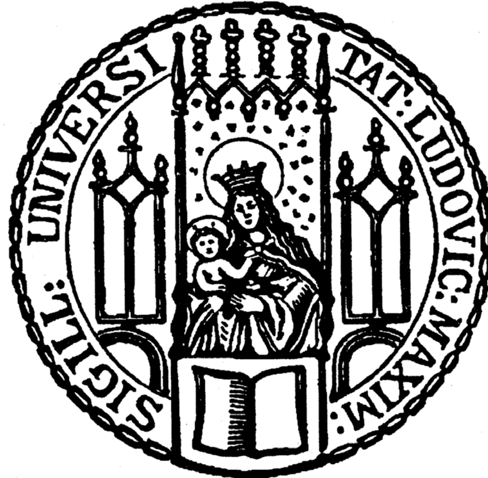
Aus dem
Klinikum der Universität München (LMU)
Abteilung für Infektions- und Tropenmedizin
(Direktor: Prof. Dr. med. Michael Hölscher)



Erregerspezifische Diagnostik tropentypischer Erkrankungen unter dem Einfluss von HIV

Kumulative Habilitationsschrift
vorgelegt von
Dr. med Inge Kroidl
(2017)

Inhaltsverzeichnis

ZUSAMMENFASSUNG……………………………………………………………………………………………………4

EINLEITUNG…….6

ZIELSETZUNG……9

DISKUSSION DER EIGENEN ARBEITEN…………………………………………………………………...........11

HIV……….11

Helminthen und HIV……………………………………………………………………………………….13

Lymphatische Filariose und HIV……………………………………………………………………….16

Tuberkulose und HIV……………………………………………………………………………………..19

EINORDNUNG DER EIGENEN ARBEITEN IN DIE LITERATUR……………………………………………….21

ABKÜRZUNGEN………………………………………………………………………………………………….....23

REFERENZEN…….24

LISTE DER FÜR DIE HABILITATION ZUSAMMENGEFASSTEN EIGENEN ARBEITEN…………………27

ORIGINALARBEITEN

ANHANG

Lebenslauf

Eigene Publikationsliste

Danksagung

Lehrtätigkeiten

Eidesstattliche Erklärung

Zusammenfassung

Zusätzlich zu den tropentypischen Erkrankungen werden die afrikanischen Länder südlich der Sahara von einer disproportionalen Ausbreitung des Human Immunodeficiency Virus (HIV) geplagt. In einer Region, die ohnehin mehr als andere unter Infektionserkrankungen leidet, wurde schon lange diskutiert, ob chronische Wurmerkrankungen zu einer erhöhten Suszeptibilität für HIV führen könnten. Helmintheninfektionen werden häufig bereits in der Kindheit erworben und können unbehandelt über Jahre im menschlichen Körper verbleiben. Da Helmintheninfektionen somit zeitlich der Lebensphase mit erhöhtem Risiko für den Erwerb einer HIV-Infektion vorausgehen, könnten sie für die HIV Epidemiologie eine Rolle spielen. Trotz vieler Hinweise, die diese Hypothese unterstützen, blieb der Beweis über lange Jahre aus. In der vorliegenden Habilitationsarbeit werden Daten einer großen prospektiven Studie in der Normalbevölkerung Süd-West-Tansanias ausgewertet und der Einfluss verschiedener Helminthen auf die Empfänglichkeit für eine HIV-Infektion untersucht. Die Infektion mit *W. bancrofti*, dem Erreger der lymphatischen Filariose (LF), die trotz adäquater Behandlung lange im menschlichen Körper bleibt, zeigte einen signifikanten Einfluss auf die HIV Inzidenz. Der Vergleich von Filarien-Infizierten mit nicht-Infizierten zeigt ein 3,2-fach erhöhtes Risiko für die HIV-Ansteckung bei den 14 bis 25-Jährigen, ein 2,4-fach erhöhtes Risiko für die 25 bis 45-Jährigen, und ein 1,2-fach erhöhtes Risiko für die über 45-Jährigen. Mehrere Konsequenzen können aus den Ergebnissen gezogen werden: Das erhöhte Risiko für den Erwerb einer HIV-Infektion kann sehr wahrscheinlich reduziert werden durch schnellere Elimination des Wurmes aus dem befallenen menschlichen Körper mit bereits verfügbaren moderneren Therapieschemata. Damit könnte ein erheblicher Beitrag zur HIV Prävention geleistet werden, der bislang nicht propagiert wurde. Die weiteren Untersuchungen zu HIV-Risikofaktoren in der Studienpopulation deckten auf, dass die verschiedenen Altersgruppen unterschiedlich empfänglich für diese Faktoren sind. Dies sollte bei Präventionsmaßnahmen berücksichtigt werden, die auf Zielgruppen zurechtgeschnitten werden. Es wurden innerhalb der großen prospektiven Studie auch intestinale Nematoden untersucht, die keinen signifikanten Einfluss auf die HIV Inzidenz zeigten. Interessant sind auch weitere Analysen, die versuchen die Hintergründe für die gesteigerte HIV Inzidenz zu beleuchten. Es wurden Veränderungen der Immunaktivierung und der Rezeptorbeladung der T-Helferzellen gefunden, die insbesondere bei Patienten infiziert mit *W. bancrofti*, aber nicht bei den mit intestinalen Helminthen Infizierten auftraten.

Die extreme Verbreitung des HI-Virus in den Ländern im südlichen Afrika hat zur Folge, dass andere Infektionen aber auch bösartige Erkrankungen in diesen Regionen an Häufigkeit zunehmen. Eine der häufigsten Ko-Infektionen in diesen Ländern ist die Tuberkulose (TB). Eine zügige und korrekte Diagnose und Therapie ist erschwert durch die paucibazilläre Natur, in der TB bei den HIV-Ko-

infizierten vorliegt. Mit zwei neueren diagnostischen Methoden, den Interferon-gamma-Release Assays (IGRA) sowie wie der Untersuchung des Lipoarabinomannan (LAM) habe ich mich näher befasst. LAM ist ein Teil der Zellwand von Mykobakterien und im Urin von Tuberkulosepatienten nachweisbar Die Verfügbarkeit von Urin und der unkomplizierte Testablauf machen diesen Test attraktiv. Die Studien der letzten Jahre hatten aber sehr schwankende Spezifitäten und recht niedrige Sensitivitäten gezeigt, was die Begeisterung über diese neue Testmethode dann doch begrenzt hat. Wir konnten in den hier beigelegten Studien in Tansania einiges zur Klärung der wechselhaften Spezifität beitragen. Die Sensitivität dieses Tests ist sehr unterschiedlich bei Personen mit bzw. ohne HIV-Infektion. Bei stark immunkompromittierten HIV-Infizierten werden tolerable Sensitivitäten erreicht, die diesen Test sinnvoll erscheinen lassen. Ebenfalls interessant als alternative diagnostische Möglichkeit für Tuberkulose sind die sogenannten Interferon-gamma Release Assays (IGRA). Wie der Tuberkulin-Haut-Test (TST) messen die IGRAs die spezifische Immunantwort gegen *M. tuberculosis* und zeigen eine aktive oder latente Tuberkulose an. Im Gegensatz zum Tuberkulin-Haut-Test reagieren die IGRAs nicht falsch positiv, bei Personen die mit dem BCG- Impfstoff geimpft wurden, oder die mit anderen (nicht-tuberkulösen) Mykobakterien (NTM) Kontakt hatten und sind somit erheblich spezifischer. Sie haben im Vergleich mit dem TST ebenfalls den großen Vorteil, dass sie korrekt negative Befunde unterscheiden können von einer immunkompromittierten Situation in denen die Tests nicht aussagefähig sind. Die HIV-Infektion führt sehr früh bereits zu einem Verlust der TB-spezifischen Gedächtniszellen. Dies beeinflusst die Interpretation eines positiven IGRAs bei den HIV/TB-Ko-infizierten und kann diagnostisch benutzt werden.

1 Einleitung

Die durch HIV ausgelöste Epidemie nahm ihren Ursprung im Westen Afrikas [1], und breitete sich dann global aus. Molekularepidemiologische Methoden legen eine Speziestransmission vom Schimpansen auf den Menschen bereits um 1920 nahe [1], aber erst 1981 trat das hierdurch ausgelöste Acquired Immunodeficiency Syndrom (AIDS) in das Blickfeld der Öffentlichkeit. Circa 78 Millionen Menschen wurden bislang infiziert, circa die Hälfte der Infizierten verstarb. Von den 36,7 Millionen Menschen, die zurzeit mit HIV infiziert sind [2], leben circa zweidrittel in Afrika südlich der Sahara. Schon lange wurden die Ursachen der disproportionalen Verbreitung des HI-Virus in dieser Region hinterfragt [3].

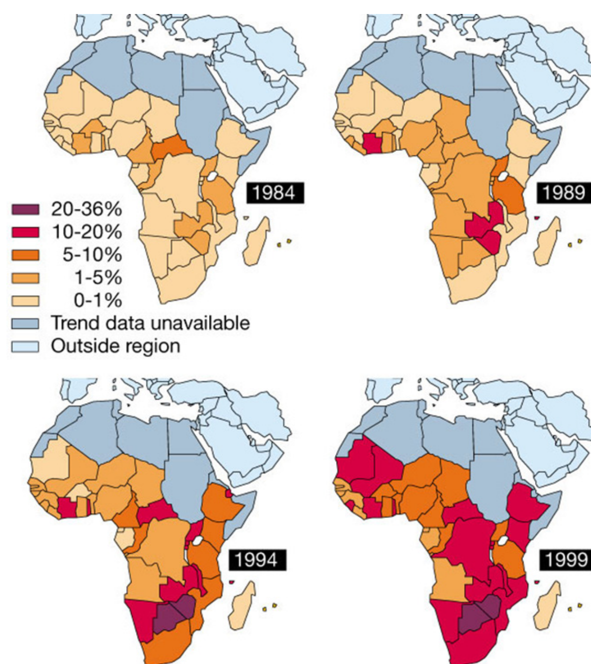


Abbildung 1: Geschätzte HIV Prävalenz in 15-49 jährigen in afrikanischen Ländern südlich der Sahara in 1984, 1989, 1994, 1999 (aus dem Artikel: The global impact of HIV/AIDS, Peter Piot, Michael Bartos, Peter D. Ghys, Neff Walker and Bernhard Schwartländer, Nature 410, 968-973 (19 April 2001)

Im Vergleich zu anderen Kontinenten, in denen die Ausbreitung von HIV erheblich langsamer verlief, leiden viele Menschen in afrikanischen Ländern an weiteren Infektionserkrankungen, wie z.B. Tuberkulose oder Wurmerkrankungen Es wurde daher bereits früh nach einer möglichen Rolle anderer Infektionen für die HIV Inzidenz und Pathogenese gefragt. Bentwich et al. formulierten 1995 die Hypothese, dass eine durch Wurminfektionen hervorgerufene Immunaktivierung sowohl die Rate der HIV-Neu-Infektionen, als auch den Krankheitsverlauf beeinflussen könnte [4-6]. Umgekehrt ist bekannt, dass die HIV-Infektion die Häufigkeit und den Verlauf anderer Infektionen verändern kann; Beispiele sind die opportunistischen Infektionen.

Das Thema dieser Habilitation sind die Interaktionen von HIV und anderen tropentypischen Infektionserregern. Dabei wird sowohl auf die Epidemiologie verschiedener Erreger und die eventuelle gegenseitige Beeinflussung, als auch auf die diagnostischen Schwierigkeiten unter dem Einfluss von HIV eingegangen.

HIV und die dadurch ausgelöste Erkrankung **AIDS** haben sich rasend schnell global ausgebreitet. Nach der Erstbeschreibung 1981 nahm die Zahl der weltweit Infizierten stetig zu. Massive Bemühungen zur Prävention der Übertragung haben in den letzten Jahren zu einer Stagnation der Zahlen geführt. Die Übertragung des Virus erfolgt über Blut, sexuelle Kontakte und von Mutter-zu-Kind. Präventionsmaßnahmen beinhalten Aufklärung über die Übertragungswege, Kondome, Zirkumzision sowie die antiretrovirale Therapie (ART) der Infizierten. Eine wirksame Impfung gegen HIV ist bislang trotz vieler Bemühungen nicht verfügfügbar. Weltweit waren in 2016 circa 50% der Infizierten mit ART behandelt, circa 77% der schwangeren Frauen erhielten eine Therapie zur Verhinderung der Mutter-zu-Kind-Übertragung. Die Zahl der Neuansteckungen liegt seit 2010 weltweit jährlich bei circa 2 Millionen und hat sich seit 2010 trotz massiver Anstrengungen nicht verringert. Weitere Informationen und mögliche Maßnahmen zur Prävention der HIV Infektion sind daher von großem Interesse.

Die sogenannten "durch-Erde-übertragenen Würmer" **(soil transmitted helminths) (STH)**, *Ascaris lumbricoides* und Hakenwürmer (*Ancylostoma duodenale* und *Necator americanus*) sowie *Trichuris trichiura* sind abhängig von verschiedenen Umweltfaktoren. Innerhalb des Menschen wandern sowohl der Spulwurm (*Ascaris lumbricoides)*, als auch die Hakenwürmer durch den Körper und passieren dabei mehrere Organe. Im Gegensatz dazu wird der Peitschenwurm (*Trichuris trichiura)* oral aufgenommen und verbleibt im Intestinaltrakt, in dem auch der Weg der anderen beiden Nematoden letztendlich mündet. Die Diagnostik der Helminthen erfolgt durch Untersuchungen des Stuhls. Die Behandlung der intestinalen Nematoden erfolgt mit Albendazol.

Eine Infektion mit dem Blut- und Gewebe-Nematoden ***Wuchereria bancrofti*** führt zur lymphatischen Filariose. Zirka 120 Millionen Menschen weltweit sind von dieser Erkrankung betroffen, circa ein Drittel von Ihnen leidet unter den Folgeerscheinungen, einer Elephantiasis oder einer Hydrozele [7, 8]. Im Gegensatz zu den intestinalen Nematoden wird *W. bancrofti* von Mücken übertragen. Diese nehmen die im Blut befindlichen Mikrofilarien auf, und übertragen nach circa 14 Tagen eine infektiöse Larve auf den Menschen. Seit dem Jahr 2000 versucht die Global Alliance for Eliminating Lymphatic Filariasis (GAELF) diese Erkrankung zu bekämpfen. Jedoch wirken die in Afrika benutzten Medikamente nur auf die Mikrofilarien, und verhindern somit die Transmission auf weitere

Menschen. Auf die adulten Würmer, die circa 12-12 Jahre im menschlichen Körper leben, wirken sie allerdings kaum.

Die **Tuberkulose** ist weiterhin eine global weit verbreitete Erkrankung. Es wird geschätzt, dass circa 2 Milliarden Menschen „latent“ mit *Mykobakterium tuberculosis* infiziert sind und circa 9 Millionen an einer aktiven Erkrankung erleiden. Seit der HIV Epidemie hat sich die Ausbreitung der Tuberkulose noch mal verstärkt. Die „latente“ TB Infektion hatte früher eine Wahrscheinlichkeit von circa 5% sich innerhalb des Lebens des Betroffenen in eine aktive Erkrankung zu verwandeln. Bei einer Ko-Infektion mit HIV rechnet man mit circa 5% aktiven Tuberkulosen pro Jahr. Die TB hat sich damit zu einer der wichtigsten opportunistischen Infektionen (OI) der HIV Infektion entwickelt. Sie kann bereits in einem frühen Stadium der HIV-Infektion auftreten, wenn andere OI noch vom Immunsystem kontrolliert werden. Dies liegt an dem frühen Eliminieren von TB-spezifischen CD4-Zellen [9, 10]. Eine aktive TB-Infektion ist bei Vorliegen einer HIV-Ko-Infektion schwerer zu diagnostizieren, weil die „Goldstandards“ der Diagnostik, wie Sputum-Ausstrich, Mykobakterien Kultur weniger sensitiv sind. Der Ausstrich, der circa 10.000 Bazillen pro ml Sputum braucht, aber auch die Kultur, die mit 10-100 vermehrungsfähigen Bazillen auskommt, tragen bei HIV-Infizierten erheblich seltener zur Diagnose bei, da die Erkrankung oft paucibazillär verläuft. Dies führt zu erheblichen Schwierigkeiten eine Tuberkulose sicher zu erkennen und zeitnah eine Behandlung zu beginnen

2 Zielsetzung:

Diese Habilitation basiert auf zwei Studienbereichen:

(i) der Untersuchungen zu epidemiologischen Parametern innerhalb einer sorgfältig randomisierten Kohorte (EMINI, SOLF) mit den folgenden Schwerpunkten:;

- Beschreibung der Prävalenz und Inzidenz verschiedener Helmintheninfektionen und HIV, sowie der Untersuchung von Risikofaktoren für die jeweiligen Erkrankungen in einer großen prospektiven Kohorte der Normalbevölkerung in Süd-West-Tansania.
- Analyse des Einflusses der HIV-Infektion auf die unterschiedlichen Helminthen.
- Analyse des Einflusses einer vorbestehenden Helminthen-Erkrankung auf die HIV-Inzidenz.

(ii) der Untersuchungen von erkrankten Kindern, die mit dem Verdacht auf eine aktive Tuberkulose in das Krankenhaus kamen (ADAT) mittels unterschiedlicher TB diagnostischer Methoden mit den folgenden Schwerpunkten:;

- Untersuchung der Sensibilität und Spezifität des Lipoarabinomannan Testes, sowie verschiedener Interferon-gamma Release Assays für die Diagnose der Tuberkulose bei Kindern.
- Analyse eines Einfluss der HIV-Infektion auf die tuberkulosespezifische Diagnostik.

Des Weiteren gingen Erfahrungen und Probleme in der Diagnostik in einer auf die Entdeckung und Beschreibung des frühen Stadiums der HIV-Infektion spezialisierten Studie (RV 217) in diese Zusammenfassung ein.

3 Wissenschaftliche Projekte

Es werden Daten von 9 publizierten PubMed gelisteten Artikel, an denen ich beteiligt war für diese Habilitation zusammengefasst. Die Daten hierfür wurden in folgenden Studien gesammelt.

ADAT (**A**ctive **D**etection of **A**ctive **T**uberculosis), eine Studie in Tansania, durchgeführt 2008-10, in der Kinder von 1-14 Jahren mit Verdacht auf Tuberkulose untersucht und behandelt wurden.
(Rolle: Co-Investigatorin, Datenanalyse)

EMINI (**E**valuate and **M**onitor the **I**mpact of **N**ew **I**nterventions), eine großangelegte Bevölkerungsstudie, durchgeführt 2006-11, die 10% der Bewohner von neun ausgewählten Distrikten der Region Mbeya, Tansania, auf HIV, TB, Malaria und Helminthen untersucht hat (Abb.1)

(Rolle: Labormanagerin, Datenanalyse)

WHIS (**W**orm **H**IV **I**nteraction **S**tudy), eine Studie (Amendement zu EMINI, 2009-12), die an ausgewählten erwachsenen Patienten die Interaktion zwischen HIV und Helminthen untersucht. Es wurden verschiedene T-Zell-Subpopulationen, sowie Aktivierungsmarker.

