Aus dem Institut für Tropenmedizin und Internationale Gesundheit
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DISSERTATION

Nebenwirkungen einer antiretroviralen
Kombinationsprophylaxe zur Verhinderung der
Mutter-Kind-Übertragung von HIV in Tansania

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Einleitung

Die Leitlinien der Weltgesundheitsorganisation (WHO) von 2006 für die Prävention der Mutter-Kind-Übertragung des Humanen Immundefizienz-Virus Typ 1 (HIV-1) für ressourcenarme Regionen empfehlen eine komplexe antiretrovirale Prophylaxe. Diese beinhaltet die Einnahme von Zidovudin (AZT) während der Schwangerschaft, eine Einmaldosis Nevirapin (NVP) zur Geburt und AZT sowie Lamivudin (3TC) ab Beginn der Wehen bis Ende der ersten postnatalen Woche. Untersucht wurden hämatologische Veränderungen, Depletion und Mutationen mitochondrialer DNA (mtDNA) sowie HIV-Resistenzentwicklung unter der Prophylaxe bei Frauen in einer ländlichen Gegend in Tansania.

Methoden

Eine Kohorte von HIV-positiven schwangeren Frauen mit AZT-Einnahme (Gruppe 1) oder ohne (Gruppe 2) wurde im Kyela District Hospital, Tansania etabliert. Blutparameter der Studienteilnehmerinnen und ihrer Neugeborenen wurden während der Schwangerschaft, der Geburt, vier bis sechs und zwölf Wochen nach der Geburt ausgewertet. Mitochondriale DNA in Plazenta und Nabelschnur wurden mittels Echtzeit-PCR quantifiziert und auf mitochondriale Deletion dmtDNA4977 hin untersucht. Allel-spezifische Echtzeit-PCR-Messung spezifisch für HIV-1-Subtypen A, C und D wurden eingesetzt, um Resistenzmutationen von AZT (K70R/T215Y/T215F), NVP (K103N/Y181C) und 3TC (M184V) bei Nachweisgrenzen von bis zu 1% zu identifizieren.

Ergebnisse

Es zeigten sich statistisch signifikante Veränderungen im Blutbild bei Frauen unter AZT-Einnahme im Verlauf der Schwangerschaft. Zur Geburt war die mediane Erythrozytenzahl signifikant niedriger und die mediane Thrombozytenzahl signifikant höher bei Frauen der Gruppe 1 im Vergleich zu Gruppe 2. Bei der Geburt zeigten Säuglinge aus Gruppe 1 zusätzlich niedrigere mediane Hämoglobinwerte und Granulozytenzahlen. Die mediane mtDNA-Konzentration in Plazenta- und Nabelschnurgewebe war signifikant höher bei AZT-exponierten Frauen und Säuglingen gegenüber nichtexponierten. Die dmtDNA4977 wurde in Plazentaproben von jeweils einer Frau pro Gruppe und in Nabelschnurgewebeproben von drei Kindern in Gruppe 2, aber in keiner von Gruppe 1 identifiziert. HIV-1-Resistenzmutationen wurden bei 20/50 Frauen erfasst, darunter 70% Minoritätenspezies. Resistenz gegenüber AZT wurden in 11/50 (22%), NVP in 9/50 (18%) und 3TC im HIV bei 4/50 Frauen (8%) gefunden. Drei Frauen zeigten Resistenzen gegen mehr als ein Medikament. Drei von sieben vertikal infizierten Säuglingen zeigten resistente Viren.

Schlussfolgerung

AZT während der Schwangerschaft sowie nach der Geburt führte zu signifikanten, jedoch überwiegend milden und transienten hämatologischen Veränderungen bei Frauen und ihren Säuglingen. Pränatale AZT-Einnahme führt nicht zu einer Erhöhung des Risikos für die Entstehung von dmtDNA4977. Unter der Kombinationsprophylaxe kam es zu weniger NVP-Resistenzmutationen im Vergleich zur Prophylaxe mit einer Einmaldosis NVP, jedoch wurden AZT-resistente HIV bei einem erheblichen Anteil der Frauen nachgewiesen.

Abstract

Introduction

WHO-guidelines from 2006 for prevention of mother-to-child transmission of HIV-1 in resource-limited settings recommend complex antiretroviral prophylaxis comprising antenatal zidovudine (AZT), nevirapine single-dose (NVP-SD) at labor onset and AZT/lamivudine (3TC) during labor and one week postpartum. We assessed drug toxicities as haematological alterations and depletion and mutation of mtDNA and analysed the emergence of minor drug-resistant HIV-1 variants in Tanzanian women following complex prophylaxis in a rural area in Tanzania.

Methods

A cohort of HIV-positive pregnant women either with AZT intake (group 1) or without AZT intake (group 2) was established at Kyela District Hospital, Tanzania. Complete blood counts of mothers and newborns were evaluated during pregnancy, birth, weeks four to six and twelve postpartum. Mitochondrial DNA levels in placentas and umbilical cords were quantified using real-time PCR. We checked for the mitochondrial deletion mtDNA4977. Allele-specific real-time PCR assays specific for HIV-1 subtypes A, C and D were developed to quantify key resistance mutations of AZT (K70R/T215Y/T215F), NVP (K103N/Y181C) and 3TC (M184V) at detection limits of 1%.

Results

We found statistically significant alterations in blood count during AZT intake in pregnancy. At delivery, the median red blood cell count was significantly lower and the median platelet count was significantly higher in women of group 1 compared to group 2. At birth, infants from group 1 showed a lower median hemoglobin level and granulocyte count. The median mtDNA levels in placentas and umbilical cords of women and infants exposed to AZT were significantly higher than in AZT-unexposed women and infants. The dmtDNA4977 was found in placentas of one woman of each group and in 3 umbilical cords of AZT-unexposed infants but not in umbilical cords of AZT-exposed infants. HIV-1 resistance mutations were detected in 20/50 women, of which 70% displayed minority species. Variants of HIV-1 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. 3/7 vertically infected infants exhibited drug-resistant virus.

Conclusion

AZT exposure during pregnancy as well as after birth resulted in significant hematological alterations for women and their newborns, although these changes were mostly mild and transient in nature. Antenatal AZT intake did not increase the risk for the common mitochondrial deletion dmtDNA4977. 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.

Einleitung

Das Risiko einer Transmission des humanen Immunodefizienz-Virus (HIV) von der Mutter zum Kind während der Schwangerschaft, zum Zeitpunkt der Geburt oder in der Stillzeit (vertikale Transmission) kann durch antiretrovirale Medikamente verringert werden. Seit 2006 wird von der Weltgesundheitsorganisation (WHO) für ressourcenschwache Regionen eine komplexe antiretrovirale Prophylaxe empfohlen, welche sich aus der Einnahme von Zidovudin (AZT) während der Schwangerschaft, einer Einmaldosis Nevirapin (single dose Nevirapin, sdNVP) zur Geburt und von AZT und Lamivudin (3TC) ab Beginn der Wehen bis zum Ende der ersten postnatalen Woche, zusammensetzt[1]. Während diese Kombinationsprophylaxe nachweislich zu einer Senkung der Transmissionsrate führt, beinhaltet sie jedoch ein Risiko für Nebenwirkungen durch Interaktionen mit humaner DNA. Zidovudin (AZT) und Lamivudin (3TC) sind Standardmedikamente aus der Gruppe der Nukleosidischen Reverse Transkriptase Inhibitoren (Nucleoside Reverse Transcriptase Inhibitors, NRTIs). Eine unerwünschte Arzneimittelnebenwirkung dieser Gruppe, insbesondere von AZT, ist die Hämatotoxizität[2]. Zudem kann AZT die Plazentaschranke überwinden und somit auf die myeloischen Zelllinien des Feten wirken. Dadurch kann es zu einer Abnahme von Hämoglobin und Granulozytenzahl führen[3]. Weiterhin interagieren die NRTIs mit der Gamma-Polymerase, einem humanen Enzym, welches für die Replikation der mitochondrialen DNA (mtDNA) verantwortlich ist. Im Zuge der Hemmung des Enzyms, aber auch durch andere Mechanismen wie oxidativen Stress kommt es zu einer Depletion der mtDNA[4,5].

Einen wichtigen Kombinationspartner in der antiretroviralen Therapie stellt das Medikament NVP aus der Gruppe der Nichtnukleosidischen Reverse Transkriptase Inhibitoren (NNRTIs) dar. Eine alleinige Gabe von sdNVP zur Transmissionsprophylaxe führt zu einer geringeren Senkung des Transmissionsrisikos und zusätzlich zu häufigem Auftreten von NVP-Resistenzmutationen als eine Kombinationsprophylaxe[6,7].

In der vorliegenden Studie wurden Nebenwirkungen der seit 2006 von der WHO empfohlenen Kombinationsprophylaxe auf Mutter und Kind in einem ressourcenarmen, ländlichen Gebiet in Tansania untersucht. Der Fokus lag dabei auf den Blutbildveränderungen, der mitochondrialen Toxizität und der Resistenzentwicklung.

Methodik

Die Studie wurde durch das Tanzanian National Institute of Medical Research, das Mbeya Region Ethical Committee und die Ethikkommission der Charité genehmigt. Die schriftliche Einverständniserklärung der Studienteilnehmerinnen nach ausführlicher Aufklärung liegt vor, alle Daten wurden streng vertraulich und anonymisiert behandelt.

Überblick

Bei der vorliegenden Studie handelt es sich um eine prospektive Verlaufsbeobachtung, die im Zeitraum vom September 2008 bis September 2009 in der südöstlich in Tansania gelegenen Region Mbeya im Kyela District Hospital durchgeführt wurde. Ziel der Studie war es, die Einführung und Umsetzung der auf antiretroviralen Medikamenten beruhenden Prophylaxe zur Verhinderung vertikaler HIV-Transmission in einem ländlichen, strukturschwachen Gebiet in Tansania zu begleiten und auf Wirkung und Nebenwirkungen untersuchen.

Standort

Mit einer HIV-Prävalenz von 9% ist Mbeya Region die Region in Tansania mit der höchsten HIV-Prävalenz[8]. Das Kyela District Hospital ist ein ländliches Krankenhaus, in dem seit 2001 Strukturen zur Prävention der vertikalen HIV-Transmission (Prevention of Mother-to-Child Transmission, PMTCT) etabliert sind. Den Empfehlungen der WHO von 2006 folgend, wurde im März 2008 im Kyela District Hospital die Prophylaxe mit sdNVP durch die oben beschriebene antiretrovirale Kombinationsprophylaxe ersetzt.

Schwangerschaftsvorsorge und PMTCT

Während des Studienzeitraums nahmen monatlich durchschnittlich 130 schwangere Frauen erstmalig eine Vorsorgeuntersuchung im Kyela Distict Hospital in Anspruch. Innerhalb dieser fanden routinemäßig Aufklärung und Beratung sowie das Testen auf eine eventuell vorliegende HIV-Infektion statt. Im Falle einer erstmalig diagnostizierten bzw. bereits bestehenden HIV-Infektion wurden CD4-Zellzahl, Transaminasen und Blutbild bestimmt. Schwangere Frauen mit einer HIV-Infektion erhielten die Empfehlung, eine Transmissionsprophylaxe durchzuführen, wenn keine Indikation für eine hochaktive antiretrovirale Therapie (HAART) vorlag. Somit waren die Kriterien für eine Transmissionsprophylaxe eine CD4-Zellzahl > 200 Zellen/µl bei

klinischem Stadium 1 und 2 nach WHO, bzw. einer CD4-Zellzahl > 350 Zellen/µl bei klinischem Stadium 3 nach WHO.

Die leitliniengerechte Prophylaxe zur Prävention vertikaler HIV-Transmission sah eine Kombination der Medikamente AZT, NVP und 3TC vor. Es wurde die Einnahme von 300mg AZT zweimal täglich ab der 28. Schwangerschaftswoche empfohlen. Mit Beginn der Wehen sollte zusätzlich die Einmalgabe von 200mg NVP stattfinden sowie 300mg 3TC und 150mg AZT eingenommen werden. In gleicher Dosis sollte die Einnahme von 3TC alle drei Stunden und von AZT alle 12h bis zum Ende der Geburt fortgesetzt werden. Danach sollten beide Medikamente in gleicher Dosis zweimal täglich für weitere sieben Tage eingenommen werden. Die Prophylaxe konnte zu einem späteren Zeitpunkt, jedoch spätestens direkt vor der Geburt, begonnen werden, falls eine frühere Vorstellung in der Klinik nicht erfolgte. Neugeborene HIV-infizierter Mütter unter Prophylaxe sollten innerhalb von 72h nach der Geburt 2mg/kg KG NVP-Sirup und über vier Wochen zweimal täglich 4mg/kg KG AZT-Sirup erhalten. Die AZT-Einnahmedauer konnte für das Neugeborene auf eine Woche verkürzt werden, wenn die Mutter innerhalb der Schwangerschaft mindestens über vier Wochen AZT eingenommen hatte.

In Kooperation mit bestehenden Strukturen der Schwangerschaftsvorsorge wurden im Studienzeitraum HIV-infizierte Frauen, welche die Kriterien für eine Transmissionsprophylaxe erfüllten, über die Möglichkeit der Teilnahme an der Studie aufgeklärt und bei Einverständnis und Erfüllung der Einschlusskriterien, welche ein Mindestalter von 18 Jahren und eine erstmalige Einnahme von antiretroviralen Medikamenten im Rahmen der Prophylaxe umfassten, in die Studie aufgenommen.

Nach Einschluss in die Studie wurden die Studienteilnehmerinnen ab der 28. Schwangerschaftswoche und somit zeitgleich mit Beginn der Prophylaxe einbestellt. Vor der ersten Medikamenteneinnahme wurde eine Blutprobe zur Bestimmung der Ausgangswerte durchgeführt, sowie ein erweiterter Fragebogen zur Erhebung von Informationen zum Gesundheitszustand und zur Soziodemographie ausgefüllt. Anschließend folgten Studientermine wöchentlich über vier Wochen und darauf folgend monatlich zur Blutentnahme und Erhebung von Informationen bezüglich Einnahme der Medikamente und Nebenwirkungen. Zur Geburt wurden eine Blutentnahme der Mutter sowie die Gewinnung von Nabenschnurblut und Gewebeproben von Plazenta und Nabelschnur durchgeführt.

Eine Woche, vier bis sechs Wochen und zwölf Wochen nach der Geburt fanden Nachsorgeuntersuchungen statt, bei denen sowohl von der Mutter aus auch vom Kind (nur im Alter von vier bis sechs Wochen und zwölf Wochen) Blutproben entnommen wurden.

Anhand der pränatalen Einnahmedauer von AZT wurden die Studienteilnehmerinnen in zwei Gruppen eingeteilt: Bei einer AZT-Einnahme in der Schwangerschaft (Mindesteinnahmedauer abhängig von der Subanalyse) wurden die Studienteilnehmerinnen der Gruppe 1, bei erstmaliger Einnahme antiretroviraler Medikamente (ARV) zur Geburt der Gruppe 2 zugeordnet.

Für die Analyse der Blutwertveränderungen innerhalb der Schwangerschaft unter AZT-Einnahme wurden Frauen eingeschlossen, die eine AZT-Einnahme von mindestens einer Woche hatten. Für den Vergleich der mitochondrialen Veränderungen zum Zeitpunkt der Geburt zwischen Frauen mit und ohne pränataler AZT-Einnahme wurde für Gruppe 1 ein Grenzwert von vier Wochen AZT-Einnahme festgelegt. In die Analyse der medikamenteninduzierten Mutationen im HIV wurden Frauen mit einer Mindesteinnahmedauer von zwei Wochen während der Schwangerschaft eingeschlossen, sowie einer sdNVP-Einnahme zur Geburt, dem Vorhandensein von Plasmaproben zur Geburt sowie zu mindestens zwei postnatalen Nachsorgeuntersuchungen.

Bei der Analyse der Säuglinge wurde die entsprechende Gruppenzuordnung beibehalten, wobei die pränatale Mindestexpositionszeit von vier Wochen ein notwendiges Kriterium zum Einschluss in Gruppe 1 für die Analyse der hämatologischen Toxizität war.

HIV-infizierte Säuglinge wurden in die Analyse mitochondrialer Veränderungen, jedoch nicht in die Auswertung hämatologischer Veränderungen einbezogen.

Transmissionsrate

Der HIV-Status der Säuglinge wurde mit Blutproben von der Geburt und von Woche vier bis sechs durch eine quantitative Polymerasekettenreaktion (PCR) bestimmt. Bei positivem Testergebnis zu einem früheren Zeitpunkt ging die HIV-Infektion auch für Woche vier bis sechs in die Analyse ein, bei negativem Testergebnis galt die HIV-Infektion für den Zeitraum vier- bis sechs Wochen nach Geburt als unbekannt.

