM RK CK GH. Wrote the paper: AH CK GH AK. Performed the field study in Uganda: KM RK GH AK.

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Dynamics of NVP-resistance development in HIV-1 of Ugandan mother-child pairs during 18 months after nevirapine single-dose exposure to reduce vertical transmission

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Introduction

A single-dose of nevirapine (NVP-SD) has been a standard regimen to prevent mother-to-child transmission of HIV-1 (PMTCT) in endemic Sub-Saharan Africa since 2001 [1]. Although the current WHO PMTCT guidelines recommend an antiretroviral triple regimen [2,3], the NVP-SD is still offered as a minimal intervention in many resource-limited countries [4]. However, exposure to NVP seems to have a negative impact on the virologic response to a subsequent NVP-containing antiretroviral treatment (ART) if started within 6 months of exposure [5,6]. Even as single dose intervention NVP frequently selects for resistance mutations, conferring cross resistance to non-nucleoside reverse transcriptase inhibitors (NNRTI), as verified for mothers [7] and infants [8-10]. Even the presence of minor drug resistant variants was reported to reduce the efficacy of a subsequent treatment in adults [11-14] and children [15,16]. In the absence of drug-selective pressure resistant virus populations fade over time [8,9,17]. However, persistence of minor resistant variants for a period of more than a year was shown in previous studies for women [18-20]. Moreover a 12-months interval after NVP-SD exposure was recommended to ensure an unrestricted treatment response to NNRTI-containing ART [6]. It is not known whether such a time interval also applies to children. Most data regarding the presence of NVP-resistant virus populations in infants are based on results of the less sensitive Sanger

Sequencing method [10] or on samples taken 4-12 weeks after birth or after treatment failure [8,15,16]. So far, the emergence and persistence of minor resistant HIV-variants in children following a NVP-SD have not been assessed. In the present study we monitored the presence of the two most commonly NVP-selected resistance mutations K103N and Y181C in the reverse transcriptase after NVP-exposure for a period of 18 months in plasma virus of Ugandan mothers and their vertically infected children. Therefore, a highly sensitive allele-specific PCR (ASPCR) assay with detection limits of <1% was applied [21]. The aim of the study was to determine the time of emergence and persistence of resistance mutations in plasma virus of the children compared to that of their mothers.

Method

Study population

During 2003-2005, 90 HIV-infected, treatment-naïve mothers and their children participated in a "prevention of mother-to-child transmission of HIV-1" (PMTCT) program at Fort Portal District Hospital, West-Uganda. In accordance with national guidelines mothers received 200mg NVP at onset of labor and children 2mg/kg NVP syrup within the first 72 hours after birth (HIVNET012 protocol) [1]. Blood samples were taken at birth, week 2-4, 6-8 and months 3, 6, 12 and 18. All women had given informed consent. The presence of NVP was confirmed in 83/90 maternal delivery blood samples [22], these 83 mothers constituted to the present and previous studies [20,23]. The 83 HIV-positive women gave birth to 86 newborns (80 single and 3 twin births); 74 (86%) infants were exclusively breastfed and 12 (14%) received replacement feeding. The study was approved by "National Council of Science and Technology of Uganda" and the ethical committee of Charite-Universitätsmedizin Berlin, Germany.

Determination of HIV infection status of infants

Since maternal HIV-1 antibodies are transferred through the placenta to the child while pregnancy, HIV-PCR has to be performed for diagnosis of HIV-infections in early pediatric blood samples. In children the maternal antibodies usually disappear 18 months after birth and uninfected infants revert to HIV-seronegative status [24]. In the present study only those pediatric specimen taken 18 months after birth were tested in two commercial HIV-1 antibody tests (Murex HIV-1.2.0, Abbott GmbH & Co. KG, Wiesbaden, Germany and Gene Screen HIV1 / 2 version 2, BioRad Laboratories, Munich, Germany). For reactive and indifferent HIV-1 antibody test results as well as samples taken before the age of 18

months (in case of death or loss to follow-up of the child), HIV-PCR was performed using the "outer" PCR of ASPCR [21]. In case of positive PCR-results all samples of the infected child were tested by PCR to determine the time of vertical transmission. Positive PCR-results for samples taken at birth indicated for "in-utero transmission" [25], a negative PCR-result for a samples taken at birth followed by positive PCR-results for samples takes at week 2-4 indicated for "intrapartum transmission" (including late in-utero and early breast feeding) and a positive PCR-result in the sample taken at week 6 (or later) but not earlier indicated for a "postpartum transmission" (via late breastfeeding).

Analysis of resistance

Since subtypes A and D are the predominant HIV-1 subtypes circulating in Uganda [26], plasma samples of HIV-positive infants and their mothers were investigated for the most common NVP-selected resistance mutations K103N (AAC and AAT codons) and Y181C (TGT codon), using the subtype-A and-D-specific ASPCR with detection limits <1% [21]. If maternal delivery sample (baseline sample; assumed to contain HIV-1 wild-type only) [27] was not available, a wild-type DNA-standard was used to determine the proportion of resistant variants in the total viral population [21]; In case of HIV-negative or lacking newborn birth sample, the corresponding maternal sample was used. Proportions of K103N mutants encoded either by codon AAC or AAT were summarized.

Population-based sequencing

Population-based sequencing was conducted using the Viroseq HIV-1 Genotyping System version 2.0 (Abbott, Wiesbaden, Germany) and the automated sequencer 3130xl Genetic Analyzer (Applied Biosystems, Darmstadt, Germany). HIV-1 subtyping was performed using the REGA HIV subtyping tool [28].

Results

Eight out of 86 infants were tested HIV-positive 6-8 weeks after delivery or even earlier (transmission rate of 9.3%). In total, ten children were infected within the study period (18 months). As one vertically infected newborn was lost to follow-up, only 9/10 HIV-positive mother-child pairs were included in the resistance analysis: Of each mother and infant 3-6 follow-up samples were available, whereas delivery samples of only seven mother-child pairs were present. According to our definition three newborns were in-utero infected, one child intrapartum and three children postpartum. For two infants time of infection could

not be identified, since samples of birth and/or 2-4 weeks after were lacking. All HIV-positive infants were breastfed. Mother-child pairs were infected with HIV-1 subtypes A1 (n=4), D (n=3), G (n=1) and K (n=1) (Table 1).

For resistance testing by ASPCR, a mean of 4.4 maternal and 3.2 infant follow up samples were investigated for K103N and Y181C mutations. In 7/9 mothers and 4/9 infants NVP-resistant HIV-1 variants could be identified during the study period. Resistant HIV-1 variants emerged 2-8 weeks after delivery in 6/7 mothers and persisted for at least six months in all but one woman. In two of these women, resistant HIV-1 was still detectable in the 12 months samples in low (0.05%) and high (100%) proportions, respectively. For 4/7 mothers resistant HIV-1 was identified as minor variants only (<5%) (Table 1).
In 3/4 children NVP-resistant mutant variants also emerged 2-8 weeks after birth while in one infant resistant virus was identified at month 3 in very low proportions (0.06%). Persistence was observed for a maximum of six months (n=1). For 2/4 infants resistant variants were detected in low proportions (<5%) (Table 1). Two of the three in-utero infected newborns displayed high proportions of resistant viruses. In contrast, in the three postpartum infected children no resistant virus was identified, although their mothers carried resistant HIV-1: In one of these mothers resistant variants were present in high proportions during a whole year postpartum, whereas in two mothers resistant virus variants were reduced to low or undetectable proportions (Table 1).

Discussion

The transmission rate in the present study is comparable to published data for the NVP-SD prophylaxis (9.3% vs. 15%) [1]. 78% HIV-positive women and 44% of their vertically infected children habored four viral variants with at least one NVP resistance mutation (K103N and/or Y181C) during the observation period. These frequencies are consistent with previous published data showing NVP-resistant HIV-variants after NVP-SD exposure in 87% of women (week 6-8) [17,19] and 46% of children (week 4-12) with [8,10]. Resistant HIV-variants emerged within 2-8 weeks after birth in maternal samples and in those of 2/3 in-utero infected infants. Since both children were infected prior to NVP-exposure at delivery, resistant variants were likely to be selected in the infant itself. Due to the long half-life of NVP, HIV is exposed to slowly decreasing NVP levels over weeks [22] providing resistance-selective conditions. For mothers with established HIV-1 infections emergence of NVP-resistance mutations 1-6 weeks after exposure is well documented [20].

For children with antenatal infections, different dynamic models of NVP-resistance development were discussed by [29] Micek *et al*: a high proportion followed by a rapid decay of NVP-resistant HIV-1 was observed in newborns with "established in-utero infection" (infection early in pregnancy; displaying high viral loads at delivery) compared to lower but stable rates of resistance in newborns with "acute in-utero infection" (infection late pregnancy; low but escalating viral loads at birth). Viremia under NVP selective pressure, as present in "acute in-utero infection", fosters proviral integration and thus archiving and persistence of NVP-resistance mutation [29]. In the present study highest proportions of resistant HIV-variants were identified 2-4 weeks after birth in 2/3 in-utero infected children indicating an "established in-utero infection". But since viral loads were not quantified, time of in-utero infection could not be categorized according Miceks´ definition.

In postpartum infected children (n=3) no resistant virus was identified, although NVP-resistance was detected in plasma virus of their mothers (Table 1). Two of three mothers carried mainly wild-type virus in plasma at the estimated times of transmission (maternal sample taken before the first HIV-positive sample of the child: month 6 and 12, respectively), while in the third mother (no 7) a high proportion of resistant plasma virus was detected (<100% Y181C at week 2-4). Nevertheless, transmission of resistant virus from this mother to her child could not be documented. Since postnatal transmission occurs via breastfeeding to 5-20% [30,31], breast milk is assumed to have had HIV wild-type (= K103K and Y181Y) only. Studies on distribution of resistant HIV-variants in plasma and breast milk have shown that different resistance patterns and proportions are present in respective body compartments [23,32].

Half of the mothers (4/7) and children (2/4) displayed resistant HIV-variants in low proportions (<5% of the total HIV-population). The presence of minor NVP-resistant variants is reported to be correlated with virological failure for mothers [11-14] and for NVP-exposed infants (n=26) of the PMTCT "Mashi" study [15]. According to that study virologic failure is also correlated with an early initiation of therapy of less than seven months after NVP-exposure [15].

In the present study. HIV resistance mutations did not persist longer in children (six months) than in their mothers (12 months). Since resistant variants may expand to become the predominant population under drug selection pressure [33] a minimum of a 12-month interval between NVP-SD and a NNRTI-containing treatment is recommended for the mothers [6]. However, applying a time interval to NVP-SD exposed children is much more

challenging: our data (despite the very small sample size) and data of Persaud *et al.* [34] (26% of children (n=19) with NVP-resistant HIV 6 months after NVP-SD exposure), suggest that the start of therapy within seven months cannot be recommended. Rather, the fact that resistant HIV-1 variants could be archived in the cellular reservoir of antenatal infected infants and thus persist for long periods of time [29,35,36] indicates for the need of extended intervals for children. On the other hand, HIV-disease progression in children is much faster than in adults: 50% of infected newborns die within the first year of life and 85% are in need of ART within the first 6 months of life [37]. The resulting interval between NVP exposure and start of ART is much shorter in children than in mothers [38]. Therefore, recommendations for an interval of more than 7 months are not realistic.

To conclude, NVP-SD and extended NVP for newborns [2,3] are widely provided as PMTCT interventions in Sub-Saharan Africa with the inherent risk of resistance development if the infant is vertically infected. Since resistance testing prior to the start of ART cannot be performed in resource-limited settings, NNRTI-containing therapy should be replaced by other antiretoviral drugs if initiated within the first month after NVP-SD. According to our results a seven months-interval seems to be sufficient to prevent a reduced NNRTI-containing treatment response.

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Table 1: Drug resistant HIV-variants in plasma of mother-child pairs detected by ASPCR

No.	Trans-mission	Sub-type		Delivery		Week 2-4		Week 6-8		Month 3		Month 6		Month 12		Month 18	
				K103N	Y181C	K103N	Y181C	K103N	Y181C	K103N	Y181C	K103N	Y181C	K103N	Y181C	K103N	Y181C
1	IU	K	Child	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	Child died	
		K	Mother	wt	wt	**2.2**	**1.1**	**1.7**	wt	wt	wt	**0.12**	wt	wt	wt	–	
2	IU	A1	Child	wt	wt	**17.8**	**18.7**	**3.6**	**1.3**	–		–		–		wt	wt
		A1	Mother	wt	wt	–		wt	**1.1**	wt	wt	wt	**3.2**	–		wt	wt
3	IU	D	Child	wt	wt	**24.0**	**4.8**	**0.8**	wt	**10.7**	wt	**47.6**	wt	wt	wt	wt	wt
		D	Mother	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
4	IP	A1	Child	–		wt	wt	wt	wt	**0.06**	wt	wt	wt	Child died		Child died	
		A1	Mother	wt	wt	wt	wt	wt	wt	wt	wt	–		–		–	
5	§	G	Child	–		–		**0.02**	wt	**0.03**	wt	Child died		Child died		Child died	
		G	Mother	–		–		**9.3**	wt	**54.8**	wt	**7.3**	wt	–		–	
6	§	A1	Child	–		–		wt	wt	wt	wt	wt	wt	–		wt	wt
		A1	Mother	–		–		–		**1.2**	wt	**0.4**	wt	–		wt	wt
7	PP	D	Child	**O**		**O**		wt	wt	wt	wt	wt	wt	–		–	
		D	Mother	wt	wt	**3.3**	**100**	**60.9**	**100**	**1.4**	**100**	wt	**100**	wt	**100**	–	
8	PP	D	Child	**O**		**O**		**O**		**O**		**O**		–		wt	wt
		D	Mother	wt	wt	**0.9**	**8.4**	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
9	PP	A1	Child	**O**		**O**		**O**		**O**		**O**		**O**		wt	wt
		A1	Mother	wt	wt	**0.05**	wt	wt	wt	n/s		wt	wt	**0.05**	wt	wt	wt

wt = no resistance mutation detected; – = no sample available; O = HIV PCR negativ; IU = in-utero transmission; IP = intrapartal transmission; PP = postpartal transmission; § = time of transmission unknown

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Short communication

Minor drug-resistant HIV type-1 variants in breast milk and plasma of HIV type-1-infected Ugandan women after nevirapine single-dose prophylaxis

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Background: Nevirapine single-dose (NVP-SD) reduces mother-to-child transmission of HIV type-1 (HIV-1), but frequently induces resistance mutations in the HIV-1 genome. Little is known about drug-resistant HIV-1 variants in the breast milk of women who have taken NVP-SD.
Methods: Blood and breast milk samples of 39 HIV-1-infected Ugandan women were taken 6–12 weeks after NVP-SD intake. Samples were analysed by population sequencing and allele-specific real-time PCR (AS-PCR) **with detection limits for NVP-resistant HIV-1 variants (K103N and Y181C) of <1% of the total viral population.**
Results: AS-PCR results for both plasma and breast milk were obtained for 19 women who constituted the final **study group (HIV-1 subtype frequencies were A1 *n*=11, D *n*=5, G *n*=2 and C *n*=1). A total of 7 (37%) and 10 (53%) women carried NVP-resistant virus in breast milk and plasma, respectively. Overall, 71% (5/7) women with NVP-resistant HIV-1 in breast milk displayed >1 drug-resistant variant. Resistance in breast milk was higher at week 6 (6/13 samples [46%]) compared with week 12 (1/6 samples [17%]). In total, 10 drug-resistant populations harbouring the K103N and/or Y181C mutation were detected in the 19 breast milk samples; 7 (70%) were caused by resistant minorities (<5% of the total HIV-1 population). In the four women with drug-resistant virus in both plasma and breast milk, the mutation patterns differed between the two compartments.**
Conclusions: Minor populations of drug-resistant HIV-1 were frequently found in breast milk of Ugandan women after exposure to NVP-SD. Further studies need to explore the role of minor drug-resistant variants in the postnatal transmission of (resistant) HIV-1.