(Rolle:, gemeinsame Auswertungen und Veröffentlichungen, Überlappung der Ergebnisse mit SOLF)
SOLF (Surveillance **o**f **L**ymphatic **F**ilariasis), eine Studie (Amendement zu EMINI, 2009-12), die die Prävalenz der lymphatischen Filariose vor und nach zwei Zyklen einer Massenbehandlung in einer der EMINI-Distrikte untersucht hat. Es wurde die gesamte Population des Distriktes über fünf Jahre untersucht. (Rolle: Antragstellerin, Projektleiterin)

RV 217 (syn. **ECHO** Early Infection HIV Cohort Study, Beginn 2009) Studie zur „HIV-Prevalence, Incidence, Cohort Retention, and Host Genetics and viral Diversity in Cohorts in East Africa and Thailand" (Rolle: Clinical Research Coordinator)

Studienort
Die Projekte wurden im Mbeya Medical Research Centre (MMRC) des National Institutes for Medical Research (NIMR) in Mbeya Tansania durchgeführt. Sie fanden unter der Leitung von Prof. Michael Hölscher, Direktor der Abteilung Tropenmedizin des Klinikum der Universität München, (LMU) und Leonard Maboko (vormaliger Direktor des NIMR-MMRC) statt. Die ersten beiden Projekte (ADAT und EMINI) wurden von Prof. Michael Hölscher initiiert. Bei der ADAT-Studie war ich für die Durchführung und Qualitätskontrolle des Quantiferon-TB-Gold und des Lipoarabinomannan Tests verantwortlich. Die beiden Projekte SOLF und WHIS waren sogenannte Ergänzungs-Studien die innerhalb der Population der EMINI-Studie durchgeführt wurden. Ich habe die SOLF Studie beim Bundesministerium für Bildung und Forschung (BMBF) beantragt und war als Projektleiterin für das Training, die Durchführung und die Auswertungen zuständig. Die Studie zur Erforschung der frühen HIV-Infektion (RV217) wurde von Dr. Merlin Robb vom Walter Reed Army Institute konzipiert und in 5 Ländern durchgeführt. Als klinische Koordinatorin war ich an der Protokollentwicklung und während der ersten zwei Jahre an der Rekrutierung und Betreuung von Studienteilnehmern beteiligt.

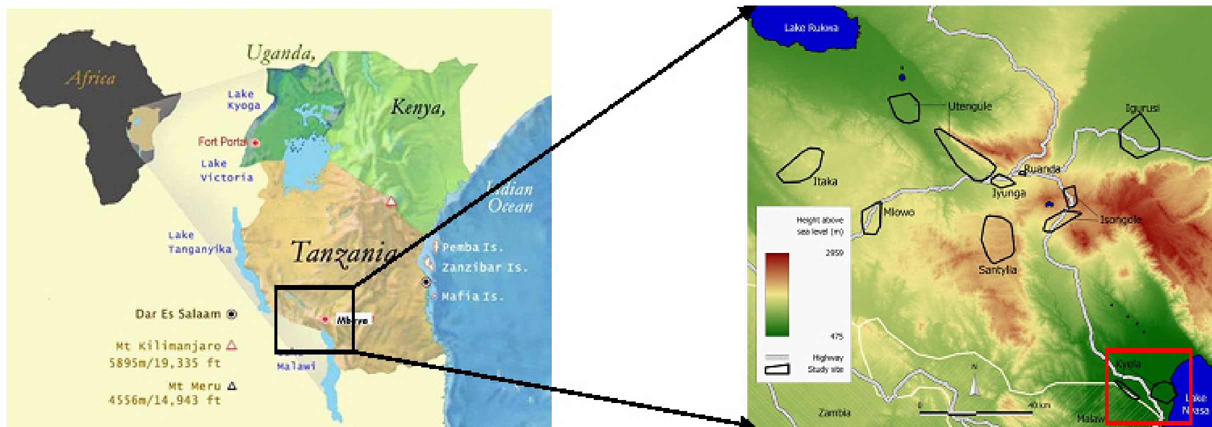


Abbildung 1: Die Region Mbeya liegt im Südwesten Tansania und grenzt an Malawi und Zambia. Das schwarze Viereck deutet das Gebiet der EMINI Studie an, das rechts vergrößert dargestellt wurde. Im rechten Bild sind die neun Gebiete der EMINI-Studie eingezeichnet. Diese unterscheiden sich geographisch erheblich, sodass verschiedene Umweltfaktoren in der Analyse erfasst und verglichen werden konnten. Mit dem roten Viereck ist das Gebiet Kyela, in welchem die Substudie SOLF stattfand, gekennzeichnet.

3 Diskussion der eigenen Arbeiten:

HIV Infektion, Probleme der Diagnostik, Frühe Infektionsphase

*Low specificity of determine HIV1/2 RDT using whole blood in south west Tanzania. **Kroidl I**, Clowes P, Mwalongo W, Maganga L, Maboko L, Kroidl AL, Geldmacher C, Machibya H, Hoelscher M, Saathoff E. PLoS One. 2012;7(6):e39529. doi: 10.1371/journal.pone.0039529.*

*Prospective Study of Acute HIV-1 Infection in Adults in East Africa and Thailand. Robb ML, Eller LA, Kibuuka H, Rono K, Maganga L, Nitayaphan S, Kroon E, Sawe FK, Sinei S, Sriplienchan S, Jagodzinski LL, Malia J, Manak M, de Souza MS, Tovanabutra S, Sanders-Buell E, Rolland M, Dorsey-Spitz J, Eller MA, Milazzo M, Li Q, Lewandowski A, Wu H, Swann E, O'Connell RJ, Peel S, Dawson P, Kim JH, Michael NL; **RV 217 Study Team** (**Kroidl I**,…).N Engl J Med. 2016 Jun 2;374(22):2120-30. doi: 10.1056/NEJMoa1508952.*

Zwei der hier beigelegten Artikel beschäftigen sich mit der **HIV-Infektion ohne begleitende Ko-Infektion.** Eine großangelegte Studie „EMINI" wurde in der Normalbevölkerung durchgeführt und war dazu gedacht, die Prävalenz von verschiedenen Infektionserkrankungen zu beschreiben. Für HIV konnte zusätzlich die Anzahl der Neuinfektionen über die 4 Jahre, in der die Studie durchgeführt wurde, beschrieben werden. Der Algorithmus zur Diagnostik der HIV-Infektion bei mehr als 18.000 Studienteilnehmern aus 4200 Haushalten sah zunächst einen sogenannten „rapid-diagnostic test" (RDT), entsprechend der nationalen tansanischen Guidelines vor. Alle positiven Ergebnisse wurden mittels ELISA und/oder Western Blot bestätigt. Um die Verschwiegenheit über das Testergebnis für die einzelnen Haushaltsmitglieder zu wahren, wurden alle HIV-Tests aus konserviertem Blut im Zentrallabor durchgeführt, aber zusätzlich Zelte der mobilen freiwilligen Beratungs- und

Testungseinheit („voluntary counseling and testing", VCT) für jeden Studienteilnehmer zu Verfügung gestellt. Sehr schnell stellte sich heraus, dass es eine hohe Anzahl von falsch positiven RDT Ergebnissen gab, insbesondere bei Kindern. Dies hatte bereits zu Besorgnis geführt, da Kinder von HIV negativen Eltern unerwartet HIV positiv getestet wurden. Es stellte sich jedoch heraus, dass der vom Staat Tansania gewählt RDT extrem sensitiv, aber wenig spezifisch war, und insbesondere bei Verwendung von Vollblut viele falsch positive Ergebnisse lieferte. Da die Blutabnahme bei kleineren Kindern vom Forschungsteam nicht genügend geübt worden war, wurde bei diesen meistens nur Fingerbeerenblut genommen, statt eine Venenpunktion durchzuführen. Die Nachtestungen ergaben, dass tatsächlich alle Ergebnisse, die zu Zweifeln geführt hatten, auf falsch positiven RDT Ergebnissen beruhten und diese häufiger bei Fingerbeerenblut (Vollblut), als bei Serumproben vorkamen. Diese Ergebnisse wurden frühzeitig mit dem tansanischen Gesundheitssystem besprochen und die Guidelines bezüglich der Wahl bzw. Kombination der HIV-Teste daraufhin auch geändert.

Die Ergebnisse der EMINI Studie, insbesondere die Ergebnisse der Testung für die HIV-Prävalenz und Inzidenz in den neun Studiengebieten wurden im Mbeya Medical Research Centre benötigt, weil weitere Studien zu HIV durchgeführt werden sollten. Es wurde eine HIV Prävalenz von 6,9% in der Studienpopulation gemessen. Die HIV-Prävalenzen betrugen 1,6% in der Altersgruppe 0-15 Jahre, 12,4% in der Altersgruppe 15-49 Jahre, und 6,8% in der Altersgruppe über 50 Jahre. Die HIV-Inzidenz betrug 1,35 auf 100 Personenjahren (95% KI 1,10-1,64).

Verschiedene Sponsoren hatten in der Region HIV-Impfstudien geplant. Zusätzlich führte das Walter-Reed-Army-Institute eine Studie zur frühen HIV Infektion durch, an der ich als klinische Forschungs-Koordinatorin für Tansania beteiligt war. Ziel der RV 217 (ECHO) Studie war es, die frühe HIV Infektion zu beschreiben, in einer Phase, in der die üblichen Tests noch negativ sind. Dies dient der Erfassung von typischen Symptomen in der frühen Phase, sowie auch der Sammlung von Blutproben, um unterschiedliche immunologische Reaktionen, die für den späteren Verlauf der chronischen HIV wichtig sind, zu erfassen. In diese Studie wurden Personen mit hohem Risiko für eine HIV-Infektion, aber bislang negativen HIV Test eingeschlossen. Mehr als 2.000 Personen mit hohem Risiko einer HIV-Infektion wurden in Tansania, Kenia, Uganda und Thailand über mehrere Jahre zweimal wöchentlich auf eine neue HIV-Infektion untersucht. Eine spezielle, hochsensitive semiquantitative DNA Nachweis-Methode namens APTIMA ermöglichte, HIV-Neuansteckungen schon bei sehr geringen Konzentrationen von circa 20 HIV-RNA-Kopien pro Milliliter Plasma nachzuweisen. Insgesamt wurden durch diese Nachweismethode 112 Personen nur wenige Tage nach der Infektion mit dem HI-Virus identifiziert. Bei diesen HIV-Infektionen im extrem frühen Stadium waren

durchschnittlich fünf Tage vom letzten negativen bis zum ersten positiven Bluttest vergangen. So konnte erstmalig der Zeitraum zwischen Infektion und dem Höchstwert der Viruslast genauer beschrieben werden, in dem es zur Ausbreitung von Zelltypen kommt, die entscheidend für den weiteren Verlauf der HIV-Infektion sind. Die Zeit vom ersten Nachweis der HIV-RNA bis zum Peak der Viruslast betrug im Mittel 13 Tage. Zwischen Maximalwert und dem Tiefstwert der Viruslast, dem sogenannten Set-point, wurden wiederum 31 Tage gemessen. Der weitere Krankheitsverlauf des HIV-infizierten Patienten wird vom Set-point bestimmt, denn die Höhe der Viruslast bleibt nach Erreichen des Set-points für eine lange Zeit gleich. Die Höhe der Viruslast zu diesem Zeitpunkt entscheidet über einen raschen oder eher langsamen Verlauf der HIV-Infektion. Informationen über Interaktionen zwischen dem Immunsystem und dem Virus, die die Höhe des Set-points beeinflussen könnten, sind daher von außerordentlicher Relevanz. Eine Analyse derart früher Zeitpunkte nach der Ansteckung ist bislang einzigartig, da frühere Studien nur Patienten einschlossen, die bereits Symptome aufwiesen oder sich schon mehrere Wochen zuvor angesteckt hatten. Inzwischen wurden Ergänzungsstudien begonnen, die die Auswirkung einer sehr früh antiretroviralen Behandlung auf den Langzeitverlauf untersuchen.

Helminthen und HIV

Im Jahr 2009 betrug die HIV-Prävalenz innerhalb der gesamten EMINI Studie 6,9%, dabei wiesen die einzelnen Studienregionen Prävalenzen zwischen 3,6% und 9.7% auf. Ab 2009 wurde in der EMINI Studie auch Stuhl und Urinproben gesammelt, um auf *S. haematobium*, *S. mansoni*, die intestinalen Helminthen *Ascaris lumbricoides*, Hakenwurm (*Necator americanus und Ancylostoma duodenale*) sowie *Trichuris trichiura* zu untersuchen. Als Ergänzungsstudie kam dann noch die Untersuchungen für *Wuchereria bancrofti* hinzu, nachdem in einer der EMINI-Studienregionen Patienten mit klinischen Zeichen der lymphatischen Filariose bemerkt worden waren. Drei der hier angegebenen Publikationen beschäftigen sich mit intestinalen Helminthen, zwei weitere mit der lymphatischen Filariose. Bei circa 60% der untersuchten Studienteilnehmer wurden ein oder mehrere unterschiedliche Helmintheninfektionen festgestellt. Die neun Regionen der EMINI Studie waren geographisch unterschiedlich gelegen. So war es nicht unerwartet, dass sowohl die HIV-Prävalenz, aber auch die Verteilung der Helminthen sehr unterschiedlich war. Im Prinzip waren vier der studierten Regionen relevant von Wurminfektionen betroffen, die anderen fünf nur sehr wenig. Es gab genau eine Studienregion, in der alle untersuchten Helminthenarten zu finden waren. Bei den folgenden Untersuchungen gab es teilweise Auswertungen der gesamten Studienpopulation, zum Teil aber auch Analysen getrennt für einzelne Regionen. Dies ist insbesondere beim Vergleich

zwischen HIV-infizierten und HIV-negativen Studienteilnehmern wichtig, da ansonsten ein verfälschtes Bild eines möglichen Einflusses entstehen könnte. Es wurden von unserer Gruppe bereits Auswertungen zu *Ascaris lumbricoides*, Hakenwurminfektionen, *Trichuris trichiura* (PloS One, in press) und *W. bancrofti* veröffentlicht. Da alle Diagnosen zu einer medikamentösen Therapie des jeweiligen Studienteilnehmers führten, konnten hauptsächlich Querschnitts-Untersuchungen durchgeführt werden, da bei gutem Therapieerfolg keine längeren Zeiten der Infektion mit einem intestinalen Helminthen definiert werden konnten.

Querschnitts (cross-sectionale) Untersuchungen zu Helminthen

Ascaris lumbricoides infection and its relation to environmental factors in the Mbeya region of Tanzania, a cross-sectional, population-based study. Schüle SA, Clowes P, ***Kroidl I****, Kowuor DO, Nsojo A, Mangu C, Riess H, Geldmacher C, Laubender RP, Mhina S, Maboko L, Löscher T, Hoelscher M, Saathoff E. PLoS One. 2014 Mar 18;9(3):e92032. doi: 10.1371/journal.pone.0092032.*

(Trichuris trichiura Infection and Its Relation to Environmental Factors in Mbeya Region, Tanzania: A Cross-Sectional, Population-Based Study. Kirsi Marjaana Manz, M.Sc.; Petra Clowes; ***Inge Kroidl;*** *Dickens O Kowuor; Christof Geldmacher; Nyanda E Ntinginya; Leonard Maboko; Michael Hoelscher; Elmar Saathoff. PONE-D-16-48704.in press)*

Hookworm infection and environmental factors in Mbeya region, Tanzania: a cross-sectional, population-based study. Riess H, Clowes P, ***Kroidl I****, Kowuor DO, Nsojo A, Mangu C, Schüle SA, Mansmann U, Geldmacher C, Mhina S, Maboko L, Hoelscher M, Saathoff E. PLoS Negl Trop Dis. 2013 Sep 5;7(9):e2408. doi: 10.1371/journal.pntd.0002408.*

Infektionen mit ***Ascaris lumbricoides*** kamen hauptsächlich in 3 Regionen der EMINI Studie vor (Ruanda 8.5%, Isongole 16.8%, Kyela 25.2 % Prävalenz). Die anderen sechs Regionen der EMINI-Studie lagen unter 1% in der Prävalenz für *A. lumbricoides*. Eine Infektion mit dieser Wurm Spezies war in der multivariaten Analyse positiv assoziiert mit Faktoren wie Dichte der Vegetation, Regenmenge und Umgebungstemperaturen. Der Peitschenwurm ***Trichuris trichiura*** kam fast ausschließlich in dem Distrikt Kyela vor, es waren dort 26.6% der Studienteilnehmer infiziert. Eine Infektion war positiv assoziiert mit der Dichte der Vegetation, aber negativ mit der Regenmenge.

Die HIV-Prävalenz in den drei Studienregionen, in denen die Ascaris Infektion hauptsächlich vorkam, war mit 9.17%, 9.74%, 9.19%, respektive, sehr ähnlich. Bezogen auf alle drei Regionen gemeinsam konnten wir bei der HIV-negativen Subgruppe Ascaris Prävalenzen von 18.9%, und bei der HIV-positiven Subgruppe von 15.2 % darstellen, und fanden somit keinen signifikanten Unterschied (p=0.210). Berücksichtigt man den Einfluss von Alter und Geschlecht in einer multivariaten logistischen Regression waren die Unterschiede noch geringer (p=0.62). Sehr ähnliche Befunde fanden wir für *Trichuris trichiura*. Da dieser fast ausschließlich in dem Distrikt Kyela vorkam, konnte

auch nur in diesem Distrikt eine Relation zu HIV untersucht werden. Im Gegensatz zu *A. lumbricoides,* der einen zweiten Peak der Prävalenz im Erwachsenenalter hat, kommt *T. trichiura* hauptsächlich bei Kindern vor (Abb. 2). Die univariate Analyse suggeriert ein selteneres Vorkommen dieses Helminthen bei HIV-infizierten Personen, da die HIV Prävalenz bei Kindern und Jugendlichen niedrig war und erst ab dem 20. Lebensjahr signifikant anstieg. In der multivariaten Analyse, die auch Alter und Geschlecht berücksichtigte war dann aber kein Unterschied mehr zu sehen (p=0.975).

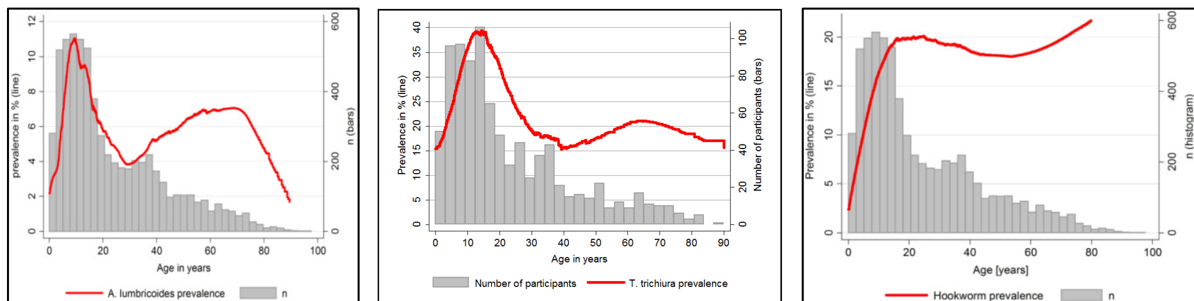


Abb 2: Abbildungen aus den jeweiligen o.g. Veröffentlichungen zur Altersverteilung der Helminthen in der EMINI-Studie. Die grauen Balken geben die Anzahl der Studienteilnehmer in der jeweiligen Altersgruppe an. Die roten Linien repräsentieren die Helminthen Prävalenz der verschiedenen Altersgruppen, links für die Spulwürmer, mittige die Peitschenwürmer, sowie rechts für die Hakenwürmer.

Die **Hakenwürmer** wurden interessanterweise hauptsächlich in den beiden Regionen mit der höchsten und der niedrigsten HIV Prävalenz gefunden. Die Analyse sowohl der einzelnen Regionen, als auch der gesamten EMINI Kohorte zeigte keinen signifikanten Einfluss von Hakenwürmern auf die Hiv Inzidenz oder Prävalenz.