Quantifizierung der mitochondrialen DNA und der 4977-bp-Deletion

Aus den Gewebeproben (jeweils 50mg Placenta- und 50mg Nabelschnurgewebe) wurde DNA mit Hilfe eines Invisorb Spin Tissue mini kit nach Herstellerangaben extrahiert. Die Konzentrationen der Kern-DNA wurden anschließend mittels Amplifikation von Genfragmenten des Telomerasegens bestimmt. Die Konzentration der mitochondrialen DNA (mtDNA) wurde durch ein spezifisch mitochondriales DNA-Fragment mittels einer Semi-nested PCR ermittelt. Die Konzentration der 4977-bp-Deletion mitochondrialer DNA (dmtDNA4977) wurde mittels einer Nested PCR bestimmt. Die PCR wurden in einem ABI 7300 Real Time PCR System

(Applied Biosystems) als Triplicates mit Hilfe der Immolase Polymerase (Bioline, Deutschland) durchgeführt. Die Analyse der mitochondrialen Veränderungen fand in Kooperation mit dem Institut für Rechtsmedizin der Christian-Albrechts-Universität Kiel statt.

Bestimmung der Viruslast und Mutationsanalyse

Medikamenteninduzierte Mutationen im HIV-1 pol Gen wurden mit Hilfe einer Allel-spezifischen PCR (ASPCR) untersucht. Nach einer ersten quantitativen PCR eines Reverse-Transkriptase (RT)-Fragmentes, welches die zu untersuchenden Codons (Codon 22-236) enthält, wurde die Viruslast bestimmt. Die zweite quantitative PCR wurde mit jeweils selektiven Primern für die zu untersuchende Punktmutation sowie Primern für die Wildtypsequenzen durchgeführt. Insgesamt erfolgten sieben ASPCR pro Probe: AZT-Resistenzmutationen (T215Y, T215F), eine frühe Mutation (K70R) mit nur geringer AZT-Resistenz, die zwei häufigsten NVP-Resistenzmutationen (K103N und Y181C) und die häufigste 3TC-Mutation (M184V).

Statistische Methoden

Die Daten zur hämatologischen Toxizität wurden mit der Statistik-Software Stata (Version 11) analysiert. Das Statistikprogramm PASW Statistics 18 (SPSS Inc., Chicago, Illinois, USA) wurde zur Analyse mitochondrialer Toxizität und Resistenzentwicklung im HIV-1 genutzt. Unterschiede in den Eigenschaften der beiden Gruppen wurden mit t-Test, Mann-Whitney-U-Test oder dem Fisher-Exact-Test analysiert. Um den Zusammenhang zwischen zwei kontinuierlichen Parametern zu untersuchen wurde die Pearson-Korrelation angewendet. Fixed-Effects-Modelle wurden verwendet, um die Entwicklung hämatologischer Werte während der AZT Einnahme in der Schwangerschaft unter Berücksichtigung der Korrelation innerhalb der gleichen Individuen und der Variabilität zwischen den Probanden zu untersuchen. Der Koeffizient wurde bei vorhandener Signifikanz im Folgenden als eine Veränderung der Parameter pro Tag angezeigt. Zur Darstellung der Ergebnisse der deskriptiven Analyse als auch der Analyse der hämatologischen Toxizität wird im Ergebnisteil der Median sowie der Interquartilsabstand (interquartile range, IQR) verwendet. Die Einordnung ARV-induzierter Toxizitäten erfolgte mit Hilfe der Einstufung des National Institute for Allergy and Infectious Disease, Division for AIDS (DAIDS)[9]. Die Häufigkeit unerwünschter Ereignisse wurde unter Verwendung des Exakten Tests nach Fisher verglichen. Frühgeburt wurde als eine Geburt vor der 37 Schwangerschaftswoche definiert. Mit Hilfe der PCR-Ergebnisse der Blutproben der Säuglinge im Alter von 29 und 60 Tagen, in Kombination mit früheren Ergebnissen und mittels

Kaplan-Meier-Schätzung wurde der kumulative Anteil infizierter Säuglinge im Alter von sechs Wochen berechnet[10]. Ein p-Wert unter 0,05 wurde als statistisch signifikant, ein p-Wert unter 0,001 als hochsignifikant definiert.

Ergebnisse

Studienpopulation

Während der Studienlaufzeit suchten 1395 Frauen die Abteilung für Schwangerenvorsorge des Kyela District Hospitals auf, wurden beraten und auf HIV getestet, wobei 220 Frauen (15.8%) als HIV-infiziert identifiziert wurden. Von den 121 Frauen, die die Einschlusskriterien für die Prophylaxe und die Studie erfüllten, nahmen 82 Frauen für mindestens eine Woche innerhalb der Schwangerschaft AZT ein. Diese Frauen, die Gruppe 1 zugeordnet wurden, hatten eine mediane CD4-Zellzahl bei Aufnahme in die Studie von 390 (IQR: 267-515) Zellen/µl. Von diesen Frauen entbanden 55 Frauen im Kyela District Hospital. Zwei Säuglinge dieser Frauen wurden zur Geburt positiv auf HIV getestet. 41 Säuglinge hatten eine pränatale AZT-Exposition von mindestens vier Wochen und wurden in die Analyse zu Blutbildveränderungen in Gruppe 1 eingeschlossen. Aus dieser Gruppe kamen 39 Mutter-Kind-Paare zu Nachsorgeuntersuchungen nach vier Wochen, und 16 Paare nach drei Monaten.

Zweiundsechzig HIV-infizierte Frauen, die zur Entbindung das Krankenhaus aufsuchten, keine ARV-Einnahme zu einem vorherigen Zeitpunkt durchführten und die Einschlusskriterien erfüllten, wurden im selben Zeitraum in der Geburtsstation in die Studie aufgenommen und Gruppe 2 zugeordnet. Die mediane CD4-Zellzahl innerhalb der Gruppe 2 war zum Zeitpunkt der Geburt 262 (IQR: 194-474) Zellen/µl. Bei vier Neugeborenen wurde für den Zeitpunkt der Geburt eine HIV-Infektion diagnostiziert. 58 Neugeborene verblieben in der Studie; von 23 Neugeborenen standen Blutproben nach einem Monat und von zwölf Neugeborenen standen Blutproben nach drei Monaten zur Analyse zur Verfügung.

Es zeigten sich keine signifikanten Unterschiede zwischen den Gruppen hinsichtlich mütterlicher CD4-Zellzahl oder soziodemografischer Variablen wie Alter, Gewicht, Bildung oder Familienstand der Mütter sowie Geburtsgewicht und -länge der Säuglinge unabhängig von den Einschlusskriterien der jeweiligen Analyse.

Transmissionsrate

Die HIV-DNA PCR wurde an Blutproben von 88 Neugeborenen zur Geburt und von 63 Neugeborenen im Alter von sechs Wochen durchgeführt. Die intrauterine Übertragungsrate auf Basis von Blutproben zum Zeitpunkt der Geburt lag bei 2,4% (Konfidenzintervall (KI): 0,35%-16,0%) für Säuglinge der Gruppe 1 und bei 8,5% (KI: 3,3%-21,1%) für Säuglinge der Gruppe 2. Die mit Hilfe der Kaplan-Meier-Schätzung ermittelte Übertragungsrate liegt für Säuglinge im Alter von sechs Wochen bei 7,7% (KI: 2,5%-22,1%) in Gruppe 1 und 12,5% (KI: 5,2%-28,5%) in der Gruppe 2.

Blutbildveränderungen

82 Frauen der Gruppe 1 hatten eine AZT-Einnahme von mindestens einer Woche und wurden in die Analysen der Blutbildveränderungen durch AZT-Einnahme aufgenommen. Die Einnahmedauer während der Schwangerschaft lag bei diesen Frauen bei 57 (IQR: 43-71) Tagen. Mit Hilfe eines Fixed-Effects-Modells konnten Wirkungen von AZT über die Zeit der Einnahme auf einzelne Blutparameter bestimmt werden. Statistisch signifikant zeigten sich Veränderungen des Mittleren Korpuskulären Volumens (MCV) (Koeffizient: 0.0636fl pro Tag, $p<0,001$), der Erythrozytenverteilungsbreite (RDW) (Koeffizient: 0,0655% pro Tag, $p<0,001$), der Thrombozytenzahl (Koeffizient: 1.0309/nl pro Tag, $p<0,001$), der Erythrozytenzahl (Koeffizient: $-0,0040 * 10^6$/µl pro Tag, $p<0,001$), der Leukozytenzahl (Koeffizient: -0.0119/nl pro Tag, $p<0,001$) und der Granulozytenzahl (Koeffizient: -0.0123/nl pro Tag, $p<0,001$). Eine Abnahme mit anschließendem Anstieg ab Tag 32 der AZT-Einnahme konnte für die Hämoglobinwerte gezeigt werden.

Der Vergleich der Gruppen 1 und 2 zur Geburt zeigte eine signifikant niedrigere Erythrozytenzahl in Gruppe 1 als in Gruppe 2 (3.66/µl vs. 4.04/µl, $p<0,05$), während das MCV (87fl vs. 79fl, $p<0,001$) und die Thrombozytenzahl (367.000/mm³ vs. 273.500/mm³, $p<0,05$) signifikant höher waren in Gruppe 1 als in Gruppe 2. Drei Monate nach der Geburt zeigten sich keine signifikanten Unterschiede.

Zur Geburt zeigten Säuglinge mit pränataler AZT-Exposition (Gruppe 1) eine signifikant geringere Hämoglobinkonzentration (13,3g/dl vs. 15,2g/dl, $p<0,001$) und eine signifikant niedrigere Erythrozytenzahl (3.7/µl vs. 4.5/µl, $p<0,001$) als Säuglinge ohne pränatale AZT-Exposition (Gruppe 2). MCV (106fl vs. 100fl, $p<0,001$) und RDW (17,1% vs. 16,2%, $p<0,01$) waren signifikant höher bei Säuglingen der Gruppe 1 als bei Säuglingen der Gruppe 2. Die Häufigkeit des Auftretens von Anämie $\geq$ Grad 1 lag unter Einbeziehung aller Säuglinge bei 26%,
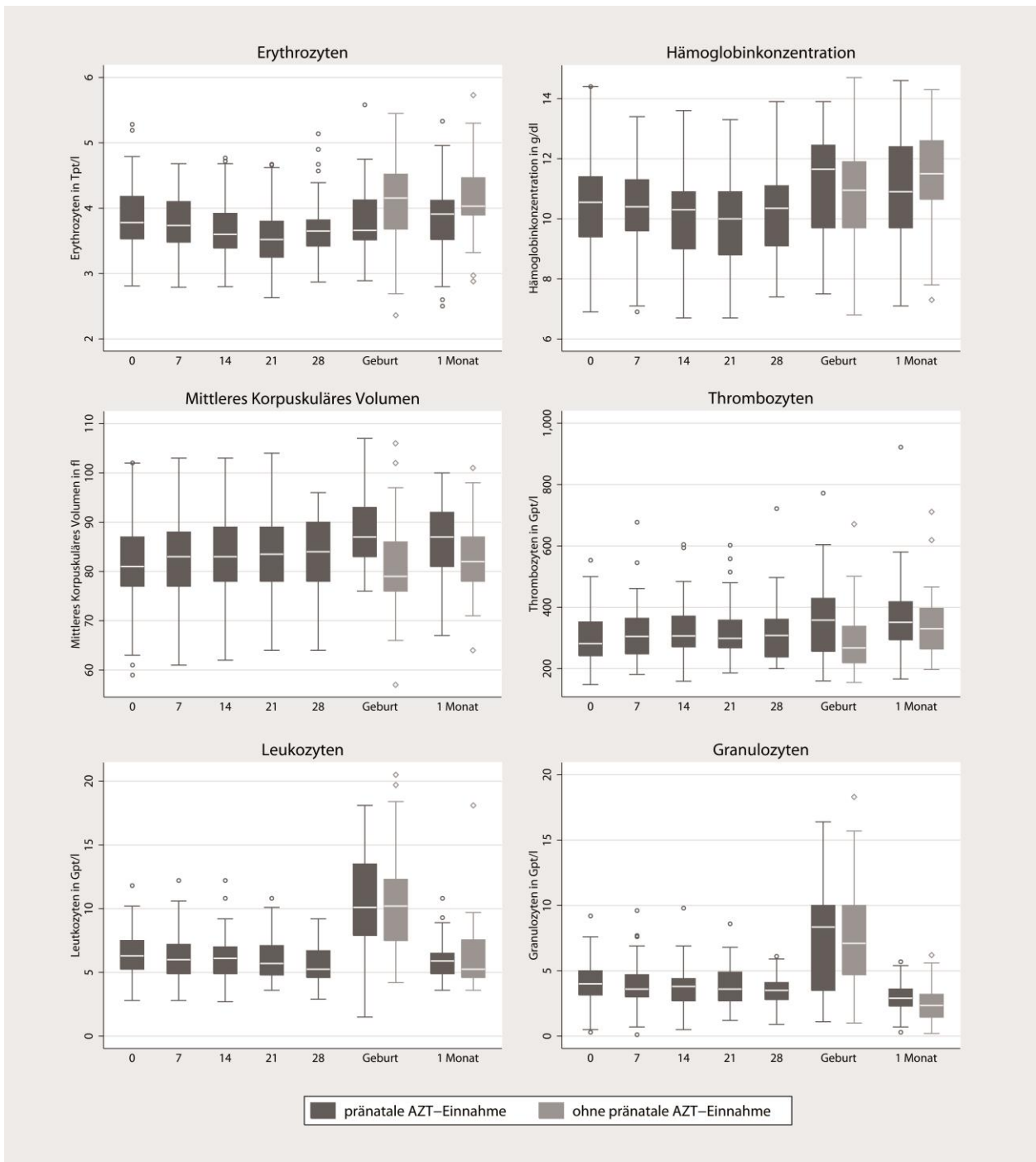
Abbildung 1: Veränderungen der Blutparameter bei Frauen der Gruppen 1 und 2. Der Verlauf der Blutparameter unter AZT-Einnahme bei Frauen der Gruppe 1 wird zum Zeitpunkt: Beginn, 7., 14., 21. und 28. Tag der AZT-Einnahme abgebildet. Für die Geburt und einen Monat nach Geburt werden zusätzlich die Werte der Gruppe 2 mit dargestellt. <u>Hämoglobin</u>: ein Abfall mit darauffolgendem Anstieg wurde für das Hämoglobin von Frauen der Gruppe 1 gezeigt. Zur Geburt gab es keinen signifikanten Unterschied zwischen beiden Gruppen; <u>MCV</u>: Unter AZT-Einnahme kam es zum Anstieg des MCV. Zur Geburt und einen Monat nach Geburt war das MCV bei Frauen der Gruppe 1 signifikant größer als bei Frauen der Gruppe 2; <u>Erythrozyten</u>: Unter AZT-Einnahme kam es zum Abfall der Erythrozytenzahl. Zur Geburt war diese signifikant niedriger bei Frauen der Gruppe 1 (3.66/µl vs. 4.16/µl; p<0.05); <u>Thrombozyten</u>: Es kam zu einem Anstieg der Thrombozyten (coef.: 1.0309/(nl*d); p<0.001) unter AZT-Einnahme. Zur Geburt war die Thrombozytenzahl signifikant größer bei Gruppe 1 als bei Gruppe 2. (367.000/mm³ vs. 267.500/mm³; p<0.05); <u>Leukozyten</u> und <u>Granulozyten</u>: Unter AZT-Einnahme kam es zu einem Abfall der Leukozyten und Granulozyten, wobei zum Zeitpunkt der Geburt kein signifikanter Unterschied dieser Parameter zwischen Gruppe 1 und Gruppe 2 bestand.

mit einer signifikant höheren Häufigkeit in Gruppe 1 (47% der Gruppe 1 vs. 11% der Gruppe 2, p<0,001). Zwei Säuglinge (6%) mit pränataler AZT-Exposition erlitten eine schwere (Grad 3) oder potenziell lebensbedrohliche (Grad 4) Anämie, während eine derart schwere Anämie in Gruppe 2 nicht vorkam.

Die mediane Hämoglobinkonzentration sank unter Einbeziehung aller Säuglinge von 14,3 (IQR: 13,0-15,5) g/dl zur Geburt auf 11,6 (IQR: 9,9-12,7) g/dl nach einem Monat und auf 10,5 (IQR: 9,9-11,4) g/dl nach drei Monaten. Im Lebensalter von einem Monat (IQR: 31-36 Tage) und von drei Monaten (IQR: 91-93 Tage) waren die Unterschiede in Hämoglobinkonzentration und Erythrozytenzahl zwischen den beiden Gruppen nicht mehr signifikant. Im Alter von einem Monat, wurde Anämie ≥ Grad 1 bei 29% aller Säuglinge (36% der Gruppe 1 vs. 20% der Gruppe 2, p=0,35) beobachtet, darunter befand sich ein Säugling aus Gruppe 1 mit schwerer Anämie. Nach drei Monaten trat eine Anämie ≥ Grad 1 bei 58% der Säuglinge ohne signifikanten Unterschied zwischen den Gruppen (46% in der Gruppe 1 vs. 73% in der Gruppe 2, p=0,24) auf.