Introduction

Mother-to-child transmission of HIV is a major concern in sub-Saharan Africa, and postnatal HIV infection through breastfeeding is estimated to account for almost one-third to one-half of all HIV infections in infants and young children [1]. Nevirapine single-dose (NVP-SD) is still widely used to reduce the transmission of HIV type-1 (HIV-1) from the mother to the child in resource-limited settings, although it is associated with a high incidence of NVP-resistant HIV-1 [2–11]. To date, most studies have focused on the development of NVP-resistant virus in plasma, and it has been shown that 19–75% of women exposed to NVP-SD harbour NVP-resistant HIV-1 variants in their blood [3–11]. Although the proportion of resistant virus in plasma fades over time, minor drug-resistant populations can persist for long periods in this compartment [5,8,9]. However, little is known about the emergence and persistence of NVP-resistant HIV-1 variants in breast milk after NVP-SD and to what extent the mutation patterns differ from plasma. This is of great importance as breastfeeding is common in many developing countries where NVP-SD is used and the presence of resistant virus in breast milk might put children at risk of acquiring drug-resistant HIV-1 through breast milk [12].

So far, only three studies have determined the emergence of NVP-resistant virus in breast milk following intake of NVP-SD. Two studies analysed breast milk of women infected with HIV-1 subtype C and showed that HIV-1 variants in breast milk emerged frequently and often differed in sequences from resistant HIV-1

variants in plasma [13,14]. Recently, a third study analysed breast milk samples of women mainly infected with HIV-1 subtypes A and D using a population-based approach with a detection limit for drug-resistant HIV-1 variants of approximately 20% [15].

Here, we describe the emergence of NVP-resistant HIV-1 variants in breast milk and plasma of Ugandan women predominantly infected with HIV-1 subtypes A and D using highly sensitive allele-specific real-time PCR (AS-PCR), capable of detecting minor drug-resistant variants representing <1% of the total viral population.

Methods

We conducted an observational cohort study that was approved by the national ethical committee of Uganda (National Council of Science and Technology). HIV-1-positive pregnant women enrolled in the national prevention of mother-to-child transmission programme were recruited at Fort Portal Hospital (Fort Portal, western Uganda) after they had given informed written consent [16]. The women had taken NVP-SD (200 mg) at the onset of labour following the HIVNET 012 protocol [2]. None of the participating women received any other antiretroviral drugs aside from NVP-SD before or during the study period. Plasma and breast milk samples were collected at delivery (baseline) and at 1, 2, 6 and 12 weeks postpartum. Participants were included in the final analysis if plasma and breast milk samples from delivery were available and follow-up samples at week 6 or 12 were amplifiable. Whenever the baseline sample was amplifiable it was used as individual threshold for the detection of NVP-resistant variants as described by Hauser *et al.* [17]; otherwise a cutoff derived from plasmid mixtures was applied. HIV-1 variants with the K103N and/or Y181C mutation in the *pol* gene were detected and quantified by two-step AS-PCR assays, as previously described [17]; the detection limits for the three mutations as estimated from plasmid controls were 0.019% (K103N:AAC), 0.013% (K103N:AAT) and 0.29% (Y181C:TGT). To compensate for the expected lower viral loads in breast milk, the sample volume for RNA extraction was increased sixfold (3 ml) as compared with plasma (0.5 ml). Viral RNA was extracted (QIAamp Viral RNA Mini Kit; Qiagen GmbH, Hilden, Germany) and reverse transcribed (SuperScript II RT; Invitrogen, Karlsruhe, Germany) according to the manufacturer's recommendations. Viral loads were determined in the outer quantitative PCR (644 base pair amplimer with coordinates 2613–3256 of HXB2; GenBank accession number K03455) using a quantified virus stock (NL4.3) as standard. The lower limit of detection for viral load was 650 copies/ml; that is, the input of 3 ml breast milk allowed the quantification of viral loads down to 220 copies/ml. For population-based sequencing of the 644 base pair product (comprising the codons 21–236 of the reverse transcriptase), the automated sequencer 3130xl Genetic Analyzer (Applied Biosystems, Darmstadt, Germany) and the B-, D- and G-sequencing primers of the ViroSeq HIV-1 Genotyping System version 2.0 (Abbott GmbH & Co. KG, Wiesbaden, Germany) were used [17]. The sequences were subtyped with the REGA HIV-1 subtyping tool [18] or by the neighbour-joining method (PHYLIP package [19]). A neighbour-joining tree was constructed by aligning the *pol* sequences with 131 subtype reference sequences from the *pol* subtype reference alignment 2008 [20] using the CLUSTALW programme implemented in BioEdit version 7.0.5 [21]. All samples were measured at least in duplicates together with no template- and HIV-1-negative controls. A neighbour-joining phylogenetic tree was calculated to control for potential sample mix-up. Sequences derived from plasma and breast milk from one patient should colocalize in clades, whereas independent HIV-1 strains should not cluster together.

Results

Blood samples and breast milk samples of 39 women were available. For 20 (51%) women, the analyses of breast milk samples were PCR-negative because viral loads were below the detection level of the assays. Samples from these women were omitted from the final analysis, and the remaining 19 women constituted the final study group. The median age was 25 years (interquartile range [IQR] 19–29) and the median parity was 2 (IQR 1–5). A total of 11 (58%) women were infected with subtype A1, 5 (26%) with subtype D, 2 (11%) with subtype G and 1 (5%) with subtype C. The median viral load in breast milk was 1,100 copies/ml (IQR 500–2,800 copies/ml; Table 1).

Overall, 7 (37%) women harboured drug-resistant HIV-1 in breast milk and 10 (53%) women in plasma (Table 1). NVP-resistant virus in breast milk was detected in 5/7 subtype A1, 0/5 subtype D, 1/2 subtype G and 1/1 subtype C samples, respectively. A total of 13 breast milk samples were collected 6 weeks and 6 were collected 12 weeks after NVP-SD intake. At week 6, drug-resistant virus was detected in 6/13 (46%) breast milk samples, whereas at week 12, 1/6 (17%) breast milk samples exhibited NVP-resistant HIV-1. In total, 10 and 13 drug-resistant populations carrying the K103N or Y181C mutation were detected in breast milk and plasma samples, respectively. Among these, 7 (70%) in the breast milk and 5 (38%) in the plasma were present as resistant minor variants defined as <5% of the total viral population.

Table 1. Drug-resistant HIV-1 variants in plasma and breast milk detected by population-based sequencing and AS-PCR 6–12 weeks after NVP-SD intake

Patient number	HIV-1 subtype	Viral load, copies/ml		Sample collection, weeks after NVP-SD		NVP-associated resistant mutations by population sequencing		K103N (AAC) by AS-PCR, %		K103N (AAT) by AS-PCR, %		Y181C (TGT) by AS-PCR,%		NVP resistance	
		Plasma	Breast milk	Plasma	Breast milk	Plasma	Breast milk	Plasma	Breast milk	Plasma	Breast milk	Plasma	Breast milk	Plasma	Breast milk
1	A1	96,000	420	6	6	wt	wt	wt	wt	wt	wt	wt	wt	No	No
2	A1	120,000	7,400	6	6	K103N	V106A	15.6	9.7	0.34	wt	wt	wt	Yes	Yes
3	D	91,000	3,400	6	6	wt	ND	6.8	wt	0.22	wt	0.62	wt	Yes	No
4	G	9,000	2,600	12	12	K103N	K103N, G190A	54.8	26.9	wt	0.03	wt	wt	Yes	Yes
5	D	95,000	14,000	6	6	wt	wt	wt	wt	wt	wt	wt	wt	No	No
6	C	21,000	2,800	12	6	Y181C	V106A	wt	0.67	wt	0.04	9.8	wt	Yes	Yes
7	G	22,000	360	12	12	wt	ND	wt	wt	wt	wt	0.5	wt	Yes	No
8	A1	19,000	1,160	6	12	wt	wt	wt	wt	wt	wt	wt	wt	No	No
9	A1	11,000	5,000	6	6	ND	wt	wt	wt	wt	wt	wt	2.6	No	Yes
10	A1	3,500	1,100	12	12	V106A	ND	wt	wt	wt	wt	wt	wt	Yes	No
11	D	2,000	500	6	6	wt	ND	wt	wt	wt	wt	wt	wt	No	No
12	A1	200,000	1,340	6	6	K103N[a], Y181C	K101E, K103N	26.1	100	35	0.04	100	wt	Yes	Yes
13	A1	4,700	340	12	12	wt	wt	wt	wt	wt	wt	wt	wt	No	No
14	D	4,600	660	6	6	Y188C	wt	19.2	wt	wt	wt	wt	wt	Yes	No
15	A1	12,000	1,000	12	6	ND	K101E	wt	wt	wt	0.02	wt	wt	No	Yes
16	A1	5,000	340	6	6	wt	wt	wt	wt	wt	wt	wt	wt	No	No
17	A1	5,400	1,400	12	12	wt	wt	1.2	wt	wt	wt	wt	wt	Yes	No
18	A1	8,100	600	6	6	wt	wt	wt	wt	wt	wt	wt	1.6	No	Yes
19	D	3,600	540	6	6	V106A	wt	wt	wt	wt	wt	wt	wt	Yes	No

[a]AAC+AAT. AS-PCR, allele-specific real-time PCR; HIV-1, HIV type-1; ND, not done; NVP, nevirapine; NVP-SD, nevirapine single-dose; wt, wild type.

In 4 (21%) women, drug-resistant virus was identified in both breast milk and plasma. In these women (patient numbers 2, 4, 6 and 12; Table 1), the mutation patterns differed between the two compartments. In 5/7 (71%) women (patient numbers 2, 4, 6, 12 and 15; Table 1) with drug-resistant virus in breast milk, >1 NVP-resistant HIV-1 variants were found in the same breast milk sample.

All samples identified as wild-type HIV-1 (K103 and Y181) by AS-PCR yielded wild-type results by population-based sequencing. Whenever population-based sequencing detected HIV-1 variants carrying the K103N and/or Y181C mutation, the AS-PCR assays confirmed the presence of mutations. Population-based sequencing with its much lower sensitivity failed to detect 15 drug-resistant variants identified by the AS-PCR assays (Table 1). By contrast, population-based sequencing detected other NVP-associated resistance mutations, such as V106A and G190A in breast milk; however, it did no identify additional women with drug-resistant HIV-1 in breast milk, as all of these women carried the K103N and/or Y181C mutation, which had already been detected by the AS-PCR assays. The neighbour-joining tree topology did not reveal any indication of sample mix-up. Only HIV-1 sequences derived from plasma and breast milk from the same individual were closely related (supported by high bootstrap values) in contrast to sequences derived from independent infections (CK *et al.*, data not shown).

Discussion

Our findings confirm previous studies reporting high frequencies of drug-resistant virus in breast milk of NVP-SD-exposed pregnant women infected with HIV-1 [13–15]. Additionally, we found that most of the detected resistance mutations were present as minor drug-resistant variants accounting for <5% of the total viral population. The effect of such minor variants on the transmission of drug-resistant HIV-1 to the infant through breastfeeding is so far unknown, and needs further exploration.

Although it has been shown that minor drug-resistant variants can persist in plasma for prolonged periods [5,8,9], their persistence in breast milk is not well characterized. Knowledge of the persistence of drug-resistant variants is important to better estimate the risk of transmission of drug-resistant HIV-1 through breastfeeding. In this study, using highly-sensitive AS-PCR assays, we found that 46% and 17% of all women harboured drug-resistant HIV-1 variants in their breast milk at 6 and 12 weeks after NVP-SD intake, respectively. These findings suggest a fading of NVP-resistant virus in breast milk over time as it was shown for plasma [5,8,9]. Recently in Uganda, Hudelson *et al.* [15] detected NVP-resistant virus in samples of 40% of women 4 weeks after NVP-SD intake using population-based sequencing. It can be assumed that the frequency of drug-resistant variants would have been even higher if they had applied a more sensitive method, such as AS-PCR assays.

The percentage of women harbouring drug-resistant variants in our study was lower compared with studies analysing HIV-1 subtype-C-infected women [13,14]. Lee *et al.* [14] found drug-resistant virus in 65% of breast milk samples of women infected with HIV-1 subtype C 8 weeks after NVP-SD exposure using a population-based approach. Similarly, drug-resistant viruses in plasma have been shown to emerge at higher frequencies in subtype C as compared with subtype A and D [4,11], which also appears to be true for breast milk.

The persistence of drug-resistant variants in breast milk over months could have an effect on the transmission of drug-resistant HIV-1. As the risk of infection increases with the duration of breastfeeding [22], it is conceivable that prolonged breastfeeding increases the risk of acquiring drug-resistant HIV-1.

To estimate the risk of acquiring drug-resistant virus through breastfeeding, the emergence of drug-resistant virus in the breast milk compartment has to be understood. Our neighbour-joining analysis showed a close relationship between virus populations found in plasma and in breast milk of the same individual; however, viral strains differed with respect to resistance mutations in the two compartments. Different mutation patterns in breast milk and plasma is a consistent finding [13,14,23]. Furthermore, HIV-1 mutation patterns can also differ between samples from the right and left breast of the same women [14]. While it has been shown that a short-course of combivir (zidovudine and lamivudine) over 1 week can prevent the formation of NVP resistances in plasma after NVP-SD intake [24], it is not known whether this holds true for other compartments, that is, for breast milk.

Caution has to be taken when generalizing the results of this study. One-half of the initially enrolled women could not be included into the final resistance analysis because we did not obtain PCR amplificates. This constitutes a common problem in the analysis of breast milk because the viral load in breast milk is considerably lower than in plasma [25]. The real lower limits of detection for the K103N and Y181C variants using AS-PCR assays depend on the respective viral load. Therefore the sensitivity of the assays for breast milk samples with low viral load is reduced compared with the theoretical sensitivity as determined by plasmid controls. Thus, the presence of minor variants in breast milk is prone to be underestimated.