Die hier beschriebenen Helmintheninfektionen werden hauptsächlich in der Kindheit erworben, je nach Art des Wurmes gibt es aber hohe Infektionsraten auch bei den Erwachsenen, z.B. bei den Hakenwürmern. In den meisten Fällen kann man davon ausgehen, dass Wurminfektionen in einem früheren Lebensabschnitt erworben werden, als eine HIV-Infektion. Bei allen Querschnitts-Betrachtungen ist aber zu berücksichtigen, dass es natürlich für den Einzelfall nicht gesichert ist, welche Infektion primär aufgetreten ist, und welche folgte. Hierfür sind **prospektive Studien** nötig. Für die meisten Helmintheninfektionen gestaltete sich dies schwierig. Die im oberen Text beschriebenen Helminthen wurden alle nach den Richtlinien der WHO therapiert, d.h. mit Albendazol gegen die intestinalen Helminthen, mit üblicherweise recht guten Erfolgsraten. Eine prospektive Untersuchung des Einflusses dieser Helminthen auf die HIV Inzidenz erwies sich daher als schwierig, da ein Zeitraum in dem der Einfluss einer Helmintheninfektion untersucht werden könnte, so nicht zu definieren war. Eine weitere Helminthenart die als Ergänzung der EMINI Studie in der Region Kyela untersucht wurde war die Blut-und Gewebsnematode ***Wuchereria bancrofti***. Im Unterschied zu den

intestinalen Nematoden befinden sich die adulten Würmer im lymphatischen System, die zur weiteren Übertragung nötigen Mikrofilarien im Blut. Ein Regierungsprogramm begann die Behandlung gegen diesen Erreger im Jahr 2009, also nachdem der EMINI Survey bereits 3 Jahre durchgeführt worden war. Wir führten daher retrospektiv Untersuchungen zur Bestimmung der Prävalenz vor Behandlung (Proben von 2007, 2008 und 2009), aber auch in den zwei Jahren nach Durchführung der Massenhandlung (2010, 2011) durch, um den Therapieerfolg unabhängig zu überprüfen. Die von den afrikanischen Ländern gewählten Präparate Albendazol und Ivermectin töten die Mikrofilarien sehr gut, und verhindern damit die weitere Transmission des Erregers. Die adulten Würmer leben jedoch unbeeinträchtigt von diesen Präparaten circa 10-12 Jahre im menschlichen Körper. Der Nachweis einer Infektion mit *W. bancrofti* gelingt durch eine Blutuntersuchung, die ein Antigen des adulten Wurmes nachweist. Wir hatten die Möglichkeit Zeiträume zu definieren in denen eine W. bancrofti Infektion vorlag (positiver Antigenbefund am Anfang und am Ende des Beobachtungszeitraumes).

Untersuchungen zum Einfluss von HIV auf LF

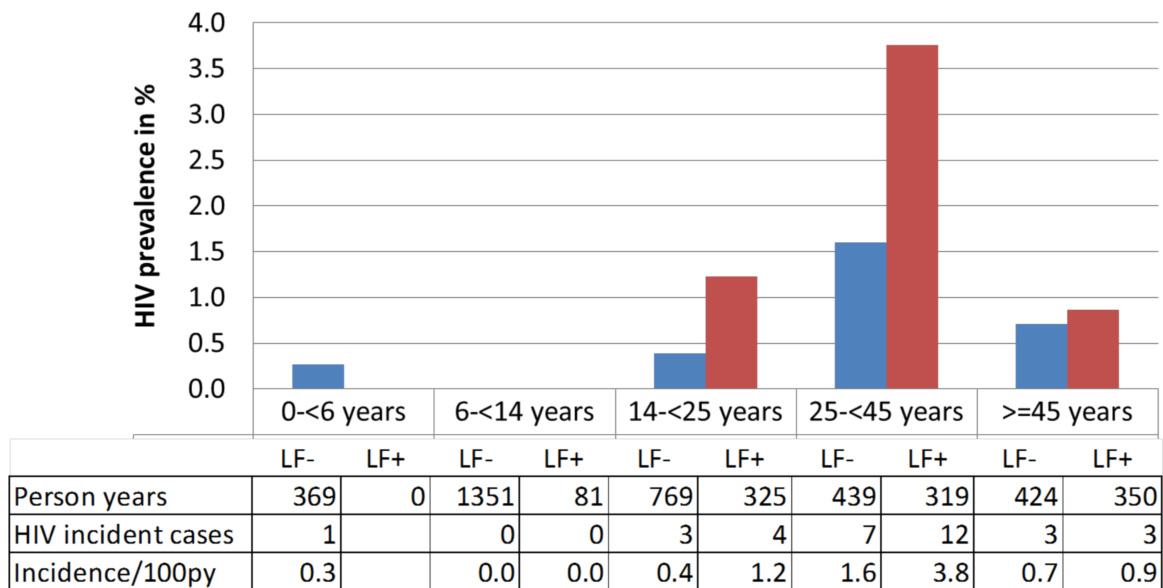
Prevalence of Lymphatic Filariasis and Treatment Effectiveness of Albendazole/ Ivermectin in Individuals with HIV Co-infection in Southwest-Tanzania. ***Kroidl I****, Saathoff E, Maganga L, Clowes P, Maboko L, Hoerauf A, Makunde WH, Haule A, Mviombo P, Pitter B, Mgeni N, Mabuye J, Kowuor D, Mwingira U, Malecela MN, Löscher T, Hoelscher M. PLoS Negl Trop Dis. 2016 Apr 12;10(4):e0004618. doi: 10.1371/journal.pntd.0004618*

In einer der neun Studienregionen von EMINI (Kyela) fielen bei den klinischen Untersuchungen Lymphödeme der Extremitäten auf, wie sie z.B. bei Infektionen mit W. bancrofti vorkommen. Für diese circa 2500 Teilnehmer wurde in den bereits gewonnenen Plasmaproben auf das zirkulierende Filarienantigen (CFA) untersucht, welches von dem adulten Wurm von W. bancrofti stammt. Der große Vorteil dieser Untersuchungsmethode ist, dass die CFA-Mengen keiner zirkadianen Rhythmik unterliegen, wie z.B. die Mikrofilarien, die nur nachts im Blut vorkommen. Im Jahr 2009 wurde die Prävalenz der LF in der EMINI-Studienregion Kyela untersucht und lag bei 24.8%. Dabei hatten Kinder unter 10 Jahren eine sehr geringe Infektionsrate von unter 3%, Erwachsene lagen bei circa 40%. Es wurde alterstratifiziert der Einfluss einer HIV-Ko-infektion auf die LF-Prävalenz untersucht. Des Weiteren wurde analysiert, ob die HIV-Erkrankung eine Veränderung der LF-Inzidenz bewirkt, oder die Ansprechrate unter Therapie verändert ist. Für keine der formulierten Fragestellungen war ein signifikanter Einfluss der HIV-Erkrankung nachzuweisen.

Untersuchungen zum Einfluss von LF auf HIV

Effect of Wuchereria bancrofti infection on HIV incidence in southwest Tanzania: a prospective cohort study. ***Kroidl I****, Saathoff E, Maganga L, Makunde WH, Hoerauf A, Geldmacher C, Clowes P, Maboko L, Hoelscher M. Lancet. 2016 Oct 15;388(10054):1912-1920. doi: 10.1016/S0140-6736(16)31252-1.*

Die EMINI Studie hat über einen Zeitraum von fünf Jahren circa 2500 Teilnehmer pro Studienregion im Alter zwischen 0 und 94 Jahren jährlich einmal aufgesucht und untersucht. Dies erlaubte Berechnungen von Neuinfektionen für die HIV Erkrankung. In dem Distrikt Kyela, in dem die lymphatische Filariose getestet wurde, konnte daher der Einfluss dieser chronischen Wurminfektion auf die HIV Inzidenz untersucht werden. Die Auswertung für HIV Risikofaktoren beschränkte sich auf Personen über 14 Jahre, um verschiedene HIV Übertragungswege, und damit unterschiedliche Faktoren, die ein Risiko darstellen könnten, nicht zu vermischen. Bei 1055 initial HIV negativen Personen aus der Studienregion Kyela, die älter als 14 Jahre waren, wurden die jährlich gesammelten Plasmaproben auf eine Infektion mit W. bancrofti und auf eine neu erworbene HIV-Infektion untersucht. In 2626 Personenjahren konnten 32 neu aufgetretene HIV-Infektionen diagnostiziert werden. Das Risiko einer HIV-Infektion war mehr als zweifach erhöht bei den LF infizierten (1,91 Fälle im Vergleich zu 0,8 Fällen pro 100 Personenjahre, Inzidenz Rate Ratio (IRR) 2,38; das 95% KI= 1,08-4,37). Es wurden drei Alterscluster getrennt untersucht. Der Vergleich von Filarien-Infizierten mit nicht-Infizierten ein 3,2-fach erhöhtes Risiko für die HIV-Ansteckung bei den 14 bis 25-Jährigen, ein 2,4-fach erhöhtes Risiko für die 25 bis 45-Jährigen, und ein 1,2-fach erhöhtes Risiko für die über 45-Jährigen (Abb. 5).



	0-<6 years LF-	0-<6 years LF+	6-<14 years LF-	6-<14 years LF+	14-<25 years LF-	14-<25 years LF+	25-<45 years LF-	25-<45 years LF+	>=45 years LF-	>=45 years LF+
Person years	369	0	1351	81	769	325	439	319	424	350
HIV incident cases	1		0	0	3	4	7	12	3	3
Incidence/100py	0.3		0.0	0.0	0.4	1.2	1.6	3.8	0.7	0.9

Abb.5 Die HIV Inzidenzen in den verschiedenen Altersgruppen bei LF-Infizierten (rote Balken) und LF-Uninfizierten (blaue Balken). Die Zahlen unter der Graphik geben Anzahl der Personenjahre und die in dieser Zeit dokumentierten HIV-Neuinfektionen an. Es gab eine Neuinfektion im Kleinkindesalter, am ehesten durch Stillen. In der Phase zwischen 6 und 14 Jahren wurde keine HIV-Neuinfektion dokumentiert. In den Altersgruppen 14-25 Jahre und 25 bis 45 Jahre zeigten die LF-Infizierten ein 3 bzw. 2,4 fach erhöhtes Risiko für eine HIV Infektion.

Des Weiteren wurde die Kohorte auf andere, üblicherweise beschriebene Risikofaktoren für HIV untersucht, um eine mögliche Beeinflussung zu identifizieren. Dabei fiel auf, dass es Faktoren mit erheblich größerem Einfluss für die HIV-Infektion gibt, z.B. das Zusammenleben mit einem HIV-Infizierten Partner (12.8-fach erhöhtes Risiko für eine HIV-Neuinfektion, linke Seite von Abb. 6). Es gab auch schützende Faktoren mit einem Risikofaktor unterhalb der 1-er Marke (siehe rechte Seite von Abb. 6)

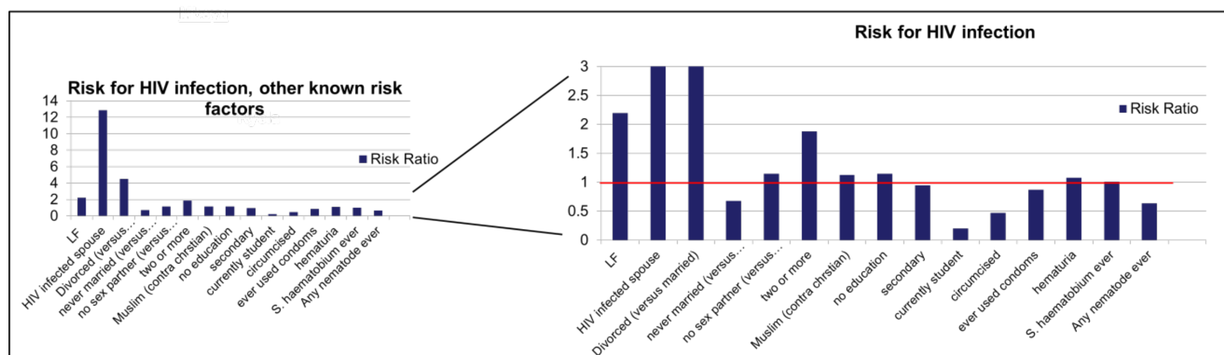


Abb. 6: Risikofaktoren für HIV. Links eine Gesamt-Darstellung, im rechten Teil der Graphik wurde der Risikobereich 0-3 vergrößert dargestellt, um feine Unterschiede besser darstellen zu können. Es wurden Risiken beschrieben, wie z.B. das Zusammenleben mit einem HIV-Infizierten Partner oder der Ehezustand „geschieden oder getrennt" im Vergleich zu „Verheirateten". Es wurden aber auch

schützende Faktoren, mit einem Risiko kleiner als 1 (all Balken unterhalb der roten Linie im rechten Bild) festgestellt.

Die Analyse der 3 Alterscluster (Adoleszenten: 14-25 Jahre, junge Erwachsene 25-45 Jahre, und ältere Erwachsene über 45 Jahre) ergab, dass eine Infektion mit W. bancrofti der wichtigste Risikofaktor für den Erwerb einer HIV-Infektion unter den Adoleszenten darstellt. Andere HIV-Risikofaktoren spielten eine relevante Rolle bei den Erwachsenen über 25 Jahren Abb.7.

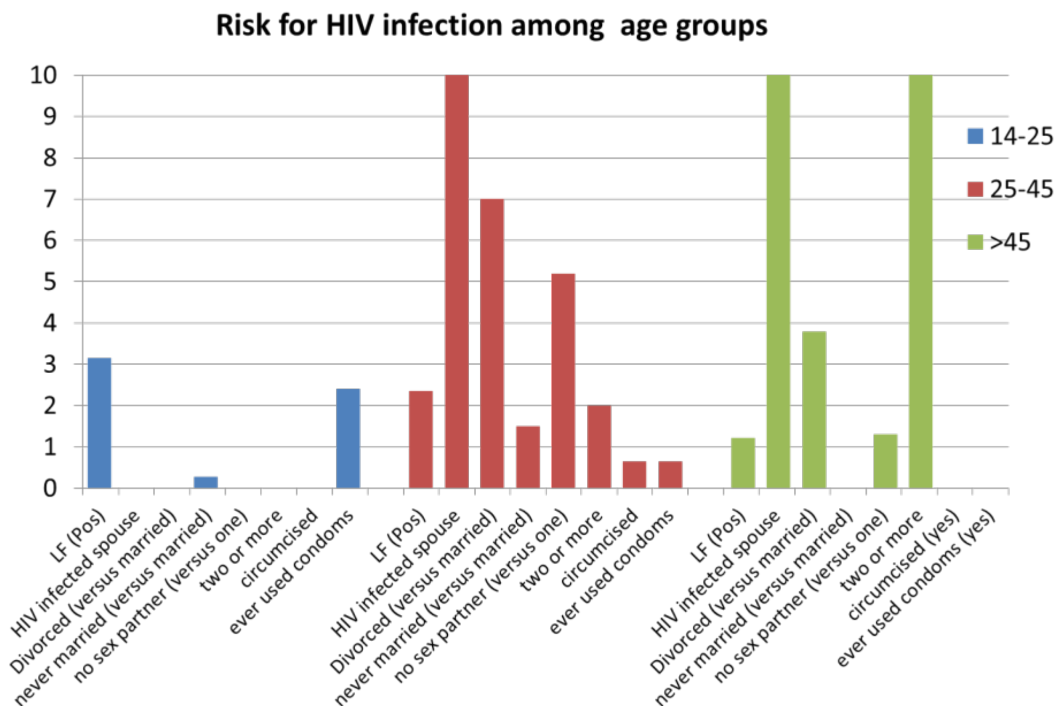


Abbildung 7: Die Faktoren, die auf ihr Risikopotential für den Erwerb einer HIV Infektion untersucht wurden sind unterschiedlich wichtig in den verschiedenen Altersclustern.

Als nächsten Schritt untersuchten wir die verschiedensten HIV-Risiko-Faktoren, ob sie die erhöhte HIV Inzidenz, die für die mit *W. bancrofti*-Infizierten gesehen wurde, beeinflussen könnten. Hiermit wurde geklärt, ob der beobachtete Einfluss der LF-Infektion auf die HIV Inzidenz sich durch andere Faktoren erklären lässt, oder ob es sich hierbei um einen eigenständigen Faktor handelt. Dazu wurden mehrere multivariate Analysen durchgeführt, die jeweils den LF-Status, die Altersgruppen, das Geschlecht sowie den zusätzlichen HIV-Risikofaktor beinhalteten. Die untersuchten Faktoren waren der eheliche Zustand, die Anzahl der sexuellen Partner, das Zusammenleben mit einem HIV-infizierten Partner, Religionszugehörigkeit, Schulbildung, Beruf, zusätzliche Infektion mit anderen Helminthen, Kondombenutzung, Zirkumzision, Art der Kontrazeption, und mehr. Bei keiner der

durchgeführten Analysen wurde der Einfluss der LF-Infektion auf das Risiko einer HIV-Neuinfektion beeinträchtigt. Das Risiko blieb immer signifikant erhöht.

Diese Ergebnisse sind von außerordentlicher Relevanz. Zum einen gelang zum ersten Mal der Nachweis in einer prospektiven Studie, dass eine vorstehende Wurmerkrankung, die völlig anders übertragen wird und mit sexuellen Praktiken und Vorlieben nicht zu tun hat, das Risiko für den Erwerb einer HIV-Infektion erhöht. Zum anderen wurde die Rolle von HIV-Risikofaktoren in den verschiedenen Altersgruppen beschrieben. Einige der HIV-Risikofaktoren, die bereits Eingang in die Präventivmedizin gefunden haben, sind noch nicht von Relevanz in diesem jungen Alter. In diesem Alter haben andere Faktoren, z.B. die LF-Infektion eine größere Wichtigkeit. Diese Erkenntnisse bieten verschiedenste Ansatzmöglichkeiten für eine Verbesserung der HIV-Prävention.

HIV und Tuberkulose

Performance of urine lipoarabinomannan assays for paediatric tuberculosis in Tanzania. ***Kroidl I****, Clowes P, Reither K, Mtafya B, Rojas-Ponce G, Ntinginya EN, Kalomo M, Minja LT, Kowuor D, Saathoff E, Kroidl A, Heinrich N, Maboko L, Bates M, O'Grady J, Zumla A, Hoelscher M, Rachow A. Eur Respir J. 2015 Sep;46(3):761-70. doi: 0.1183/09031936.00003315.*

Reasons for false-positive lipoarabinomannan ELISA results in a Tanzanian population. ***Kroidl I****, Clowes P, Mwakyelu J, Maboko L, Kiangi A, Rachow A, Reither K, Jung J, Nsojo A, Saathoff E, Hoelscher M. Scand J Infect Dis. 2014 Feb;46(2):144-8. doi: 10.3109/00365548.2013.853133.*

QuantiFERON®-TB gold in-tube performance for diagnosing active tuberculosis in children and adults in a high burden setting. Rose MV, Kimaro G, Nissen TN, ***Kroidl I****, Hoelscher M, Bygbjerg IC, Mfinanga SG, Ravn P. PLoS One. 2012;7(7):e37851.*

Drei der hier vorgelegten Arbeiten befassen sich mit der Diagnostik der **Tuberkulose**. Im Rahmen meiner Tätigkeit in Tansania war ich auch für die Durchführung und Beurteilung neuer Diagnostika zuständig. Im Allgemeinen führt die HIV-Erkrankung zu einer erschwerten Beurteilbarkeit von z.B. serologischen Untersuchungen. Auch die Sputum-Diagnostik für Tuberkulose ist deutlich weniger sensitiv bei Vorliegen einer Ko-infektion mit HIV. Diese Einschränkung trifft nicht zu für den Test auf Lipoarabinomannan (LAM), einen Bestandteil der Zellwand von Mykobakterien. Im Gegenteil, LAM ist im Urin von Tuberkulosepatienten deutlich besser nachweisbar bei HIV-Ko-infizierten Patienten, im Vergleich zu den ausschließlich an TB erkrankten Patienten. Dies konnten wir in einer pädiatrischen

Studie in Tansania bestätigen (ADAT). Bei HIV-negativen TB-Patienten wird die Durchführung des LAM Tests daher überhaupt nicht mehr empfohlen. Im Gegensatz dazu stehen die Interferon-Gamma Release Assays, die ein funktionierendes Immunsystem brauchen um ein aussagekräftiges Ergebnis zu produzieren. Drei verschiedene IGRAs (Quantiferon TB-Gold, T-Spot.TB und ein in-house ELISPOT) wurden in der ADAT-Studie getestet und mit dem konventionellen Tuberkulin –Haut-Test (TST) verglichen. Es konnte allgemein eine etwas bessere Sensitivität bei HIV negativen Patienten im Vergleich zu den mit HIV-infizierten demonstriert werden. Für beide Gruppen konnten die IGRAs jedoch empfohlen werden. Die ELISPOT Techniken und der Quantiferon TB Gold waren deutlich besser als der Tuberkulin Skin Test, sowohl bei HIV-positiven, als auch bei HIV-negativen Studienteilnehmern. Es gab einige weitere interessante Aspekte. Bei den IGRAs gibt es Untersuchungen, die nicht auswertbar sind, weil die positive Testkontrolle keine ausreichenden Werte erzielt hat. Diese nicht auswertbaren Tests für TB sind signifikant assoziiert mit Mangelernährung und Mortalität. Es sollte deshalb nach kurzer Zeit eine Wiederholung des IGRA durchgeführt werden, um zu einem auswertbaren Ergebnis zu kommen. In dieser Hinsicht, sind die IGRAs dem konventionellen Hauttest deutlich überlegen, denn dieser kann nicht zwischen „korrekt“ negativen Ergebnissen und dem geschilderten Problem unterscheiden.