Die Granulozytenzahl zur Geburt war signifikant niedriger bei Säuglingen der Gruppe 1 als bei Säuglingen der Gruppe 2 (5.0/nl in Gruppe 1 vs. 7.3/nl in Gruppe 2, p<0,05). Zu diesem Zeitpunkt wurde eine Granulozytopenie ≥ Grad 1 bei 37% aller Säuglinge beobachtet, mit signifikant häufigerem Auftreten in Gruppe 1 (52% in Gruppe 1 vs. 26% in Gruppe 2, p<0,05).
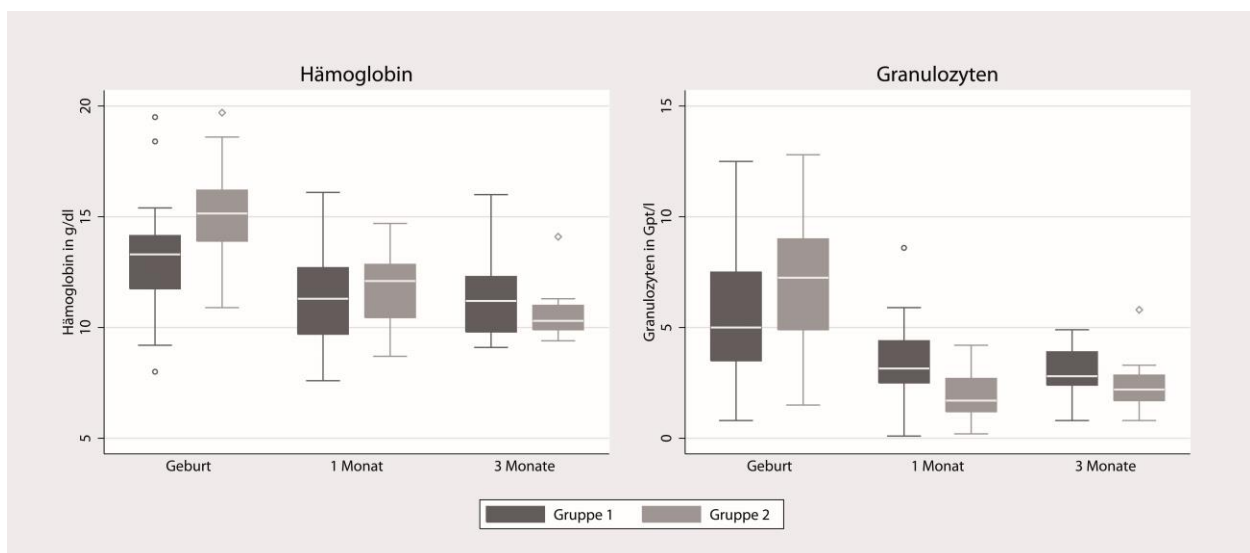

Abbildung 2. Hämoglobin und Granulozytenzahl der Säuglinge der Gruppen 1 und 2 zur Geburt und im Alter von einem und von drei Monaten. Zur Geburt ist das mediane Hämoglobin signifikant niedriger in Gruppe 1 im Vergleich zu Gruppe 2 (13,2 g/dl vs 15,2 g/dl, p=0,001). Im Alter von einem und von drei Monaten sind die Unterschiede im medianen Hämoglobin zwischen den beiden Gruppen nicht signifikant. Die mediane Granulozytenzahl ist zur Geburt signifikant niedriger bei Säuglingen nach intrauteriner AZT Exposition (5.1/nl in Gruppe 1 vs 7.3/nl in der Gruppe 2, p, 0,05). Nach einem Monat ist der Median der Granulozytenzahl signifikant niedriger in Gruppe 2 im Vergleich zu Gruppe 1 (3.2/nl in Gruppe 1 vs 1.7/nl in der Gruppe 2, p=0,001). Es wurden keine statistisch signifikanten Unterschiede in der Granulozytenzahl im Alter von drei Monaten beobachtet.

Eine Toxizität ≥ Grad 3 wurde bei 18% der Säuglinge der Gruppe 1 im Vergleich zu 9% in Gruppe 2 (p=0,31) beobachtet. Die Granulozytenzahl aller Säuglinge betrug 6,2 (IQR: 3,9-8,6) /nl zur Geburt und sank auf 2,8 (IQR: 1,6-3,4) /nl im Lebensalter von einem Monat und auf 2,7 (IQR: 1,8-3,3) /nl im Alter von drei Monaten. Nach einem Monat war die Granulozytenzahl signifikant niedriger in Gruppe 2 als in Gruppe 1 (3.2/nl in Gruppe 1 vs. 1.7/nl in Gruppe 2, p=0,001). In diesem Alter hatten 19% aller Säuglinge eine Granulozytopenie ≥ Grad 1, mit einem signifikant häufigeren Auftreten in Gruppe 2 (9% in Gruppe 1 gegenüber 33% in der Gruppe 2, p=0,039). Ein Granulozytopenie ≥ Grad 3 wurde bei 6% der Säuglinge in Gruppe 1 und bei 10% der Säuglinge in Gruppe 2 (p = 1.0) beobachtet.

Mitochondriale Veränderungen

Für die Analyse mitochondrialer Veränderungen standen für den Zeitpunkt der Geburt Plazenta- und Nabelschnurgewebeproben von 30 Frauen und Neugeborenen der Gruppe 1 (Einnahmedauer von bzw. pränatale Exposition mit AZT: Median 56 Tage, IQR: 43-70 Tage) und von 53 Frauen und Neugeborenen der Gruppe 2 zur Verfügung.

Die Konzentration mtDNA war signifikant höher in Plazentagewebeproben bei Frauen der Gruppe 1 (311 Kopien pro Zelle, IQR: 166-475 Kopien pro Zelle) im Vergleich zu Frauen der Gruppe 2 (187 Kopien pro Zelle, IQR: 115-352 Kopien pro Zelle, p=0,021). Auch in den Nabelschnurgewebeproben von Neugeborenen der Gruppe 1 (190 Kopien pro Zelle, IQR: 121-323 Kopien pro Zelle) war die Konzentration mtDNA deutlich höher als in denen von Neugeborenen der Gruppe 2 (127 Kopien pro Zelle, IQR: 70-234 Kopien pro Zelle, p=0.037). Es zeigte sich keine Korrelation der pränatalen AZT-Einnahmedauer mit dem Ausmaß der Depletion mtDNA in Plazentagewebeproben (p=0,95) und Nabelschnurgewebeproben (p=0,76) bei alleiniger Betrachtung von Gruppe 1-Frauen und -Neugeborenen.

Die dmtDNA4977 wurde nur selten in den untersuchten Plazentagewebeproben vorgefunden (1/24 Gruppe 1 vs. 1/43 Gruppe 2, p=1,0) und in diesen Fällen war der Anteil mtDNA4977 an der gesamten mtDNA nur gering (2.9E-05 Gruppe 1, 2.6E-05 Gruppe 2).

In Nabelschnurgewebeproben von Neugeborenen der Gruppe 1 zeigten sich keine Mutationen (0/23), wohingegen bei 6.4% (3/47) der Neugeborenen der Gruppe 2 dmtDNA4977 nachgewiesen werden konnte (p=0.55). Der prozentuale Anteil an der gesamten mtDNA lag im Bereich von 9.4E-04 bis 1.4E-02 und war somit höher als in den Plazentagewebeproben.

Mutation im HIV-1-pol-gen

Plasmaproben von 50 Frauen sowie von sieben HIV-infizierten Säuglingen wurden in die Analyse zur Mutationsentwicklung aufgenommen. Die mediane AZT-Einnahmedauer innerhalb dieser Subpopulation der Frauen betrug 53 (IQR: 39–64) Tage. 37 Frauen (74%) gebaren im Kyela District Hospital. 34 dieser Frauen nahmen während der Geburt AZT und 3TC ein. Für eine weitere Woche nach der Geburt nahmen 41 Frauen AZT sowie 3TC und fünf Frauen nur AZT ein. Insgesamt nahmen 86% (43/50) der Frauen mindestens einmal 3TC ein.

Vor Prophylaxebeginn betrug die mediane maternale HIV-1-Viruslast innerhalb dieser Gruppe $1.25*10^4$ (IQR: $4.4*10^3$–$4.5*10^4$) Kopien/ml, zur Geburt $2.9*10^3$ (IQR $1.4*10^3$–$6.8*10^3$) Kopien/ml, ein bis zwei Wochen postpartum $1.7*10^3$ (IQR: $1.3*10^3$–$5.8*10^3$) Kopien/ml, vier bis sechs Wochen postpartum $1.2*10^4$ (IQR: $6.3*10^3$–$3.7*10^4$) Kopien/ml und 12–16 Wochen postpartum $2.56*10^4$ (IQR: $1.2*10^4$–$3.7*10^4$) Kopien/ml. Die maternale Viruslast war im Vergleich zum Ausgangswert jeweils signifikant niedriger zur Geburt und ein bis zwei Wochen postpartum (p jeweils 0.001).

28% (14/50) der Frauen waren mit HIV-1 Subtyp A1, 68% (34/50) mit HIV-1 Subtyp C und 4% (2/50) mit Subtyp D infiziert. In keiner der 50 Ausgangsproben konnte eine medikamenteninduzierte HIV-Mutation nachgewiesen werden. Nach Einnahme der Prophylaxe wurden bei 40% der Frauen Resistenzen im HIV nachgewiesen. Darunter waren 45% AZT-Resistenzen, 35% NVP-Resistenzen, 5% 3TC-Resistenzen und 15% Resistenzen, welche zwei oder drei Medikamente betrafen.

Zur Geburt wurden AZT-resistente HIV-Virusvarianten in fünf der 50 (10%) Frauen identifiziert. Bei dem Vergleich der medianen AZT-Einnahmedauer der fünf Frauen mit AZT-
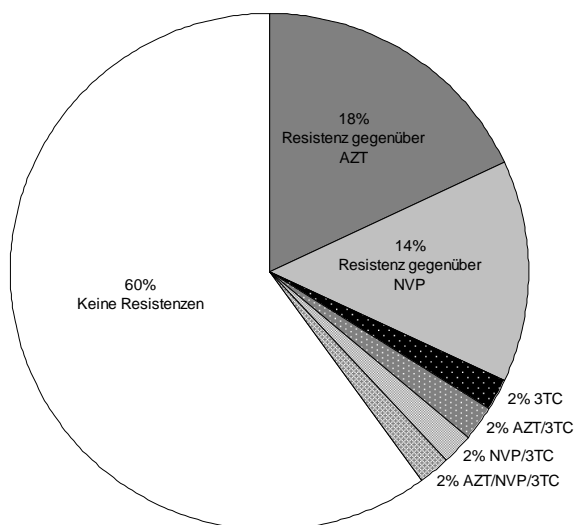

Abbildung 3. Resistenzen im HIV-1-pol-gen nach Einnahme der Prophylaxe

resistenten Viren zur Geburt (77 Tage) mit jenen 45 Frauen ohne AZT-resistente Viren (50 Tage), zeigte sich kein signifikanter Unterschied (p=0.2, Mann-Whitney-U-Test). AZT-resistente HIV-Virusvarianten traten jedoch zur Geburt signifikant häufiger bei Frauen mit pränataler AZT-Einnahme von mindestens zehn Wochen (3/10 = 33%) auf, als bei Frauen mit einer kürzen AZT-Einnahme (2/40 = 5%, p=0.048, Exakter Test nach Fisher).

Die Reduktion der Viruslast vom Beginn der Prophylaxe bis zur Geburt war, unter Einbeziehung aller Frauen, 0.6 $\log_{10}$. Es konnte jedoch gezeigt werden, dass es bei Frauen, bei denen AZT-resistente Virenvarianten nachgewiesen wurden, bis zur Geburt zu einer signifikant geringeren Abnahme der Viruslast im Vergleich zu Frauen ohne AZT-resistente Virusvarianten kam (p=0.045, Mann-Whitney-U-Test). Dementsprechend hatten Frauen mit AZT-resistenten HIV eine deutlich höhere Viruslast zur Geburt (29400 Kopien/ml) im Vergleich zu Frauen ohne AZT-resistente HIV (2680 Kopien/ml; p=0.021, Mann-Whitney U-Test). Zudem zeigten Frauen mit AZT-resistenten Viren eine niedrigere CD4-Zellzahl (331 Zellen/µl) gegenüber Frauen ohne diese (406 Zellen/µl, p=0.077, Mann-Whitney U-Test).

NVP-Resistenzmutationen K103N und/oder Y181C im HIV-Genom wurde in postpartalen Proben von neun der 50 Frauen (18%) nachgewiesen, wobei der Anteil an der Gesamt-RNA bei sieben der neun Frauen 5% nicht überstieg.

Diskussion

In dieser prospektiven Beobachtungsstudie wurden Wirkung und Nebenwirkungen der seit 2006 von der WHO empfohlenen antiretroviralen Transmissionsprophylaxe aus AZT, sdNVP und 3TC im Zeitraum von September 2008 bis September 2009 untersucht. Der Fokus der Arbeit liegt auf der Transmissionsrate, der hämatologischen und mitochondrialen Toxizität sowie auf der Resistenzentwicklung, die im Rahmen der Studie ermittelt wurden.

Transmissionsrate

Die mit Hilfe der Kaplan-Meier-Schätzung berechnete Übertragungsrate für ein Alter von sechs Wochen betrug 7,7% bei Säuglingen der Gruppe 1 und 12,5% bei Säuglingen der Gruppe 2. Zwei randomisierte, doppelblinde, Placebo-kontrollierte Studien haben zuvor bereits gezeigt, dass AZT, verabreicht in den letzten vier Wochen der Schwangerschaft, zu einer Verringerung der Übertragungsrate auf 14,7% im Vergleich zu 24,8% bei der Vergleichsgruppe mit Placebo im Alter von sechs Wochen führt[11]. Bei einer Studie in Uganda stellte sich bei alleiniger Gabe von sdNVP eine Transmissionsrate von 11,8% im Alter von sechs Wochen heraus[12]. Die

Ergebnisse von Dabis et al. zeigen weiterhin eine Reduktion des Transmissionsrisikos auf 6,5% bei Kombination einer vierwöchigen AZT-Einnahme mit sdNVP[12]. Diese Ergebnisse stehen mit den Sudienergebnissen im Einklang.

Hämatologische Toxizität

Die Untersuchung der Blutwerte der Frauen mit AZT-Einnahme während der Schwangerschaft ergab signifikante Blutwertveränderungen mit einem signifikanten Abfall der Granulozytenzahl und der Erythrozytenzahl. Die Hämoglobinwerte fielen in den ersten vier Wochen der AZT-Einnahme und stiegen dann wieder an. Die vorübergehende Abnahme von Hämoglobin findet Übereinstimmung mit den Ergebnissen von Briand et al[2]. Weiterhin zeigte sich ein signifikanter Anstieg von MCV, RDW und Thrombozytenzahl. Bei der Geburt gab es keinen signifikanten Unterschied im Hämoglobin zwischen den Gruppen. MCV, RDW und Thrombozytenzahl waren signifikant höher und die Erythrozytenzahl signifikant niedriger bei Frauen der Gruppe 1 zur Geburt.

Es ist bekannt, dass es während der Schwangerschaft zu einer Abnahme der Thrombozytenzahl kommen kann[13]. Unser Fixed-Effects-Modell zeigte jedoch eine Erhöhung der Thrombozytenzahl während der AZT-Einnahme bei den schwangeren Frauen. Weiterhin konnte eine deutlich höhere Thrombozytenzahl in Gruppe 1 im Vergleich zu Gruppe 2 zum Zeitpunkt der Geburt beobachtet werden. Dies deutet darauf hin, dass es unter Einnahme von AZT zu einer Erhöhung der Thrombozytenzahlen bei HIV-infizierten schwangeren Frauen kommen kann.

Die Vergleichbarkeit der beiden Gruppen wurde analysiert, indem auf Unterschiede in Krankheitsverlauf (mit Hilfe von CD4-Zellzahl) sowie soziodemographischen Eigenschaften (u.a. Alter, Gewicht, Familienstand, Ausbildung) zwischen den Gruppen geprüft wurde. Es gab keine signifikanten Abweichungen hinsichtlich dieser Variablen. Dennoch kann nicht ausgeschlossen werden, dass andere, hier nicht berücksichtigte Faktoren, wie z.B. die Inanspruchnahme von Leistungen des Gesundheitssystems, einen Einfluss auf die Ergebnisse haben.