In conclusion, we have shown that minor drug-resistant variants frequently emerge in breast milk

of Ugandan women infected with HIV-1 who took NVP-SD. Considering that breastfeeding is still the only option for many women in areas with a high HIV-1 prevalence, the exact role of minor drug-resistant variants in the postnatal transmission of HIV-1 should be subject to further investigations.

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Disclosure statement

The authors declare no competing interests.

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Emergence of Minor Drug-Resistant HIV-1 Variants after riple ntiretroviral Prophylaxis for Prevention of Vertical HIV-1 ransmission

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Abstract

Background: WHO-guidelines for prevention of mother-to-child transmission of HIV-1 in resource-limited settings recommend complex maternal antiretroviral prophylaxis comprising antenatal zidovudine (AZT), nevirapine single-dose (NVP-SD) at labor onset and AZT/lamivudine (3TC) during labor and one week postpartum. Data on resistance development selected by this regimen is not available. We therefore analyzed the emergence of minor drug-resistant HIV-1 variants in Tanzanian women following complex prophylaxis.

Method: 1395 pregnant women were tested for HIV-1 at Kyela District Hospital, Tanzania. 87/202 HIV-positive women started complex prophylaxis. Blood samples were collected before start of prophylaxis, at birth and 1–2, 4–6 and 12–16 weeks postpartum. Allele-specific real-time PCR assays specific for HIV-1 subtypes A, C and D were developed and applied on samples of mothers and their vertically infected infants to quantify key resistance mutations of AZT (K70R/T215Y/T215F), NVP (K103N/Y181C) and 3TC (M184V) at detection limits of <1%.

Results: 50/87 HIV-infected women having started complex prophylaxis were eligible for the study. All women took AZT with a median duration of 53 days (IQR 39–64); all women ingested NVP-SD, 86% took 3TC. HIV-1 resistance mutations were detected in 20/50 (40%) women, of which 70% displayed minority species. Variants with AZT-resistance mutations were found in 11/50 (22%), NVP-resistant variants in 9/50 (18%) and 3TC-resistant variants in 4/50 women (8%). Three women harbored resistant HIV-1 against more than one drug. 49/50 infants, including the seven vertically HIV-infected were breastfed, 3/7 infants exhibited drug-resistant virus.

Conclusion: Complex prophylaxis resulted in lower levels of NVP-selected resistance as compared to NVP-SD, but AZT-resistant HIV-1 emerged in a substantial proportion of women. Starting AZT in pregnancy week 14 instead of 28 as recommended by the current WHO-guidelines may further increase the frequency of AZT-resistance mutations. Given its impact on HIV-transmission rate and drug-resistance development, HAART for all HIV-positive pregnant women should be considered.

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Introduction

Mother-to-child transmission of HIV-1 in resource-limited settings accounts for almost 16% of all new HIV-1 infections in Sub-Saharan Africa [1]. Antiretroviral drugs for HIV-1-infected pregnant women and their infants are an essential component in reducing mother-to-child transmission of HIV-1. The non-nucleoside reverse transcriptase inhibitor (NNRTI) nevirapine (NVP) has been widely applied as single dose (NVP-SD) prophylaxis at the onset of labor [2]. However, due to the low genetic barrier of NVP even a single dose frequently induces viral resistance [3–10], thus compromising the success of subsequent NNRTI-containing highly active antiretroviral treatment (HAART) if initiated within 6–12 month after prophylaxis [11–13]. To reduce viral resistance as well as to further lower the vertical transmission risk of HIV-1, the WHO guidelines for the prevention of mother-to-child transmission (PMTCT) of 2006 and 2010 [14,15] recommend complex antiretroviral prophylaxis. This is composed of antenatal zivoduvine (AZT) for three (2006) or six months (2010), NVP-SD at labor onset and AZT/lamivudine (3TC) during labor and for one week postnatally. In 2008, complex prophylaxis was recommended by the national Tanza-

nian PMTCT guidelines as preferred PMTCT regimen [16]. Monotherapy of antiretroviral drugs, however, inherently involves the risk of drug resistance development. Selection of AZT-resistant virus during prenatal AZT monotherapy might decrease the efficacy of future AZT-containing prophylactic and therapeutic regimens. Furthermore, as both NVP and 3TC rapidly select for drug-resistant virus, dual- or multi-resistant HIV-1 variants could emerge. Even minor drug-resistant HIV-1 variants representing small proportions of the total viral population can impair virological outcome of HAART [17–24]. Hence, it is mandatory to characterize the resistance development including minority species following complex prophylaxis, which to our knowledge has not been assessed for the WHO-recommended complex prophylaxis regimen. The aim of this study was to evaluate the emergence of HIV-1 variants resistant against AZT, NVP and/or 3TC following complex antiretroviral prophylaxis in a rural district hospital in Kyela, Mbeya Region, Tanzania. For this purpose, we developed, evaluated and applied highly sensitive allele-specific PCR (ASPCR) assays enabling the detection and quantification of three key mutations for AZT resistance (K70R, T215Y and T215F), the two most common NVP-associated resistance mutations (K103N and Y181C) and the most frequent 3TC-selected mutation M184V in the *pol* open reading frame with a detection limit of <1% [25,26]. ASPCR assays were adapted for HIV-1 subtypes A, C and D which are common in Sub-Saharan Africa and prevalent in Mbeya Region, Tanzania [27]. Subsequently, blood specimens from HIV-1-infected pregnant Tanzanian women and their vertically infected infants who had taken complex antiretroviral prophylaxis were analyzed.

Materials and Methods

Ethics Statement

Ethical approval was obtained from the local Mbeya Medical Research and Ethics Committee, the National Institute for Medical Research of Tanzania and the ethical committee of Charité – Universitätsmedizin Berlin in Germany. We obtained informed written consent from all participants involved in our study.

Clinical samples and study design

The present study analyzes the HIV-1 resistance development in HIV-1-infected Tanzanian women and their infants as part of an observational study at Kyela District Hospital, Mbeya Region between October 2008 and September 2009 [28]. In March 2008, complex antiretroviral prophylaxis was introduced as the standard PMTCT regimen at Kyela District Hospital. According to WHO PMTCT guidelines from 2006 [14] and National Tanzanian PMTCT guidelines [16], women were offered complex antiretroviral prophylaxis composed of AZT starting in gestational week 28 (2×300 mg per day), or as soon as possible thereafter, followed by NVP-SD (200 mg) at labor onset and AZT (300 mg) every three hours plus 3TC (150 mg) every 12 hours during labor, followed by a one week postpartum course of AZT (2×300 mg per day) and 3TC (2×150 mg per day). Infants received NVP-SD (2 mg/kg) within 72 hrs after birth and AZT (4 mg/kg per day) for one week. In case the mother had taken antenatal AZT for less than four weeks, the infant received postnatal AZT for four weeks. Blood samples were collected before start of AZT prophylaxis, during pregnancy, at delivery and at 1–2, 4–6, and 12–16 weeks postnatally.

202 of 1395 (14.5%) pregnant women tested for HIV-1 during antenatal care were HIV-1 positive. 122 HIV-positive women were included in the observational study as they fulfilled the following eligibility criteria: no HAART, no clinical or immunological indication to start HAART, i.e. CD4 cell count >=200 cells/mm3 and clinical categories A or B according to CDC classification, age >=18 years, absence of other severe diseases including psychiatric disorders, written informed consent [28]. Eventually, 87 of the 122 eligible women started AZT prophylaxis during pregnancy [28]. Women and if applicable their HIV-infected infants were included in the resistance analysis if they had taken AZT in pregnancy for at least two weeks, if they had taken NVP at labor onset, and if a delivery sample and at least two postnatal (1–2 weeks, 4–6 weeks and/or 12–16 weeks) plasma samples were available. In the case of home delivery, the last antenatal specimen was used as "delivery sample". Additionally, a baseline sample prior to AZT intake had to be amplifiable in order to establish an individual cut-off for resistance detection [29]. No woman received any other antiretroviral drugs during the study period. Children of the study cohort were breastfed.

Detection and quantification of drug-resistant HIV-1

Drug-resistant mutations in the *pol* open reading frame of HIV-1 were detected by ASPCR which is an established and widely used method for the analysis of minor drug-resistant HIV-1 variants [5,29–33]. The assay is composed of two consecutive real-time PCRs. The outer real-time PCR amplified a reverse transcriptase (RT) fragment comprising the codons of interest (codons 22 to 236 of the RT) and was also used for quantification of viral load. The inner ASPCR was composed of one real-time PCR reaction with discriminatory ability for mutant sequences using selective primers and one generic real-time PCR reaction amplifying both wild-type and mutant sequences using non-selective primers (Table 1). For each resistance mutation, an individual inner ASPCR assay had to be designed. In total, seven ASPCR assays were performed per sample: two AZT mutations confering high level resistance (T215Y, T215F) and one early AZT mutation (K70R) confering only low level resistance but indicating for emergence of AZT-resistance; additionally the two most common NVP-selected resistance mutations (K103N and Y181C) and the most frequent 3TC-selected mutation M184V were analysed [34,35] (details in Materials and Methods S1).

Vertical transmission of HIV-1

The HIV-status of newborns was determined by RT-PCR of blood specimens collected 4–6 weeks after birth using the above described outer PCR. Infants with a positive PCR result at 4–6 week were defined to be HIV-infected whereas infants with a negative PCR result were assumed to be not HIV-infected. If the 4–6 week sample was lacking, an earlier blood sample from delivery or week 1–2 was analysed. If the earlier sample was PCR-positive, the child was considered to be HIV-infected 4–6 weeks after birth as well; if the earlier blood sample was PCR-negative, the infant was excluded from calculation of transmission rate as the HIV status week 4–6 after birth could not be determined.

Population-based sequencing and determination of HIV-1 subtype

For population-based sequencing of the 644 bp product generated by outer PCR, the automated sequencer 3130xl Genetic Analyzer (Applied Biosystems, Darmstadt, Germany) and the HIV SEQ MIX B, D and G of the Viroseq HIV-1 Genotyping System version 2.0 (Abbott, Wiesbaden, Germany) were applied. To exclude sample mix-up and to confirm vertical HIV-1 transmission, phylogenetic analysis of maternal and infant sequences generated by population-based sequencing was performed using

Table 1. Oligonucleotide sequences of primers used in outer and allele-specific PCR (ASPCR).

Assay and primer name	Nucleotide sequence	Nucleotide position (HXB2)	Fragment size (bp)
Outer-PCR			
HIV-TZ FOR	5′- AAACAATGGCCATTRACAGARGA-3′+	2613–2635	
HIV-TZ REV	5′- GGATGGAGTTCATAICCCATCCA-3′−	3234–3256	644
K70R ASPCR			
TZ-K70 FOR 1	5′- GCIATAAARAARAARGACAGYACTC-3′+	2733–2757	
TZ-K70R FOR 2	5′- GCIATAAARAARAARGACAGYACTCG-3′+	2733–2758	
TZ-K70 REV	5′- CCCACATCYAGTACTGTYACTGATTT-3′−	2859–2884	152
K103N ASPCR			
TZ-K103 FOR	5′- GGCCTGAAAATCCATAYAAYACTCC-3′+	2701–2725	
TZ-K103 REV1	5′- CCCACATCYAGTACTGTYACTGATTT-3′−	2859–2884	
TZ-K103N(C) REV3	5′- CCCACATCYAGTACTGTYACTGATTGG-3′−	2858–2884	
TZ-K103N(T) REV4	5′- CCCACATCYAGTACTGTYACTGATTGA-3′−	2858–2884	184
Y181C ASPCR			
TZ-Y181/M184 FOR	5′- AAATCAGTRACAGTACTRGATGTRGG-3′+	2859–2884	
TZ-Y181 REV1	5′- ATCCTACATACAARTCATCCATRTATTGA-3′−	3092–3120	
TZ-Y181C REV3	5′- ATCCTACATACAARTCATCCATRTATTGCC-3′−	3091–3120	262
M184V ASPCR			
TZ-Y181/M184 FOR	5′- AAATCAGTRACAGTACTRGATGTRGG-3′+	2859–2884	
TZ-M184 REV1	5′- TCAGATCCTACATAYAARTCATCCA-3′−	3101–3124	
TZ-M184V REV3	5′- TCAGATCCTACATAYAARTCATCIGC-3′−	3098–3124	266
T215Y/F ASPCR			
TZ-T215 FOR	5′- CACAGGGATGGAAAGGATCACC-3′+	2998–3019	
TZ-T215 REV1	5′- CTTCTGATGYTTYTTGTCTGGIGT-3′−	3185–3205	
TZ-T215Y REV3	5′- CTGATGYTTYTTGTCTGGIGTCTA-3′−	3182–3205	
TZ-T215F REV4	5′- CTGATGYTTYTTGTCTGGIGTCAA-3′−	3182–3205	
TZ-T215F REV5	5′- CTGATGYTTYTTGTCTGGIGTTAA-3′−	3182–3205	208

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the neighbor joining method (Bioedit 7.0.9) [36]. HIV-1 subtyping of the *pol* sequence was performed using the REGA HIV-1 subtyping tool [37].

Statistical analysis

The non-parametric Mann-Whitney U test was used to assess significant differences between two independent samples whereas the Wilcoxon signed-rank test was used to analyze repeated measurements. Chi-Square test or Fisher's exact test were applied to analyze the independence of categorical variables. Testing of significant correlations between two continuous variables was done by Pearson's correlation coefficient. For descriptive analysis, median and interquartile ranges (IQR) were calculated. Two-sided tests were used and $p<0.05$ was considered statistically significant. Drug-resistant HIV-1 variants carrying the K103N (AAC) mutation and the K103N (AAT) mutation were summed to obtain the total proportion of virus carrying the K103N mutation. Statistical analysis was carried out using PASW Statistics 18 (SPSS Inc., Chicago, Illinois, USA).