Einen weiteren interessanten Aspekt boten die Verlaufsuntersuchungen der Patienten der ADAT-Studie, die 12 Monate nach Beginn der TB-Therapie durchgeführt wurden. Bei HIV-negativen Menschen bleiben nach einer erfolgreichen TB-Therapie immer TB-spezifische CD 4 Zellen zurück, die nach Stimulation mit TB-spezifischen Antigenen Interferon-gamma produzieren. Es ist daher anhand eines IGRA-Ergebnisses unmöglich zu sagen, ob die TB ausgeheilt ist. Dies ist anders bei HIV-positiven Patienten, die keine antiretrovirale Therapie bekommen. Bei dieser Subgruppe von Patienten verschwanden die TB-spezifischen CD4 Zellen unter Therapie, sodass die meisten von Ihnen circa 12 Monate nach Therapie Beginn einen negativen IGRA aufwiesen. (diese Auswertungen sind bislang nur auf der Welt-TB-Konferenz vorgetragen, Veröffentlichung geplant). Dieses Phänomen kann diagnostisch genutzt werden.

4 Einordnung der eigenen Studienergebnisse in die Literatur

Der mögliche Einfluss von chronischen Infektionen auf die Empfänglichkeit für HIV wurde lange diskutiert. Breits in den 1990iger Jahren fiel bei Querschnittsstudien an äthiopischen Immigranten in Israel auf, dass es eine Korrelation zwischen Helminthen Prävalenz und HIV gab [4-6, 11-15]. Auch in-vitro Studien deuteten auf eine erhöhte Suszeptibilität der T-Zellen von Helminthen-infizierten Personen hin [16, 17]. Prospektive Studien zum weiteren Beweis der Hypothese wurden durchgeführt, brachten aber keine schlüssigen Ergebnisse. Das mag auch daran liegen, dass die meisten Helminthen mit einer einmaligen Therapie gut zu behandeln sind und somit ein langfristiger Einfluss schwer zu untersuchen ist. Der Nachweis einer erhöhten HIV Inzidenz bei Wurminfizierten gelang nun erstmalig bei einer Infektion mit *W. bancrofti* [18], bei den weiteren untersuchten Helminthen war kein Zusammenhang mit HIV dokumentiert worden. Nur wenige Studien hatten sich bereits mit der Ko-infektion der lymphatischen Filariose und HIV befasst, bislang auch nur in Querschnittsbeobachtungen. Nielsen et al. hatte 2006 eine positive Assoziation zwischen LF und HIV beschrieben [19], konnte dies aber in Folgestudien nicht unterlegen [20]. Kein Unterschied in der Prävalenz und der Wurmlast war in Querschnittsstudien in Indien [21] und Malawi [22] gesehen worden. Auch die Behandlung der LF war gleichermaßen erfolgreich bei HIV Infizierten [23, 24]. Dies konnten wir in unseren Auswertungen bestätigen [25].

Die Ergebnisse bezüglich der durch Filarien erhöhten HIV-Inzidenz haben weitere Forschungsansätze zu Folge. In Kooperation mit der ebenfalls in Mbeya durchgeführten WHIS Studie konnten bereits Analysen zu T-Zellaktivierungsmarkern und weiteren Faktoren, die eine Rolle spielen könnten, durchgeführt werden (Auswertungen laufen zurzeit). Die WHIS Studie hatte unterschiedliche Level von Aktivierung bei *Trichuris trichiura*- im Vergleich mit Hakenwurm-Infizierten beschrieben [26, 27]. Ob die beobachtete erhöhte HIV Inzidenz bei LF-Infizierten sich auf ein regionalübliches Niveau zurück entwickelt und somit die LF-Therapie als Präventions-Maßnahme gegen HIV anzusehen ist, ist ein weiteres Studienfeld. LF gehört zu den so genannten „neglected tropical diseases“ (NTD). Die Therapie bzw. Elimination dieser Erkrankung könnte forciert werden, um damit als Nebeneffekt die HIV-Inzidenz zu senken. Eine Reihe von Studien hat in den letzten Jahren die Wirksamkeit von Medikamenten, bzw. Medikamentenkombinationen auch gegen den adulten Wurm von *W. bancrofti* getestet [28-33]. Diese bieten die Möglichkeit *W.bancrofti* nach circa 6-12 Monaten, statt den 12 Jahren, aus dem menschlichen Körper zu eliminieren und somit das erhöhte Risiko für HIV erheblich schneller zu reduzieren. Studien mit dieser Fragestellung sind in Planung.

Bezüglich der Untersuchung der neuen Diagnostika im Bereich der TB-HIV Interaktion konnten wir einen wertvollen Betrag zur Wertigkeit des LAM Testes leisten. Die LAM Testes waren in der Literatur sehr unterschiedlich bewertet worden [34-37]. Wir konnten maßgeblich zur Beschreibung von

Problemen bei der Diagnostik [38] und weitere Informationen zur Sensibilität und Spezifität bei richtigem Gebrauch beitragen [39]. Eine Besonderheit bei der Tuberkulose HIV-Ko-Infektion ist der Verlust von TB spezifischen T-Helferzellen, solange keine aktive TB vorliegt, oder die HIV Infektion mit antiretroviraler Medikation therapiert wird. Dies wurde von unserem Institut beschrieben [9, 10] und wird in der weiteren Untersuchung von HIV/TB Ko-Infizierten genauer untersucht [40, 41]. Weitere Studien zum prospektiven Wert der IGRAs bei ART naiven HIV-Infizierten Individuen sind im Gange (AFRICOS-Studie).

5 Abkürzungen

95% KI	95% Konfidenzintervall
ADAT	Akkronym der Studie: „Active Detection of Active Tuberculosis”
AIDS	Acquired Immune Deficiency Syndrom
ART	Antiretroviral Treatment
CD4	Synonym für T-Helferzellen (Cluster of Differentiation 4)
CIH	Center for International Health an der Universität München
ECHO	Akkronym der Studie: „Early HIV Infection”= RV 217
EMINI	Akkronym der Studie: „Evatuating and Monitoring the Impact of New Interventions
HIV	Human Immunodeficiency Virus
IGRA	Interferon Gamma Release Assay
IRR	Incidence Rate Ratio
LAM	Lipoarabinomannan
LF	Lymphatische Filariose
MMRC	Mbeya Medical Research Centre
NTD	Neglected Tropical Diseases
OI	Opportunistische Infektion
RV 217	Akkronym der Studie: „Early Infection HIV Cohort”==ECHO
SOLF	Akkronym der Studie: „Surveillance Of Lymphatic Filariasis”
STH	Soil Transmitted Helminths
TB	Tuberkulose
TST	Tuberculin Skin Test
WHIS	Akkronym der Studie: „Worms-HIV-Interaction-Study”
VCT	Voluntary Counseling and Testing

6 Referenzen

1. Faria NR, Rambaut A, Suchard MA, Baele G, Bedford T, Ward MJ, Tatem AJ, Sousa JD, Arinaminpathy N, Pepin J, Posada D, Peeters M, Pybus OG, Lemey P. HIV epidemiology. The early spread and epidemic ignition of HIV-1 in human populations. Science 2014: 346(6205): 56-61.

2. UNAIDS. Global report: UNAIDS report on the global AIDS epidemic 2016. Geneva: Joint United Nations Programme on HIV/AIDS (UNAIDS).

3. Piot P, Bartos M, Ghys PD, Walker N, Schwartlander B. The global impact of HIV/AIDS. Nature 2001: 410(6831): 968-973.

4. Bentwich Z, Kalinkovich A, Weisman Z. Immune activation is a dominant factor in the pathogenesis of African AIDS. Immunology today 1995: 16(4): 187-191.

5. Bentwich Z, Kalinkovich A, Weisman Z, Borkow G, Beyers N, Beyers AD. Can eradication of helminthic infections change the face of AIDS and tuberculosis? Immunology today 1999: 20(11): 485-487.

6. Bentwich Z, Kalinkovich A, Weisman Z, Grossman Z. Immune activation in the context of HIV infection. Clinical and experimental immunology 1998: 111(1): 1-2.

7. Dissanayake S, Watawana L, Piessens WF. Lymphatic pathology in Wuchereria bancrofti microfilaraemic infections. Trans R Soc Trop Med Hyg 1995: 89(5): 517-521.

8. Dreyer G, Noroes J, Figueredo-Silva J, Piessens WF. Pathogenesis of lymphatic disease in bancroftian filariasis: a clinical perspective. Parasitol Today 2000: 16(12): 544-548.

9. Geldmacher C, Ngwenyama N, Schuetz A, Petrovas C, Reither K, Heeregrave EJ, Casazza JP, Ambrozak DR, Louder M, Ampofo W, Pollakis G, Hill B, Sanga E, Saathoff E, Maboko L, Roederer M, Paxton WA, Hoelscher M, Koup RA. Preferential infection and depletion of Mycobacterium tuberculosis-specific CD4 T cells after HIV-1 infection. The Journal of experimental medicine 2010: 207(13): 2869-2881.

10. Geldmacher C, Schuetz A, Ngwenyama N, Casazza JP, Sanga E, Saathoff E, Boehme C, Geis S, Maboko L, Singh M, Minja F, Meyerhans A, Koup RA, Hoelscher M. Early depletion of Mycobacterium tuberculosis-specific T helper 1 cell responses after HIV-1 infection. J Infect Dis 2008: 198(11): 1590-1598.

11. Bar-Yehuda S, Weisman Z, Kalinkovich A, Vonsover A, Zlotnikov S, Jehuda-Cohen T, Bentwich Z. High prevalence of HIV-specific immunity in seronegative Ethiopian immigrants in Israel. AIDS 1997: 11(1): 117-118.

12. Bentwich Z, Weisman Z, Moroz C, Bar-Yehuda S, Kalinkovich A. Immune dysregulation in Ethiopian immigrants in Israel: relevance to helminth infections? Clinical and experimental immunology 1996: 103(2): 239-243.

13. Kalinkovich A, Borkow G, Weisman Z, Tsimanis A, Stein M, Bentwich Z. Increased CCR5 and CXCR4 expression in Ethiopians living in Israel: environmental and constitutive factors. Clinical immunology 2001: 100(1): 107-117.

14. Kalinkovich A, Weisman Z, Bentwich Z. Chemokines and chemokine receptors: role in HIV infection. Immunol Lett 1999: 68(2-3): 281-287.

15. Kalinkovich A, Weisman Z, Leng Q, Borkow G, Stein M, Greenberg Z, Zlotnikov S, Eitan S, Bentwich Z. Increased CCR5 expression with decreased beta chemokine secretion in Ethiopians: relevance to AIDS in Africa. J Hum Virol 1999: 2(5): 283-289.

16. Gopinath R, Ostrowski M, Justement SJ, Fauci AS, Nutman TB. Filarial infections increase susceptibility to human immunodeficiency virus infection in peripheral blood mononuclear cells in vitro. J Infect Dis 2000: 182(6): 1804-1808.

17. Shapira-Nahor O, Kalinkovich A, Weisman Z, Greenberg Z, Nahmias J, Shapiro M, Panet A, Bentwich Z. Increased susceptibility to HIV-1 infection of peripheral blood mononuclear cells from chronically immune-activated individuals. AIDS 1998: 12(13): 1731-1733.

18. Kroidl I, Saathoff E, Maganga L, Makunde WH, Hoerauf A, Geldmacher C, Clowes P, Maboko L, Hoelscher M. Effect of Wuchereria bancrofti infection on HIV incidence in southwest Tanzania: a prospective cohort study. Lancet,Aug 3 pii: S0140-6736(16)31252-1 doi: 101016/S0140-6736(16)31252-1 [Epub ahead of print] 2016.

19. Nielsen NO, Simonsen PE, Magnussen P, Magesa S, Friis H. Cross-sectional relationship between HIV, lymphatic filariasis and other parasitic infections in adults in coastal northeastern Tanzania. Trans R Soc Trop Med Hyg 2006: 100(6): 543-550.

20. Nielsen NO, Friis H, Magnussen P, Krarup H, Magesa S, Simonsen PE. Co-infection with subclinical HIV and Wuchereria bancrofti, and the role of malaria and hookworms, in adult Tanzanians: infection intensities, CD4/CD8 counts and cytokine responses. Trans R Soc Trop Med Hyg 2007: 101(6): 602-612.

21. Talaat KR, Kumarasamy N, Swaminathan S, Gopinath R, Nutman TB. Filarial/human immunodeficiency virus coinfection in urban southern India. Am J Trop Med Hyg 2008: 79(4): 558-560.

22. Tafatatha T, Taegtmeyer M, Ngwira B, Phiri A, Kondowe M, Piston W, Molesworth A, Kayuni N, Koole O, Crampin A, Horton J, French N. Human Immunodeficiency Virus, Antiretroviral Therapy and Markers of Lymphatic Filariasis Infection: A Cross-sectional Study in Rural Northern Malawi. PLoS Negl Trop Dis 2015: 9(6): e0003825.

23. Talaat KR, Babu S, Menon P, Kumarasamy N, Sharma J, Arumugam J, Dhakshinamurthy K, Srinivasan R, Poongulali S, Gu W, Fay MP, Swaminathan S, Nutman TB. Treatment of W. bancrofti (Wb) in HIV/Wb coinfections in South India. PLoS Negl Trop Dis 2015: 9(3): e0003622.

24. Tafatatha TT, Ngwira BM, Taegtmeyer M, Phiri AJ, Wilson TP, Banda LG, Piston WN, Koole O, Horton J, French N. Randomised controlled clinical trial of increased dose and frequency of albendazole and ivermectin on Wuchereria bancrofti microfilarial clearance in northern Malawi. Trans R Soc Trop Med Hyg 2015: 109(6): 393-399.

25. Kroidl I, Saathof E, Maganga L, Clowes P, Maboko L, Hoerauf A, Makunde WH, Haule A, Mviombo P, Pitter B, Mgeni N, Mabuye J, Kowuor D, Mwingira U, Malecela MN, Loscher T, Hoelscher M. Prevalence of Lymphatic Filariasis and Treatment Effectiveness of Albendazole/ Ivermectin in Individuals with HIV Co-infection in Southwest-Tanzania. PLoS Negl Trop Dis 2016: 10(4): e0004618.

26. Chachage M, Podola L, Clowes P, Nsojo A, Bauer A, Mgaya O, Kowour D, Froeschl G, Maboko L, Hoelscher M, Saathoff E, Geldmacher C. Helminth-associated systemic immune activation and HIV co-receptor expression: response to albendazole/praziquantel treatment. PLoS Negl Trop Dis 2014: 8(3): e2755.

27. Chachage M, Pollakis G, Kuffour EO, Haase K, Bauer A, Nadai Y, Podola L, Clowes P, Schiemann M, Henkel L, Hoffmann D, Joseph S, Bhuju S, Maboko L, Sarfo FS, Eberhardt K, Hoelscher M, Feldt T, Saathoff E, Geldmacher C. CD25+FoxP3+ memory CD4 T cells are frequent targets of HIV infection in vivo. J Virol 2016.

28. Hoerauf A. New strategies to combat filariasis. Expert Rev Anti Infect Ther 2006: 4(2): 211-222.

29. Hoerauf A. Filariasis: new drugs and new opportunities for lymphatic filariasis and onchocerciasis. Curr Opin Infect Dis 2008: 21(6): 673-681.

30. Hoerauf A, Mand S, Fischer K, Kruppa T, Marfo-Debrekyei Y, Debrah AY, Pfarr KM, Adjei O, Buttner DW. Doxycycline as a novel strategy against bancroftian filariasis-depletion of Wolbachia endosymbionts from Wuchereria bancrofti and stop of microfilaria production. Med Microbiol Immunol 2003: 192(4): 211-216.

31. Hoerauf A, Marfo-Debrekyei Y, Buttner M, Debrah AY, Konadu P, Mand S, Adjei O, Buttner DW. Effects of 6-week azithromycin treatment on the Wolbachia endobacteria of Onchocerca volvulus. Parasitol Res 2008: 103(2): 279-286.

32. Hoerauf A, Specht S, Buttner M, Pfarr K, Mand S, Fimmers R, Marfo-Debrekyei Y, Konadu P, Debrah AY, Bandi C, Brattig N, Albers A, Larbi J, Batsa L, Taylor MJ, Adjei O, Buttner DW. Wolbachia endobacteria depletion by doxycycline as antifilarial therapy has macrofilaricidal activity in onchocerciasis: a randomized placebo-controlled study. Med Microbiol Immunol 2008: 197(3): 295-311.

33. Thomsen EK, Sanuku N, Baea M, Satofan S, Maki E, Lombore B, Schmidt MS, Siba PM, Weil GJ, Kazura JW, Fleckenstein LL, King CL. Efficacy, Safety, and Pharmacokinetics of Coadministered Diethylcarbamazine, Albendazole, and Ivermectin for Treatment of Bancroftian Filariasis. Clin Infect Dis 2016: 62(3): 334-341.

34. Tessema TA, Hamasur B, Bjun G, Svenson S, Bjorvatn B. Diagnostic evaluation of urinary lipoarabinomannan at an Ethiopian tuberculosis centre. Scand J Infect Dis 2001: 33(4): 279-284.

35. Hamasur B, Bruchfeld J, Haile M, Pawlowski A, Bjorvatn B, Kallenius G, Svenson SB. Rapid diagnosis of tuberculosis by detection of mycobacterial lipoarabinomannan in urine. J Microbiol Methods 2001: 45(1): 41-52.

36. Dheda K, Davids V, Lenders L, Roberts T, Meldau R, Ling D, Brunet L, van Zyl Smit R, Peter J, Green C, Badri M, Sechi L, Sharma S, Hoelscher M, Dawson R, Whitelaw A, Blackburn J, Pai M, Zumla A. Clinical utility of a commercial LAM-ELISA assay for TB diagnosis in HIV-infected patients using urine and sputum samples. PLoS One 2010: 5(3): e9848.