In der Analyse hatten Neugeborene mit intrauteriner AZT-Exposition (Gruppe 1) zur Geburt signifikant niedrigere Hämoglobinwerte sowie signifikant häufiger eine Anämie (Grad $\geq$ 1) als Säuglinge ohne AZT-Exposition (Gruppe 2). Diese Ergebnisse stimmen mit denen von Connor et al.[14] und Sperling et al.[15] überein, die ebenfalls ein signifikant niedrigeres Hämoglobin bei Säuglingen nach intrauteriner AZT-Exposition feststellten. In der vorliegenden Studie waren die Unterschiede im Hämoglobin zwischen den Gruppen bei Säuglingen im Alter von einem Monat nicht mehr signifikant. Dies lässt sich auf eine schnellere Hämoglobinabnahme bei Säuglingen

der Gruppe 2 nach der Geburt zurückführen. Diese nehmen vier Wochen AZT und 3TC ein, während Säuglinge der Gruppe 1 diese Medikamente nur eine Woche einnehmen. Die Häufigkeit des Auftretens von Anämie $\geq$ Grad 1 betrug 29% unter Einbeziehung der Säuglinge beider Gruppen im Alter von einem Monat. Es zeigte sich keine signifikante Diskrepanz zwischen den beiden Gruppen. Im Gegensatz dazu ermittelten Briand et al. signifikante Unterschiede in der Häufigkeit einer Anämie zwischen Säuglingen mit dreitägiger und sechswöchiger postnataler AZT-Exposition. Interessanterweise erwies sich dieser Effekt als unabhängig von der intrauterinen Exposition[2].

Bei der Geburt hatten Säuglinge der Gruppe 1 signifikant niedrigere Granulozytenzahlen sowie häufiger eine Granulozytopenie $\geq$ Grad 1. Dieser Effekt der intrauterinen AZT-Exposition bei Säuglingen, einschließlich der Persistenz der verringerten Granulozytenzahl bis zu 18 Monate nach der Geburt, wurde bereits beschrieben[16].

Säuglinge der Gruppe 2 zeigten nach der postnatalen einmonatigen AZT-Einnahme im Vergleich zu Säuglingen der Gruppe 1 mit einer postnatalen AZT-Einnahme von nur einer Woche eine signifikant niedrigere Granulozytenzahl sowie ein signifikant häufigeres Auftreten von Granulozytopenie $\geq$ Grad 1. Im Alter von einem Monat hatten 9% der Säuglinge eine schwere Granulozytopenie ($\geq$ Grad 3). Diese ist am ehesten auf eine prä- und postpartale AZT-Exposition zurückzuführen.

Zum Zeitpunkt der Geburt stellten Säuglinge der Gruppe 2 eine Kontrollgruppe zu Gruppe 1 hinsichtlich der Nebenwirkungen der AZT-Einnahme dar. Limitierend für die Analyse der Blutwerte zu den Nachsorgeuntersuchungen war jedoch die unterschiedliche postpartale Medikamenteneinnahme der Säuglinge, da sich die Unterschiede der Blutwerte dadurch nicht mehr eindeutig der pränatalen AZT-Einnahme zuordnen lassen können. Die Etablierung einer entsprechenden Vergleichsgruppe hätte jedoch den Rahmen unserer Studie, die die in der Praxis angewendeten Regime begleitete, überschritten.

Eine weitere Limitierung unserer Studie ist die hohe Dropout-Rate während der Verlaufskontrollen. Dies ist ein häufiges Problem von PMTCT-Projekten in ressourcenarmen Regionen. Bei einer Evaluation von PMTCT-Programmen in Uganda von Aloua et al. stellten sich mangelndes Verständnis für den Nutzen der Nachsorgeuntersuchungen sowie der Kindstod als führende Gründe für hohe Dropout-Raten heraus[17]. Für unsere Erhebungen liegen uns leider keine Daten über die Entwicklung der Säuglinge, welche nicht zu den Verlaufskontrollen erschienen sind, vor.

Mitochondriale Depletion

Sowohl AZT als auch HIV-1 können toxisch auf Mitochondrien wirken und zur Reduktion von mtDNA führen[18-21]. In unserer Studie zeigte sich eine signifikant höhere Menge von mtDNA in AZT-exponierten als in unexponierten Müttern und Kindern. Dies steht im Einklang mit den Ergebnissen von Aldrovandi et. al[22], die ebenfalls eine erhöhte Konzentration mtDNA in AZT-exponierten Mutter-Kind-Paaren mit vergleichbarer Krankheitsprogression nachweisen konnten. Die pränatale Exposition mit NRTIs innerhalb der Studien unterschied sich jedoch innerhalb der Studien. Gegenläufige Ergebnisse erzielten Poirier et al., in deren Analyse von Kindern HIV-infizierter Mütter eine Verringerung der Menge an mtDNA bei intrauterin AZT-exponierten gegenüber nichtexponierten Säuglingen auffiel. Die Studie ist jedoch nur bedingt vergleichbar, da Viruslast und CD4-Zellzahl zwischen den Gruppen deutlich variierten[23].

In unserer Analyse konnten wir nur ein sehr seltenes Auftreten von dmtDNA4977 in den Plazentaproben HIV-infizierter Mütter feststellen (1/24 Gruppe 1 vs. 1/43 Gruppe 2). Das geringe Vorkommen steht im Einklang mit vorangegangenen Studien, welche keine dmtDNA4977 in Plazentaproben entdecken konnten[24].

Bei unserer Analyse kam dmtDNA bei drei von den insgesamt 70 untersuchten Nabelschnurgewebeproben vor, wobei alle drei betroffenen Säuglinge keine vorangegangene AZT-Exposition hatten. Das Auftreten von dmtDNA ist altersabhängig und in Säuglingen ungewöhnlich. Eine Studie, die den Nachweis von dmtDNA in verstorbenen Neugeborenen erbrachte, führte dies auf perinatale Hypoxie oder ein erhöhtes Sauerstoffangebot bei intensivmedizinischer Therapie zurück[25]. In der vorliegenden Studie konnte gezeigt werden, dass die Mutation auch in Säuglingen HIV-infizierter Mütter vorliegt, wobei die Menge an dmtDNA4977 gering war und im Verhältnis zur gesamten Menge an mtDNA im Bereich zwischen 0.00094% und 0.014% lag. Auffallend ist, dass alle Säuglinge mit entsprechender Mutation keine pränatale AZT-Exposition hatten. Statistisch stellte sich dies nicht als signifikant heraus. Andere Einflüsse, wie z.B. Alkohol- oder Nikotinkonsum der Mutter, könnten hierbei eine Rolle spielen.

Die toxische Wirkung von AZT auf mtDNA spiegelt sich nicht in den Ergebnissen wider. Dies könnte daran liegen, dass hier im Gegensatz zu anderen Studien nur eine kurze Exposition mit AZT stattfand und die Frauen erstmalig ARVs im Rahmen der Prophylaxe einnahmen. Weiterhin kann nicht ausgeschlossen werden, dass AZT über eine Herunterregulierung der Expression von TNF-alpha, welches bei HIV-Erkrankten erhöht sein kann und nachweislich eine schädigende Wirkung auf Mitochondrien hat, protektiv wirkt[24]. Faktoren wie der Wirkstoff (z.B. d4T oder

AZT), das analysierte Material (PBMC oder Gewebe), oder der Krankheitsprogress könnten die unterschiedlichen Ergebnisse erklären.

Resistenzentwicklung

Mit Hilfe der hochsensitiven Methode der ASPCR wurde bei 22% Frauen Resistenzen im HIV-Genom nachgewiesen. Unter der Kombinationsprophylaxe lässt sich im Vergleich zur alleinigen Gabe von sdNVP zur Geburt eine erhebliche Reduktion von NVP-Resistenzen feststellen. Dem gegenüber steht das Vorkommen von AZT-resistenten Virusvarianten bei einem erheblichen Anteil der Frauen. In vorangegangen Studien konnte gezeigt werden, dass ein fortgeschrittenes Stadium der HIV-Erkrankung sowie niedrige CD4-Zellenzahlen mit einem erhöhten Auftreten von AZT-Resistenzen assoziiert sind[26,27]. Dies entspricht unserem Ergebnis, dass Frauen, bei denen AZT-resistente Viren zur Geburt nachgewiesen wurden, eine zehnfach höhere mediane Viruslast sowie eine niedrigere CD4-Zellzahl aufwiesen, als Frauen ohne AZT-resistente Viren. In der aktuellsten WHO-Leitlinie (2010) wird eine AZT-Prophylaxe ab einem Grenzwert für die CD4-Zellzahl von 350 Zellen/µl anstelle von 200 Zellen/µl (Leitlinien von 2006) empfohlen. Dies könnte dazu beitragen, die Entstehung von AZT-resistenter HIV-1 zu reduzieren. Weiterhin bestätigen die Ergebnisse frühere Studien, die nachwiesen, dass eine längere AZT-Einnahme die Häufigkeit einer AZT-Resistenzentwicklung erhöht. In dieser Studie zeigte sich ein signifikanter Unterschied bei einem Grenzwert für die AZT-Einnahme von zehn Wochen. Die empfohlene Verlängerung der AZT-Einnahme während der Schwangerschaft, welche durch die Vorverlegung des empfohlen Prophylaxebeginns von Woche 28 auf Woche 14 stattfindet, könnte somit das Auftreten von Resistenzentwicklungen erhöhen.

Schlussfolgerung

Die nachgewiesenen hämatologischen Veränderungen bei Frauen und Säuglingen unter der 2006 empfohlenen antiretroviralen Transmissionsprophylaxe waren überwiegend von geringem Ausmaß und begrenzter Dauer. Zudem konnte keine Depletion mtDNA oder das vermehrte Auftreten von dmtDNA4977 festgestellt werden. Auch zeigte sich eine Abnahme der Nevirapinresistenz im Vergleich zu Daten, die über eine Prophylaxe mit ausschließlich sdNVP vorliegen. Allerdings sollte bei Planung, Implementierung und Durchführung der antiretroviralen Prophylaxe in ressourcenarmen Gebieten berücksichtigt werden, dass die Überwachung hämatologischer Nebenwirkungen während der antiretroviralen Prophylaxe ein entscheidender Faktor für die Sicherheit darstellt, da, wenn auch selten, schwerwiegende hämatologische Nebenwirkungen auftreten können. Weiterhin sollte bei der Auswahl des Regimes bedacht

werden, dass obwohl die Nevirapinresistenz bei der Kombinationsprophylaxe im Vergleich zur Prophylaxe mit sdNVP geringer ist, eine Zunahme von AZT-Resistenz zu beobachten ist, welche sich erschwerend auf nachfolgende Therapie oder wiederholte Prophylaxe auswirken kann.

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Eidesstattliche Versicherung

„Ich, Judith Ziske, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema:

„Nebenwirkungen einer antiretroviralen Kombinationsprophylaxe zur Verhinderung der Mutter-Kind-Übertragung von HIV in Tansania"

selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Datum   Unterschrift

Anteilserklärung an den erfolgten Publikationen

Judith Ziske hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1:

Ziske J, Kunz A, Sewangi J, Lau I, Dugange F, Hauser A, Kirschner W, Harms G, Theuring S, Hematological Changes in Women and Infants Exposed to an AZT-Containing Regimen for Prevention of Mother-to-Child-Transmission of HIV in Tanzania, PloS one 2013

Beitrag im Einzelnen:

- Mitarbeit in der Planung zur Umsetzung der Studie
- Supervision der Studiendurchführung in Tansania, Rekrutierung der Studienteilnehmerinnen
- Probenabnahme und Probenverarbeitung, Datenerhebung, Datenaufbereitung und statistische Auswertung
- Erstellen der Erstversion des Manuskriptes und Vorbereitung der Publikation

Publikation 2:

Kunz A, von Wurmb-Schwark N, Sewangi J, **Ziske J**, Lau I, Mbezi P, Theuring S, Hauser A, Dugange F, Katerna A, Harms G, Zidovudine Exposure in HIV-1 Infected Tanzanian Women Increases Mitochondrial DNA Levels in Placenta and Umbilical Cords, PloS one 2012

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- Mitarbeit in der Studiendurchführung in Tansania: Rekrutierung der Studienteilnehmerinnen, Probenabnahme und Probenverarbeitung, Datenerhebung, Datenaufbereitung
- Mitwirken bei der Manuskripterstellung

Publikation 3:

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

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- Mitwirken an der Manuskripterstellung

Unterschrift, Datum und Stempel des betreuenden Hochschullehrers/der betreuenden Hochschullehrerin

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Druckexemplar Publikation 1

Ziske J, Kunz A, Sewangi J, Lau I, Dugange F, Hauser A, Kirschner W, Harms G, Theuring S

Hematological Changes in Women and Infants Exposed to an AZT-Containing Regimen for Prevention of Mother-to-Child-Transmission of HIV in Tanzania

PLOS ONE

Hematological Changes in Women and Infants Exposed to an AZT-Containing Regimen for Prevention of Mother-to-Child-Transmission of HIV in Tanzania

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Abstract

Introduction: Tanzanian guidelines for prevention of mother-to-child-transmission of HIV (PMTCT) recommend an antiretroviral combination regimen involving zidovudine (AZT) during pregnancy, single-dosed nevirapine at labor onset, AZT plus Lamivudine (3TC) during delivery, and AZT/3TC for 1–4 weeks postpartum. As drug toxicities are a relevant concern, we assessed hematological alterations in AZT-exposed women and their infants.

Methods and Materials: A cohort of HIV-positive women, either with AZT intake (n = 82, group 1) or without AZT intake (n = 62, group 2) for PMTCT during pregnancy, was established at Kyela District Hospital, Tanzania. The cohort also included the infants of group 1 with an *in-utero* AZT exposure ≥4 weeks, receiving AZT for 1 week postpartum (n = 41), and infants of group 2 without *in-utero* AZT exposure, receiving a prolonged 4-week AZT tail (n = 58). Complete blood counts were evaluated during pregnancy, birth, weeks 4–6 and 12.

Results: For women of group 1 with antenatal AZT intake, we found a statistically significant decrease in hemoglobin level, red blood cells, white blood cells, granulocytes, as well as an increase in red cell distribution width and platelet count. At delivery, the median red blood cell count was significantly lower and the median platelet count was significantly higher in women of group 1 compared to group 2. At birth, infants from group 1 showed a lower median hemoglobin level and granulocyte count and a higher frequency of anemia and granulocytopenia. At 4–6 weeks postpartum, the mean neutrophil granulocyte count was significantly lower and neutropenia was significantly more frequent in infants of group 2.

Conclusions: AZT exposure during pregnancy as well as after birth resulted in significant hematological alterations for women and their newborns, although these changes were mostly mild and transient in nature. Research involving larger cohorts is needed to further analyze the impact of AZT-containing regimens on maternal and infant health.

Introduction

Mother-to-child transmission of HIV has become a relatively rare event in most resource-rich countries, where vertical transmission nowadays occurs in less than 2% of cases [1]. This decline is based on a combination of several strategies, including early maternal diagnosis through routine counseling and HIV testing during antenatal care (ANC), provision of antiretroviral therapy (ART) or of antiretroviral (ARV) prophylaxis, elective Caesarean section and the complete avoidance of breastfeeding. The high requirements for this complex range of measures, such as access for women to a health care system, broad coverage of HIV testing among pregnant women, CD4 cell count monitoring, or affordable and sustainable replacement feeding [2], make it difficult to successfully reduce mother to child transmission of

HIV (PMTCT) in resource-limited countries. Indeed, in 2010, ARV coverage for PMTCT was only about 50% in sub-Saharan Africa [3]. The implementation of a single-dose (sd) administration of the non-nucleoside reverse transcriptase inhibitor nevirapine (NVP) to mothers and babies in resource-poor countries has been a considerable step forward in PMTCT. However, although representing a simple, feasible and cost-effective regimen [4], a major problem of sdNVP is the high risk of inducing drug-resistant HIV variants. It has been shown that the addition of nucleoside reverse transcriptase inhibitors, such as Zidovudine (AZT) and Lamivudine (3TC), can significantly reduce this risk [5]. Furthermore, combining several drugs is more effective in reducing HIV-transmission and can result in transmission rates as low as 6.5% at six weeks postpartum [6].