Results

Sample characteristics

Of 87 women having started complex prophylaxis, 50 women fulfilled the eligibility criteria and were included in the resistance analysis, together with their seven vertically HIV-infected infants. Median baseline characteristics before start of prophylaxis were: age 28 years (IQR 26–30), HIV-1 viral load 1.25×10^4 copies/mL (IQR 4.4×10^3–4.5×10^4) and CD4 cell counts of 390 cells/mm^3 (IQR 260–492). The median maternal viral load was 2.9×10^3 copies/mL (IQR 1.4×10^3–6.8×10^3) at delivery, 1.7×10^3 copies/mL (IQR 1.3×10^3–5.8×10^3) 1–2 weeks postpartum, 1.2×10^4 copies/mL (IQR 6.3×10^3–3.7×10^4) 4–6 weeks postpartum and 2.5×10^4 copies/mL (IQR 1.2×10^4–3.7×10^4) 12–16 weeks postpartum. Compared to baseline viral load, maternal viral loads at delivery and 1–2 weeks postpartum were significantly lower (both $p<0.001$) but reached similar levels at 4–6 weeks ($p=0.45$) and at 12–16 weeks ($p=0.54$) postpartum, respectively. Women received AZT during pregnancy for a median of 53 days (IQR 39–64). Thirty-seven (74%) women delivered at Kyela District Hospital whereas 13 (26%) women delivered at home or in another health facility. Regardless of the place of delivery, all women took NVP-SD before birth. Thirty-four of 37 women who delivered at Kyela District Hospital received intrapartum AZT/3TC. Forty-one women took AZT/3TC postpartum for one week, while another five women took AZT but not 3TC postpartum. In total, 86% (43/50) of women took at least one dose of 3TC. Forty-four (88%) infants received NVP-SD after birth, including all 37 newborns born at Kyela District Hospital and 7/13 infants born at another place. Forty-five (90%) newborns took AZT postnatally; 42 of whom for one week and three for four weeks. Forty-nine of 50 infants including all HIV-infected infants were breastfed. 28%

(14/50) of the women were infected with HIV-1 subtype A1, 68% (34/50) with subtype C and two women (4%) with subtype D. None of the 50 baseline samples exhibited preexisting drug-selected mutations in the RT as determined by population sequencing.

Quantification of HIV-1 RNA by outer PCR

A standard curve was calculated from eight independent runs ($r^2 = 0.992$, standard deviation 0.004) by using defined concentrations of HIV-1 NL4.3 virus ranging from 6.5×10^1–10^7 copies/ml (details in Materials and Methods S1). The lower limit of detection for HIV-1 RNA was 650 copies/ml.

226 maternal samples (mean 4.5 samples per woman) were available, of which 211 were successfully amplified and quantified in the outer PCR, including 50/50 baseline samples, 48/50 delivery samples, 37/46 1–2 weeks samples (which displayed the lowest viral load), 47/49 4–6 weeks samples and 29/31 12–16 weeks samples. Out of the seven vertically HIV-1-infected newborns, 11/15 available samples were amplifiable in the outer PCR.

Evaluation of ASPCR assays

Accuracy, precision, sensitivity and specificity of ASPCR. Accuracy, precision and sensitivity (detection limit) of all ASPCR assays are shown in Table 2. The coefficient of variation as measurement of inter-assay precision did not exceed 47% (range 12%–47%, data not shown). The lower detection limit for evidence of minor drug-resistant HIV-1 variants was 0.99% for K70R, 0.04% for K103N (AAC), 0.01% for K103N (AAT), 0.35% for Y181C, 0.63% for M184V, 0.33% for T215Y and 0.42% for T215F (Table 2). Specificity for HIV-1 wild-type controls was 100% for all ASPCR assays.

Some maternal ASPCR results had to be excluded from analysis due to polymorphisms in primer binding sites (details in Materials and Methods S1); this affected two women for K103N analysis, one woman for Y181C analysis and six women for K70R analysis.

Detection limit for drug-resistant HIV-1 in samples with low viral load

The sensitivity of ASPCR assays for detection of drug-resistant HIV-1 correlates with the input viral load. In order to avoid false positive results, we established a threshold considering the respective viral load of any given sample (see Materials and Methods S1). The lower detection limit for drug-resistant HIV-1 variants was 0.17% for samples with 10^4 copies/ml and 0.97% for samples with 10^3 copies/ml. If the calculated proportion of drug-resistant HIV-1 fell below the calculated threshold, it was considered to be false positive and presence of HIV-1 wild type was assumed; this affected the detection of K103N and T215Y only once.

Emergence of drug-resistant HIV-1 variants in Tanzanian women

In total, 20/50 (40%) women exhibited drug-resistant virus during the observation period (Table 3), including 13/34 (38%) women infected with HIV-1 subtype C, 6/14 (43%) women with subtype A1 and 1/2 with subtype D. Genotypic mutations associated with decreased susceptibility to AZT were detected in 11/50 (22%) women (7/50 (14%) containing K70R alone and 4/50 (8%) with T215Y/F mutation) whereas 9/50 (18%) women harbored NVP-resistant virus (K103N and/or Y181C). In 4/50 (8%) women a 3TC-resistance mutation (M184V) was identified, of these 3/50 (6%) developed drug-resistant HIV-1 strains against more than one drug (Figure 1).

In 5/20 women, drug-resistant variants were already detectable at delivery and all of these women carried HIV-1 with AZT-selected resistance mutations only. In 4/20 women, resistant virus was detectable for the first time 1–2 weeks after delivery and in 11/20 women resistant variants were not present before weeks 4–6. 50% of the women with HIV-1 resistance still exhibited drug-resistant virus at week 12.

The first AZT-selected mutation emerging was the K70R, which was detectable at delivery in 5/50 women in proportions of 2%–28%. The shortest interval between the start of AZT prophylaxis and detection of the K70R mutation was 28 days (Table 3, no 3). T215Y and T215F mutations mostly emerged later and were measurable 1–6 weeks postpartum in 4/50 (8%) women in low proportions of 0.5%–3.9%. One woman displayed both AZT resistance mutations K70R and T215F in the viral genome, which were present already at delivery and persisted throughout the observation period at low frequencies (Table 3, no 5).

The total median viral load reduction from baseline to delivery was 0.6 $\log_{10}$; women with AZT-resistant virus at delivery displayed significantly lower reduction (0.1 $\log_{10}$) compared to women without AZT resistance at delivery ($p = 0.045$, Mann-Whitney U-test). Accordingly, women with AZT-resistant virus at delivery displayed significantly higher median viral load at delivery (29400 copies/ml) compared to women without AZT resistance at delivery (2680 copies/ml; $p = 0.021$, Mann-Whitney U-test). Furthermore, women exhibiting AZT-resistant virus at delivery had lower CD4 cell counts at baseline (331 cells/mm^3) versus women without AZT resistance (406 cells/mm^3); this difference marginally failed to reach statistical significance ($p = 0.077$, Mann-Whitney U-test).

Table 2. Accuracy, inter-assay variability and detection limit of ASPCR assays to detect drug-resistant HIV-1 variants calculated from 7–9 independent experiments.

Input mutant allele (%)	Measured mean mutant allele (% ± standard deviation)													
	K70R (AGA)		K103N (AAC)		K103N (AAT)		Y181C (TGT)		M184V (GTG)		T215Y (TAC)		T215F (TTC)	
100	110	±33.6	115	±48.9	102	±20.4	108	±23.7	112	±24.5	116	±40.9	115	±31.5
10.0	9.35	±2.74	10.2	±2.59	10.9	±3.94	9.28	±2.72	8.38	±1.02	9.17	±2.63	11.7	±5.40
1.00	1.11	±0.42	0.85	±0.23	1.07	±0.42	1.12	±0.39	1.11	±0.22	1.09	±0.46	1.01	±0.47
0.10	0.29	±0.08	0.12	±0.05	0.10	±0.03	0.30	±0.08	0.27	±0.03	0.12	±0.06	0.11	±0.06
0	0.19	±0.08	0.01	±0.01	0.01	±0.01	0.08	±0.06	0.23	±0.03	0.05	±0.03	0.09	±0.04
Detection limit (%)	0.99		0.04		0.01		0.35		0.63		0.33		0.42	

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Table 3. Drug-resistant HIV-1 variants in plasma samples of 20/50 women after complex antiretroviral prophylaxis as analyzed by allele-specific PCR (ASPCR).

No	Sub-type	Viral load delivery (cop/ml)	Antenatal AZT-intake (days)	Results of population sequencing and ASPCR							
				Delivery		Week 1–2		Weeks 4–6		Weeks 12–16	
				popseq	ASPCR	popseq	ASPCR	popseq	ASPCR	popseq	ASPCR
1	C	1,546	58	K70R	13% K70R		wt		-		wt
2	C	29,400	77	K70R	11% K70R	wt	0.7% M184V		wt		-
3	A1	97,450	28	K70R	14% K70R		wt	wt	5.4% K70R		-
4	A1	7,915	81	K70R	28% K70R		wt	K70R	14% K70R		wt
5	C	37,800	81	wt	2.0% K70R 0.5% T215F	K65R	0.5% T215F	wt	2.3% K70R	wt	0.7% T215F
6	A1	4,806	43		wt	wt	0.5%T215Y		wt		wt
7	A1	6,400	87		wt	wt	10% K103N	wt	0.8%Y181C		-
8	C	3,790	49		wt	wt	0.4% Y181C	wt	1.3% K103N		-
9	C	21,800	14		wt	wt	0.6% M184V	wt	3.4% K103N		wt
10	A1	3,455	95		wt		wt	wt	4.9% K70R		-
11	C	1,002	92		wt		wt	wt	2.7% K70R		-
12	C	1,079	33		wt		-	wt	0.8% T215F		wt
13	C	4,625	32		wt		wt	wt	3.9% T215Y		wt
14	A1	646	65		wt		wt	wt	2.1% K103N		-
15	C	2,150	67		wt		wt	wt	3.4% K103N		-
16	C	2,875	49		wt		wt	K103NY181CV106A	36% K103N 20% Y181C 0.6% M184V	K103N	12% K103N 4.0% K70R
17	D	1,480	48		wt		-		wt	wt	0.2% K103N
18	C	1,258	38		wt		-		wt	wt	0.4% Y181C
19	C	1,055	56		wt		wt		wt	G190A	1.5% Y181C
20	C	47,050	56		wt		wt		wt	wt	1.0% M184V

wt = wild-type HIV-1.
- = no sample/not amplifiable.
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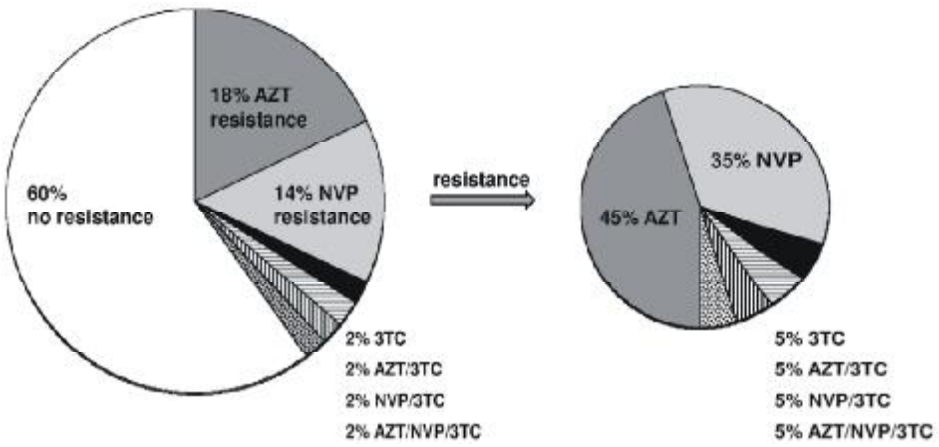

Figure 1. Distribution of drug-resistant HIV-1 variants after complex antiretroviral prophylaxis in 50 Tanzanian women.
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The median number of days of antenatal AZT intake did not differ significantly between the five women who displayed AZT resistance mutation at delivery (77 days) and the 45 women without AZT-resistance at delivery (50 days; p = 0.20, Mann-Whitney U-test). However, the frequency of AZT resistance at delivery differed significantly in women with antenatal AZT intake of at least 10 weeks (3/10 = 33%) as compared to women who took antenatal AZT for less than 10 weeks (2/40 = 5%; p = 0.048, Fisher's exact test).

NVP resistance mutations K103N and/or Y181C were detected in postpartum samples of nine (18%) women, but the proportion of resistant variants never exceeded 5% during the study period in 7/9 (78%) of these women. In 2/9 (22%) women higher proportions were detectable (Table 3, nos. 7, 16). One of these women (no. 16) did take NVP-SD and AZT/3TC during labor, but did not receive the postpartum AZT/3TC-tail to avoid NVP-resistance development. This woman exhibited dual-resistant virus against NVP and 3TC at week 4–6 and dual-resistant virus against NVP and AZT at month three. 3/9 women who had not taken AZT and/or 3TC postpatum (Table 3, nos. 16, 17, 19) developed NVP-resistance compared to 6/41 women who took the postpartal tail correctly (p = 0.33, Fisher's exact test).

The 3TC-resistance mutation M184V was detected in four women (8%) in low proportions of 0.6%–1.0% and was no longer detectable in 3/4 women at week 12–16.

In 70% (14/20) of the women who developed drug-resistant HIV-1 variants the relative proportions of resistant populations never exceeded 5% during the whole study period. The range of proportions of drug-resistant HIV-1 variants was 0.2–36% for K103N mutants, 0.4–20% for Y181C mutants, 0.6–1.0% for M184V mutants, 2.0–28% for K70R mutants, 0.5–3.9% for T215Y mutants and 0.5–0.8% for T215F mutants, respectively. In total, 34 drug-resistant variants were detected; out of these, 12 were present in proportions <1%, 12 in proportions of 1–5%, and 10 in proportions of >5%.

Altogether, complex prophylaxis resulted in the development of drug resistance in 40% of HIV-infected women. Out of these, 45% carried HIV-1 with AZT-resistance mutations, 35% showed NVP single drug-resistance, 5% 3TC single drug-resistance and 15% dual or triple drug-resistance in the viral genome (Figure 1). A longer duration of antenatal AZT intake seemed to increase the risk for selection of AZT-resistance mutations. In most women drug-resistant virus was present as minority species only.

Vertical transmission and emergence of drug-resistant HIV-1 variants in infected infants

Blood specimens collected 4–6 weeks after birth were available for 47/50 newborns; 5 were tested to be HIV-positive (no. 5, 6, 13, 21, 22; Table 4). In three additional cases, the 4–6 week sample was lacking, and an earlier sample (taken at delivery, 3 days or 2 weeks postpartum) was analyzed respectively: two of these samples were HIV-PCR positive, those infants were therefore assumend to be HIV-1 infected (no. 23, 24; Table 4). The third child was HIV-PCR negative, this infant was excluded from calculation of the transmission-rate. The overall HIV-transmission rate 4–6 weeks after birth was 14.3% (7/49 infants).