37. Reither K, Saathoff E, Jung J, Minja LT, Kroidl I, Saad E, Huggett JF, Ntinginya EN, Maganga L, Maboko L, Hoelscher M. Low sensitivity of a urine LAM-ELISA in the diagnosis of pulmonary tuberculosis. BMC Infect Dis 2009: 9: 141.

38. Kroidl I, Clowes P, Mwakyelu J, Maboko L, Kiangi A, Rachow A, Reither K, Jung J, Nsojo A, Saathoff E, Hoelscher M. Reasons for false-positive lipoarabinomannan ELISA results in a Tanzanian population. Scand J Infect Dis 2014: 46(2): 144-148.

39. Kroidl I, Clowes P, Reither K, Mtafya B, Rojas-Ponce G, Ntinginya EN, Kalomo M, Minja LT, Kowuor D, Saathoff E, Kroidl A, Heinrich N, Maboko L, Bates M, O'Grady J, Zumla A, Hoelscher M, Rachow A. Performance of urine lipoarabinomannan assays for paediatric tuberculosis in Tanzania. Eur Respir J 2015: 46(3): 761-770.

40. Rose MV, Kimaro G, Kroidl I, Hoelscher M, Bygbjerg IC, Mfinanga SM, Ravn P. Evaluation of QuantiFERON microtube, using 0.9 mL blood, for diagnosing tuberculosis infection. Eur Respir J 2013: 41(4): 909-916.

41. Rose MV, Kimaro G, Nissen TN, Kroidl I, Hoelscher M, Bygbjerg IC, Mfinanga SG, Ravn P. QuantiFERON(R)-TB gold in-tube performance for diagnosing active tuberculosis in children and adults in a high burden setting. PLoS One 2012: 7(7): e37851.

DANKSAGUNG

Ich möchte allen Studienteilnehmern und Studienteilnehmerinnen danken für Ihre Beteiligung an den Untersuchungen. Hinter den schönen Veröffentlichungen steckt viel Arbeit eines großen Teams und ich möchte meinen Kollegen in Tansania und München danken für die fröhliche und fruchtbare Zusammenarbeit. Folgende Forschungseinrichtungen haben finanziell die hier dargelegten Arbeiten unterstützt:

Europäische Union

Bundesministerium für Bildung und Forschung

Henry M. Jackson Foundation for the Advancement of Military Medicine

United Stated Department of Defense

Persönlich danken möchte ich meinen Professoren Michael Hölscher und Thomas Löscher, die mich sowohl in der klinischen, als auch in der Forschungstätigkeit beraten und unterstützt haben.

Liste der für die Habilitation zusammengefassten eigenen Arbeiten mit Impact Faktor (IF)

1. **Kroidl I**, Saathoff E, Maganga L, Makunde WH, Hoerauf A, Geldmacher C, Clowes P, Maboko L, Hoelscher M. Effect of Wuchereria bancrofti infection on HIV incidence in southwest Tanzania: a prospective cohort study. Lancet 2016: 388(10054): 1912-1920. **(IF: 44,00)**

2. **Kroidl I**, Saathof E, Maganga L, Clowes P, Maboko L, Hoerauf A, Makunde WH, Haule A, Mviombo P, Pitter B, Mgeni N, Mabuye J, Kowuor D, Mwingira U, Malecela MN, Loscher T, Hoelscher M. Prevalence of Lymphatic Filariasis and Treatment Effectiveness of Albendazole/ Ivermectin in Individuals with HIV Co-infection in Southwest-Tanzania. PLoS Negl Trop Dis 2016: 10(4): e0004618. **(IF: 4,45)**

3. Schule SA, Clowes P, **Kroidl I**, Kowuor DO, Nsojo A, Mangu C, Riess H, Geldmacher C, Laubender RP, Mhina S, Maboko L, Loscher T, Hoelscher M, Saathoff E. Ascaris lumbricoides infection and its relation to environmental factors in the Mbeya region of Tanzania, a cross-sectional, population-based study. PLoS One 2014: 9(3): e92032. **(IF: 4,17)**

4. Riess H, Clowes P, **Kroidl I**, Kowuor DO, Nsojo A, Mangu C, Schule SA, Mansmann U, Geldmacher C, Mhina S, Maboko L, Hoelscher M, Saathoff E. Hookworm infection and environmental factors in mbeya region, Tanzania: a cross-sectional, population-based study. PLoS Negl Trop Dis 2013: 7(9): e2408. **(IF: 5,32)**

5. **Kroidl I**, Clowes P, Mwalongo W, Maganga L, Maboko L, Kroidl AL, Geldmacher C, Machibya H, Hoelscher M, Saathoff E. Low specificity of determine HIV1/2 RDT using whole blood in south west Tanzania. PLoS One 2012: 7(6): e39529. **(IF: 4,82)**

6. Robb ML, Eller LA, Kibuuka H, Rono K, Maganga L, Nitayaphan S, Kroon E, Sawe FK, Sinei S, Sriplienchan S, Jagodzinski LL, Malia J, Manak M, de Souza MS, Tovanabutra S, Sanders-Buell E, Rolland M, Dorsey-Spitz J, Eller MA, Milazzo M, Li Q, Lewandowski A, Wu H, Swann E, O'Connell RJ, Peel S, Dawson P, Kim JH, Michael NL, **RV 217 Study Team** (**Kroidl I**,…and more). Prospective Study of Acute HIV-1 Infection in Adults in East Africa and Thailand. N Engl J Med 2016: 374(22): 2120-2130. **(IF: 59,56)**

7. **Kroidl I**, Clowes P, Reither K, Mtafya B, Rojas-Ponce G, Ntinginya EN, Kalomo M, Minja LT, Kowuor D, Saathoff E, Kroidl A, Heinrich N, Maboko L, Bates M, O'Grady J, Zumla A, Hoelscher M, Rachow A. Performance of urine lipoarabinomannan assays for paediatric tuberculosis in Tanzania. Eur Respir J 2015: 46(3): 761-770. **(IP: F,33)**

8. **Kroidl I**, Clowes P, Mwakyelu J, Maboko L, Kiangi A, Rachow A, Reither K, Jung J, Nsojo A, Saathoff E, Hoelscher M. Reasons for false-positive lipoarabinomannan ELISA results in a Tanzanian population. Scandinavian journal of infectious diseases 2014: 46(2): 144-148. **(IF: 1,69)**

9. Rose MV, Kimaro G, Nissen TN, **Kroidl I**, Hoelscher M, Bygbjerg IC, Mfinanga SG, Ravn P. QuantiFERON(R)-TB gold in-tube performance for diagnosing active tuberculosis in children and adults in a high burden setting. PLoS One 2012: 7(7): e37851. **(IF: 4,82)**

THELANCET-D-16-04773R2

PII TO BE REQUESTED

Embargo: August 2, 2016—23:30 (BST)

Effect of *Wuchereria bancrofti* infection on HIV incidence in southwest Tanzania: a prospective cohort study

Inge Kroidl, Elmar Saathof, Lucas Maganga, Williams H Makunde, Achim Hoerauf, Christof Geldmacher, Petra Clowes, Leonard Maboko, Michael Hoelscher

Summary

Background **The past decades have seen an ongoing controversial debate about whether the immune activation induced by helminths has an effect on the susceptibility of individuals to HIV. In view of this, we assessed the effect of lymphatic filariasis, a chronic helminth disease elicited by *Wuchereria bancrofti*, on HIV incidence in southwest Tanzania.**

Methods **In this population-based cohort study, we enrolled a geographically stratified randomly chosen sample of about 10% of the households in nine distinct sites in southwest Tanzania. All household members present were followed up and tested for HIV and circulating filarial antigen, an indicator of *W bancrofti* adult worm burden. Our main outcome of interest was HIV incidence in participants with or without lymphatic filariasis.**

Findings **Between May 29, 2006, and June 16, 2011, we enrolled 4283 households with roughly 18 000 participants. Of these, 2699 individuals from Kyela district participated in at least one round of the EMINI study. In the 1055 initially HIV-negative adolescents and adults with clearly defined lymphatic filariasis status, 32 new HIV infections were observed in 2626 person-years. HIV incidence in lymphatic filariasis-positive participants (1·91 cases per 100 person-years) was significantly higher than the incidence in lymphatic filariasis-negative participants (0·80 cases per 100 person-years). The age-adjusted and sex-adjusted incidence rate ratio was 2·17 (95% CI 1·08–4·37, p=0·0300). Lymphatic filariasis status remained an independent and significantly relevant risk factor for HIV infection when controlled for other known risk factors such as sexual behaviour and socioeconomic factors.**

Interpretation **To our knowledge, this is the first prospective study demonstrating a significantly increased risk of acquiring HIV for lymphatic filariasis-infected individuals. Immunological studies and interventional treatment studies that eliminate the adult worms and not only the microfilaria are needed to follow up on the results presented.**

Funding **European Union as part of EuropAid; German Ministry of Education and Research; German Center for Infection Research.**

Published **Online** August 2, 2016 http://dx.doi.org/10.1016/PII

See **Online/Comment** http://dx.doi.org/10.1016/PII

Division of Infectious Diseases and Tropical Medicine, Medical Center of the University of Munich, Munich, Germany (I Kroidl MD, E Saathof PhD, C Geldmacher PhD, Prof M Hoelscher PhD); **National Institute of Medical Research, Mbeya Medical Research Centre, Mbeya, Tanzania** (I Kroidl, L Maganga MD, P Clowes MD, L Maboko PhD, Prof M Hoelscher); **German Center for Infection Research (DZIF), partner site Munich, Munich, Germany** (I Kroidl, E Saathof, C Geldmacher, Prof M Hoelscher); **National Institute of Medical Research, Tanga, Tanzania** (W H Makunde MD); **Institute of Medical Microbiology, Immunology and Parasitology, University Hospital of Bonn, Bonn, Germany** (Prof A Hoerauf PhD); **and German Center for Infection Research (DZIF), partner site Bonn-Cologne, Bonn-Cologne, Germany** (Prof A Hoerauf)

Correspondence to: Inge Kroidl, Division of Infectious Diseases and Tropical Medicine, Medical Center of the University of Munich, Munich 80802, Germany **ikroidl@lrz.uni-muenchen.de**

Introduction

The disproportionately high prevalence of HIV in communities of sub-Saharan Africa has led to the hypothesis that concomitant parasitic infections might increase the risk of HIV transmission.[1,2] The foundation of this premise has been based on cross-sectional studies of Ethiopian immigrants in Israel since correlations were observed between the prevalence of helminth and HIV infections.[3] An underlying mechanism for this occurrence is that the immune milieu induced by helminth infections could facilitate HIV acquisition.[4] In those participants who were predominantly infected with soil-transmitted helminths, immunological changes were characterised by a dominant T-helper-2 cell response and broad T-cell activation.[5,6] Moreover, an increased susceptibility for HIV in peripheral blood mononuclear cells of these immigrants was found through in vitro experimentation.[7]

Further support that helminths affect HIV susceptibility stems from several cross-sectional studies[8] that have reported a positive association of urogenital schistosomiasis with HIV prevalence. By contrast with soil and vector-transmitted helminths, infections with *Schistosoma haematobium* are located in the urogenital tract and it seems that the disruption of the mucosal barrier is the prevailing factor for increased susceptibility to the virus.[9] With regards to filarial nematode infections, no studies have addressed the effect on HIV incidence in endemic communities. Lymphatic filariasis is a chronic helminth disease that infects 120 million people worldwide and is elicited by *Wuchereria bancrofti* or *Brugia* species.[10] Adult worms reside in the lymphatic system for many years, producing the transmission life-stage microfilariae. Although most individuals with lymphatic filariasis remain asymptomatic, presenting either patent (with microfilariae) or latent (without microfilariae) states,[11] some patients do develop severe pathology (hydrocele or lymphoedema).[12] Mass drug administration programmes focus on the interruption of transmission with drugs that target microfilariae. An interesting facet of filarial nematodes is the requirement of the endosymbiont *Wolbachia* spp for their survival and reproduction. Indeed, this has become the Achilles' heel of filariae because treatment with tetracyclines is additionally macrofilaricidal and can revert and improve pathology.[13] Before larger treatment programmes started in 2001, lymphatic filariasis was present in most of the 21 regions of Tanzania, with a reported prevalence in

Research in context

Evidence before the study

We searched PubMed on April 15, 2016, for studies published in English using the search terms "lymphatic filariasis", "*Wuchereria bancrofti*", and "HIV" with no restrictions on publication date. We found few manuscripts describing co-infections of both diseases, which have been cited and discussed in this manuscript. Helminth infections have been suggested as one of the factors driving the HIV/AIDS epidemic in sub-Saharan Africa. However, not many studies have examined the interaction of *W bancrofti*, a mosquito-transmitted filarial nematode, and HIV, and all of them have focused on already co-infected individuals. A cross-sectional study in north Tanzania suggested a higher HIV prevalence in *W bancrofti*-positive individuals, but a follow-up study of the same group did not provide evidence for an interaction of lymphatic filariasis with HIV. Recent reports from India, Zimbabwe, and south Tanzania did not show any difference in lymphatic filariasis prevalence or circulating filarial antigen levels in HIV-positive compared with HIV-negative individuals. However, none of the published reports focused on the effect of lymphatic filariasis on HIV transmission.

Added value of the study

The risk for HIV infection is determined by multiple factors, most of which are behavioural. This study describes an increased HIV infection risk caused by a helminth infection (lymphatic filariasis). Our findings open up new opportunities for prevention, especially in the high-HIV-risk group of young adults in developing countries. Furthermore they support the primarily laboratory-based hypothesis that an immune-activated host is more receptive for HIV infection, which if investigated further might improve our understanding of the physiopathology during primary HIV infection.

Implications of all the available evidence

The adult worm of *W bancrofti* lives for 10–12 years in the lymphatic system and is not killed by single-dose treatment. By contrast, it takes up to 10 years of annual mass treatment to produce a lasting reduction in lymphatic filariasis prevalence in an affected area. Lymphatic filariasis eradication programmes in the past decade have focused on the reduction of transmission but made only limited efforts to cure *W bancrofti* infection, although recently an active therapy has become available. Lymphatic filariasis, together with other helminth infections, belongs to the 17 neglected diseases as defined by WHO. Our findings add another argument to push neglected diseases, in this case filarial infection, into the focus of global strategies, as they create not only morbidity but in addition generate an increased risk of acquiring HIV.

coastal regions of up to 44% (799 of 1829 in Tanga, Tanzania).[14] In the southern regions of Tanzania, mass drug administration programmes began in 2009. With regards to HIV, several governmental surveys have documented prevalence.[15] The third population-based Tanzanian HIV/AIDS and Malaria Indicator Survey 2011/2012 found a countrywide HIV prevalence of 5% in Tanzanian adults between 15 and 49 years of age, and a 9% prevalence for the same age group in the Mbeya region. Possible effects of lymphatic filariasis infection on the prevalence or pathology of HIV have been previously studied.[16–19] A large cross-sectional prevalence study in northern Tanzania reported an increased HIV prevalence in individuals infected with *W bancrofti*.[17] However, a follow-up study in the same group did not support this association.[16] Recent studies[18,20] from Malawi and Tanzania reported no evidence that HIV infection affected lymphatic filariasis epidemiology. Because no prospective study focusing on HIV susceptibility in individuals with lymphatic filariasis has been published to date, we did a large cohort study in individuals from the Mbeya region of Tanzania, an area highly endemic for both diseases.[15]

Methods

Study design and participants

The EMINI study was a population-based cohort study done to assess and monitor the effect of future interventions done in the area of infectious disease control (eg, HIV, tuberculosis, malaria, neglected tropical diseases). We enrolled a geographically stratified random sample of about 10% of the households in nine distinct sites. There were no exclusion criteria except for unwillingness to participate or to donate a biological sample. The overarching aim of the cohort was to describe the epidemiology of HIV, tuberculosis, malaria, and other infectious diseases and to generate baseline data for the planning and conduct of future randomised trials. Following the advice of the local and international ethical boards that approved the study, reporting of HIV results and medical care was offered by an independent team on a voluntary and anonymous basis. To ensure privacy, the study team remained masked to any HIV data. Surveillance data of lymphatic filariasis were generated as part of a substudy of EMINI (SOLF) that was initiated in 2009 to describe the effect of mass drug administration (ivermectin and albendazole) on filarial prevalence in the general population of Kyela region between 2009 and 2011. Filarial antigen testing was done on all participants of EMINI in the Kyela district at all visits on stored samples. Although the number of included households remained unchanged over the years, the family members participating in the study varied. The EMINI and SOLF studies were approved by both the National Ethical Committee/Medical Research Coordinating Committee of the National Institute for Medical Research, Tanzania, and the Mbeya Medical Research and Ethics Committee. All adult participants

provided written informed consent before enrolment in the study. Parents provided consent for children younger than 18 years of age and children above the age of 12 years signed their own assent form, together with their parents' consent.

Procedures

In five annual surveys the households were visited by the study team and all household members present at that time answered a structured questionnaire and donated blood, urine, stool, and sputum (if suspected for tuberculosis). Unused samples were stored for further analysis. Additionally, participants were interviewed to accumulate data on sociodemographic and behavioural factors. Interview data were collected with handheld computers and downloaded into an Access database on return to the institute. The characterisation of the socioeconomic status is described in the appendix. Laboratory data were recorded on paper, double entered into an Access database, and then compared with the original to check for discrepancies. Results for the ELISA tests for Lymphatic filariasis and HIV were directly imported into the database.

A commercially available ELISA (Og4C3 serum ELISA, TropBio, Townsville, Australia) was used to detect the circulating filarial antigen in 100 µL of the collected sera (appendix).[20] HIV screening was done with a rapid diagnostic test (Determine HIV 1/2 test, Abbott Laboratories, Abbott Park, IL, USA, in 2007; HIV 1/2 STAT-PAK Dipstick Assay, Chembio Diagnostics Systems, Medford, NY, USA, in 2008; and SD-Bioline *HIV-1/2 3·0,* Standard Diagnostics, Kyonggi-do, South Korea, from 2009 to 2011). All positive rapid test results were confirmed with an ELISA HIV test (Enzygnost Anti HIV 1/2 Plus, DADE-Behring, Marburg, Germany) and subsequent western blot (MPD HIV Blot 2.2, MP Biomedicals, Geneva, Switzerland) if results were discordant.

Negative rapid diagnostic tests from the previous annual survey, which again showed a negative rapid diagnostic test result in the following survey, were regarded as confirmed negative. For all HIV incident cases, the negative result of the previous year was confirmed by an ELISA HIV test, according to the above described testing algorithm. For children younger than 2 years, HIV testing was done by nucleic acid amplification test (PCR, Amplicor, Roche, Randburg, South Africa) with EDTA plasma.[21]

To measure HIV incidence and related variables, we used observation periods to define exposure time—ie, the time between two consecutive visits for the same participant (observation period) was only considered as exposure time if the participant both had an HIV result at the beginning and at the end of the respective period and if the result at the beginning was negative. Univariable and multivariable models included person-time for observation periods with defined lymphatic filariasis status until the earliest of the following points: the end of follow-up after about 4 years, the date when the participant was last visited, or the estimated date of HIV seroconversion, taken as the midpoint between the last negative and the first positive HIV test result.