Since 2006, the World Health Organization (WHO) PMTCT guidelines for resource-poor settings follow those findings and recommend a sequential combination prophylaxis, including antenatal AZT intake, sdNVP during labor and intra/postpartum AZT/3TC. Despite the clear advantages of this regimen in terms of efficacy and reduction in NVP resistance, it has nevertheless several drawbacks. As drug intake is supposed to last from pregnancy until the postpartum period, requiring different drugs at specific points in time, this prolonged and complex process can make adherence difficult [2,7]. Another important issue is that a combination prophylactic regimen obviously results into a much higher drug burden for mothers and infants than the previously recommended regimen. Previous retro- and prospective studies have shown that AZT interferes with hematopoiesis, resulting in decreased levels for several cell lineages in pregnant women [8,9]. Other studies have shown an impact on hematopoiesis with varying persistence in infants exposed to AZT *in utero* or postnatally [10,11,12,13]. Connor *et al.* [12] and Sperling *et al.* [14] found a transiently lower hemoglobin level that resolved within the first six to 12 weeks of age. Regarding other cell lineages, especially granulocytes, Le Chenadec *et al.* found that decreased levels persisted up to the age of 18 months [11]. As granulocytes are crucial for the immune response, with deficiency often leading to severe bacterial infections, and anemia can have life-threatening potential, monitoring is a crucial aspect, particularly in settings where treatment options are limited.

The United Republic of Tanzania, one of the poorest countries in the world [15], is also one of the countries most affected by the global HIV/AIDS epidemic. The general HIV prevalence is estimated to be 6%, while the prevalence of HIV in pregnant women is estimated to be 10% in major urban areas and 6% in less densely populated regions [16]. In 2008, Tanzania changed its PMTCT standard recommendation from sdNVP to a combination regimen in accordance with the 2006 WHO guidelines.

The aim of this study was to assess the potential hematological toxicity of the combination PMTCT regimen in women and infants in a peripheral setting in Tanzania.

Methods

Ethics Statement

The study was approved by the Tanzanian National Institute of Medical Research, by the Mbeya Region Ethical Committee, and by the Ethical Commission of Charité-Universitätsmedizin Berlin. Written informed consent was obtained from all participants, and all data remained confidential.

Setting, Procedures and Recruitment

An observational prospective follow-up study was conducted from September 2008 until September 2009 at the Kyela District Hospital (KDH) in Mbeya Region, Tanzania to accompany the introduction of ARV combination prophylaxis for MTCT with regard to feasibility and adherence [7]. Within the larger frame of this research, we performed a sub-study assessing hematological alterations linked to this regimen in mothers and infants.

Mbeya is among the regions in Tanzania with the highest HIV prevalence, estimated at around 9% of the general population [17]. KDH is a rural health facility in the south of Mbeya Region. Each month, approximately 130 pregnant women attend the ANC services of KDH for the first time. This hospital has been providing PMTCT services supported by the German Agency for International Cooperation since 2001. Combination ARV prophylaxis was introduced in KDH in March 2008 following the updated 2008 Tanzanian Guidelines.

Routine PMTCT procedures at KDH include voluntary HIV counseling and testing for all new antenatal care (ANC) clients visiting KDH. CD4 cell counts were performed for pregnant women identified as HIV-positive to assess their eligibility for either ART or ARV prophylaxis. Women with CD4 cell counts above 200 cells/mm^3 (and therefore not requiring ART for their own health) were eligible for ARV prophylaxis. Administration of AZT was initiated at week 28 of gestation or anytime thereafter. During delivery, the women received sdNVP, AZT and 3TC and were given a take-home postpartum tail of AZT for seven days. Women first identified as HIV-positive at the time of delivery, who therefore had no previous ARV intake, were also offered intra/postpartum ARV prophylaxis. Infants received sdNVP within 72 hours after birth and AZT syrup for one week if the mother had taken AZT for at least four weeks during pregnancy and for four weeks if the mother had taken AZT for less than four weeks.

The cohort included HIV-positive pregnant ART-naïve women aged 18 years or older who attended the ANC clinic and/or delivered in the maternity ward at KDH during the study period, who were eligible for ARV prophylaxis according to the guidelines and who had given informed consent. Women enrolled in ANC with pre-delivery AZT intake were assigned to group 1, and those who had no ARV prophylaxis during pregnancy but received drugs during delivery at the KDH maternity ward were assigned to group 2.

Group 1-women were included for the analysis of hematological changes during pregnancy. Infants of women participating in the study were eligible for analysis if they had received prophylaxis according to the guidelines, i.e. group 1-infants with postnatal AZT intake of one week and group 2-infants with four weeks (see graph 1). In accordance with the WHO guidelines for postnatal AZT prophylaxis in infants, the analysis of adverse effects in group 1-infants only included those with at least four weeks *in utero* exposure.

Samples and Data Collection

Data on the socio-demographic and clinical background of the women were collected through standardized, structured questionnaires for the stages of ANC, delivery and postpartum follow-up. The questionnaires were developed and pretested by the authors. Self-observed adverse events, adherence to ARV prophylaxis, and concomitant drug use were recorded at regular intervals throughout pregnancy (group 1), delivery (group 1 and 2) and during the postpartum period (groups 1 and 2). Maternal blood samples from group 1 were taken weekly in the first month of AZT intake, monthly throughout pregnancy, once at delivery, and at months one and three post-delivery. Blood was drawn from group 2 participants at delivery and again monthly during the first three months postpartum. Laboratory analysis included full blood counts for all samples, and CD4 cell counts were conducted with samples taken during the first ANC visit (group 1) and at delivery (groups 1 and 2). The comparability of the groups was confirmed with regard to the socio-demographic situation, CD4 cell count as well as malarial symptoms and malarial prophylaxis.

For the infants, cord blood was taken at delivery and further blood samples were taken during follow-up visits at one and three months of age. Full blood cell counts were performed for both mothers and infants. HIV-PCR was performed with samples from newborns taken at delivery and at one month of age and HIV-infected infants were excluded from the analysis as HIV infection itself causes hematological changes.

Sample taking and data collection in questionnaires and forms was performed by staff within the respective wards at KDH; a trained study nurse supervised this process.

Statistical Analysis

Data was analyzed using the statistical software Stata version 11. Descriptive analysis of maternal baseline information was performed to characterize the study population. Baseline characteristics of both groups were compared using the student's t-test, Mann-Whitney-U-test or Fisher's exact test.

Fixed effects models were used to follow the development of maternal hematological values during AZT intake, taking into account correlation within the woman herself and the variability between women. Coefficients are expressed as an alteration of parameter per day if the linear model was significant. Student's t-test for independent variables was used to compare hematological values of women by group at birth and to compare hematological parameters of infants at birth and one month of age. For data at three months of age, Mann-Whitney-U-test was performed, taking the dropout rate into account. To classify ARV induced toxicities, tables from the Division of AIDS (DAIDS) were used for grading the severity of adverse events in adults and infants [18]. At birth, hemoglobin levels between 10 g/dl and 8.5 g/dl were defined as mild anemia (grade 1); 8.4 g/dl to 7.5 g/dl as moderate anemia (grade 2); 7.4 g/dl to 6.5 g/dl as severe anemia (grade 3) and hemoglobin levels less than 7.4 g/dl as potentially life threatening anemia (grade 4). Granulocyte counts between 5/nl and 4/nl were defined as grade 1 toxicity; less than 4/nl to 3/nl as grade 2 toxicity; less than 3/nl to 1.5/nl as grade 3 toxicity and counts less than 1.5/nl as grade 4 toxicity. For follow-up visits, toxicity thresholds for granulocyte counts and hemoglobin levels were adjusted to age according to the DAIDS tables. Frequencies of adverse events were compared using Fisher's exact test. Prematurity was defined as delivery before 37 weeks of gestation. The Kaplan-Meier approach was used to estimate the cumulative proportion of infected infants at six weeks of age, representing short term efficacy [19]. As recommended by the Ghent Group [19,20], any PCR result collected from infants between 29 and 60 days of age in combination with earlier results were used for this estimation. A p-value below 0.05 was considered statistically significant.

Results

Study Population

During the study period, 1395 pregnant women were counseled and tested for HIV infection at the ANC clinic of KDH [7]. HIV infection was diagnosed in 220 women (15.8% of all tested), of whom 121 met the eligibility criteria for this study. The study population during all stages of observation is explained in Figure 1.

Eighty-two women had a pre-delivery AZT intake of at least one week and were therefore assigned to group 1. The median CD4 cell count at enrolment in this group was 390 (inter-quartile range [IQR]: 267 to 515) cells/mm^3 and the median gestational age at start of AZT intake was 29.1 (IQR: 28.0 to 32.2) weeks. Hematological parameters and the specifications of adverse events of these 82 women were analyzed during pregnancy. Fifty-five women of group 1 delivered at KDH with 41 infants born within this group having been exposed to AZT *in utero* for at least four weeks and identified as HIV-negative at birth. The median duration of AZT exposure was 57 (IQR: 43 to 71) days during pregnancy and the median CD4 cell count in mothers at delivery in this group was 350 (IQR: 252 to 490) cells/mm^3. Thirty-nine of the 41 mother-infant pairs returned for follow-up after one month. Two of the 39 infants were tested HIV positive at that point and were excluded from the analysis. At one month of age, 37 infants remained for assessing hematological alterations. Sixteen group 1-infants were available for blood analysis upon their three month return visit.

In the same period, 62 women meeting the inclusion criteria were enrolled at the time of delivery and assigned to group 2. The median CD4 cell count in these women was 262 (IQR: 194 to 474) cells/mm^3. Four infants of this group were identified as HIV-positive at delivery and were excluded from the cohort, leaving 58 infants available for the analysis at the time of birth. Twenty-three HIV-negative infants remained for analysis at one month of age, and 12 were returned for follow-up at three months.

Baseline characteristics of both groups, including age, weight, years of education, marital status and malarial symptoms did not differ significantly among the two groups (tables 1 and 2).

Hematological Alterations in Women

Eighty-two women in group 1 had an AZT intake of at least one week and were included in the analyses of blood alterations. Coefficients for alterations in blood components over time were estimated with a fixed effects model for the duration of AZT intake. Mean corpuscular volume (MCV) (coef.: 0.0636fl per day, p<0.001), red distribution width (RDW) (coef.: 0.0655% per day, p<0.001) and platelet count (coef.: 1.0309/nl per day, p<0.001) increased significantly over the time of AZT intake. Red blood count (RBC) (coef.: −0.0040*10^6/µl per day, p<0.001), white blood cell count (WBC) (coef.: −0.0119/nl per day, p<0.001) and granulocyte count (coef.: −0.0123/nl per day, p<0.001) decreased significantly over time. A significant decrease with a subsequent increase after day 32 of AZT intake was shown for hemoglobin values (p<0.05). Comparing group 1 and 2 at the point of delivery, differences between median hemoglobin and median granulocyte count were not statistically significant. Median red blood cell count was significantly lower in women of group 1 (3.66/µl vs. 4.04/µl, p<0.05), whereas MCV (87fl vs. 79fl, p<0.001) and median platelet count (367000/mm^3 vs. 273500/mm^3, p<0.05) were significantly higher in group 1-women with antenatal AZT intake. No significant differences in hematological parameters were observed at one month and three months post-delivery. Detailed results are shown in the supplementary Figure S1.

Hematological Alterations in Infants

At birth, infants of group 1 with prenatal AZT exposure showed significantly lower median hemoglobin (13.3 g/dl vs. 15.2 g/dl, p<0.001) and median RBC (3.7/µl vs. 4.5/µl, p<0.001) levels compared to those of group 2. The median MCV (106fl vs. 100fl, p<0.001) and RDW (17.1% vs. 16.2%, p<0.01) were significantly higher in infants exposed to AZT during the prenatal period. At birth, the overall frequency of anemia ≥ grade 1 was 26%, with a significantly higher frequency in infants with antenatal AZT exposure (47% group 1 vs. 11% group 2, p<0.001). Two infants with prenatal AZT exposure had severe (grade 3) or potentially life threatening (grade 4) anemia (6% of group 1-infants) at birth compared to zero in group 2.

The median hemoglobin concentrations declined in all infants from 14.3 (IQR: 13.0 to 15.5) g/dl at birth to 11.6 (IQR: 9.9 to 12.7) g/dl at one month of age and to 10.5 (IQR: 9.9 to 11.4) g/dl at three months of age. At one month (IQR: 31–36days) and at three months (IQR: 91–93 days) of age, differences in median hemoglobin and median RBC between both groups were no longer significant.

At one month of age, anemia ≥ grade 1 was observed in 29% of all infants (36% group 1 vs. 20% group 2, p = 0.35), with one of the group 1-infants (3%) showing severe anemia. At three month
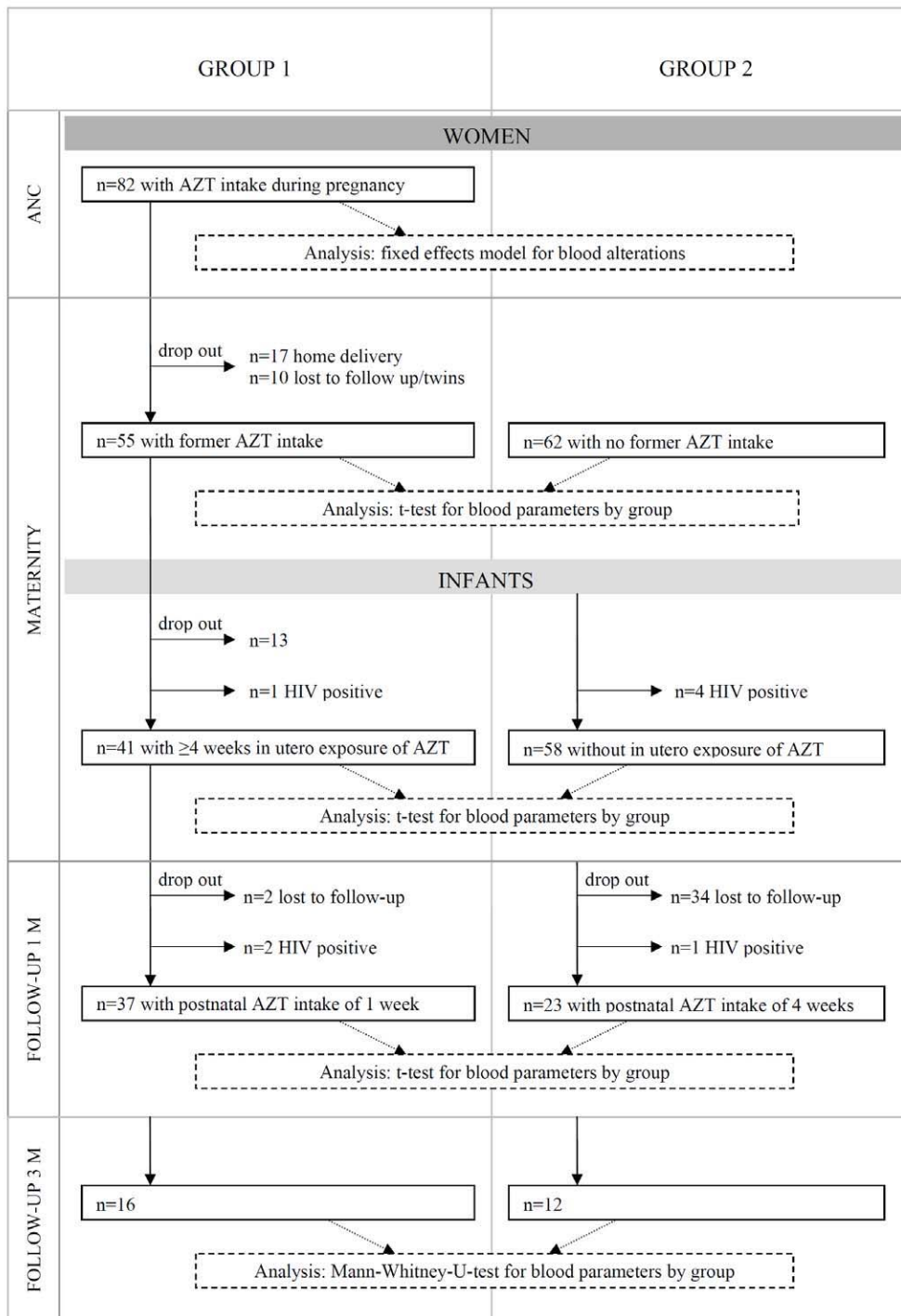
Figure 1. Flow chart of study cohort. Study population and applied statistical tests during antenatal care visits, delivery and follow-up visits at one month and three months post-delivery.
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of age, the overall anemia rate ($\geq$ grade 1) was 58% (46% in group 1 vs. 73% in group 2-infants, p = 0.24).

The median granulocyte count at birth was significantly lower in infants with AZT exposure during pregnancy (5.0/nl in group 1 vs. 7.3/nl in group 2, p<0.05). At birth, granulocytopenia $\geq$ grade 1 was observed in 37% of all infants with a significantly higher

frequency in group 1-infants (52% in group 1 vs. 26% in group 2, p<0.05). Toxicity $\geq$ grade 3 was observed in 18% of group 1-infants compared to 9% of group 2-infants, p = 0.31.