Vertical transmission was proven by phylogenetic analysis of maternal and infant HIV-1 sequences (data not shown). We did not observe a correlation between the vertical transmission risk of HIV-1 with either maternal CD4 cell count at enrolment, viral load at delivery or viral load reduction during pregnancy (p = 0.131; p = 0.388; p = 0.360, Mann-Whitney U-test) or with the presence of AZT-resistant HIV-1 variants (p = 0.546, Fisher's exact test). All children were at least exposed to maternal NVP-SD during delivery, and 44/50 (88%) infants took an additional dose of NVP postnatally. Eleven plasma samples of the seven HIV-infected infants were amplifiable in outer PCR and were available for subsequent ASPCR assays (Table 4). Three of 7 infants developed drug-resistant virus (Table 4, nos. 5, 21 and 22). Two infants (nos. 21 and 22) developed NVP-resistant HIV variants while both mothers exhibited wild-type virus only during the observation time. To one of these infants (no. 22) neither postnatal NVP nor AZT was administered, but the child developed high proportions of NVP-resistant virus at week 4–6. The third newborn (no. 5) carried resistant virus against AZT (K70R) and NVP (K103N) 4–6 weeks after birth; the mutation K70R was also detectable in the maternal delivery sample.

Results of population-based sequencing and comparison with ASPCR results

Population-based sequencing was conducted on all maternal and infant samples with drug resistance mutations as determined by ASPCR (n = 34, Table 3 and Table 4) and additionally on 27 samples without indication of drug-resistant virus in the ASPCR (data not shown).

In all samples harboring resistant virus in proportions >20% according to ASPCR assays, population-based sequencing confirmed the presence of drug-resistant virus, and the presence of mutations as identified by population-based sequencing was always detected in the ASPCR assays (Table 3). All samples without detectable drug-resistant HIV-1 or with drug-resistant variants in proportions <= 10% in the ASPCR were identified to contain HIV wild-type only by population sequencing (Table 3).

We also checked population sequences for additional AZT/3TC/NVP-selected resistance mutations like M41L, D67N, K70R, L210W, T215Y/F and K219QE for AZT, K65R for 3TC and L100I, K101P, V106A/M, V108I, Y188C/L/H and G190A for NVP. Additional mutations in the HIV-1 genome were detected in three women: One woman each harbored the V106A (together with K103N, Y181C and M184V), the K65R (together with T215F) and the G190A (together with Y181C) mutation, respectively (Table 3, nos. 5, 16, 19).

Discussion

Since 2006, WHO PMTCT guidelines recommend complex antiretroviral prophylaxis with AZT monotherapy during pregnancy, NVP-SD at labor onset, AZT/3TC during labor and for one week after delivery [14,15]. Since AZT monotherapy and usage of drugs with low genetic barriers like NVP and 3TC might facilitate the formation of drug resistance, we aimed at monitoring the emergence and persistence of key resistance mutations selected by AZT, NVP and 3TC in 50 Tanzanian women from enrolment (before start of prophylaxis) up to three months postpartum. To our knowledge, this is the first study analyzing drug-resistance including minority species in women who had taken the WHO recommended complex prophylaxis.

AZT resistance

Emergence of AZT-resistant virus after starting AZT monotherapy during pregnancy has been reported to be low with less than 3% occurrence [38,39]. Applying our highly sensitive ASPCR assays capable of detecting minority species <1%, we detected HIV-1 with AZT-resistance mutations in a much higher proportion of women (11/50 = 22%). However, population-based sequencing, detecting minor variants in proportions only above 20%, revealed AZT-resistance mutations (K70R) in HIV-1 of only 4 women (8%). Furthermore, the women in our study displayed

Table 4. Drug-resistant HIV-1 variants in plasma samples of seven children HIV-1 infected by vertical transmission as analyzed by allele-specific PCR (ASPCR).

No	Sub-type	Mother/child	Maternal CD4 count (cells/μl)	Maternal viral load (cop/ml)	Ante-natalAZT (days)	Drug intake during labor	Drug intake postnatal	Results of ASPCR			
								delivery	week 1–2	week 4–6	week 12–16
5	C	mother	344	37,800	81	NVP-SD	AZT/3TC	2.0% K70R° 0.5% T215F°	0.5% T215F°	2.3% K70R°	0.7% T215F°
		child					NVP-SD AZT	-	-	15% K70R * 3.4% K103N°	2.7% K70R°
6	A1	mother	572	4,806	43	NVP-SD AZT/3TC	AZT/3TC	wt	0.5%T215Y°	wt	wt
		child					NVP-SD AZT	n/a	-	wt	n/a
13	C	mother	678	4,625	32	NVP-SD AZT/3TC	AZT/3TC	wt	wt	3.9% T215Y°	wt
		child					NVP-SD AZT	wt	-	wt	wt
21	A1	mother	231	14,850	33	NVP-SD AZT	AZT/3TC	wt	wt	wt	-
		child					NVP-SD AZT	-	-	0.9% K103N° 2.5% Y181C°	-
22	C	mother	211	1,720	60	NVP-SD	-	wt	n/a	wt	-
		child					-	-	-	12% K103N° 12% Y181C°	-
23	C	mother	612	2,110	20	NVP-SD AZT/3TC	AZT/3TC	wt	wt	wt	wt
		child					NVP-SD AZT	n/a	wt	-	wt
24	A1	mother	200	5,385	46	NVP-SD AZT/3TC	AZT/3TC	wt	wt	wt	-
		child					NVP-SD AZT	n/a	wt #	-	-

wt = wild-type HIV-1.
n/a = not amplifiable.
- = no sample.
= sample collected at day 3.
* = also detected by population-based sequencing.
° = not detected by population-based sequencing.
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lower CD4 cell count levels (median: 390 cells/mm^3) compared to the relatively immunocompetent women in other studies (median: >500 cells/mm^3) [38,39]. Advanced disease stage and low CD4 cell counts have been shown to be associated with a higher frequency of AZT-resistance [40,41]. This is in accordance with our finding, that women carrying virus variants with AZT-selected mutations at delivery displayed a 10fold higher median viral load compared to women without AZT resistance mutation at delivery ($p = 0.021$, Mann-Whitney U-test). Furthermore, these women tended to display lower CD4 cell counts (median: 331 cells/mm^3) in comparison to women without AZT resistance mutations (median: 406 cells/mm^3; $p = 0.077$, Mann-Whitney U-test). In the most recent WHO guidelines (2010), AZT prophylaxis is recommended to start at a higher CD4 cell count level of 350 cells/mm^3 instead of 200 cells/mm^3 as in the previous 2006 guidelines. This might contribute to reduced emergence of AZT resistant HIV-1.

The shortest interval between start of AZT exposure and the emergence of AZT-selected mutation K70R was 28 days only. AZT resistance mutations were detected more frequently in HIV-1 of women who had taken AZT during pregnancy for longer than 10 weeks. In fact, in 30% of these women HIV carried AZT-resistance mutations at delivery. It is well known from other studies that the duration of AZT intake is associated with resistance development [40,42,43].

K70R was the most frequently observed AZT mutation in samples taken at delivery ($n = 5$), while T215Y and T215F mutations mostly emerged later during the observation period. In fact, the K70R mutation is considered to be an early AZT mutation and indicates the emergence of AZT-resistance followed by M41L, T215Y/F and L210W [34]. This might be due to the fact that for K70R one base substitution is sufficient (AAA/AAG to AGA/AGG) while for T215Y and F two base mutations are required (ACC to TAC = >T215Y or TTC = >T215F) [34]. 7/11 women with HIV-1 carrying AZT-selected mutants displayed the K70R mutation in proportions of 3%–28%, whereas T215Y/F-carrying virus was harbored in lower proportions of 0.5%–3.9% by four women. It is important to note that the K70R mutation affecting HIV-1 of 7/50 (14%) women confers low level resistance towards AZT, whereas T215Y and T215F mutations affecting virus of 4/50 (8%) women result in high-level resistance [34,35]. While emergence of K70R is transient, AZT-resistant mutation T215Y is reported to persist for several months up to more than one year even after AZT discontinuation [44–46].

Antenatal AZT is supposed to reduce in-utero HIV-1 transmission. So far, it is not fully understood how exactly AZT is preventing in-utero transmission. Viral load reduction by AZT in pregnancy has been shown to be modest with -0.24 $\log_{10}$ and -0.3 $\log_{10}$ by Sperling [47] and Clarke [48] and with -0.6 $\log_{10}$

in our study. Therefore, since AZT readily crosses the placenta [49] it is rather conceivable that the child is at least also protected by pre- and post-exposure prophylaxis than by the maternal viral load reduction at delivery.

Since the AZT resistance mutation T215Y was shown to persist for several month [44–46], resistant variants could be re-selected if exposed to prophylactic AZT in future pregnancies or during subsequent AZT-containing HAART if initiated within this period after AZT exposure. This is of special importance for Sub-Saharan African populations as many women give birth to more than one child; AZT mutations may accumulate over time if AZT is used during consecutive pregnancies.

Our results are conflicting with the WHO statement that "the available evidence suggests that the time-limited use of AZT monotherapy during pregnancy for prophylaxis (for approximately six months, or less) should not be associated with a significant risk of developing AZT resistance" [15]. Compared to 2006, WHO guidelines from 2010 recommend to prepone the start of antenatal AZT to week 14 instead of week 28 [14,15], corresponding to a 6-month AZT monotherapy. According to our findings, prolongation of antenatal AZT may increase the frequency of AZT-resistant virus.

NVP and 3TC resistance

NVP-selected resistance mutations that cause cross-resistance to other NNRTIs are a major concern as NNRTIs are cornerstones of first-line HAART in resource-constrained settings. According to WHO guidelines, AZT/3TC should be taken by women for seven days postpartum to counteract the long presence of subtherapeutic NVP concentrations due to NVP's long half-life. NVP resistance was detected in 18% in our study group, which is a remarkable reduction compared to up to 87% after NVP-SD intervention [10]. The efficacy of postpartum short-course AZT/3TC-tails in reducing NNRTI resistance after intrapartum NVP-SD has indeed been shown in other studies [50,51]. In our study group, 8% of women exhibited 3TC-resistant virus in very low proportions of <1% only. The M184V mutation results in complete resistance to 3TC and the presence of postpartum M184V in proportions >20% has been correlated to subsequent treatment failure using 3TC-containing HAART [52]. However, the clinical and virological relevance of 3TC-resistant virus in low proportions is not known. Moreover, M184V is known to be rapidly lost upon withdrawal of 3TC.

Multiple drug resistance

In three women, resistant virus against more than one drug emerged during the observation period. The main risk factor for resistance development in general is incomplete adherence. The most severely affected woman with respect to HIV-1 resistance development (Table 3, no. 16) did not take AZT/3TC postpartum; it seems reasonable to assume that this fostered resistance development. It could be argued that the resistance development in this woman cannot be attributed to the effect of complex prophylaxis as it was not taken correctly. However, this might as well realistically reflect the existing conditions in rural settings and the challenges to adhere to a complex drug regimen.

Minor drug resistance

In 70% (14/20) of the women with development of drug-resistant HIV-1, the resistant variants never exceeded proportions of 5%. The clinical relevance of these minority species is not fully understood and controversially discussed [17–24,53]. There is evidence that minor drug-resistant variants can re-emerge in subsequent regimens leading to failure of salvage therapy [21]. While Metzner et al. [53] reported of successful treatment despite pre-existing minor K65R, K103N and M184V-variants in German Truvada cohort, several other studies have shown that the presence of drug-resistant minor variants increased the risk for subsequent treatment failure for NNRTI- [18–24], protease inhibitor- [17,54,55] and AZT-containing treatment [56]. While a single NNRTI-resistance mutation confers high-level resistance to some NNRTIs (an association with virologic failure in efavirenz-containing regimen was found for K103N variants at frequencies of >= 0.5% by Halvas et al. [57]), resistance to PI and AZT requires an accumulation of several mutations [58]. It is not yet fully understood at which threshold minor resistant viral populations may become clinically relevant. Furthermore, the threshold might be different for each resistance mutation and also depend on the subsequent treatment regimen. More evidence-based data are necessary to determine the role of minor drug-resistant HIV-1 in the response to antiretroviral therapy.

Vertical transmission and emergence of drug-resistant HIV-1 variants in infected infants

The overall transmission rate in this study cohort of 50 mother-infant pairs 4–6 weeks after delivery was 14.3% and thus unexpectedly high. Neither a low CD4 cell count nor a high viral load at delivery in the transmitting mothers could be identified as transmission risk factors. Of 50 infants, all but one were breastfed, including all HIV-infected infants. We could not define the exact time of transmission for 4/7 infants due to lacking samples of delivery and/or of week 1–2. However, at least 3/7 children were born HIV uninfected (HIV-PCR was negative in the delivery sample). We therefore assume that postpartal transmission via breastmilk is the main reason for the high transmission rate.

Three of 7 infants developed drug-resistant HIV-1. In 2/3 newborns with NVP-resistant variants, mutations most likely emerged in the infants as both mothers exhibited wild-type HIV-1 only during the observation period. One infant, who did not take AZT and NVP postnatally (no. 22) exhibited NVP-resistant virus in high proportions at week 4–6 which was selected most likely by the maternal NVP dose. NVP rapidly crosses the placenta, resulting in high NVP concentrations in the infant's blood at birth [59,60]. Postnatal NVP dosing of the infant only slightly elevated the NVP levels in infants [61]. Therefore an infant whose mother has taken NVP-SD during labor can develop NVP-resistant virus even without postnatal ingestion of NVP.

Conclusions

Although complex antiretroviral prophylaxis decreased NVP-selected resistance compared to NVP-SD alone, HIV-1 with AZT-resistance mutations emerged in a substantial proportion of women. This may impact negatively future AZT-containing prophylaxis and HAART of the mother. In accordance with Katzenstein [62], we believe that it should be considered to substitute AZT monotherapy in pregnancy by HAART. There is growing evidence that starting HAART regardless of CD4 cell count level is highly beneficial for all HIV-infected individuals [63–66]. Additionally, HAART during pregnancy seems to be safe and advantageous for maternal and infant health [67–70] although it is important to further monitor the long-term effects of antiretroviral drugs on HIV-exposed but uninfected children [71]. In the light of the accumulating knowledge on the detrimental nature of untreated HIV-1, it seems justified to treat this infectious disease as soon as it is diagnosed instead of delaying medication until destructions of immune functions have taken place. Therefore, we advocate for HAART for *all* HIV-positive pregnant women; this equals "option B" in WHO guidelines of

2010 [15]. However, beyond that HAART should be considered lifelong and not be stopped after delivery, as discontinuation increases the risk of future treatment failure when restarting HAART [72]. This approach would minimize the risk of HIV-1 transmission and of resistance development, would allow breast-feeding and have an overall beneficial impact on HIV-1-infected mothers and their children.

Supporting Information

Materials and Methods S1
(DOC)

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Author Contributions

Analyzed the data: AH IL JZ CK AK. Contributed reagents/materials/analysis tools: CK GH. Wrote the paper: AH JS PM FD IL JZ ST CK GH AK. Designed the experiments/the study: AH IL CK GH AK. Collected data/did experiments for the study: AH JS PM FD IL JZ ST AK. Enrolled patients: JS PM FD IL JZ.