See Online for appendix

Outcomes

Our main outcome of interest was HIV incidence in participants with or without lymphatic filariasis. For lymphatic filariasis, we used a conservative status definition which excluded observation periods where the lymphatic filariasis status changed during the period, meaning that we only retained periods with stable lymphatic filariasis status where lymphatic filariasis test results at the beginning and at the end were either both positive or both negative. To test the results obtained with the above conservative lymphatic filariasis status definition for robustness, we also used a more liberal

	Person-years	HIV incidence	Incidence per 100 person-years	Univariable model*		Multivariable model†	
				IRR (95% CI)	p value	IRR (95% CI)	p value
Lymphatic filariasis status							
Negative	1632	13	0·80	1	··	1	··
Positive	994	19	1·91	2·38 (1·17–4·84)	0·0163	2·17 (1·08–4·37)	0·0300
Sex							
Female	1333	18	1·35	1	··	1	··
Male	1293	14	1·08	0·80 (0·40–1·62)	0·5416	0·78 (0·38–1·57)	0·4801
Age (years)							
14 to <25*	1095	7	0·64	1	··	1	··
25 to <45	758	19	2·51	3·87 (1·62–9·22)	0·0023	3·49 (1·46–8·38)	0·0050
≥45	773	6	0·78	1·21 (0·41–3·63)	0·7302	1·01 (0·32–3·19)	0·9824
Socioeconomic rank (per unit)	··	··	··	0·96 (0·84–1·10)	0·5577	0·95 (0·84–1·08)	0·4738

Univariable and multivariable results from log binomial regression models with robust variance estimates to adjust for within-person clustering only in person-periods with stable lymphatic filariasis status. Socioeconomic rank was calculated based on the participant's household according to socioeconomic status score, calculated from various proxy variables. IRR=incidence rate ratio. *Univariable results are from analysing each variable in a separate model. †Multivariable results including all variables shown.

Table 1: **Association of lymphatic filariasis status and other covariates with HIV incidence**

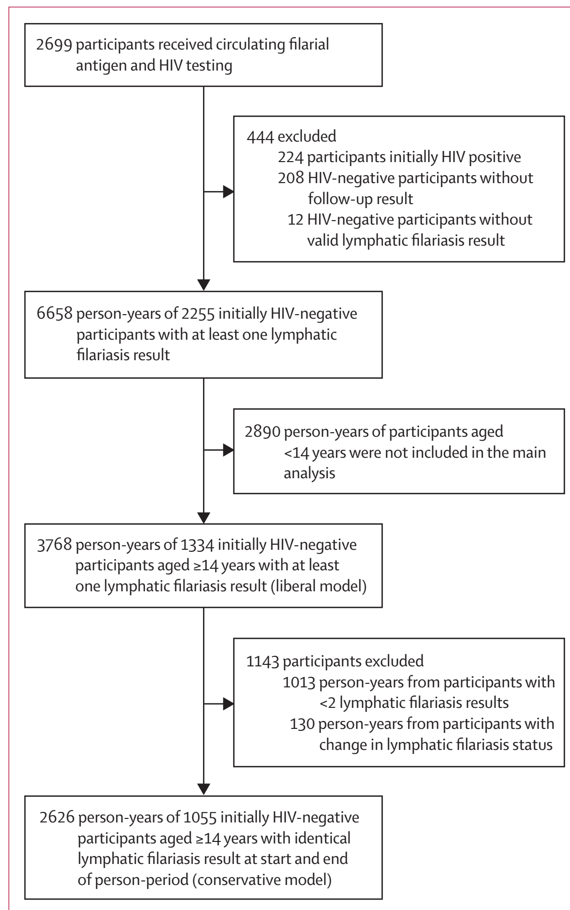


Figure 1: **Flow chart showing study participants with person-years and exclusion criteria for defining the liberal and conservative case models for lymphatic filariasis**

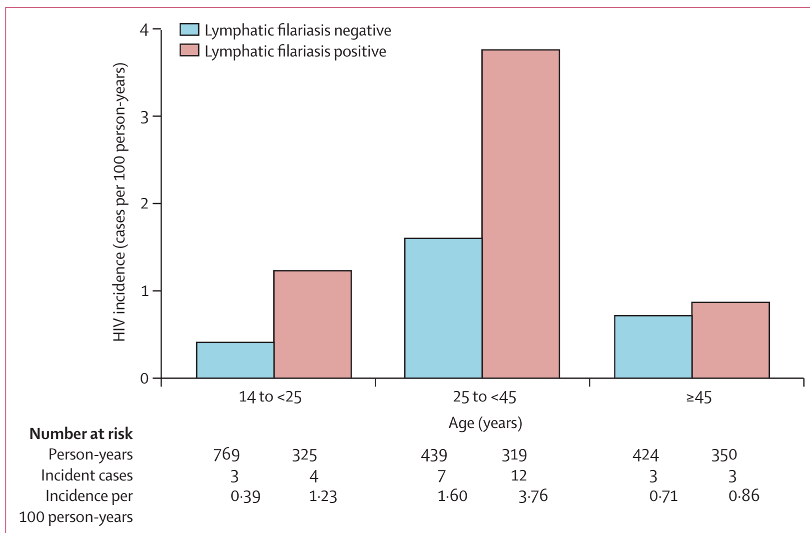


Figure 2: **HIV incidence by age and lymphatic filariasis status (conservative definition)**
For each age group the person-years and number of incident cases are given for the lymphatic filariasis-negative and lymphatic filariasis-positive groups. For the age group of 6 to <14 years, no incident cases were noted for both groups despite the high number of person-years.

definition, which used the maximum binary lymphatic filariasis result that had been obtained for each participant over the whole course of the study, meaning that participants with at least one positive lymphatic filariasis result were regarded as positive for lymphatic filariasis according to this definition.

Statistical analysis

Our primary analysis used log binomial regression models to assess the association of stable lymphatic filariasis infection with HIV incidence univariably and adjusted for sex, age, and socioeconomic status. Robust variance estimates were used to adjust for within-participant clustering and person-time was accounted for by including its natural logarithm as an offset into the model. Lymphatic filariasis infection status, age, and other covariates that were subject to change over time were included in our analyses as time-varying covariates.

To examine the potentially confounding effect of other known risk factors for HIV infection (eg, sex, marital status, living with a discordant partner, male circumcision, etc), we analysed the unadjusted association of HIV incidence with each of these risk factors separately for each age group to identify potential differences in these associations at different ages. Furthermore, we ran separate binomial regression models with the same specifications as previously mentioned that each included lymphatic filariasis infection status, age, and only one of these risk factors respectively.

To examine the results of the primary analysis (table 1) for robustness, we used the Cox proportional-hazards model to estimate hazard ratios (HRs; appendix) with the conservative stable lymphatic filariasis status definition, using robust variance estimates adjusted for within-participant clustering. A Kaplan-Meier plot was used to visualise the cumulative HIV incidence during the course of the study. Furthermore we estimated univariable and multivariable log binomial regression models with similar specifications as the main model, but with the more liberal maximum lymphatic filariasis status definition (appendix). Observations where HIV incidence or lymphatic filariasis status could not be ascertained were excluded from subsequent analyses (figure 1).

Regarding covariates used in the main model (table 1), sex and age information was available for every participant. Household socioeconomic status information was available for all participants and for most of the five annual visits. If this information was missing for one of the visits we used the information from the previous visit, or, if this was unavailable, from the following visit. Since all other covariates which were used in additional analyses (tables 2, 3; appendix) were categorical, we created a separate category for missing values which was included in our analyses. Stata/SE software, version 14.1 was used for all statistical analyses and to draw graphs.

 www.thelancet.com **Published online August 2, 2016 http://dx.doi.org/10.1016/PII**

	Aged 14 to <25 years			Aged 25 to <45 years			Aged ≥45 years		
	Person-years	Incidence	IRR (95% CI)	Person-years	Incidence	IRR (95% CI)	Person-years	Incidence	IRR (95% CI)
Lymphatic filariasis status during person-period									
Negative*	769	0·39	1·00	439	1·60	1·00	424	0·71	1·00
Positive	325	1·23	3·16 (0·53– 21·5)	319	3·76	2·36 (0·86–7·06)	350	0·86	1·21 (0·16–9·05)
HIV status of spouse									
HIV negative*	93	2·15	1·00	549	0·91	1·00	412	0·24	1·00
HIV positive	6	0·00	0·00 (0·00– 78·3)	51	11·87	13·04 (3·32–54·0)	24	12·34	50·77 (4·08–2665)
No spouse	963	0·52	0·24 (0·04–2·53)	130	4·62	5·07 (1·29–21·0)	279	0·72	2·95 (0·15–174)
Data missing/NA	32	0·00	0·00 (0·00–15·5)	28	7·21	7·92 (0·75–48·4)	59	0·00	0·00 (0·00–274)
Marital status									
Never married	894	0·45	0·28 (0·05–1·88)	74	2·72	1·53 (0·16–7·01)	15	0·00	0·00 (0·00–46·3)
Married*	185	1·62	1·00	619	1·78	1·00	444	0·90	1·00
Divorced/seperated	13	0·00	0·00 (0·00–34·4)	32	12·41	6·99 (1·62–23·6)	30	3·38	3·76 (0·08–38)
Widowed	3	0·00	0·00 (0·00–147)	32	6·19	3·48 (0·38–16·0)	285	0·35	0·39 (0·01–3·94)
Number of sexual partners within the last year									
None	29	0·00	0·00 (0·00–11·2)	20	10·22	5·17 (0·56–23·7)	201	0·50	1·33 (0·02–104)
One*	381	1·57	1·00	556	1·98	1·00	267	0·37	1·00
Two or more	83	0·00	0·00 (0·00–3·90)	155	3·86	1·95 (0·59–5·76)	67	4·45	11·90 (0·96–624)
Data missing/NA	602	0·17	0·11 (0·00–0·87)	27	0·00	0·00 (0·00–8·34)	237	0·42	1·13 (0·01–88·3)
Circumcised (male)									
No*	561	0·53	1·00	286	2·10	1·00	293	1·37	1·00
Yes	57	0·00	0·00 (0·00–24)	73	1·37	0·65 (0·01–5·36)	23	0·00	0·00 (0·00–19·1)
NA (female)	476	0·84	1·57 (0·27–10·7)	399	3·01	1·43 (0·50–4·65)	457	0·44	0·32 (0·03–2·23)
Ever used condom (male)									
No*	275	0·73	1·00	444	2·93	1·00	682	0·88	1·00
Yes	229	1·75	2·40 (0·34–26·6)	302	1·99	0·68 (0·21–1·92)	56	0·00	0·00 (0·00–10·3)
Missing/NA (female)	590	0·17	0·23 (0·00–4·48)	12	0·00	0·00 (0·00–12·0)	35	0·00	0·00 (0·00–16·8)

Unadjusted values were used for the IRR calculation. Socioeconomic rank was calculated based on the participant's household according to socioeconomic status score, calculated from various proxy variables. IRR=incidence rate ratio per 100 person-years. NA=not applicable. *Reference strata.

Table 2: **Influence of different factors on HIV incidence stratified by age**

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. ES, IK, and MH had full access to all the data in the study and IK and MH had final responsibility for the decision to submit for publication.

Results

The EMINI study was conducted between May 29, 2006, and June 26, 2011. We enrolled 4283 households with roughly 18 000 participants. At the Kyela site, about 88% of participants of each previous year were followed up in the consecutive year. Kyela was visited five times between Feb 16, 2007, and June 10, 2011 (once a year). 2699 individuals from Kyela district participated in at least one round of the EMINI study (figure 1). 51% of participants were female, and the overall median age of the cohort was 15·4 years (IQR 7·2–31·1). 224 (8%) participants tested positive for HIV and were excluded from the analysis. The HIV age distribution was typical for a medium-high incidence country before introduction of antiretroviral therapy, with peak incidence in the reproductive age group (appendix).[20] The prevalence of lymphatic filariasis infection was 26% (691 of 2673), with a low prevalence in young children (13 [3%] of 489 children younger than 6 years), rising rapidly to an average prevalence of 41% (520 of 1280) in HIV-negative and 37% (72 of 193) in HIV-positive individuals older than 14 years. Valid lymphatic filariasis results and at least one follow-up HIV result were obtained from 2255 initially HIV-negative participants. The participants were followed up for 1–4 years (mean 2·95; SD 0·997), leading to 6658 person-years of observation, during which 48 study participants seroconverted to HIV (figure 1).

Three different age clusters were distinguished representing different possible routes of HIV transmission (0 to <6 years, 6 to 14 years, and older than 14 years). Among infants and toddlers younger than 6 years (986 person-years), the group of potential transmission through breastfeeding, we observed four incident HIV infections, of which one was in an infant with lymphatic

	Risk ratio lymphatic filariasis infection	p value for lymphatic filariasis infection	Years of exposure	Incident HIV infections	Risk ratio of the additional factor (95%CI)	p value
None (only adjusted for age)	2·20	0·0332	··	··	··	··
Spouse HIV status	2·39	0·0250				
HIV negative*	··	··	1054	8	1·00	··
HIV positive	··	··	81	9	12·88 (4·72–35·1)	<0·0001
No spouse in household	··	··	1372	13	2·61 (0·82–8·33)	0·1051
Data missing/NA	··	··	118	2	3·80 (0·76–19·0)	0·1047
Marital status	2·14	0·0387	··	··	··	··
Never married	··	··	982	6	0·68 (0·17–2·76)	0·5867
Married*	··	··	1249	18	1·00	··
Separated/divorced	··	··	75	5	4·48 (1·55–13·0)	0·0057
Widowed	··	··	320	3	1·15 (0·26–5·09)	0·8569
Number of sexual partners last year	2·07	0·0459	··	··	··	··
None			249	3	1·15 (0·26–5·04)	0·8521
One*	··	··	1204	18	1·00	··
Two or more	··	··	306	9	1·88 (0·84–4·20)	0·1263
Data missing/NA	··	··	866	2	0·26 (0·05–1·24)	0·0899
Religion	2·17	0·0359				
Christian*	··	··	2408	28	1·00	··
Muslim	··	··	63	1	1·13 (0·15–8·53)	0·9079
Data missing/NA	··	··	155	3	1·93 (0·61–6·11)	0·2636
Education	2·06	0·0500	··	··	··	··
None	··	··	537	6	1·15 (0·41–3·22)	0·7917
Any primary*	··	··	1202	22	1·00	··
Secondary and above	··	··	112	2	0·95 (0·22–4·18)	0·9507
Currently a student	··	··	774	2	0·20 (0·04–1·04)	0·0550
Occupation	2·03	0·0494	··	··	··	··
Farmer*	··	··	1719	28	1·00	··
Student	··	··	671	0	†	··
Other	··	··	235	4	1·03 (0·36–3·00)	0·9533
Circumcised (male)	2·22	0·0286	··	··	··	··
No*	··	··	1139	13	1·00	··
Yes	··	··	153	1	0·47 (0·06–3·36)	0·4482
NA (female)	··	··	1333	18	1·19 (0·58–2·44)	0·6426
Ever used condom	2·04	0·0544	··	··	··	··
No*	··	··	1401	21	1·00	··
Yes	··	··	587	10	0·87 (0·41–1·87)	0·7242
Data missing/NA	··	··	637	1	0·14 (0·02–1·15)	0·0673
Used birth control	2·22	0·0272	··	··	··	··
None*	··	··	379	7	1·00	··
Pill, injection, or implant	··	··	421	9	0·78 (0·26–2·30)	0·6464
Other	··	··	93	1	0·43 (0·05–3·43)	0·4229
NA (male)	··	··	1293	14	0·53 (0·20–1·38)	0·1960
Data missing/NA	··	··	440	1	0·18 (0·02–1·57)	0·1203
Haematuria ever	2·20	0·0331	··	··	··	··
Negative*	··	··	2059	24	1·00	··
Positive	··	··	566	8	1·08 (0·49–2·34)	0·8534

(Table 3 continues on next page)

filariasis. No seroconversions were observed in 1904 person-years from individuals between 6 and 14 years of age. 44, presumably sexual, HIV transmissions occurred during 3768 person-years in 1334 adolescents and adults older than 14 years. For stringency, the primary analysis was only done for observation periods that had the same lymphatic filariasis result at the start and end of a period (figure 1). With use of the "conservative" lymphatic filariasis status definition, 2626 person-years from 1055 individuals were analysed. Lymphatic filariasis-negative individuals contributed 1632 person-years, lymphatic filariasis-positive individuals contributed 994 person-years. Of the initially depicted 44 HIV infections in individuals older than 14 years, 32 occurred during the 2626 person-years with stable lymphatic filariasis status, 13 in lymphatic filariasis-negative (0·80 cases per 100 person-years) and 19 in lymphatic filariasis-positive individuals (1·91 cases per 100 person-years), suggesting a 2·38 times increased risk of HIV infection for individuals infected with *W bancrofti* compared to those who are uninfected (95% CI 1·17–4·84, p=0·0163; table 1).

Because our assessments focused on newly acquired HIV infections through sexual contact, we further categorised age into 14 years to older than 25 years, 25 years to younger than 45 years, and older than 45 years, which reflects the personal situations of participants (eg, marital status). With use of these groups, we assessed the effect of factors describing sociodemographic status or sexual behaviour on HIV incidence (table 2, appendix). Although most of those factors were more relevant in the middle and older age groups, lymphatic filariasis infection was one of the most important risk factors in the early years (14–25 years) of sexual activity (risk ratio [RR] 3·16, 95% CI 0·53–21·5, p=0·0750; table 2, figure 2). In this age group, the increased incidence risk was also reflected by a more than doubled prevalence of HIV in individuals with lymphatic filariasis (12 [8%] of 147) compared with individuals without lymphatic filariasis (11 [4%] of 303; RR 2·25, 95% CI 1·02–4·97, p=0·0406; appendix).

To control for potential confounding factors, we estimated the association of HIV incidence with stable lymphatic filariasis status in a multivariable log binominal regression model adjusted for sex, age, and socioeconomic status (table 1). The adjusted incidence rate ratio was 2·17 (95% CI 1·08–4·37, p=0·0300). Additionally, separate binomial regression models were done that included lymphatic filariasis status, age, and one other of the risk factors for HIV. A significant effect on HIV incidence could be found for the factors "living with an HIV-positive partner", "having more than one sex partner", and "being divorced or separated" (table 2). Factors which had no significant effect on the HIV incidence were education, religion, and occupation (appendix), and being circumcised (for males) or use of condoms (table 2). Further analysis with separate

binominal regression models, which included lymphatic filariasis status and age with one of the risk factors (table 3) showed that lymphatic filariasis infection remained a stable risk factor for new HIV infections.

Furthermore, we used a Kaplan-Meier plot to examine whether the duration of lymphatic filariasis infection differentially affected the risk of HIV infection. Supporting the findings from the regression analysis, the Kaplan-Meier plot showed that the HIV-infection hazard continues to be increased in individuals with lymphatic filariasis over the duration of the study (figure 3). To check the results of our primary analysis (table 1) for robustness, we also applied a Cox proportional-hazard model (appendix) and another multivariable log binomial regression model using the more liberal lymphatic filariasis case definition (figure 1), where all 44 HIV seroconversions, which occurred during 3768 person-years in 1334 adolescents or adults, were included (appendix). Compared with the main dataset (table 1), both of these additional models showed similar, statistically significant findings, strengthening the primary result of a significantly increased risk for HIV acquisition in individuals with lymphatic filariasis.

	Risk ratio lymphatic filariasis infection	p value for lymphatic filariasis infection	Years of exposure	Incident HIV infections	Risk ratio of the additional factor (95%CI)	p value
(Continued from previous page)						
Schistosoma haematobium ever†	2·20	0·0333	..	..	..	..
Negative*	..	..	2238	27	1·00	
Positive	..	..	387	5	1·01 (0·39–2·62)	0·9840
Any intestinal nematode ever‡§	2·25	0·0290	..	..	..	..
Negative*	..	..	1139	18	1·00	..
Positive	..	..	987	9	0·64 (0·28–1·46)	0·2842
Data missing	..	..	499	5	0·80 (0·30–2·15)	0·6577

All models included lymphatic filariasis infection, the respective risk factor, and age as covariates (results for age not shown). *Reference strata. †*S haematobium* eggs have been visualised at some timepoint during the study. All diagnosed infections have been treated with praziquantel. ‡*Trichuris trichiura, Ascaris lumbricoides,* or hookworm. §Infections with intestinal nematodes have been diagnosed through Kato Katz technique at some timepoint during the study. All diagnosed infections have been treated with albendazole.