The overall median granulocyte count was 6.2 (IQR: 3.9–8.6)/nl and decreased to 2.8 (IQR: 1.6–3.4)/nl at one month of age and to 2.7 (IQR: 1.8–3.3)/nl at three months of age. At one month, the

Table 1. Baseline characteristics of mothers of group 1 and 2.

	Group 1	Group 2	p
	Enrolment in antenatal clinic/ pre-delivery AZT intake	Enrolment in maternity ward/no pre-delivery AZT intake	
Total number of pregnant women included	82	62	
Pregnancy week at start of AZT intake [median (IQR)]	29.1 (28.0–32.2)		
CD4 at enrolment in antenatal clinic [median (IQR) cells/µl)]	390 (267–515)		
Place of delivery [no. (%)]:			
Maternity ward	55 (67%)	62 (100%)	
Home delivery	17 (21%)[a]	0	
Lost to follow-up	10 (12%)[a]	0	
Marital status: married [%]:	74.5%	76.7%	0.96[b]
Years of education [median (IQR) years]	7 (7–7)	7 (7–7)	0.20[b]
Travel minutes to hospital [median (IQR)]	30 (30–60)	30 (30–60)	0.35
Household number [median (IQR)]	4 (3–5)	3 (2–5)	0.51
Number of children [median (IQR)]	2 (1–3)	1 (0.5–2)	0.40
Age at enrolment [median (IQR) years]	28 (24–30)	25 (23–29)	0.58
Weight at enrolment [median (IQR) kg]	60 (54–65)	57 (53–65)	0.64
Height [median (IQR) cm]	158 (154–160)	157 (152–160)	0.93
Gravida [median (IQR)]	3 (2–3)	3 (2–3)	0.61
Para [median (IQR)]	2 (1–2)	2 (1–3)	0.98

[a]Excluded from below socio-demographic comparison.
[b]Mann-Whitney-U-test; all other compared by t-test.
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Table 2. Baseline characteristics of infants of group 1 and 2.

	Group 1	Group 2	p
	Enrolment in antenatal clinic/ pre-delivery AZT intake	Enrolment in maternity ward/no pre-delivery AZT intake	
No AZT intake during pregnancy [no]		62	
At least four weeks antenatal AZT/no twins/available blood count results [no]	42		
HIV positive children at delivery [% (no)]	2.4% (1/41)	8.5% (4/47)	
Children enrolled in study [no]	41	58	
CD4 of mothers at delivery [median (IQR) cells/µl; no.]	350 (252–490); 25	262 (194–474); 40	0.26
Duration of prenatal AZT exposure [median (IQR) days]	57 (43–71)	0	
Duration of AZT syrup intake of infants [days]	7	28	
Mode of delivery: section [%]	7.5	11.1	0.41[b]
Preterm infants [%]	9.8	3.5	0.20[b]
Sex: female [%]	46.3	54.4	0.28[b]
Weight [median (IQR) g]	3100 (2840–3450)	3200 (2900–3500)	0.67
Apgar newborns at 1 minute	9 (8–9)	9 (8–9)	0.63[a]
Apgar newborns at 5 minutes	10 (10–10)	10 (10–10)	0.28[a]
Frequency of symptom maternal fever at delivery [%]	2.4	0	0.42
Median no. of sulphadoxine-pyrimeth. doses during pregnancy	2 (2–2)	2 (1–2)	0.10[a]

[a]Mann-Whitney-U-test.
[b]Fisher's exact test; all other compared by t-test.
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median granulocyte count was significantly lower in group 2-infants compared to group 1-infants (3.2/nl in group 1 vs. 1.7/nl in group 2, p = 0.001). At this age, 19% of all infants presented with granulocytopenia ≥ grade 1, with a significantly higher frequency in group 2-infants (9% in group 1 vs. 33% in group 2, p = 0.039). Toxicity ≥ grade 3 was observed in 6% of group 1-infants and 10% of group 2-infants, p = 1.0.

The hematologic parameters of infants in group 1 and group 2 are summarized in table 3 and figure 2. Frequency of anemia and granulocytopenia are shown in table 4.

Transmission Rate

HIV-DNA PCR was performed in 88 infant blood samples at birth and in 63 infant blood samples at six weeks of age.

The *in utero* transmission rate tested at birth was 2.4% (CI: 0.35–16.0) in group 1-infants and 8.5% (CI: 3.3–21.1) in group 2-infants. The transmission rate estimated by Kaplan-Meier at six weeks of age was 7.7% in group 1 (CI: 2.5–22.1) and 12.5% (CI: 5.2–28.5) in group 2-infants.

Discussion

Focusing on hematological parameters in pregnant women and their infants up to the age of three months in a rural Tanzanian setting, this study was conducted to assess the impact of antiretroviral prophylaxis involving treatment of both mothers and infants with AZT.

Our evaluation was performed within the frame of an observational study in which all patients received antiretroviral prophylaxis as recommended by WHO guidelines. The two subgroups of our cohort were comparable with regard to the clinical and socio-demographic variables tested.

During AZT intake, pregnant women showed a significant decline in granulocyte count and RBC. Hemoglobin decreased within the first four weeks of AZT intake and then increased. Concurrently, a significant increase of MCV, RDW and platelet count was observed. However, at birth, there was no significant difference in hemoglobin between women taking AZT during pregnancy (group 1) and those did not (group 2). The values for MCV, RDW and platelet count were significantly higher in group 1-women at birth and RBC counts were significantly lower.

The transient decrease of hemoglobin is in agreement with previous publications. Briand *et al.* [8] demonstrated a suppression of hemoglobin levels in pregnant women taking AZT. Comparing short versus long exposure during pregnancy, a slight difference in hemoglobin level persisted until the time of delivery whereas differences in the other values resolved by this time.

It is known that platelet counts may generally decrease during pregnancy [21]. However, our fixed effects model showed an increase during AZT treatment of pregnant women, and a significantly higher platelet count in group 1- compared to group 2-women was observed at the time of delivery. This indicates that AZT treatment may increase platelet counts in HIV-infected pregnant women.

AZT has been shown to cross the placenta, reaching a cord-to-maternal blood level ratio of 0.8 [22] and to affect myeloid and erythroid cell lines [23], resulting in a decrease in hemoglobin levels and the number of granulocytes.

Our study found significantly lower hemoglobin levels in infants with intrauterine AZT exposure (group 1) at birth. The effect on hemoglobin was associated with a significantly higher frequency of
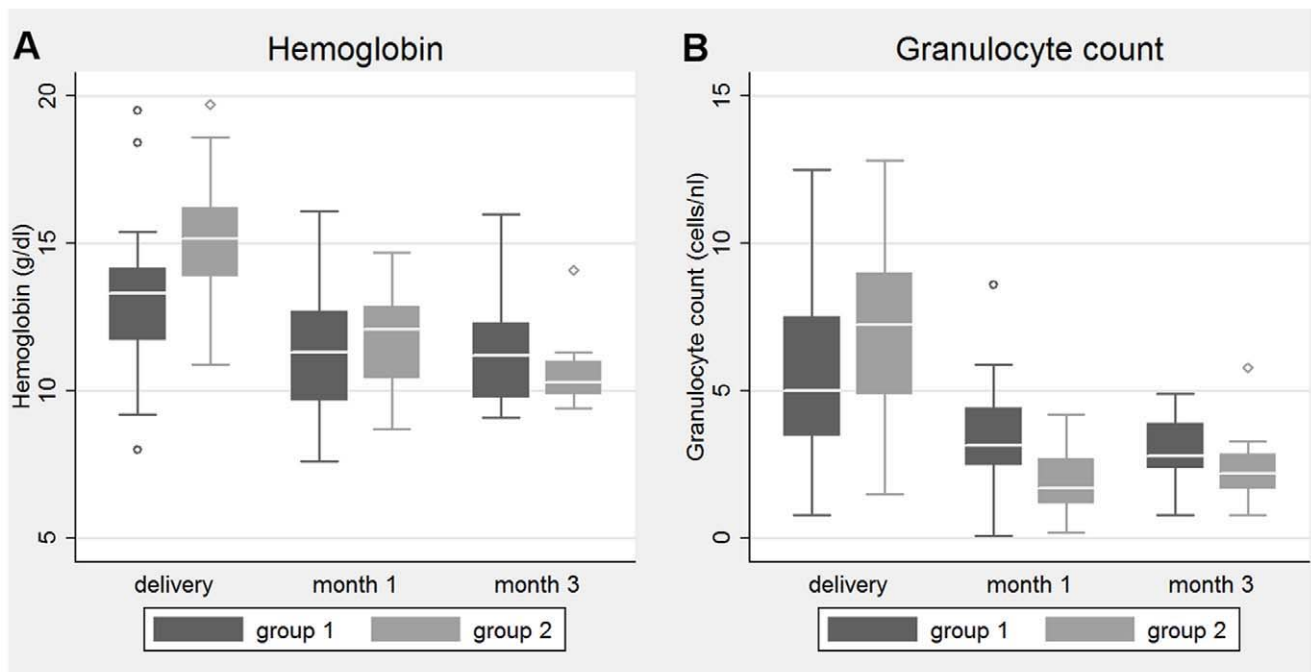

Figure 2. Hemoglobin and granulocyte count in infants at birth, one month and three months of age. A. At birth, group 1-infants with prenatal AZT exposure presented with significantly lower median hemoglobin (13.2 g/dl vs. 15.2 g/dl, p<0.001) than group 2-infants. At one month and at three months of age, differences in median hemoglobin between both groups were no longer significant. B. The median granulocyte count at birth was significantly lower in infants with AZT exposure during pregnancy (5.1/nl in group 1 vs. 7.3/nl in group 2, p<0.05). At one month of age, the median granulocyte count was significantly lower in group 2-infants compared to group 1-infants (3.2/nl in group 1 vs. 1.7/nl in group 2, p = 0.001). No statistically significant differences in granulocyte count were observed at three months of age.
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Table 3. Hematological parameters of infants by group at birth, 4–6 weeks of age and 12 weeks of age.

	Birth			Week 4–6 (IQR: 31–36; n = 62)			Week 12 (IQR: 91–93; n = 31)		
	group 1	group 2	p	group 1	group 2	p	group 1	group 2	P[a]
RBC, median	3.7 (3.2–4.0)	4.5 (4.2–4.8)	**<0.001**	3.6 (3.0–4.0)	3.7 (3.4–4.1)	0.58	4.4 (4.1–4.5)	4.1 (3.9–4.5)	0.18
(IQR), 10^6/uL			n = 78			n = 51			n = 24
Hemoglobin, median	13.3 (11.8–14.2)	15.2 (13.9–16.2)	**<0.001**	11.3 (9.7–12.7)	12.1 (10.5–12.9)	0.49	11.2 (9.8–12.3)	10.3 (9.9–11.0)	0.6
(IQR), g/dl			n = 78			n = 51			n = 24
MCV, median	106 (101–114)	100 (97–105)	**0.001**	95 (89–101)	92 (89–94)	0.27	74 (70–81)	75 (72–78)	0.47
(IQR), fl			n = 78			n = 51			n = 24
RDW, median	17.1 (16.2–18.1)	16.2 (15.6–16.9)	**0.008**	17.1 (16.8–18.3)	15.7 (14.8–17.1)	**0.002**	15.9 (15.6–16.9)	16.0 (14.6–16.6)	0.54
(IQR), %			n = 75			n = 51			n = 24
WBC, median	10.4 (8.2–13.6)	12.8 (10.9–17.1)	0.07	9.5 (7.6–11.5)	8.5 (6.3–10.9)	0.35	10.6 (8.8–14.7)	8.9 (6.4–11.4)	0.11
(IQR),/nL			n = 79			n = 53			n = 25
Granulocytes, median	5.0 (3.5–7.5)	7.3 (4.9–9.0)	**0.042**	3.2 (2.5–4.4)	1.7 (1.2–2.7)	**0.001**	2.8 (2.4–3.9)	2.2 (1.7–2.9)	0.18
(IQR),/nL			n = 79			n = 53			n = 25
Lymphocytes, median	4.0 (3.5–6.2)	4.9 (3.2–6.4)	0.42	4.8 (3.9–7.3)	5.3 (4.1–8.3)	0.62	6.8 (5.9–10.2)	6.1 (3.7–7.7)	0.18
(IQR),/nL			n = 79			n = 53			n = 25
Monocytes, median	1.0 (0.6–1.6)	1.3 (0.7–1.9)	0.42	0.9 (0.7–1.3)	0.9 (0.7–1.3)	0.73	0.8 (0.6–1.4)	0.8 (0.5–1.1)	0.16
(IQR),/nL			n = 79			n = 53			n = 25
Platelets, median	375 (303–436)	326 (245–386)	0.09	397 (248–493)	420 (216–533)	0.79	440 (284–568)	403 (215–569)	0.64
(IQR),/nL			n = 76			n = 49			n = 23

[a]Mann-Whitney-U-test, all other compared by t-test.
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anemia grade ≥1 at birth. These results agree with those of Connor *et al.* and Sperling *et al.* [12,14], who both showed significantly lower levels of hemoglobin in infants exposed *in utero* to AZT. In our study, differences in hemoglobin were no longer significant by the age of one month, which is most likely the result of differences in postnatal AZT intake and a faster decrease of hemoglobin in group 2-infants, again agreeing with Connor *et al.* and Sperling *et al.* The overall rate of anemia ≥ grade 1 was 29% with no significant difference between the groups at one month of age. In contrast, Briand *et al.* found significant differences in the

frequency of anemia between infants with three days or six weeks of postnatal AZT intake at the age of six weeks. Interestingly, this effect was found to be independent of *in utero* exposure [24].

At birth, infants of group 1 had significantly lower granulocyte counts, accompanied by a significantly higher frequency of granulocytopenia ≥ grade 1. This effect of *in utero* exposure to AZT in infants, including persistence of decreased granulocyte counts up to 18 months after birth, has been described previously [11].

Table 4. Frequency of anemia and granulocytopenia in infants of group 1 and 2.

Anemia	Birth (n = 78)			Month 1 (n = 51)			Month 3 (n = 24)		
	group 1	group 2	P[a]	group 1	group 2	P[a]	group 1	group 2	P[a]
grade ≥1	15 (46.9%)	5 (10.9%)	**0.001**	11 (35.5%)	4 (20.0%)	0.35	6 (46.2%)	8 (72.7%)	0.24
grade ≥3	2 (06.3%)	0 (00.0%)	0.17	1 (03.2%)	0 (00.0%)	1	0 (00.0%)	0 (00.0%)	1

Granulocytopenia	birth (n = 79)			month 1 (n = 53)			month 3 (n = 25)		
	group 1	group 2	P[a]	group 1	group 2	P[a]	group 1	group 2	P[a]
grade ≥1	17 (51.5%)	12 (26.1%)	**0.033**	3 (09.4%)	7 (33.3%)	**0.039**	1 (07.7%)	2 (16.7%)	0.59
grade ≥3	6 (18.2%)	4 (08.7%)	0.31	2 (06.3%)	2 (09.5%)	1	0 (00.0%)	0 (00.0%)	1

[a]Fisher's exact test.
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After receiving AZT for one month in the postnatal period, group 2-infants had a significantly lower granulocyte count and significantly higher frequency of granulocytopenia ≥ 1 than group 1-infants having a postnatal AZT intake of just one week. By the age of one month, 9% of infants had developed a severe granulocytopenia ($\geq$ grade 3), most likely due to *in utero* and postpartal exposure to AZT.

Throughout this study, alterations in the mothers' and infants' blood parameters were carefully monitored. However, it must be kept in mind that the routine health care in low-income countries only rarely includes monitoring of hemoglobin and granulocyte counts during AZT-intake. Despite PCR-HIV testing of infants blood samples at the age of one month being common, economic or logistic constraints preclude the regular monitoring of blood components. It is therefore important to include this lack of monitoring in debates about the potential toxicity of ARVs in the context of peripheral, resource-limited settings.

The transmission rate estimated by Kaplan-Meier was 7.7% in group 1 and 12.5% in group 2-infants at 6 weeks of age. Two randomized, double-blind, placebo-controlled trials have shown that AZT administered in the last four weeks of pregnancy is effective in reducing the transmission rate to 14.7% compared to 24.8% with placebo at six weeks of age [25]. Dabis *et al.* [6] have shown that combining the four-week course of AZT with sdNVP further reduces the six week probability of transmission to 6.5%, a finding largely consistent with our own.

To ensure comparability of the two groups, we checked for differences in disease progression in terms of CD4-cell counts, as well as with regards to socio-demographic aspects such as age, weight, marital status or years of education and found that none of these factors differed significantly between groups 1 and 2. Nevertheless, it cannot be ruled out that there may be other unconsidered factors such as health-seeking behavior that might vary between the two groups and act as confounders.