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Drug-resistant HIV-1 in cellular DNA of Tanzanian women following WHO-recommended antiretroviral prophylaxis for vertical transmission

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Introduction

Antiretroviral prophylaxis for mother-to-child transmission (PMTCT) of HIV-1 has proven efficacy in resource-limited countries. However, a major drawback is the emergence of resistant HIV-1 strains, extensively shown after nevirapine single-dose (NVP-SD) [1,2]. In the absence of selective drug pressure, resistant variants decrease to undetectable levels in plasma [3,4]. However, mutant HIV-variants integrate into the host DNA of infected cells and persist as provirus [5,6]. Resistance in HIV-DNA was still identified 15 months after NVP-SD intake in maternal peripheral blood mononuclear cells (PBMC) [7] and is assumed to persist for years in the host genome as “latent reservoir” [6].

We recently reported the emergence of minor drug-resistant variants in plasma virus of HIV-1 infected Tanzanian women having taken triple antiretroviral prophylaxis according the 2006 WHO recommendations [8]. We identified resistant variants in plasma of 40% (20/50) of women using highly sensitive allele-specific real-time PCR (ASPCR) [9]. However, little is known about archived drug-resistant variants including minorities in the DNA of PBMCs after triple PMTCT prophylaxis. Therefore, the aim of this study was to quantify resistant strains in HIV-1 DNA from PBMC samples of HIV-1 infected Tanzanian women, and to compare resistance patterns and frequencies of resistant strains with the results from corresponding plasma virus [9].

Methods

HIV-1 infected treatment-naive, pregnant Tanzanian women were recruited within an observational study at Kyela District Hospital (KDH), Mbeya Region, Tanzania. According to the national Tanzanian PMTCT guidelines 2008 [10] and WHO 2006 recommendations [8] women received triple combination regimen starting AZT exclusively in gestational week 28, NVP-SD at labor onset and AZT/3TC during labor for seven days postpartally. EDTA-blood samples, collected before initiation of prophylaxis and at 1-2, 4-6 and 12-16 weeks postpartum were processed into plasma and buffy coat/PBMC sample each. Signed informed consent was obtained from all subjects prior to enrolment. The study was approved by the National Institute for Medical Research of Tanzania, Mbeya Medical Research Ethical Committee and the ethical committee of Charite-Universitätsmedizin Berlin, Germany.

We previously described the drug-resistance mutations in plasma virus of 50 Tanzanian women [9]. The cohort for the present study consisted of women with buffy coat-samples taken before initiation of drug-prophylaxis, at 4-6 weeks and/or 12-16 weeks after delivery, as well as HIV-DNA loads of at least 2,000 copies/ml buffy coat yielding a detectable ASPCR-product.

Human genomic DNA was isolated from 200µl buffy coat using the Qiagen QIAmp DNA Blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer´s instructions. HIV-DNA copies in genomic DNA were quantified using TaqMan real-time PCR (HIV-1 LTR region). A minimum of 100 copies was used for the HIV-1 subtype A-, D- and C-specific ASPCR assays to detect key resistance mutations selected by AZT (K70R, T215Y/F), 3TC (M184V) and NVP (K103N and Y181C) in reverse transcriptase (RT) with detection limits of <1% as described previously [9]. Samples with ASPCR-detected resistance mutations were additionally sequenced by population-based sequencing of the RT amplicon (for details see supplementary material).

Results

36/50 women previously being tested for drug-resistant plasma virus [9] met the inclusion criteria for HIV-DNA resistance analysis. The median baseline characteristics at antiretroviral treatment initiation were 29 years of age (IQR 26-32), a CD4-cell count of 395 cells/ml (IQR 260-454), a plasma viral load of 21,725 copies/ml (IQR 6,816-60,950) and a median cellular HIV-1 load of 92 DNA copies/µg PBMC-DNA (IQR 53-161). Women took AZT during pregnancy for a median of 54 days (IQR 36-75), NVP-SD intake was documented for all women and intra- and/or postpartal AZT/3TC intake for 32/36

(89%) women. Viral load was reduced to a median of 1,747 copies/ml plasma (IQR 1,265-5,649) at week 1-2 and increased again to 15,123 copies/ml plasma (IQR 8,830-63938) at week 4-6 (Fisher's exact test: both $p<0.005$). 67% (24/36) women were infected with subtype C and 33% (12/36) with HIV-1 subtype A1 (Table 1). Previous ASPCR analysis of corresponding plasma samples (week 4-6 and 12-16) detected resistant virus variants in 15/36 (42%) women: in 8/36 (22%) selected by AZT (5x K70R, 3x T215Y/F); in 5/36 (14%) by NVP (K103N and/or Y181C) and in 2/36 (6%) by 3TC (M184V) (Table1) [9].

Resistance testing of PBMC-DNA by ASPCR was performed for 32 samples of week 4-6 and for 11 samples of week 12-16 with a median HIV-DNA-input of 123 copies/ASPCR (IQR 111-145). Resistance mutations in HIV-DNA were detected in 10/36 (28%) women. AZT-selected mutations were detected in DNA from 9/36 (25%) women (4x K70R, 5x T215Y/F +/- K70R), NVP-selected resistance mutations (K103N and/or Y181C) in 3/36 (8%) and 3TC-selected resistance (M184V) only in 1/36 (3%) woman. Three women carried dual-resistant HIV-DNA (2x to AZT(K70R)/NVP and 1x AZT(T215Y)/3TC) (Figure 1). Six of overall 16 resistance mutations were present in minorities ($\leq$5%; Table 1).

Resistant HIV-1 variants were detected in 17/54 (31%) plasma samples and 11/43 (27%) buffy coat samples (Fisher's exact test: $p=0.67$) and proportions of women with resistant HIV-1 variants selected by AZT, NVP and 3TC were similar in both blood compartments (Figure 2). In four women HIV-resistance mutations were detected in both blood compartments, while in 15 women HIV-resistance was found neither in plasma nor in PBMCs. In six women, resistant HIV-variants were exclusively identified in PBMCs (Table 1) and in 12 women in plasma virus only. However, proportions of drug-resistant HIV were very low ($\leq$5 %) in plasma virus population of these 12 women [9]. Six women revealed resistant HIV-1 in both blood compartments if considering as well the plasma samples taken at delivery-2 weeks postpartum. In all cases, HIV-1 mutation patterns were similar in both blood compartments, although detected in samples taken at different time points (Table 1).

Discussion

In the present study, resistance mutations were identified in HIV-1 PBMC-DNA in a high proportion (28%) of HIV-infected Tanzanien women after antiretroviral triple prophylaxis. NVP-resistance mutations were detected in HIV-DNA of 8% of women, which is a considerable lower proportion compared to 52.3% detected six weeks after exposure to NVP-SD only [11]. The efficacy of NVP-resistance reduction through AZT/3TC

administration after intake of NVP-SD was frequently shown for plasma virus [12] and seems to apply also for resistance in cell-associated HIV-DNA. In contrast, AZT-selected resistance mutations were identified in HIV-DNA in PBMCs of one quarter of the women (25%). Especially the highly resistant T215Y/F was present in a substantial number of women (14%). Additionally, the early AZT-selected mutation K70R was detected in HIV-DNA of 11% of women, which, is indicative for the emergence of AZT-resistance despite it is associated with low phenotypic resistance [13]. Although the median antenatal AZT intake of 7.5 weeks (54 days) was shorter than recommended by the National Tanzanian and WHO guidelines at that time (12 weeks) [9], AZT resistance mutations were selected in high frequencies. Prolongation of antenatal AZT intake up to 26 weeks, as advocated by the latest WHO 2010 guidelines [14], may further increase resistance development .

Furthermore, comparable proportions of AZT, 3TC and NVP resistance mutations in HIV from both blood compartments as well as similar resistance patterns in corresponding plasma and PBMC samples may reflect the ongoing process of viral replication and proviral integration into the womens´ genome. This assumption is supported by maternal plasma viral loads increasing to 10-fold higher levels (viral rebound) one month after ARV discontinuation: viral rebound in the presence of resistant variants enhances the integration of resistant genomes in the cellular reservoir. Although the exact rate of HIV-1 infected CD4+ cells returning to a resting state as memory cells (latent reservoir) is not yet clear [6], at least a part of the resistant provirus will be archived for the life span of the cell [5]. Under the selective pressure of subsequent treatments, these cells may be re-activated to release resistant virus, possibly resulting in an increased risk of treatment failure [5,6,16]. Indeed, Wind-Rotolo *et al.* [17] detected replication competent provirus carrying resistance mutations of re-activated PBMCs in 8% of women beyond six months after NVP-SD intake, and women with detectable NVP-resistance in HIV-DNA were significantly more likely to experience virologic failure at initiation of NVP-containing treatment according to Jourdain *et al.* [18].

In conclusion, since resistance mutations persist longer in cellular DNA than in plasma RNA [6,7,19], PBMCs are an useful additional source about the total dimension of resistance development after antiretroviral prophylaxis for vertical HIV-transmission. According to our findings, exclusively AZT intake in pregnancy, lasting over months, increases AZT-resistance development with a risk of integration into the host genome during viral rebound after discontinuation of the prophylactic antiretroviral regimen.

Persistence of resistance in HIV-DNA of PBMCs may negatively impact subsequent maternal AZT-containing treatment. Hence, in the context of resistance development, full antiretroviral therapy during and after pregnancy, as recommended by WHO 2010 guidelines (Option B), should be considered as a preferable PMTCT drug regimen.

Acknowledgments

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Table 1: Drug resistant HIV-variants in plasma and PBMCs detected by ASPCR

No	HIV-1 subtype	Antenatal AZT-intake (days)	Proportion of resistance mutation				
			delivery-2weeks	4-6 weeks		12-16 weeks	
			Plasma	Plasma	PBMC	Plasma	PBMC
046	C	40	wt	wt	3.7% T215F	wt	-
081	C	57	-	wt	19% K103N*	wt	-
093	A1	105	wt	wt	-	wt	10% K70R
098	C	91	wt	wt	wt	wt	7.7% T215F
130	A1	33	12% K70R*	wt	1.5% K70R	-	-
02#	C	77	11% K70R* 0.7% M184V	wt	-	-	18% K70R* 44% T215F* 6.7% M184V
11#	C	92	wt	2.7% K70R	1.2% K70R 13% K103N*	-	-
10#	A1	95	wt	4.9%K70R	5.5% K70R 1.2% T215F	-	-
16#	C	49	wt	36% K103N* 20% Y181C* 0.6% M184V	26% K103N*	4.0% K70R 12%K103N*	1.2% K70R 11% K103N*
13#	C	32	wt	3.9% T215Y	wt	wt	1.3% T215Y
05#	C	81	2.0% K70R 0.5% T215F	2.3% T215F	wt	0.7% T215F	wt
03#	A1	28	14% K70R*	5.4% K70R	wt	-	-
04#	A1	81	28% K70R*	14% K70R*	-	wt	wt
07#	A1	87	10% K103N	0.8% Y181C	wt	-	-
08#	C	49	0.4% Y181C	1.3% K103N	wt	-	-
09#	C	14	0.6% M184V	3.4% K103N	-	wt	wt
14#	A1	65	wt	2.1% K103N	wt	-	-
15#	C	67	wt	3.4% K103N	wt	-	-
12#	C	33	wt	0.8% T215F	wt	wt	-
19#	C	56	wt	wt	wt	1.5% Y181C	wt
20#	C	56	wt	wt	wt	1.0% M184V	wt

- = sample not available according inclusion criteria
wt = no resistance mutation detected
* = mutation also detected by Sanger Sequencing
# = patient numbers according Hauser et al. PLoS ONE 2012;7:e32055

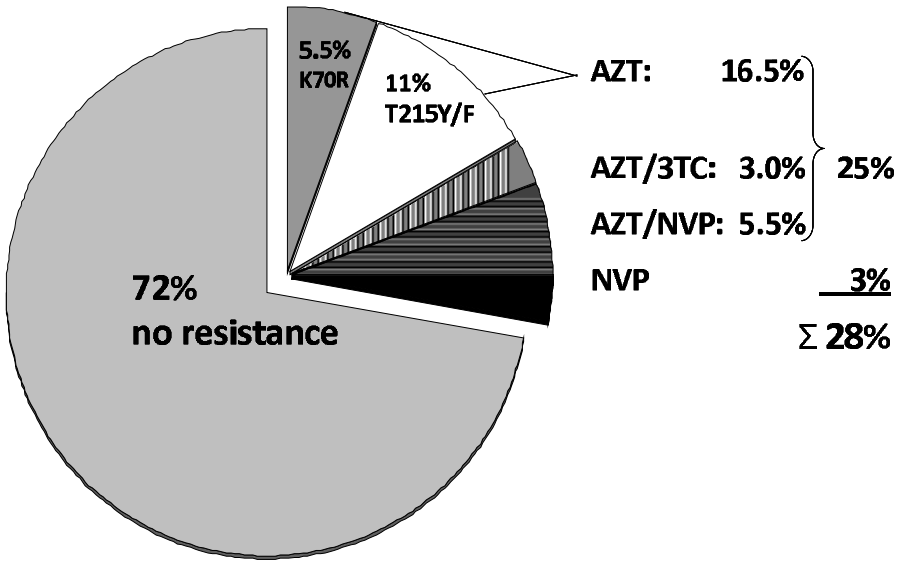

Figure 1: Drug-resistant HIV-1 DNA was detected in PBMCs of 10/36 (28%) women: AZT-selected in 9/36 (25%) women NVP-selected in 3/36 (8%) women and 3TC-selected in 1 (3%) woman; these were present as dual-resistant HIV-DNA in 3 women (2x AZT /NVP and 1x AZT /3TC