Table 3: **Effect of HIV risk factors on the association between lymphatic filariasis and HIV incidence**

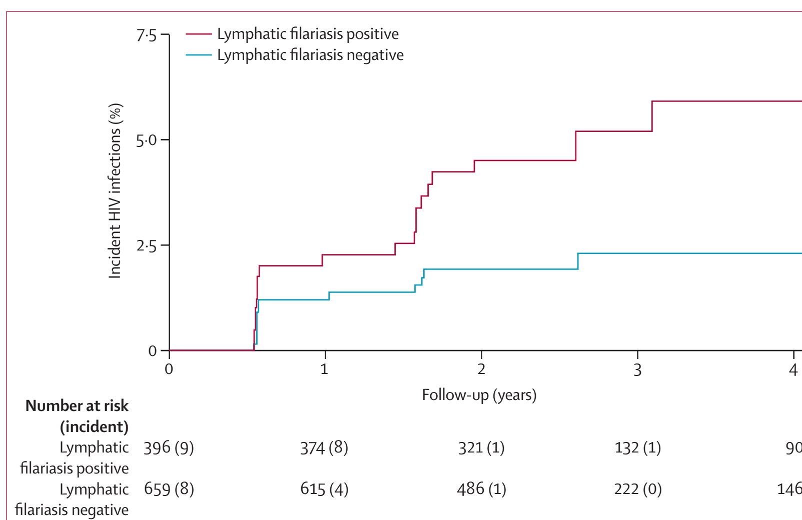


Figure 3: **Kaplan-Meier plot of HIV incidence by stable lymphatic filariasis status in participants older than 14 years**

Discussion

Our study shows a significantly increased risk of acquiring HIV in individuals infected with lymphatic filariasis. Our data were obtained in an area with high prevalence for both HIV and lymphatic filariasis infection at a time when antiretroviral treatment was not widely available, and mass drug administration only began in 2009, affecting the last 2 years of the study.[15,20] The most dominant association between lymphatic filariasis and HIV was in the adolescent group where more than a three-fold increase in HIV incidence was observed. This was also reflected in the HIV prevalence, which for this age group was significantly higher in those with lymphatic filariasis compared with those with no lymphatic filariasis. Although lymphatic filariasis infection was one of the most relevant risk factors in the years of first sexual activity, we show that known HIV risk factors such as marital status or the number of sexual partners are important for older adults.

A limitation of this study is its observational character, which precludes firm conclusions about the causal effect of lymphatic filariasis infection on HIV incidence. However, most conceivable confounders would have a socioeconomic dimension, which should have been apparent in our various models. We have carefully addressed possible confounding effects of major known risk factors for HIV and show that these factors did not have any effect on the influence of lymphatic filariasis on HIV incidence. Other limitations include missing lymphatic filariasis results for some individuals and losses to follow-up, which might both have biased our findings. However, we addressed this issue by using separate models with the conservative and the liberal lymphatic filariasis case definitions, which showed similar, statistically significant, results. The conservative model avoids bias that might be due to lymphatic filariasis misclassification, at the cost of introducing bias by excluding large amounts of person-time from analysis, whereas the "liberal" model makes full use of the available person-time, but introduces misclassification bias for the lymphatic filariasis exposure variable. The fact that both models are similar shows that our results are robust to both types of bias.

Potential causes for the increased HIV acquisition in individuals with lymphatic filariasis might exist in the interaction between the filarial parasite and the host's immune system. Helminth infections have been postulated to modulate the level of systemic immune

activation that could facilitate early viral dissemination.[4,22,23] Individuals with lymphatic filariasis can present distinct clinical conditions, which differ regarding the activation status of the immune system. Patients with lymphoedema show an increased proinflammatory Th17 profile,[12] whereas asymptomatic individuals display either T-cell hyporesponsiveness with high numbers of microfilariae, or increased immune responses in amicrofilaraemic individuals.[11] However, lymphatic filariasis is known to be associated with changes in the human immune system profile that could alleviate early HIV transmission events. For example, lymphatic filariasis infection-associated changes in the cytokine profile of peripheral blood mononuclear cells are linked to increased susceptibility to in-vitro HIV infection.[24] Furthermore, because macrophage populations have differing potential to mediate the transmission of HIV-1 to CD4-positive T cells, further studies should investigate whether the expansion of such populations in individuals with lymphatic filariasis[25] also facilitates HIV acquisition.

The effect of nematode infections on HIV transmission has been discussed for more than three decades. Previous cross-sectional studies that concentrated on coinfected individuals in Tanzania suggested an association,[17] although this could not be confirmed in follow-up studies.[16,18,20,26] Despite the difficulties in conducting longitudinal studies to dissect the effect of filariae on HIV transmission, we have been able to follow a large cohort of individuals from a high HIV incident setting over several years and show for the first time a significant effect of an ongoing lymphatic filariasis infection on the acquisition of HIV.

To reduce the susceptibility to HIV, a change in lymphatic filariasis treatment recommendations might help. Commonly used dual combinations of drugs administered for mass drug administration (diethylcarbamazine, albendazole, or ivermectin) in lymphatic filariasis kill microfilariae but only partly affect adult worms. Treatment concepts that aim to kill adult worms, such as doxycycline[27] or diethylcarbamazine, albendazole, and ivermectin in triple combination[28] (currently only used outside Africa because of contra-indication of diethylcarbamazine in onchocerciasis) might more quickly diminish the described increased susceptibility to HIV. Future studies should address the question of whether antifilarial treatment reduces HIV incidence in individuals with lymphatic filariasis. If true, this would be an important instrument to reduce HIV-infection in areas where lymphatic filariasis is prevalent.

Contributors

MH, ES, and LMab planned and received funding for the EMINI study. IK, LMag, LMab, WHM, AH, and MH planned and secured funding for the SOLF study. IK, LMag, PC, ES, and LMab were involved in the conduct and supervision of EMINI and SOLF. WHM, AH, and CG were involved in training for SOLF and ongoing scientific discussion. IK and ES analysed the data and take responsibility for the integrity of the data and the accuracy of the analysis. IK and ES drafted the initial manuscript and received major input from MH, AH, PC, and CG. All authors were involved in the critical revision of the manuscript.

Declaration of interests

We declare no competing interests.

Acknowledgments

We thank all EMINI participants for their willingness to be part of the study, and the EMINI field and laboratory teams for their contribution. We thank Joseph Mabuye, Neema Mgeni, Antelmo Haule, Prisca Mviombo, Jacklina Mhidze, and Bettina Pitter for performing lymphatic filariasis-specific laboratory work, Dickens Kowuor for data management, and Upendo Mwingira, Mwelecele N Malecela, and Laura E Layland for helpful discussions and critical assessment of the manuscript. This study was funded by the European Union as part of EuropAid (SANTE/2004/078-545/130; SANTE/2006/129-931) and the German Ministry of Science (BMBF, grant 01KA0904). AH was supported by the German Research Foundation (DFG grant PF 673/4-1). IK, ES, CG, and MH acknowledge support from the German Center for Infection Research.

References

1 Grant AD, Djomand G, De Cock KM. Natural history and spectrum of disease in adults with HIV/AIDS in Africa. *AIDS* 1997; **11** (suppl B): S43–54.
2 Van de Perre P. The epidemiology of HIV infection and AIDS in Africa. *Trends Microbiol* 1995; **3:** 217–22.
3 Bar-Yehuda S, Weisman Z, Kalinkovich A, et al. High prevalence of HIV-specific immunity in seronegative Ethiopian immigrants in Israel. *AIDS* 1997; **11:** 117–18.
4 Lawn SD, Butera ST, Folks TM. Contribution of immune activation to the pathogenesis and transmission of human immunodeficiency virus type 1 infection. *Clin Microbiol Rev* 2001; **14:** 753–77.
5 Bentwich Z, Weisman Z, Moroz C, Bar-Yehuda S, Kalinkovich A. Immune dysregulation in Ethiopian immigrants in Israel: relevance to helminth infections? *Clin Exp Immunol* 1996; **103:** 239–43.
6 Kalinkovich A, Weisman Z, Leng Q, et al. Increased CCR5 expression with decreased beta chemokine secretion in Ethiopians: relevance to AIDS in Africa. *J Hum Virol* 1999; **2:** 283–89.
7 Shapira-Nahor O, Kalinkovich A, Weisman Z, et al. Increased susceptibility to HIV-1 infection of peripheral blood mononuclear cells from chronically immune-activated individuals. *AIDS* 1998; **12:** 1731–33.
8 Kjetland EF, Ndhlovu PD, Gomo E, et al. Association between genital schistosomiasis and HIV in rural Zimbabwean women. *AIDS* 2006; **20:** 593–600.
9 Mbabazi PS, Andan O, Fitzgerald DW, Chitsulo L, Engels D, Downs JA. Examining the relationship between urogenital schistosomiasis and HIV infection. *PLoS Negl Trop Dis* 2011; **5:** e1396.
10 Hotez PJ, Kamath A. Neglected tropical diseases in sub-saharan Africa: review of their prevalence, distribution, and disease burden. *PLoS Negl Trop Dis* 2009; **3:** e412.
11 Arndts K, Deininger S, Specht S, et al. Elevated adaptive immune responses are associated with latent infections of *Wuchereria bancrofti*. *PLoS Negl Trop Dis* 2012; **6:** e1611.
12 Babu S, Bhat SQ, Pavan Kumar N, et al. Filarial lymphedema is characterized by antigen-specific Th1 and th17 proinflammatory responses and a lack of regulatory T cells. *PLoS Negl Trop Dis* 2009; **3:** e420.
13 Taylor MJ, Makunde WH, McGarry HF, Turner JD, Mand S, Hoerauf A. Macrofilaricidal activity after doxycycline treatment of Wuchereria bancrofti: a double-blind, randomised placebo-controlled trial. *Lancet* 2005; **365:** 2116–21.
14 Simonsen PE, Magesa SM, Dunyo SK, Malecela-Lazaro MN, Michael E. The effect of single dose ivermectin alone or in combination with albendazole on *Wuchereria bancrofti* infection in primary school children in Tanzania. *Trans R Soc Trop Med Hyg* 2004; **98:** 462–72.
15 Tanzania NIMR. Tanzania HIV/AIDS malaria indicator survey (2011/2012). https://dhsprogram.com/pubs/pdf/AIS11/AIS11.pdf (accessed March 10, 2016).
16 Nielsen NO, Friis H, Magnussen P, Krarup H, Magesa S, Simonsen PE. Co-infection with subclinical HIV and *Wuchereria bancrofti*, and the role of malaria and hookworms, in adult Tanzanians: infection intensities, CD4/CD8 counts and cytokine responses. *Trans R Soc Trop Med Hyg* 2007; **101:** 602–12.

17 Nielsen NO, Simonsen PE, Magnussen P, Magesa S, Friis H. Cross-sectional relationship between HIV, lymphatic filariasis and other parasitic infections in adults in coastal northeastern Tanzania. *Trans R Soc Trop Med Hyg* 2006; **100:** 543–50.
18 Tafatatha T, Taegtmeyer M, Ngwira B, et al. Human immunodeficiency virus, antiretroviral therapy and markers of lymphatic filariasis infection: a cross-sectional study in rural northern Malawi. *PLoS Negl Trop Dis* 2015; **9:** e0003825.
19 Talaat KR, Kumarasamy N, Swaminathan S, Gopinath R, Nutman TB. Filarial/human immunodeficiency virus coinfection in urban southern India. *Am J Trop Med Hyg* 2008; **79:** 558–60.
20 Kroidl I, Saathof E, Maganga L, et al. Prevalence of Lymphatic Filariasis and treatment effectiveness of albendazole/ivermectin in individuals with hiv co-infection in southwest-Tanzania. *PLoS Negl Trop Dis* 2016; **10:** e0004618.
21 Kroidl I, Clowes P, Mwalongo W, et al. Low specificity of determine HIV1/2 RDT using whole blood in south west Tanzania. *PLoS One* 2012; **7:** e39529.
22 Fauci AS, Pantaleo G, Stanley S, Weissman D. Immunopathogenic mechanisms of HIV infection. *Ann Intern Med* 1996; **124:** 654–63.
23 Weissman D, Barker TD, Fauci AS. The efficiency of acute infection of CD4+ T cells is markedly enhanced in the setting of antigen-specific immune activation. *J Exp Med* 1996; **183:** 687–92.
24 Gopinath R, Ostrowski M, Justement SJ, Fauci AS, Nutman TB. Filarial infections increase susceptibility to human immunodeficiency virus infection in peripheral blood mononuclear cells in vitro. *J Infect Dis* 2000; **182:** 1804–08.
25 Babu S, Kumaraswami V, Nutman TB. Alternatively activated and immunoregulatory monocytes in human filarial infections. *J Infect Dis* 2009; **199:** 1827–37.
26 Talaat KR, Babu S, Menon P, et al. Treatment of W. bancrofti (Wb) in HIV/Wb coinfections in South India. *PLoS Negl Trop Dis* 2015; **9:** e0003622.
27 Hoerauf A. Filariasis: new drugs and new opportunities for lymphatic filariasis and onchocerciasis. *Curr Opin Infect Dis* 2008; **21:** 673–81.
28 Thomsen EK, Sanuku N, Baea M, et al. Efficacy, safety, and pharmacokinetics of coadministered diethylcarbamazine, albendazole, and ivermectin for treatment of bancroftian filariasis. *Clin Infect Dis* 2016; **62:** 334–41.

RESEARCH ARTICLE

Prevalence of Lymphatic Filariasis and Treatment Effectiveness of Albendazole/ Ivermectin in Individuals with HIV Co-infection in Southwest-Tanzania

Inge Kroidl[1,2☯]*, Elmar Saathof[1,3☯], Lucas Maganga[2], Petra Clowes[1,2], Leonard Maboko[2], Achim Hoerauf[4,5], Williams H. Makunde[6], Antelmo Haule[2], Prisca Mviombo[2], Bettina Pitter[1,2], Neema Mgeni[2], Joseph Mabuye[2], Dickens Kowuor[2], Upendo Mwingira[7], Mwelecele N. Malecela[7], Thomas Löscher[1], Michael Hoelscher[1,2,3]

1 Division of Infectious Diseases and Tropical Medicine, Medical Center of the University of Munich (LMU), Munich, Germany, **2** National Institute of Medical Research (NIMR)-Mbeya Medical Research Centre (MMRC), Mbeya, Tanzania, **3** German Center for Infection Research (DZIF), Munich, Germany, **4** German Center for Infection Research (DZIF), Bonn-Cologne, Germany, **5** Institute of Medical Microbiology, Immunology and Parasitology, Bonn, Germany, **6** National Institute of Medical Research (NIMR), Tanga, Tanzania, **7** National Institute of Medical Research (NIMR), Dar es Salaam, Tanzania

☯ These authors contributed equally to this work.
* ikroidl@lrz.uni-muenchen.de

OPEN ACCESS

Citation: Kroidl I, Saathof E, Maganga L, Clowes P, Maboko L, Hoerauf A, et al. (2016) Prevalence of Lymphatic Filariasis and Treatment Effectiveness of Albendazole/ Ivermectin in Individuals with HIV Co-infection in Southwest-Tanzania. PLoS Negl Trop Dis 10(4): e0004618. doi:10.1371/journal.pntd.0004618

Editor: Johan van Griensven, Institute of Tropical Medicine, BELGIUM

Received: September 5, 2015

Accepted: March 18, 2016

Published: April 12, 2016

Copyright: © 2016 Kroidl et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The EMINI study was funded by the European Commission (SANTE/2004/078-545/130 and SANTE/2006/129-931). The SOLF study was supported by a grant from the German Ministry of Science (BMBF, grant number 01KA0904). AHo and WHM acknowledge support by the German Research Council, DFG, grant PF 673/4-1. The funding source had no role in the design, conduct, analysis, or interpretation of the study, nor in writing the report or submitting the paper for publication.

Abstract

Background

Annual mass treatment with ivermectin and albendazole is used to treat lymphatic filariasis in many African countries, including Tanzania. In areas where both diseases occur, it is unclear whether HIV co-infection reduces treatment success.

Methodology

In a general population study in Southwest Tanzania, individuals were tested for HIV and circulating filarial antigen, an indicator of *Wuchereria bancrofti* adult worm burden, before the first and after 2 consecutive rounds of anti-filarial mass drug administration.

Principle Findings

Testing of 2104 individuals aged 0–94 years before anti-filarial treatment revealed a prevalence of 24.8% for lymphatic filariasis and an HIV-prevalence of 8.9%. Lymphatic filariasis was rare in children, but prevalence increased in individuals above 10 years, whereas a strong increase in HIV was only seen above 18 years of age. The prevalence of lymphatic filariasis in adults above 18 years was 42.6% and 41.7% (p = 0.834) in HIV-negatives and–positives, respectively. Similarly, the HIV prevalence in the lymphatic filariasis infected (16.6%) and uninfected adult population (17.1%) was nearly the same. Of the above 2104 individuals 798 were re-tested after 2 rounds of antifilarial treatment. A significant reduction in the prevalence of circulating filarial antigen from 21.6% to 19.7% was found after treatment (relative drop of 8.8%, McNemar´s exact p = 0.036). Furthermore, the post-treatment

Competing Interests: The authors have declared that no competing interests exist.

reduction of CFA positivity was (non-significantly) larger in HIV-positives than in HIV-negatives (univariable linear regression p = 0.154).

Conclusion/Significance

In an area with a high prevalence for both diseases, no difference was found between HIV-infected and uninfected individuals regarding the initial prevalence of lymphatic filariasis. A moderate but significant reduction in lymphatic filariasis prevalence and worm burden was demonstrated after two rounds of treatment with albendazole and ivermectin. Treatment effects were more pronounced in the HIV co-infected subgroup, indicating that the effectiveness of antifilarial treatment was not reduced by concomitant HIV-infection. Studies with longer follow-up time could validate the observed differences in treatment effectiveness.

Author Summary

Parasite infections and HIV show large geographical overlap in sub-Saharan Africa and could hence potentially interact in co-infected individuals. In a general-population study conducted in Southwest Tanzania, we found high prevalence of both, lymphatic filariasis and HIV, with 42.5% of the adult population infected with *Wuchereria bancrofti* and 16.8% infected with HIV. Seven percent of the adults were infected with both pathogens. When adjusting for age, there was no statistically significant difference in initial prevalence or worm burden between HIV-positive and negative participants. For 798 individuals test results for both diseases were available in 2009, before and in 2011, after 2 rounds of treatment against lymphatic filariasis. Between 2009 and 2011, a significant drop of prevalence and worm burden in infected individuals were observed, which was more pronounced in the HIV co-infected subgroup. Hence, HIV co-infection does not seem to negatively affect lymphatic filariasis treatment programmes.

Introduction

Lymphatic Filariasis (LF) is a mosquito-borne disease caused either by *Wuchereria bancrofti* which is distributed throughout the tropics, or *Brugia malayi and Brugia timori*, both limited to Southeast-Asia. It is estimated that 120 million people world-wide are infected with one of these pathogens, and 1 billion are at risk to acquire LF during their lifetime [1]. Before larger treatment programmes started, LF was present in most of the 21 regions of Tanzania with up to 63.8% of individuals testing positive for circulating filarial antigen, a marker for LF infection [2]. Since the year 2000 the "Global Alliance to Eliminate Lymphatic Filariasis" uses annual mass drug administration (MDA), with the aim to control and ultimately eliminate the disease [3]. The campaign of the Tanzanian National Lymphatic Filariasis Elimination Programme (NLEFP) commenced in 2001 in the coastal regions of Tanzania. In the Mbeya district in Southwest-Tanzania the treatment programme started in October 2009 with the annual distribution of albendazole (400mg) and ivermectin (150–200μg/kg). Ivermectin is considered to be mainly microfilaricidal [4], for albendazole an effect on the release of intrauterine antigen components of the adult worm was described [5]. Some studies report on the treatment effectiveness of the combination of albendazole and ivermectin after 12-month: in Ghana a significant reduction in circulating filarial antigen (CFA) levels but no measurable reduction of CFA

prevalence was described in 370 individuals receiving both drugs [6, 7]. A longitudinal study from Northern Tanzania showed only small reductions of CFA positivity after two annual drug distributions (from 53.3% to 51.4%), but a significant drop to 44.9% and 19.6% after four and seven years of treatment, respectively [8].