At the point of birth, group 2-infants, who had no prior AZT exposure during pregnancy, represent a true control group for analyzing the influence on group 1- infants of AZT exposure during pregnancy. However, during follow-up visits at one and three months of age, infants of both groups had different pre- as well as postnatal ARV exposure and are not strictly comparable. It is a limitation of this study that alterations in blood parameters at these time points can therefore not explicitly be assigned to either part of the regimen. However, establishing a control group with no AZT exposure at all would have exceeded the frame of a study that was primarily aimed at describing the hematological effects of PMTCT regimens actually administered in practice.

A further limitation of our study is the high drop-out rate of infants throughout the follow-up period. Loss to follow-up is a common problem for PMTCT services in resource-limited settings [26,27]. In addition to the parents failing to understand the importance of follow-up visits, especially in the absence of illness, infant death is a major reason for loss to follow-up, as described by Ahoua *et al.* [28]. In our study, no information about the development of infants lost to follow-up exists and cases of undiagnosed virus transmission, side effects or mortality among such infants cannot be ruled out.

In conclusion, this study revealed that AZT exposure during and after pregnancy can cause significant hematologic alterations in women and infants. However, it was also shown that the side effects observed were generally transient and predominantly mild

in nature. However, the few cases of severe hematologic toxicity ($\geq$ grade 3) in infants should be taken into serious consideration when planning and implementing antiretroviral PMTCT interventions in structure-limited settings, where surveillance of blood alterations is often not standard practice. Our results demonstrate that monitoring side effects during antiretroviral PMTCT regimens is a crucial factor for intervention safety, and should be a constituent part for any such services. Further research involving larger cohorts and longer follow-up periods are needed to further analyze the impact of regimens involving AZT on maternal and infant health.

Supporting Information

Figure S1 Blood values in women of groups 1 and 2 during AZT intake Figures show selected blood values in group 1- and 2-women during AZT intake. Time points are: initiation of AZT intake (day 0), 7th, 14th, 21st, 28th day of AZT intake, delivery and one month post-delivery. The period between 28th day of AZT intake and delivery differs between women, depending on the gestational stage at initiation of AZT. Group 1-women had antenatal AZT intake, group 2-women were included at delivery and had no antenatal AZT intake. Sample sizes were: $n \geq 70$ at beginning, $n \geq 70$ at 7th day, $n \geq 56$ at 14th day, $n \geq 62$ at 21st day, $n \geq 41$ at 28th day of AZT intake. At delivery, sample sizes were $n \geq 30$ (group 1-women) and $n \geq 54$ (group 2 women); at one month post-delivery, sample sizes were $n \geq 39$ (group 1-women) and $n \geq 26$ (group 2-women). A. A decrease and a subsequent increase in hemoglobin values was shown in group 1- women. No significant difference between the median hemoglobin levels of group 1- and 2-women was observed at birth and one month post-delivery. B. Mean corpuscular volume increased with AZT intake and resulted in statistically significant higher values at delivery (87fl vs. 79fl, $p<0.001$) in group 1-women. C. Red blood count decreased in the first weeks of AZT intake. Median red blood count was significantly lower in group 1-women ($3.66/\mu l$ vs. $4.04/\mu l$, $p<0.05$) at delivery. There was no statistically significant difference at 1 month post-delivery. D. Platelet count increased during the time of AZT intake. At delivery, the median platelet count was significantly higher in women with antenatal AZT intake ($367.000/mm^3$ vs. $273.500/mm^3$, $p<0.05$), although by one month post-delivery the difference was no longer significant. E and F. White blood counts and granulocyte counts decreased during AZT intake. Comparing group 1 and 2 at delivery and one month post-delivery, differences in the median white blood count and median granulocyte count were not statistically significant. (TIF)

Acknowledgments

Author Contributions

Revision of manuscript: JZ AK JS IL FD AH WK GH ST. Conceived and designed the experiments: JZ AK JS IL FD GH ST. Performed the experiments: JZ JS IL FD. Analyzed the data: JZ AK IL AH WK ST. Contributed reagents/materials/analysis tools: JZ AK JS FD GH. Wrote the paper: JZ AK JS IL FD AH WK GH ST.

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Druckexemplar Publikation 2

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Zidovudine exposure in HIV-1 infected Tanzanian women increases mitochondrial DNA levels in placenta and umbilical cords

PLoS one

Zidovudine Exposure in HIV-1 Infected Tanzanian Women Increases Mitochondrial DNA Levels in Placenta and Umbilical Cords

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Abstract

Background: Zidovudine (AZT) constitutes part of the recommended regimens for prevention and treatment of HIV-1 infection. At the same time, AZT as well as HIV-1 infection itself may induce mitochondrial damage. In this study, we analyzed the impact of prenatal AZT-exposure on mitochondrial alterations in HIV-infected women and their infants.

Methods: Mitochondrial DNA (mtDNA) levels in placentas of HIV-1 infected Tanzanian women with and without prenatal AZT exposure, and in the umbilical cords of their AZT-exposed/unexposed infants were quantified using real-time PCR. Furthermore, we checked for the most common mitochondrial deletion in humans, the 4977 base pair deletion (dmtDNA4977) as a marker for mitochondrial stress.

Results: 83 women fulfilled the inclusion criteria. 30 women had been treated with AZT (median duration 56 days; IQR 43–70 days) while 53 women had not taken AZT during pregnancy. Baseline maternal characteristics in the two groups were similar. The median mtDNA levels in placentas and umbilical cords of women (311 copies/cell) and infants (190 copies/cell) exposed to AZT were significantly higher than in AZT-unexposed women (187 copies/cell; $p = 0.021$) and infants (127 copies/cell; $p = 0.037$). The dmtDNA4977 was found in placentas of one woman of each group and in 3 umbilical cords of AZT-unexposed infants but not in umbilical cords of AZT-exposed infants.

Conclusions: Antenatal AZT intake did not increase the risk for the common mitochondrial deletion dmtDNA4977. Our data suggests that AZT exposure elevates mtDNA levels in placentas and umbilical cords possibly by positively influencing the course of maternal HIV-1 infection.

Introduction

HIV-positive pregnant women can decrease the risk for in-utero vertical HIV transmission by intake of antiretroviral drugs (ARVs). Zidovudine (AZT) during pregnancy is a frequently used and WHO- recommended drug regimen [1]. However, it has been proven in human and animal studies that Nucleoside Reverse Transcriptase Inhibitors (NRTIs) like AZT can cause mitochondrial damages including depletion of mitochondrial DNA (mtDNA) [2–10].

One underlying mechanism of AZT-induced mitochondrial toxicity is the inhibition of human DNA polymerase gamma [11–12], the enzyme needed for replication of mtDNA. Other assumed mechanisms include increased mitochondrial oxidative stress, introduction of mtDNA mutations, negative effects on nucleotide phosphorylation and mitochondrial gene expression, depletion of L-carnitine and inhibition of the mitochondrial bioenergetic machinery [13–17].

However, also HIV-1 infection itself causes mitochondrial damage, like depletion of mtDNA and decreased activities of the mitochondrial respiratory chain complexes [18–21]. HIV-1 has been shown to induce mitochondrial toxicity in several ways: by loss of mitochondrial membrane potential, by increase of reactive oxygen species and through different mechanisms of the viral proteins Vpr, Tat and HIV protease [22].

In humans, the mitochondrial toxicity of antenatal NRTI-exposure was determined by measuring different mitochondrial parameters like the emergence of clinical mitochondriopathy or death [23–25], quantification of mtDNA [18,26–29], analysis of

mtDNA mutations [30] or expression of mitochondrial respiratory chain proteins [27].

Studies indicating NRTI-induced mitochondrial toxicity include a detailed analysis by Barret [23], who found a higher incidence of neuro-mitochondrial diseases in NRTI-exposed infants compared to NRTI-unexposed infants; Divi [28] found a decrease of mtDNA in umbilical cords of infants of HIV-positive mothers exposed to Combivir compared to infants of HIV-negative women. Shiramizu [26] measured lower mtDNA contents in placenta and cord blood of HIV-positive women following NRTI-exposure in comparison to HIV-negative individuals. Torres [30] detected a higher frequency of mtDNA mutations in umbilical cords of HIV-positive AZT exposed infants compared to HIV-negative infants.

In contrast, McComsey [27] identified increased mtDNA levels without changes in expression of mitochondrial respiratory chain proteins in infants of HIV-positive mothers having taken NRTIs compared to NRTI-unexposed infants of HIV-negative mothers. Williams [31] did not detect lower mental or motor functioning scores in HIV-exposed, uninfected infants who were in-utero exposed to ARVs including NRTIs compared to those unexposed to ARVs during pregnancy. Accordingly, two large cohort studies did not discover an increased risk for death or clinical manifestations suggestive of mitochondrial abnormalities in NRTI-exposed infants [24–25].

The only two studies comparing mtDNA levels exclusively among HIV-positive mothers and their infants came to contradictory conclusions. In blood samples of HIV-positive mothers and infants with and without prenatal AZT exposure, Poirier [29] found lower mtDNA levels in AZT-exposed infants, whereas Aldrovandi [18] identified higher mtDNA levels in women and newborns with antenatal AZT exposure.

Altogether, it has not been clarified whether the net effect of short-course AZT for drug-naive HIV-1 infected pregnant women and their infants is a positive or a negative one with regard to mitochondriopathy. In the present study, we therefore quantified the mtDNA content in placentas of HIV-1 positive women with and without antenatal AZT exposure and in umbilical cords of their AZT exposed/unexposed infants. Furthermore, we checked for the most common mitochondrial deletion in humans, the 4977 base pair deletion (dmtDNA4977) as a marker for mitochondrial stress [32].

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 Charitè-Universitätsmedizin Berlin, Germany. All participants had given written informed consent, and data and samples were treated strictly confidentially.

Clinical Samples

The present study is a sub-evaluation of an observational study analyzing feasibility and adherence regarding combination prophylaxis for the prevention of mother-to-child transmission of HIV-1 (PMTCT) at Kyela District Hospital (KDH), Mbeya Region, Tanzania between October 2008 and September 2009 [33].

According to the WHO guidelines from 2006 and the National Tanzanian PMTCT guidelines from 2007 [34–35], HIV-1 positive women without treatment indication (CD4 cell count >200 cells/µl) took AZT, starting in gestational week 28

$(2\times300$ mg per day), or anytime thereafter followed by single-dosed Nevirapine (200 mg) at labor onset and AZT (300 mg) every three hours, plus Lamivudine (150 mg) every 12 hours during labor; additionally, the mother took postnatal AZT/Lamivudine for one week. Newborns received single-dosed Nevirapine after birth and AZT for 1 week [33].

PMTCT clients who had taken antenatal AZT for at least 4 weeks and who delivered at KDH within the study period were eligible for this sub-study if placenta and/or umbilical cord specimens were available, if the mother delivered a singleton, and if both mother and infant were alive 48 hours after birth. The same eligibility criteria applied for HIV-1 positive women delivering at KDH who had not taken AZT during pregnancy, constituting the control group. These women were offered the same intrapartum and postpartum drug regimen as described above. Since the half-life of mtDNA in mammalian cells is several days [36], ARV exposure during labor and thus shortly before the samples were taken should not affect mtDNA levels and the presence of the dmtDNA4977 deletion. Aliquots of placenta and umbilical cords of HIV-1 positive women and their infants were sampled at delivery, frozen and stored at $-20°C$ for future DNA extraction. For the detection and quantification of HIV-1 RNA viral load in newborns, a plasma sample from delivery was used and analyzed by RT-PCR according to our previously published protocol [37]. Socio-demographic data, AZT intake and maternal and newborn parameters were documented using specific questionnaires during antenatal care and at delivery [33].

Quantification of Mitochondrial DNA and the Mitochondrial 4977 bp Deletion (dmtDNA4977)

DNA extraction. 50 mg of each tissue (placenta and umbilical cord) were subjected to DNA extraction using the Invisorb Spin tissue mini kit. Samples were incubated at $52°C$ overnight and vortexed in an Invisorb Gyrator to intensify the lysis process (both STRATEC Molecular, former Invitek, Berlin, Germany). DNA was eluted in 55 µl elution buffer and stored at $4°C$ or analyzed immediately by PCR.

Real-time PCR for absolute quantification of nuclear DNA. Nuclear DNA was quantified as previously described [38]. Briefly, a 98 bp fragment of the telomerase gene (forward primer: 5'-GGC ACA CGT GGC TTT TCG-3', reverse primer: 5'-GGT GAA CCT CGT AAG TTT ATG CAA-3') was used to specifically amplify nuclear DNA. Dilutions of control DNA (Promega, Mannheim, Germany) were prepared (100 ng, 10 ng, 2.5 ng, 1.0 ng, 0.5 ng, 0.1 per µl) and used as standards assuming approximately 1,500 haploid copies of the telomerase gene in 10 ng total control DNA [39]. Every sample was amplified in triplets using 2 µl of pure DNA extract. The thermal cycling program was: 10 s at $50°C$ and 10 min at $95°C$ for enzyme activation (allowing an automated hot start PCR), 40 cycles of denaturation for 30 s at $94°C$ and 60 s annealing at $65°C$ on an ABI 7300 Real Time PCR System (Applied Biosystems). This approach allowed calculation of the amount of DNA/µl in ng and the number of cells/µl.

Real-time PCR for absolute quantification of mitochondrial DNA. Absolute quantification of dmtDNA4977 and total mtDNA was done as presented in [40] with some changes. To test the precision and reliability of the real time PCR, the primer pair L15/H16 (np: L3304-L3328/H3564-H3539 in [41] was employed to produce synthetic ND1 targets of 260 bp in length, which were used as specific template molecules for undeleted wildtype mtDNA in the subsequent PCRs. These ND1 targets were purified from primer sequences by ultrafiltration through Centricon 30 membranes and quantified after gel

electrophoresis and detection on a gel imaging system (Geldoc, Biorad). Then, the molecules were serially ten-fold diluted from 10^6 to 1 copies/10 µl, and mixed with 100 ng mouse DNA (15,000 haploid genome equivalents) per 10 µl each to simulate the complexity of the human genome.

For quantification of dmtDNA4977, a 238-bp fragment was amplified using the primer pair L35/H45 (np: L8285-L8310/ H13499-H13475, [41]). These primers flank the breakpoints of the common deletion and preferentially amplify dmtDNA4977 under short cycle conditions. Known numbers of the purified 238-bp products served as targets for quantification of specifically deleted molecules in our DNA samples. The standard preparation was done in the same way as described for total mtDNA. For detection of specific PCR products, we used FAM-labeled probes for wildtype and VIC-labeled probes for dmtDNA4977. For sample analysis, a real time duplex-PCR was performed using standard mixtures of 1, 10, 100 and 1000 dmtDNA4977 specific fragments on a background of 106 wildtype specific template molecules. Each amplification was done in triplets. PCR was performed using a standard Immuno buffer and an Immolase polymerase at a concentration of 1 U/25 µl per reaction (both Bioline Germany). The concentrations of the primers, magnesium chloride, and dNTPs were 0.0002 mM, 1.5 mM, and 0.2 mM per dNTP, respectively. Dimethylsulfooxide (DMSO, SIGMA, Steinheim, Germany) was added as an additive in a concentration of 2%.

Statistical Analysis

For statistical analysis, the non-parametric Mann-Whitney U test was used to assess significant differences with regard to continuous variables between two independent samples whereas the 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. Levels of mtDNA in placenta and umbilical cord were logarithmically transformed and presented as box and whisker plots. For descriptive analysis, median and interquartile ranges (IQR) were calculated. Two-sided tests were used and P<0.05 was considered as statistically significant. Statistical analysis was carried out using PASW Statistics 18 (SPSS Inc., Chicago, Illinois, USA).

Results

Sample Characteristics

In total, 83 women and their infants fulfilled the inclusion criteria. Samples of 30 women having taken AZT antenatally for a median duration of 56 days (IQR 43–70 days) and their infants were compared with samples of 53 women without pre-delivery AZT exposure and their infants. No significant differences between the two groups could be observed with regard to maternal CD4 cell count or socio-demographic variables like age, weight, education or marital status of the mother or infant's birth weight and length (table 1).

The proportion of HIV-1 infected newborns at birth was similar in the two groups; 4/44 AZT-unexposed infants versus 2/30 AZT-exposed infants (p = 1.0). Since the exclusion of data of HIV-1 infected infants did not change the results (data not shown) we decided to keep the data of HIV-infected infants in the analysis.