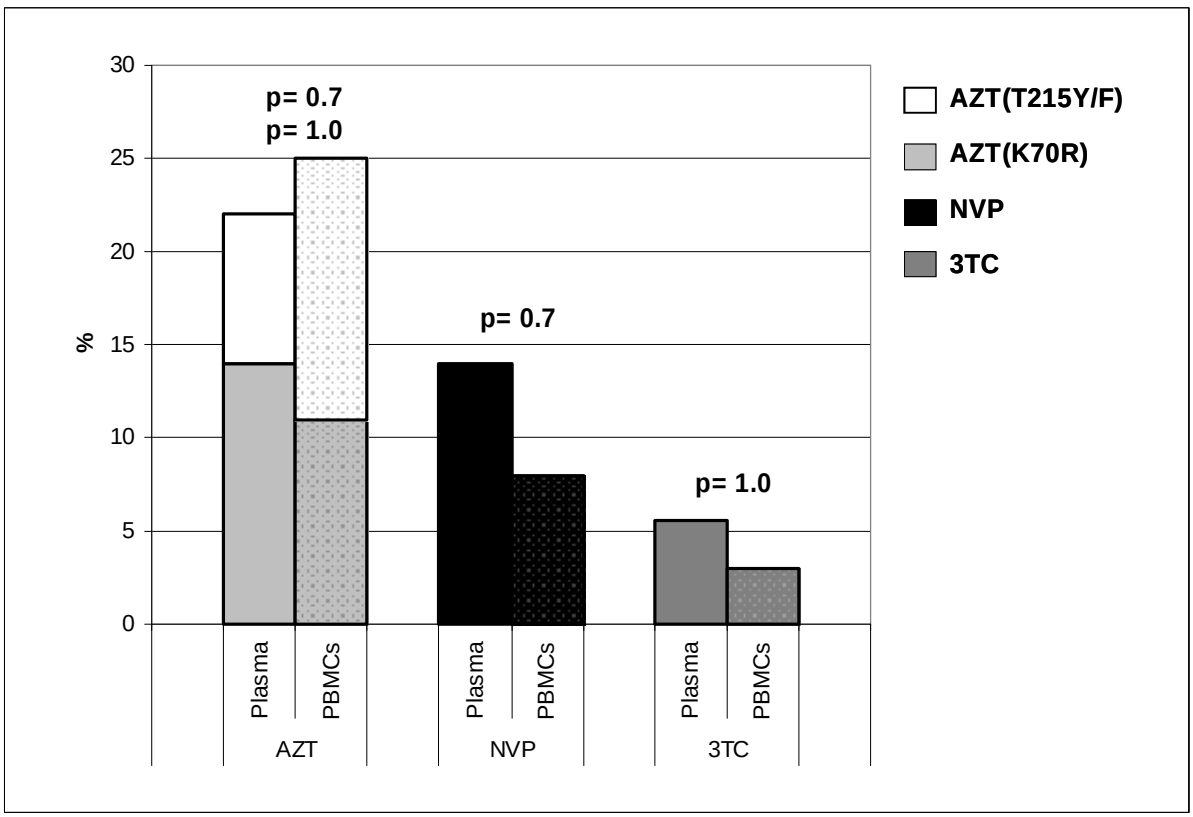

Figure 2: Proportions of women with AZT-, 3TC- or NVP-resistant HIV-1 were not significantly different in corresponding plasma and PBMC samples using allele-specific real-time PCR

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supplementary material:

Quantification of HIV proviral DNA

Proviral DNA copies were quantified in DNA extracted from buffy coat by TaqMan real-time PCR of the HIV-1 LTR genomic region using an HIV-1 DNA concentration standard: defined copies of HIV-1 plasmid DNA "pNL4-3ΔPRT5" [1] were spiked into HIV-1 negative human DNA (0.5µg/µl); two sets of DNA standards were prepared to allow accurate quantification of samples with high and low HIV-copy number: set A with 100, 75, 50, 25,10, 5, 1 copies and set B with a serial dilution ranging from 10^7 to 10^1 copies of pNL4-3ΔPRT5. TaqMan PCR was performed in the Stratagene Mx3005P Real-Time QPCR System (Agilent Technologies, Waldbronn, Germany) using subtype generic primers LTR524s: 5´-CTC AAT AAA GCT TGC CTT G-3´ (nt 524-542) and LTR625as: 5´GCG CCA CTG CTA GAG AT (nt 625-641) spanning HIV-LTR-region (nt 524-641 in HXB2 Acc No K03455) and resulting in a 118bp PCR product. The subtype-generic TaqMan probe TM-LTR3: Fam - TCT GAG GGA TCT CTA GYT ACC AGA-BHQ-1 (nt 578-601) was oriented in antisence direction. TaqMan PCR was performed with 5µl PBMC-DNA extracted from maternal buffy coat samples or DNA-standard with 1.1µM primer LTR524s and LTR625as each, 110nM probe TM-LTR3, 5.5mM Mg^{2+} in 2x SuperMix-UDG (Invitrogen) in a total volume of 20µl. PCR reaction was incubated for 5 min at 50°C, 7 min at 94°C and 45 cycles of 15 sec at 94°C and 30 sec at 55°C. The assay was linear for a DNA input ranging rom 10^1 to 10^7 copies/reaction; all samples could be successfully quantified within this range. The standard curve was generated by the serially diluted DNA concentration standards amplified in the same run. All measurements were performed in duplicate and the mean was used for subsequent analysis.

Amplification HIV-1 *pol* DNA-template for the ASPCR-assays

The amplification of HIV-1 *pol* DNA as template for the different ASPCR-assays (= "outer" PCR product) was performed by a semi-nested PCR using previously described first round primers (TZ-FOR and TZ-REV) amplifying a 644 bp *pol* fragment and second round primers (TZ-K103-FOR and TZ-REV) amplifying a 556 bp *pol* fragment comprising the codons 70, 103, 181, 184 and 215 of the RT (nt 2071-nt 3256 in HXB2; Acc No K03455) [2]. A minimum of 100 HIV-1 DNA copies per maternal sample were used as DNA input for amplification in the first-round PCR resulting in a minimal detection limit of 1%. 2µl of first-round PCR products were used for the second-round PCR. First round and second round PCR were performed in PCR conditions as previously described for the "outer" PCR [2] with two minor differences: (A) first round PCR was done in a volume of

50µl with 12.5 µl genomic DNA and (B) the cycle number in the second round PCR was reduced to 20 cycles.

The result of the pre-treatment sample was used for calculation of the individual cut-off [3]. Depending on the DNA-copy input in ASPCR, a detection limit was calculated for each sample. If the calculated proportion of drug-resistant HIV-1 fell below the samples´ threshold, it was assumed to be HIV-1 wild type.

Population-based sequencing

For population-based sequencing of the semi-nested PCR product (556 bp) the automated sequencer 3130xl Genetic Analyzer (Applied Biosystems, Darmstadt, Germany) and the HIV SEQ MIX B, D and G of the Viroseq HIV-1 Genotyping System version 2.0 (Abbott, Wiesbaden, Germany) were used. To exclude sample mix-up phylogenetic analysis of maternal sequences generated by population-based sequencing were performed using the neighbor joining method (Bioedit 7.0.9, data not shown).

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Minor drug-resistant HIV-1 variants in Tanzanian women after antiretroviral prophylaxis for vertical transmission detected by ultra-deep amplicon sequencing and allele-specific real-time PCR

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Introduction

Sanger population sequencing is routinely used for genotypic resistance testing of HIV-1 protease and reverse transcriptase (RT) [1,2]. However, only mutant variants present at levels above 20% in the viral quasispecies of the patient can be detected by this method. In the past, more sensitive methods have revealed the presence of resistant variants in the virus population at frequencies lower than 20%. These methods were based on real-time PCR using mutant-specific oligonucleotides (allele-specific real-time PCR, ASPCR) with detection limits below 1% [3-6]. However, a separate PCR-assay has to be established for each resistance position of interest, which limits the number of mutations that can be analyzed: Additional resistance-associated or compensatory mutations present in the same genome are not detected. A more recently developed "ultra-deep sequencing" (UDS) method is also able to detect and quantify viral minorities. In contrast to the ASPCR, the amplicon-based UDS provides information about all mutations in the genomic region analyzed that differ from the wild type. This method has been used for drug-resistance testing in the HIV-1 protease and RT [7-12] or to predict the HIV-1 co-receptor usage [13-15] with sensitivities of less than 1%. In a recent study, minority variants with frequencies as low as 0.05% in the viral quasispecies were detected successfully [16].

In the present study, a pre-screened panel of plasma samples with ASPCR-detected resistance mutations in HIV-1 was investigated by amplicon-based UDS using the GS FLX System (Roche 454 Life Sciences) for the presence of additional drug-resistance mutations in the viral RT. The plasma samples were collected from Tanzanian women who had received an antiretroviral regimen to prevent mother-to-child transmission (MTCT) of HIV-1. According to the WHO 2006 guidelines to reduce MTCT in resource-limited countries, the regimen consisted of antepartal zidovudine (AZT) and nevirapine (NVP) at onset of labor as a single dose (SD) and intra-and postpartum AZT/lamivudine (3TC) for 7 days. This regimen was assumed to reduce the vertical transmission rate to <5% [17] and to lower the development of drug resistance compared to the NVP-SD regimen [18-20]. However, we have previously demonstrated the presence of minor resistant HIV-variants selected by AZT/3TC/NVP in the plasma of 40% (20/50) of women within 1-16 weeks after delivery detected by ASPCR [21]. In 70% of these women, resistant virus variants were present as minor variants at frequencies below 5% of the total virus population. Based on these results the aim of the present study was to directly compare the performance of ASPCR and UDS using the same sample aliquot.

Materials and methods:

The study cohort

In a previous study, 13/50 women who had taken antiretrovial triple prophylaxis were found to carry HIV-1 variants with at least one regimen-selected resistance mutation at week 4-6 after delivery [21]. These 13 women were included in the sub-study to compare performance of ASPCR and amplicon-based UDS. The previous study revealed HIV-variants with the AZT-selected mutations K70R and/or T215Y/F in seven of 13 women, NVP-selected mutations K103N and/or Y181C in five women and dual-resistant HIV-populations (K103N/Y181C and M184V) selected by NVP and 3TC in one woman (Table 2). In two women only, HIV-1 resistance mutations were also detected by Sanger sequencing (Table 3). HIV-1 subtype C was identified in 62% (8/13) and subtype A1 in 38% (5/13) of the women (Table 1). To perform the amplicon-based UDS and the ASPCR-assays, back-up samples of the previously analyzed plasma samples taken at week 4-6 were used for RNA extraction. Viral loads were determined in an “outer-PCR” as an integral part of the ASPCR procedure as described previously [21,22].

Allele-specific real-time PCR

A subtype A, D and C specific ASPCR was etablished for seven resistance-associated key mutations selected by AZT, 3TC and NVP-regimen (K70R, T215Y/R for AZT, M184V for 3TC and K103N and Y181C for NVP) with detection limits below 1% as described in Hauser *et al.* [21,22].

Amplicon-based UDS

The principle of the amplicon-based UDS is described briefly. First, reverse transcribed PCR-amplicons of the HIV genome are generated. Sense and antisense amplicon PCR-primers consist of a) amplicon-specific sequences, b) sample-specific barcode sequences called "multiplex identifier" (MID) allowing parallel sequencing of sample mixtures [23] and c) an adaptor-sequence at the 5'-end providing the primer-binding sites in the emulsion PCR and for sequencing. Resulting PCR-amplicons are attached to capture beads (theoretically one amplicon per capture bead) during the subsequent emulsion-PCR (water-in-oil emulsion = “micro-reactor" PCR) and clonaly amplified, resulting theoretically in 10^7 clonal DNA copies of a single amplicon per bead. Beads are isolated from each other in a "picotiter plate" (PTP) and clonal amplicons are sequenced in parallel by pyrosequencing.

Each bead provides a single sequence (read). The total number of reads obtained per genome position is termed "coverage". The number of mutant reads within the total number of reads provides the relative proportion (%) of mutant.

In the present study the 13 back-up plasma samples were analyzed within the Roche 454 RTPV3 Alpha site study researcher run omitting the envelope-genome region. Subtype-generic barcoded primers to amplify protease and RT and most of the reagents were obtained from Roche 454 as part of the RTPV3 Alpha site study. GS FLX Titanum Chemistry was used to increase the read length to 400-500 bases. A 500µl aliquot of each back-up sample was availabe and viral RNA was extracted according to manufacturer's instruction (Viral RNA-Extraction Kit, Qiagen, Hilden; Germany). Following the Roche HIV RTPV3-Alpha Site Study protocol, 13µl of RNA (130µl plasma equivalent) were reverse transcribed using the “4R-” and “5R”-primers, resulting in two overlapping cDNA-fragments of HIV's protease and RT coding region (nt 2200-3400 of HXB2, Acc No K03455). 3µl of each of the two cDNA-fragments were used in subsequent PCRs with primers provided in ready-to-use microtiter plates. Six overlapping amplicons, "RTP1" to "RTP6", were generated by PCR spanning protease codons 10-99 and RT-codons 1-251. After amplification, PCR products at concentrations below 5ng/µl (Quant-iT^{TM} PicoGreen$^{®}$dsDNA Reagent and Kit, Invitrogen, Darmstadt, Germany) were further analyzed (Agilent 2100 Bioanalyzer, Agilent Technologies, Böblingen, Germany). PCR products with a molar ratio of primer-dimer to amplicon above 3:1 were excluded from UDS. Subsequently, amplicons RTP1-6 from each sample were pooled in equimolar proportions. Missing RTPs were compensated with one or two overlapping amplicons since all regions were covered by more than one amplicon. Clonal amplification on beads (emulsion PCR), bead isolation (breaking) and sequencing was performed according to manufacturer’s protocol for the GS FLX System (Roche 454 Life Sciences). Resulting sequences were matched to the entire protease-RT-sequence of HXB2 using the “Amplicon Variant Analyzer”-software (AVA). Variants differing from the wild type at defined resistance positions were identified and their relative proportion to the total number of sequences was calculated. Only AZT, 3TC and NVP-selected resistance-associated mutations were considered in this analysis.

For generation of amplicons, an initial HIV RNA-input of 2,000 copies per 10µl RNA eluate is recommended [24] equivalent to a viral load of 20,000 copies/ml of the back-up samples. A median viral load of 21,000 copies/ml (IQR 4,300-58,500) was determined for the 13 maternal back-up samples. To permit valid quantification of minorities down to 1%,

a coverage of at least 50 mutant reads within 5000 total reads generated by primers of both orientations is recommended [25]. In this study, the validity criteria were modified to allow the sensitivity UDS and ASPCR to be compared: a minor variant was considered to be valid if found to be present in at least 10 of 800 or more total reads. For each RT-resistance mutation detected by ASPCR the corresponding number of mutant reads in UDS at the same position was considered even if present in less than 10 reads (Table 2).

Results

By amplicon-based UDS a median of 4 (IQR 1.5-5, totally 33) regimen-selected resistance variants ($\geq$ 10 mutant reads) were detected in 9/13 women (Table 1). Four of 13 women carried HIV-1 variants with NVP-resistance mutations and one with 3TC-resistant virus, while AZT/NVP dual-resistant virus populations were detected in 4/13 women. Resistant variants with AZT-selected mutations were identified at frequencies in the range of 0.3% to 5.4%, NVP-resistant variants at 0.3% to 42% and of 3TC-resistant variant at 1.1%. In 5/9 women, only minor resistant variants were detected, at frequencies below 5% of the total virus population.

A total of 53/78 amplicons were amplified (mean: 4 per sample; 13/13 RTP1, 9/13 RTP2, 9/13 RTP3, 4/13 RTP4, 10/13 RTP5 and 8/13 RTP6, Table 1). Amplification failed for 24/78 RTPs but 20 of these could be compensated by other RTPs. Successfull generation of amplicons significantly correlated with viral loads above 20,000 cop/ml (category 1: <20,000 cop/ml; category 2: >= 20,000 cop/ml; Fisher's exact test: p = 0.0141). Since 28/48 RTPs were amplified from subtype C-isolates and 12/30 from subtype A1-isolates, amplification success did not depend on the HIV-1 subtype (Fisher's exact test: p = 0.1626). The RTPs could be sequenced with 144-5582 reads per sample. Coverage of the entire RT-genomic region (AS 1-251 of RT) was achieved in 10/13 samples, with the 3'RT region downstream of the AS position 90 or 179 missing from the other three. No resistant variants were detected in these 3 samples (No. 4, 9, 14; Table 1) but their presence cannot be excluded for missing regions.