Levels of mtDNA in Placenta and Umbilical Cord

The median mtDNA level was significantly higher in women exposed to AZT (311 copies per cell, IQR 166–475) compared to women without AZT-exposure (187 copies per cell, IQR 115–352;

p = 0.021). Accordingly, the median mtDNA level was significantly higher in umbilical cords of infants exposed to AZT (190 copies per cell, IQR 121–323) compared to infants without AZT-exposure (127 copies per cell, IQR 70–234; p = 0.037). The box and whisker plots of mtDNA levels in placentas of HIV-1 infected women and in umbilical cords of their infants according to antenatal AZT-exposure are shown in figure 1. Restricting the analysis to women having taken AZT during pregnancy, we did not find a correlation between the duration of antenatal AZT intake in days and mtDNA levels in placenta (p = 0.95) and umbilical cord (p = 0.76).

Frequency of dmtDNA4977 in Placenta and Umbilical Cord

The mitochondrial 4977-bp deletion was rarely found in placental tissues (1/43 AZT- unexposed women versus 1/24 AZT-exposed women, p = 1.0). These two women displayed the mtDNA4977 at very low proportions with mutant DNA in percentage of the total DNA being 2.6E-05 (woman unexposed to AZT and 2.9E-05 (woman exposed to AZT).

In umbilical cords of infants, the dmtDNA4977 was detectable in 3/47 (6.4%) infants without AZT exposure and in no infant with prenatal AZT exposure (0/23); this was not statistically different (p = 0.55). The proportion of mutant DNA in percentage was higher in umbilical cords than in the placental tissues: 9.4E-04, 6.9E-03 and 1.4E-02.

In no case, the dmtDNA4977 was detected in both placenta and umbilical cord of a mother-child-pair.

Discussion

There is a high level of evidence from animal and human studies that both AZT and HIV-1 can cause mitochondrial toxicity and reduce mtDNA levels [2–10,18–22].

As a main finding, our study detected higher mtDNA levels in AZT-exposed mother-child- pairs compared to unexposed ones. This is in accordance with findings by Aldrovandi [18], who analyzed samples of HIV-positive mothers with similar HIV-1 disease progression but differing antenatal exposure towards NRTIs; higher mtDNA levels in the AZT-exposed mother-infant group were detected even five years later. Contradictingly, Poirier et al. [29] achieved results indicating lower mtDNA levels in the AZT-exposed infants. Yet, this study is only partly comparable because both infant groups differed remarkably in the HIV-1 disease progression of their mothers, with those having taken AZT displaying a tenfold higher viral load and >twofold lower CD4 cell count levels [29].

We analyzed mtDNA levels in placentas and umbilical cords instead of PBMCs. While mtDNA levels in PBMCs do not necessarily correlate with mtDNA levels in other tissues or with clinical signs of mitochondriopathy [42–44], placentas and umbilical cords seem to reflect mitochondrial changes induced by AZT: antenatal AZT–exposure led to decreased mtDNA levels in placentas and umbilical cords of fetal patas monkeys, which was correlated with an increase in mitochondrial morphological changes [6,9]. This suggests that mtDNA levels in placenta and umbilical cord reflect mitochondrial damage and are thus suitable markers of potential mitochondrial toxicity following AZT-exposure.

To our knowledge, this study is the first to analyze the presence and the relative amount of dmtDNA4977, the most common mitochondrial mutation, in placentas of HIV-infected women and umbilical cords of their infants with and without exposure to ARVs. In our study, dmtDNA4977 detection was rare and could

Table 1. Demographic and clinical characteristics of HIV-1 infected women and their infants with or without AZT exposure during pregnancy.

Characteristics	n	No antenatal AZT (n = 53) % or median (IQR)	n	Antenatal AZT (n = 30) % or median (IQR)	p-value
Duration of AZT intake, days	53	No intake	30	56 (43–70)	
Maternal age, years	51	25 (23–29)	30	28 (25–30)	0.12
Maternal weight, kg	46	59 (55–68)	29	61 (56–65)	0.38
Education, years	45	7 (6–7)	26	7 (7–7)	0.26
Marital status, married	50	72	28	75	0.77
Gravidity	51	3 (2–3)	30	3 (2–3.3)	0.54
Parity	51	2 (1–2)	30	2 (1–2.3)	0.68
Maternal CD4 count at delivery, uL	36	307 (184–462)	19	402 (272–492)	0.10
Prematurity (<37 wk gestation)	51	3.9	30	13.3	0.19
Mode of delivery, caesarean section	49	6.1	30	6.7	1.0
Apgar score at 1 minute	41	9 (8–9)	29	9 (8.5–9)	0.08
Female sex of infant	50	58	30	40	0.12
Infant birth weight, g	48	3100 (2800–3300)	29	3200 (2950–3500)	0.30
Child length, cm	42	48 (46–50)	27	48 (46–50)	0.71
Child head circumference, cm	42	35 (34–36)	27	36 (34–36)	0.11

only be detected in placentas of a single AZT-exposed and AZT-unexposed woman each, at very low proportions. This finding is consistent with other studies [45–46] which did not find the dmtDNA4977 in human placenta samples.

The occurence of mtDNA4977 has been shown to be age-dependent and was undetectable in tissue biopsies of children [47–48]. However, the deletion has been identified in brain, liver, kidney, heart, and muscle samples taken at autopsy of deceased neonates [49]. The authors speculate that mtDNA4977 could be generated by perinatal hypoxia or temporary oxygen oversaturations during the intensive care of the neonates.

Here, we demonstrate that mtDNA4977 can also be observed in newborns of HIV-1 infected women. We identified the dmtDNA4977 in umbilical veins of 3/70 (4.3%) infants, all unexposed to AZT. The amount of dmtDNA4977 out of the total mitochondrial DNA was low and varied between 0.00094% and 0.014%. As there was no significant difference between AZT-exposed and AZT-unexposed newborns (p = 0.55), we suggest that our findings could be explained by the influence of external factors other than AZT. It is well known that mitochondrial mutagenesis depends on many different factors such as alcohol [38,50] or nicotine [51]. While these stressors are known to damage mitochondrial DNA there are also findings on protective factors, e. g. green tea [52] or other dietary components. We did, however, not investigate such possibly influencing factors. It is also imaginable that the difference was not significant due to low sample size. On the other hand, since none of the AZT-exposed infants displayed the deletion, we cannot rule out the possibility that AZT has a benefical impact with regard to the emergence of the mtDNA4977.

Altogether, we did not find evidence for an increased risk resulting from AZT for the most common mitochondrial deletion in placenta or umbilical cord tissues of HIV-1 infected women and their infants.

There is no doubt that AZT can cause mitochondrial toxicity. However, the mitochondrial toxicity of AZT may be counter-balanced by the positive effect of AZT on maternal HIV-1 infection. Compared to individuals under ARV long-term treatment, the situation may be different in pregnant, drug-naive women taking AZT for a short period only. Generally, HIV-infection of the mother has a profound derogatory effect on the cell-mediated immunity and T-cell maturation of the infant [53]. In HIV-positive individuals, the inflammatory cytokine tumor necrosis factor alpha (TNF-alpha) is elevated [54–55] which also applies to placental trophoblastic cells [56–57]. However, increased TNF-alpha levels lead to mitochondrial DNA damage including mtDNA depletion [58–60]. Interestingly, AZT has been shown to down-regulate the expression of TNF-alpha in placental tissue [61]; this mechanism could prevent HIV-induced mitochondrial damage and explain the higher mtDNA levels in placenta and umbilical cord samples of AZT-exposed women and infants as observed in our study.

Accordingly, it has been shown that mtDNA levels in PBMCs of HIV-positive adults and infants increase after start of ARV treatment. This finding has been interpreted by some authors as restorative effect due to suppression of HIV-1 infection and by others as over-replication to compensate for mitochondrial dysfunction [21,44,62]. However, we believe that mtDNA over-replication as a sign for mitochondrial dysfunction is unlikely in our study, as it has been shown that antenatal AZT exposure leads to decreased mtDNA levels but increased mitochondrial morphological damage in placenta and umbilical cords of fetal patas monkeys [6,9].

There is a wide variety of factors influencing the mitochondrial toxicity of NRTIs, like the type of NRTI (e.g. d4T or AZT), the material to be analyzed (e.g PBMCs or tissues), mitochondrial outcome parameter (e.g mtDNA or activities of mitochondrial respiratory chain complexes) or stage of HIV-1 disease; this could explain the somewhat ambiguous results of the studies conducted so far.

There are also technical issues involved. Contamination with platelets is one possible confounder of measuring mtDNA
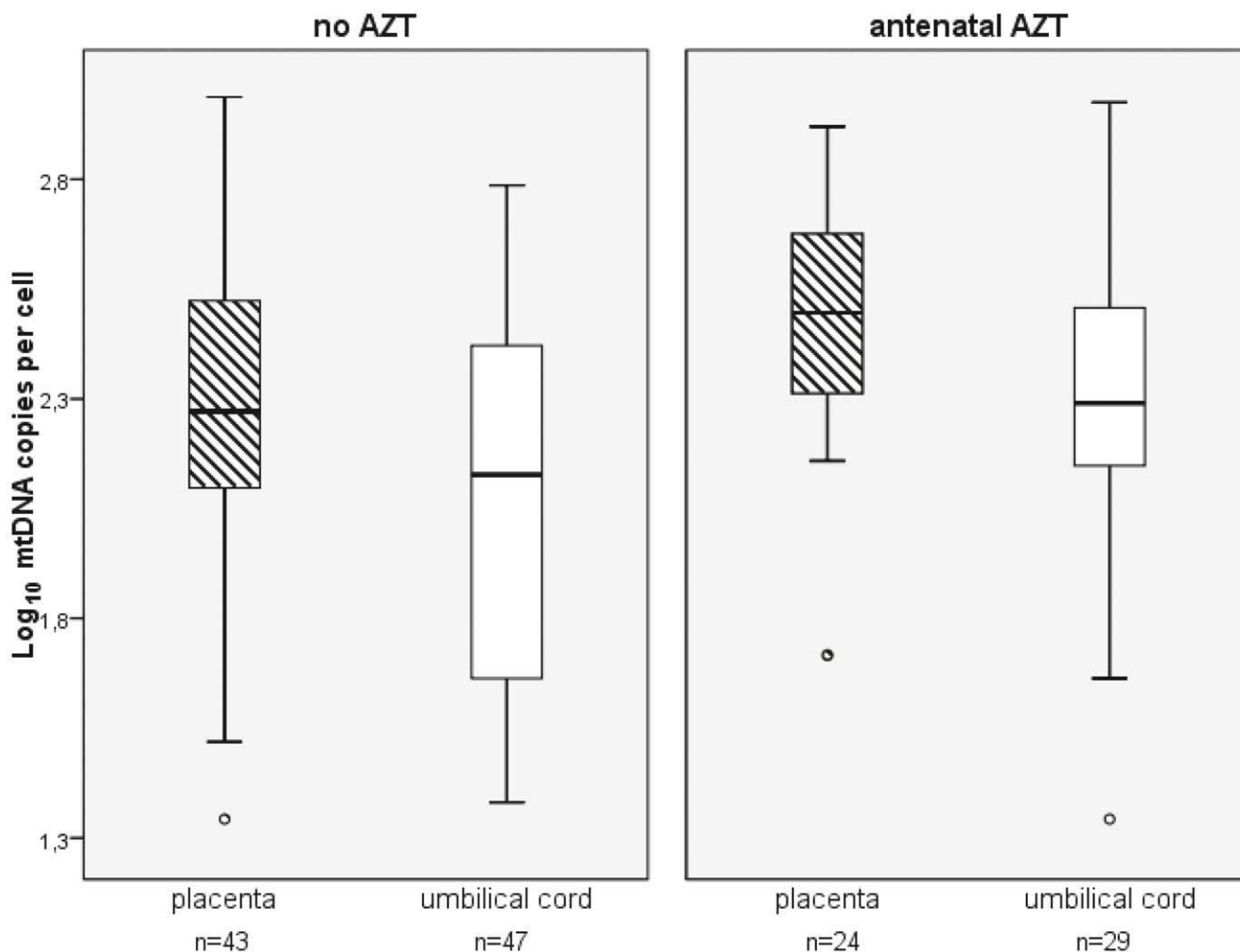
Figure 1. Box/whisker plots of mitochondrial DNA levels in AZT-exposed and AZT-unexposed placenta and umbilical cords. Box and whisker plots show mitochondrial DNA (mtDNA) levels in placentas of HIV-1 infected women and in umbilical cords of their infants according to exposure to antenatal AZT. The median mtDNA level was significantly higher in women exposed to AZT compared to women without AZT-exposure. Accordingly, the median mtDNA level was significantly higher in umbilical cords of infants exposed to AZT compared to infants without AZT-exposure.
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content in PBMCs by real-time PCR, as platelets are not completely removed during standard Ficoll gradient separation method [63–64]. In real time PCR, the mtDNA/nDNA-ratio is calculated; as platelets do contain mtDNA, but not nDNA, every increase of platelets leads automatically to a higher mtDNA/nDNA-ratio resulting in higher calculated mtDNA levels.

Furthermore, other factors like human mitochondrial DNA mutations and haplotypes may influence the development of HIV- and NRTI-associated mitochondrial dysfunction [65–70]. There are nine known European mitochondrial DNA haplotypes, whereas the greatest and still insufficiently characterized variety of mitochondrial haplotypes can be found in Africa; one study revealed 105 haplotypes with 75 forming a single, unique African haplogroup L [71]. Since we analyzed samples from Tanzanian women, it cannot be excluded that other populations harboring different mitochondrial DNA haplotypes react differently towards AZT-exposure.

In conclusion, in our setting of relatively immunocompromized drug-naive pregnant women from rural Tanzania, antenatal AZT intake seemed to improve mitochondrial parameters in the women and their infants.

Acknowledgments

Author Contributions

Conceived and designed the experiments: A. Kunz NWS JS PM JZ IL GH. Performed the experiments: A. Kunz NWS JS JZ IL PM FD AH A. Katerna. Analyzed the data: A. Kunz NWS ST GH. Contributed reagents/materials/analysis tools: NWS JS GH FD GH. Wrote the paper: A. Kunz NWS JS ST AH GH. Revision of manuscript draft: A. Kunz NWS JS JZ IL PM ST AH FD A. Katerna GH.

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Druckexemplar Publikation 3

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

Emergence of Minor Drug-Resistant HIV-1 Variants after Triple Antiretroviral Prophylaxis for Prevention of Vertical HIV-1 Transmission

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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.

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 $6.5 \times 10^1 – 10^7$ 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	

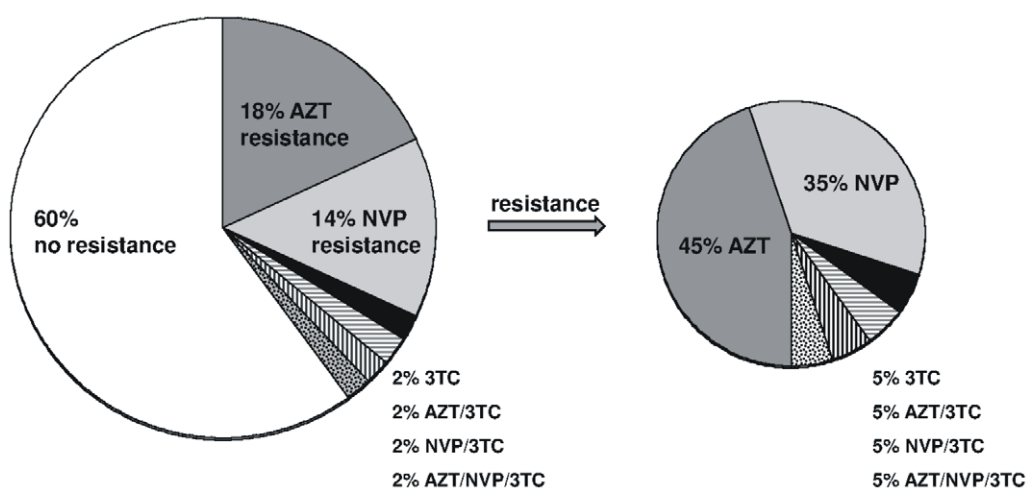doi:10.1371/journal.pone.0032055.t002

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 mono-therapy 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/mm³) compared to the relatively immunocompetent women in other studies (median: >500 cells/mm³) [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/mm³) in comparison to women without AZT resistance mutations (median: 406 cells/mm³; 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/mm³ instead of 200 cells/mm³ 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 log₁₀ and −0.3 log₁₀ by Sperling [47] and Clarke [48] and with −0.6 log₁₀

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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Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht angezeigt.

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Publikationsliste

Ziske J, Kunz A, Sewangi J, Lau I, Dugange F, Hauser A, Kirschner W, Harms G, Theuring S. Hematological Changes in Women and Infants Exposed to an AZT-Containing Regimen for Prevention of Mother-to-Child-Transmission of HIV in Tanzania. PloS one 2013;8:e55633.

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