By ASPCR, HIV-1 resistance mutations were detected in ten of the 13 back-up samples compared to nine by amplicon-based UDS. The ASPCR-detected resistance mutations in 10 samples (11 mutations) could also be detected in 6 samples (7 mutations) by UDS. The minor variants were identified at very similar frequencies by both methods (Table 2). Compared to ASPCR, an additional 22 resistance mutations in nine samples were detected

by UDS. The presence of the key mutations (K70R, K103N, Y181C, M184V, T215Y/F) detected by UDS (n=9) was confirmed by ASPCR in all but one case: Y181C identified by UDS with 0.3% was not detected by ASPCR while another Y181C mutation quantified by UDS with 0.4%, was borderline by ASPCR at a frequency just below the ASPCR detection limit of <0.35% [21] (Table 2). Both methods missed previously identified ASPCR mutations in 3 samples, although in 2 of these samples additional resistance mutations (K65R, F227L) were detected by UDS.

Altogether, for 9/13 samples ASPCR results were reproduced by UDS. For the other four samples the ASPCR results could not be confirmed by UDS (Table 2). Three of the samples failing by UDS had low viral loads compared to the others (3,400-11,000 copies/ml versus 15,000-650,000 copies/ml) that also resulted in a very low coverage at the respective position in two samples (144 and 801 reads, Tabelle 2). The discrepancy in the fourth sample concerned RT position 103 where a K103R-mutation was identified by UDS and K103N by ASPCR (Table 2).

In summery, based on the UDS results, virus populations with NVP-resistance were identified in 62% (8/13), 3TC-resistance in 8% (1/13) and AZT-resistance mutations in 31% (4/13) of women. Dual-resistant (AZT/NVP) virus populations were present in 31% (4/13) of these samples. Based on ASPCR results, NVP-resistant virus variants were identified in 31% (4/13), 3TC-resistance in 8% (1/13) and AZT-resistance mutations in 38% (5/13) of women. No dual resistant virus was detected by ASPCR (Table 3). Resistant virus was present at proportions frequencies below 5% in 5/9 mothers by UDS and in 8/10 women by ASPCR.

Discussion:

Samples of 13 Tanzanian women pre-screened in a previous study [21] by ASPCR for HIV-1 genomes carrying AZT, 3TC and NVP-selected resistance mutations were investigated by UDS for additional antiretroviral regimen-selected resistance mutations in the HIV RT-region. The amplicon-based UDS and a sensitive ASPCR were performed in parallel using the same RNA preparation. The results obtained using the two methods were compared.

Amplicon-based UDS

In the amplicon-based UDS (Roche 454 RTPV3 Alpha Site Study researcher run) a median of 4 (IQR: 1.5-5) AZT, 3TC and/or NVP-selected resistance mutations was detected in 9 of the 13 investigated back-up samples collected from mothers 4-6 weeks after giving birth. Although the presence of the resistance mutations previously identified by Hauser *et al.* using ASPCR [21] was used as an inclusion criterion for this sub-study, in 4 of the 13 samples no resistant HIV variant could be identified by UDS. There are a number of potential reasons for this as discussed below.

The generation of amplicons is an essential and critical step in the UDS-workflow. In this study only two-thirds of the amplicons were amplified successfully. Statistical analysis revealed a significant correlation between viral load and amplicon generation of RTP1-6. Samples with viral loads higher than 20,000 copies/ml were amplified more efficiently than samples with viral loads below 20,000 copies/ml. In fact, all four samples with no detectable resistance mutations by UDS had low viral loads (≤ 11.000 copies/ml), the coverage was predominantly low and several amplicons were not generated. As a consequence, for three of the four samples the RT sequence was shorter (RT codon 1-90 or 1-179 instead of codon 1-252) and no sequence was obtained in the 3'-region of RT. This lack could be a result of a lower "5R" primer-efficiency in cDNA-synthesis (affecting amplicons RTP4-6). Additionally, the less efficient amplification of RTP-4 (4/13) was probably the consequence of the larger fragment size as compared to the other amplicons. The primer design can be considered as generic for HIV-1 subtypes A and C since they were amplified with the same efficiency. The consequence of the larger RTP-4 fragment size as well as the correlation between viral load and amplicon generation has also been discussed by John *et al.* [26] in the Roche 454 HIV RTPV3 Alpha Site Study study run. According to our data, a minimum input of 20,000 copies/ml should be considered to be an assay cut-off for UDS to guarantee the amplification of all 6 amplicons, confirming Roche's recommendation of using at least 2,000 copies per 10µl RNA eluate [24].

Reliable detection of minorities of 1% and below should be based on a coverage of at least 50/5000 sequence reads at the position of interest [25]. In the present study this coverage was not achieved for any position. Therefore, a lower cut off based on a lower number of mutant reads was defined to allow a comparison between ASPCR and UDS. Using these criteria, frequencies of mutant HIV-1 down to 0.3% could be quantified. However, due to the innate error rate of reaction enzymes [27] and the inherent limitations at homopolymeric sequences in HIV-1 in the pyrosequencing biochemistry, a sensitivity below 1% has to be

considered critical [8,10]. The cut off used in this study would be too low for patient resistance testing and therapy advice. Additional studies are therefore needed to validate the experimental detection limit for minorities of HIV-1 variants.

Comparison of amplicon-based UDS and ASPCR results

The number of regimen-selected resistance mutations detected by UDS was three times higher than those detected by ASPCR (33 versus 11). Seven of the 11 ASPCR-detected resistance mutations were detected at similar frequencies by UDS while the remaining four were not detected (Table 2). As discussed above, three resistant variants detected exclusively by ASPCR were from plasma samples with low viral loads. A viral load below 20,000 copies/ml was concluded to be the main reason for a less effective PCR-amplification and a low "coverage" in the earlier UDS discussion. In contrast, the ASPCR is designed as a "nested" PCR with a more sensitive detection limit of 650 copies/ml [21] and is therefore more sensitive in amplicon generation than UDS. Accordingly, in two of the three low viral load samples the same resistance mutations were detected in the previous study by ASPCR and Sanger sequencing but not by UDS [21]. These two resistance mutations were therefore assumed to truly exist in the viral genome. The third mutation was present as a minor variant of 1% in the back-up sample, a proportion that is far below the detection limit of Sanger sequencing and near the experimental ASPCR-detection limit of 0.97% for samples with viral loads of $1x10^3$ copies/ml [21]. Discrepant ASPCR results between the previous study and the back-up sample can be explained in all cases by the fact that the proportion of resistant variant was near the detection limit (samples no. 5, 9, 12, 16). At concentrations close to the detection limit, Poisson distribution can result in different results being obtained with independent aliquots drawn from the same sample. The fourth discrepant result was a K103N mutation detected by ASPCR whereas a K103R substitution was identified by UDS. While the K103R mutant was encoded in the sequence “AGA”, the K103N mutant is based on the “AAC” codon (wild type: “AAA”). Since the selective K103N-primer for the ASPCR-assay is a reverse primer with a “C”-base at the 3´-terminal [21] there is no obvious explanation why the AGA codon sequence was primed by the K103N-selective ASPCR-primer. However, as another UDS-detected K103R-mutation (AGA-codon) (no.13) was not found by ASPCR, mispriming by the K103N-specific ASPCR-primer seems unlikely. Indeed, ASPCR may be prone to errors as a result of polymorphisms in the primer binding sites that limit the success of the assay [21,22].

Despite the small number of patient samples analyzed in this comparative study some conclusions can be drawn about the general benefits or limitations of the two methods. By ASPCR, only a third of the UDS-detected resistance mutations were identified, revealing the limitation of ASPCR in its restriction to a limited number of mutations. Analyzing an amplicon by UDS provides more detailed information of resistance mutations in the complete genome fragment compared to the ASPCR. While the presence of virus with AZT- and 3TC-selected resistance mutations in women was detected in similar proportions by UDS and ASPCR (31% versus 38% and 8% versus 8%, respectively), the presence of NVP-selected resistance mutations was underestimated by ASPCR (31%) compared to UDS (62%) (Fisher's exact test p=0.24). UDS detected additional NVP-resistance mutations at other positions in the RT (some even in proportions of over 5%). Consequently, UDS revealed: a) more resistance mutations at proportions higher than 5% (4/9 mothers by UDS and 2/10 mothers by ASPCR) and b) the presence of dual-resistant HIV-populations in four of 13 women (31%) that were not identified by ASPCR (Fisher's exact test p= 0,10). On the other hand, UDS is limited by the less sensitive PCR: a minimum input of 20,000 copies/ml has to be available for the generation of amplicons and to obtain sufficient coverage. As a result, a greater number of key mutations were detected by ASPCR than by UDS in this study.

Both methods facilitate the detection of resistant virus populations at frequencies down to 1%, but the clinical relevance of these minor variants is not yet clear. However, sensitive methods are essential to assess the prevalence of minor resistant variants and their impact on treatment outcome. Methodological advances in UDS-technology will definitely offer the ability to routinely monitor the presence of minor variants in the HIV quasispecies.

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Table 1: Detection of minor resistant HIV-variants (AZT, 3TC und NVP) by UDS

Pat No.	Sub type	Viral load (cop./ml)	Evaluated region AS pos in RT	Reads in evaluated region	Total reads /mut	Reads /mut	mut%	Resistance -mutation	Drug-resistance
3	A1	650,000	1-252	1076-3579	3605 3106 1076 1076	12 63 14 22	0.3 2.0 1.3 2.0	M41L K70R K101E V108I	AZT AZT NVP NVP
4	A1	11,000	1-090	801-3806	-	-	-	-	-
5	C	43,000	1-252	1074-3849	1665	18	1.1	K65R	3TC
7	A1	4,500	1-252	658-4994	-	-	-	-	-
8	C	74,000	1-252	1731-3868	1787 1787 1781 1781 1905 2731	108 134 12 11 10 10	6.0 7.5 0.7 0.6 0.5 0.4	K101E K103N V106A V106M D67G Y181C	NVP NVP NVP NVP AZT NVP
9	C	1,400	1-179	1715-2342	-	-	-	-	-
10	A1	15,000	1-252	1506-5582	1507 1507 1506 1506	53 81 20 59	3.5 5.4 1.3 3.9	D67G K70R L100I V108I	AZT AZT NVP NVP
11	C	4,100	1-252	1421-2841	1935 3285 3268	20 11 24	1.0 0.3 0.7	K70R Y181C F227L	AZT NVP NVP
12	C	37,000	1-252	1144-2724	1602	15	0.9	F227L	NVP
13	C	250,000	1-252	1870-4030	1489 1462	132 57	8.9 3.9	K103R F227L	NVP NVP
14	A1	3,400	1-179	144	-	-	-	-	-
15	C	21,000	1-252	1203-4994	1203 1202 1202 3769 3769 3772 3772	43 16 43 12 57 40 13	3.6 1.3 3.6 0.3 1.5 1.1 0.3	K101E K103R V106M Y188C G190A P225H F227L	NVP NVP NVP NVP NVP NVP NVP
16	C	21,000	1-252	845-4012	845 845 845 3151 3150	355 69 83 358 49	42.0 8.2 9.8 11.3 1.6	K103N K103T V106A Y181C G190A	NVP NVP NVP NVP NVP

- = no detection; AZT = Zidovudine; 3TC = Lamivudine; NVP = Nevirapine

Table 2: Detection of minor resistant HIV-variants by UDS, allele-specific PCR and Sanger-sequencing

Pat No. (#)	Results of previous analysis (#)			Results of analysis of reserve plasma sample					
	Pop seq	ASPCR mutants (%)		Virus load (cop./ml)	ASPCR mutants	(%)	UDS mutants	(%)	Mutant reads
3		K70R	5.4	650,000	M41L K70R K101E V108I	na 2.1 na na	M41L K70R K101E V108I	0.3 2.0 1.3 2.0	12 63 14 22
4	K70R	K70R	14	11,000	K70R	12	K70R	-	0
5		K70R	2.3	43,000	K65R K70R	na -	K65R K70R	1.1 -	18 0
7		Y181C	0.8	4,500	Y181C M184V	- 1.0	Y181C M184V	- -	0 0
8		K103N	1.3	74,000	K101E K103N V106A V106M D67G *Y181C	na 3.4 na na na 0.3	K101E K103N V106A V106M D67G Y181C	6.0 7.5 0.7 0.6 0.5 0.4	108 134 12 11 10 10
9		K103N	3.4	1,400	K103N	-	K103N	ns	0
10		K70R	4.9	15,000	D67G K70R L100I V108I	na 1.8 na na	D67G K70R L100I V108I	3.5 5.4 1.3 3.9	53 81 20 59
11		K70R	2.7	4,100	K70R Y181C F227L	2.2 - na	K70R Y181C F227L	1.0 0.3 0.7	20 11 24
12		T215F	0.8	37,000	T215F F227L	- na	T215F F227L	0.0 0.9	1* 15
13		T215Y	3.9	250,000	K103R T215Y F227L	na 0.4 na	K103R T215Y F227L	8.9 0.4 3.9	132 6* 57
14		K103N	2.1	3,400	K103N	2.0	K103N	ns	0
15		K103N	- - 3.4 - - - - -	21,000	K101E K103R K103N V106M Y188C G190A P225H F227L	na na 4.8 na na na na na	K101E K103R K103N V106M Y188C G190A P225H F227L	3.6 1.3 - 3.6 0.3 1.5 1.1 0.3	43 16 0 43 12 57 40 13
16	K103N V106A Y181C	K103N Y181C M184V	36 20 0.6	21,000	K103N K103T V106A Y181C M184V G190A	33 na na 5.8 - na	K103N K103T V106A Y181C M184V G190A	42.0 8.2 9.8 11.3 - 1.6	355 69 83 358 0 49

= according to Hauser et al. 2012; - = no diction; na = not analysed; ns = no sequence generated (coverage=0);
* = below assay cut off

Table 3: Resulting prognosis for drug-resistance

Pat No.	Pop seq	ASPCR	UDS
3	-	AZT	AZT NVP
4	AZT	AZT	-
5	-	-	3TC
7	-	3TC	-
8	-	NVP	NVP AZT
9	-	-	-
10	-	AZT	AZT NVP
11	-	AZT	NVP AZT
12	-	-	NVP
13	-	AZT	NVP
14	-	NVP	-
15	-	NVP	NVP
16	NVP	NVP	NVP

- = no detection
AZT = Zidovudine
3TC = Lamivudine
NVP = Nevirapine

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