In South Western Tanzania, both LF and HIV are public health concerns. The HIV prevalence in the country has been documented in several national surveys [1, 9, 10]. The third population based Tanzanian HIV/AIDS and Malaria Indicator Survey in 20011/2012 (THMIS) revealed a country-wide HIV prevalence of 5.1% in Tanzanian adults between the age of 15 and 49 years, and a prevalence of 9.0% for this age-group in Mbeya Region [10]. Large scale distribution of antiretroviral (ART) drugs was initiated in Tanzania in 2005. At the time of our study, ART was not widely available in Southwest Tanzania. [10–12].

Local differences in initial prevalence, coverage of treatment programs, co-infection with other pathogens, etc. can all affect treatment success, thus careful surveillance of the programs is necessary to control the infection. [1, 13–17]. Only few manuscripts focus specifically on the possible interaction of HIV with LF and most of these use cross-sectional data [18–21]. Only one recently published study investigates the treatment effectiveness of MDA drugs in HIV/LF co-infected individuals [22], but focusses on changes in CD4 and HIV viral load after antifilarial treatment in selected HIV-positive individuals. No study concentrated on the antifilarial treatment effectiveness of MDA drugs in HIV/LF co-infected individuals.

Our study assesses LF prevalence in the Mbeya Region, before and after the governmental eradication program reached the area and examines the potential impact of HIV co-infection on LF treatment.

Methods

Study population and study design

Data were collected during the SOLF cohort-study (**S**urveillance **o**f **L**ymphatic **F**ilariasis, http://www.mmrp.org/projects/basic-research/solf.html) in the Kyela district/Mbeya region in Southwest Tanzania which was conducted at the National Institute for Medical Research (NIMR)—Mbeya Medical Research Centre (MMRC) between 2009 and 2011. The study was embedded into the population based EMINI (**E**valuation and **M**onitoring of the **I**mpact of **N**ew **I**nterventions, http://www.mmrp.org/projects/cohort-studies/emini.html) cohort study, which was carried out in 9 selected communities in the Mbeya region (Fig 1) from 2006 to 2011. More than 170,000 inhabitants from ~42,000 households of these communities were registered and 10% of households randomly selected to participate in the study. No additional households entered the surveillance, but some new participants entered through birth or marriage into included household.

Ethical considerations

The SOLF study was approved by the Mbeya Medical Research and Ethics Committee and the Tanzanian National Institute for Medical Research—Medical Research Coordinating Committee as an amendment to the EMINI cohort study. Prior to enrolment, each EMINI participant had provided written informed consent regarding study participation. Parents consented for their children below 18 years of age. In addition, children above the age of 12 years signed their own assent form.

Data collection

Data and samples from participants in the Kyela site of the EMINI study were collected annually from 2007 until 2009. During the last two surveys (2010 and 2011) only half of the study

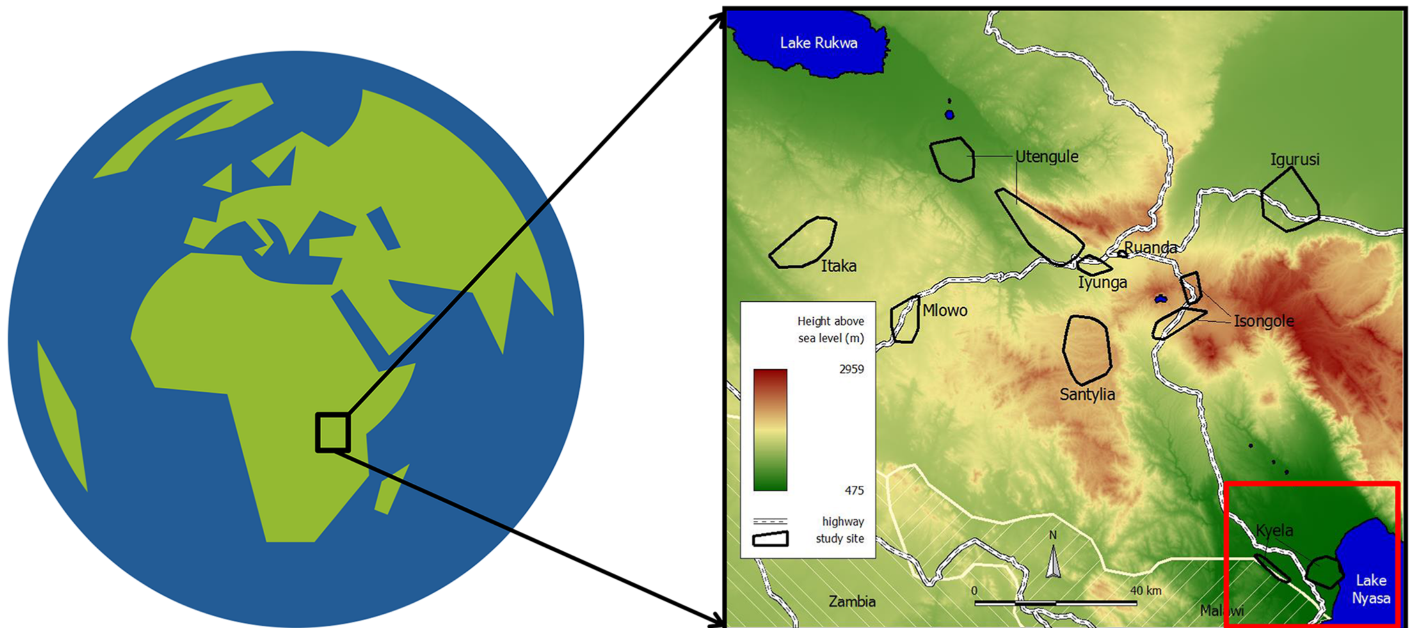


Fig 1. Study area. The study was conducted in the southwestern part of Tanzania (black rectangle). A general population study was performed in nine study areas (black polygons). Data for this study were collected in Kyela (red rectangle) situated close to Lake Nyassa. (Globe Icon by Sev, https://openclipart.org).

doi:10.1371/journal.pntd.0004618.g001

households were visited in each year. During each visit, which took place between 8 am and 2 pm, blood, urine and stool samples were collected from each participant. Samples from 2,165 participants from March 2009 were used to estimate the prevalence of LF directly before the government treatment program commenced in Kyela in October 2009. In March 2011, 18 month after the first and 6 month after the second delivery of antifilarial treatment, samples from 1,010 participants were used to evaluate treatment impact.

Sample processing

From each study participant, 2.7 ml of blood was collected during morning hours in EDTA tubes and immediately stored at 4°C. Cells and plasma were separated within 24 hours and subsequently stored at -80°C. All laboratory tests were performed at NIMR-MMRC, Mbeya Tanzania.

HIV diagnosis

HIV testing was performed using the SD-Bioline HIV-1/2 3.0 (Standard Diagnostics, Kyonggi-do, South Korea) rapid diagnostic test (RDT). Negative RDT results from one survey, followed by another negative RDT result in a subsequent survey, were regarded as confirmed negative and not further tested. All positive results were confirmed using an ELISA HIV test (Enzygnost Anti HIV 1/2 Plus, DADE-Behring, Marburg, Germany), and tested by Western blot (MPD HIV Blot 2.2, MP Biomedicals, Geneva, Switzerland) if discordant. For all HIV incident cases, the negative result of the previous year, as well as the new positive results was confirmed by the testing algorithm described above. For children below the age of two years, HIV testing was done by PCR. Further details are described elsewhere [23]. Because confidential disclosure of the HIV-status could not be ensured during household visits, we did not inform participants about their HIV status. Instead they were offered voluntary counseling and testing by an

independent team, which was travelling with our study team, who provided referral to the local care and treatment center, to everyone who was tested positive.

Filarial antigen testing

A commercially available ELISA (TropBio Og4C3 serum ELISA, Townsville, Australia) was used to detect circulating filarial antigen (CFA) using 100 μl of the collected sera. The Og4C3 antibody detects *Wuchereria bancrofti* antigen with high specificity (98.5%) and no known cross-reaction to *Onchocerca volvulus*, *Brugia spp*., *Mansonella*, *Dracunculus medinensis*, *Ascaris lumbricoides* or *Strongyloides stercoralis* [24]. Sensitivity varies between 73% [25] and 100% [26], but was found 97.9% in individuals carrying microfilariae [24]. CFA is secreted by fully developed *W*. *bancrofti* adults and can be found at similar levels during day and night. Antigen levels thus reflect the *W*. *bancrofti* worm burden. The measurement of CFA with the Trop Bio ELISA is semi-quantitative; seven control tubes with standardized amounts of antigen are supplied and allow an estimation of the filarial antigen levels in the analysed plasma according to the measured optical density (OD). LF test results were considered negative, indeterminate or positive if the OD was <0.2, ≥0.2 and ≤0.3, or >0.3 respectively.

Statistics

Statistical analyses were performed using Stata statistics software (version 14; Stata Corp., College Station, TX). Pearson´s chi-squared test was used to compare binominal outcomes between groups and to compare CFA positivity before and after treatment in all participants. McNemar´s exact test for paired data was used to compare CFA positivity before and after treatment in those individuals who participated in both surveys. The non-parametric Wilcoxon rank sum test was used to compare selected baseline characteristics of continuous variables, since none of these was normally distributed. In order to examine the association of LF infection with HIV status and other potentially important covariates we performed uni- and multi-variable log link binomial regression analyses with robust variance estimates.

Results

In March 2009, before the first national MDA commenced, valid CFA results were obtained from 2,104 individuals (Table 1). Indeterminate results were found for the 61 of the tested 2,165 samples (2.8%). Their median age was 16.6 years (range 0–94, IQR: 8.8 to 34), and 51.0% were female. Only 4 (1.6%) of the 245 children below the age of 5 years were CFA-positive; LF prevalence started to rise in participants above 10 years and was 42.3% in adults above 18 years of age (Fig 2). When including all age groups, 24.8% of the study population were CFA-positive with a trend to higher prevalence in males (26.5%) than in females (23.1%, chi-squared $p = 0.074$). In the adult population above 18 years the difference in CFA-positivity between males (47.3%) and females (38.0%) was significant (chi-squared $p = 0.003$).

In March 2011, 18 months after the first MDA and six months after the second, ~50% of the initially included households were revisited for interviews and blood sample collection. Some scheduled participants were not found in 2011, and some new individuals had entered the visited households (see study population and design). In addition to an analysis where the data of all participants form each Survey (= open cohort) are evaluated, which reflects more a cross sectional design, a second analysis included only the 798 individuals who actively participated in both years of the surveillance longitudinally (= closed cohort). The numbers of participants is shown in Table 1. Of the 974 valid test results in 2011, 19.7% were CFA-positive, leading to a calculated prevalence reduction of 5.1% (24.8% vs. 19.7%, chi-squared $p = 0.002$) when

Table 1. Study participants and LF prevalence in 2009 and 2011.

	2009			2011		
	all n	LF-positive n	LF-prevalence %	all n	LF-positive n	LF-prevalence %
open cohort						
all*	2,104	521	24.8	974	192	19.7
HIV-negative	1,905	450	23.6	878	166	18.9
HIV-positive	187	69	36.9	91	25	27.5
closed cohort						
all**	798	172	21.6	798	157	19.7
HIV-negative	723	151	20.9	723	140	19.4
HIV-positive	69	19	27.5	69	15	21.7

*12 individuals without valid HIV result

**6 individuals without valid HIV result

Data for all study participants from 2009 and 2011 are shown in the upper part of the table (= open cohort). Only 798 individuals participated in both years of the surveillance (= closed cohort) their data are shown in the lower part.

doi:10.1371/journal.pntd.0004618.t001

including all subjects who participated in at least one survey (Table 1, open cohort). In the analysis of samples from 798 individuals who actively participated in both surveys (Table 1, closed cohort), a lower prevalence reduction (21.6 to 19.7%, McNemar´s exact p = 0.036) was measured (Fig 3).

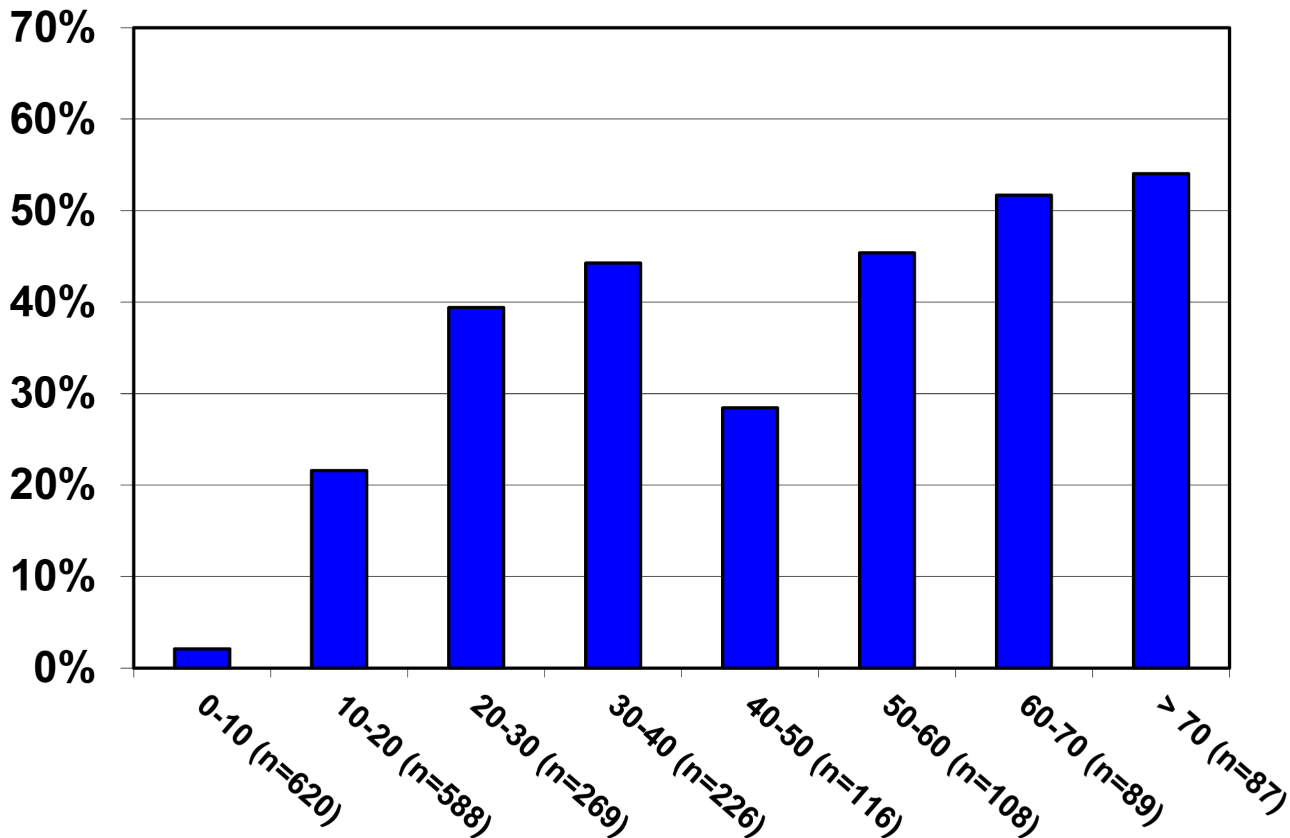


Fig 2. Baseline CFA prevalence by age. Numbers of participants for each age group are given in parenthesis.

doi:10.1371/journal.pntd.0004618.g002

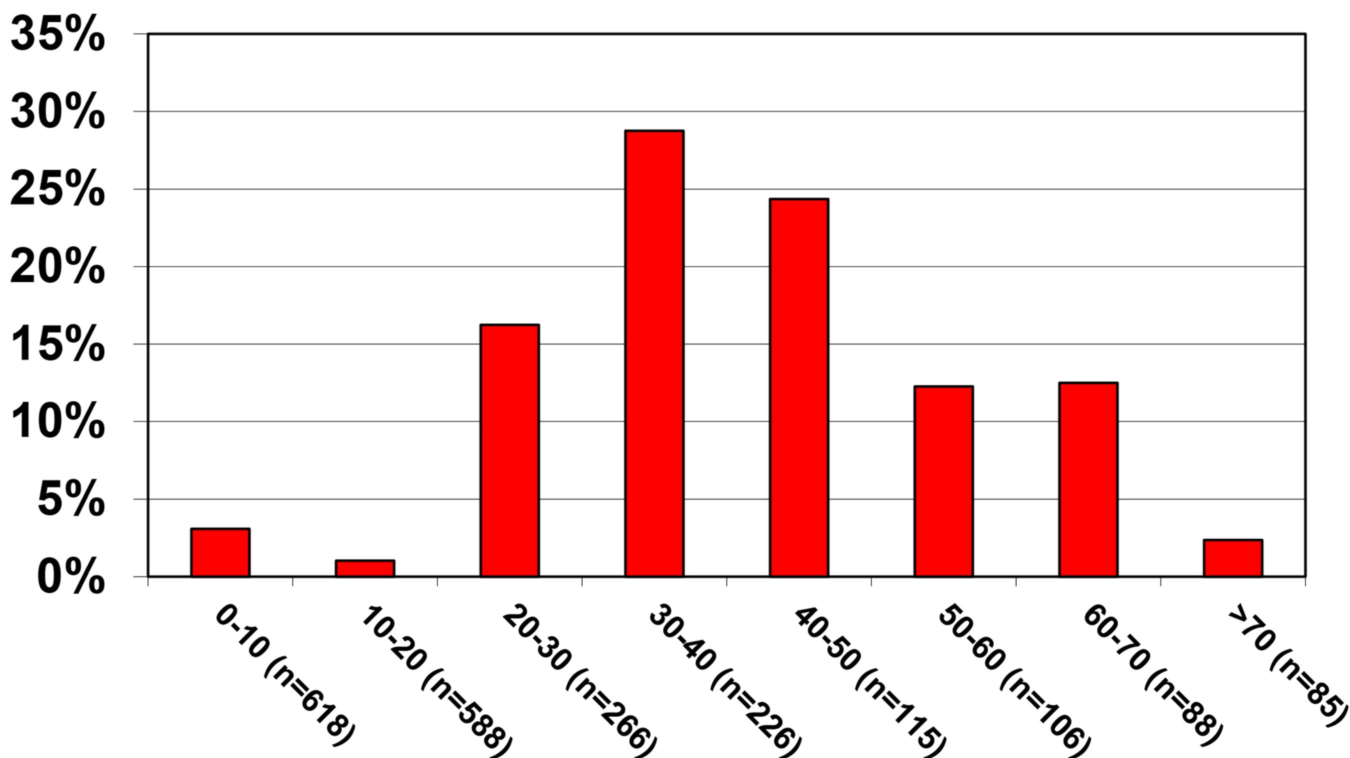


Fig 3. HIV prevalence by age. Numbers of participants for each age group are given in parenthesis (for 12 individuals no valid HIV result was available).

doi:10.1371/journal.pntd.0004618.g003

At baseline the overall HIV prevalence in our study cohort was 8.9%, with a prevalence of only 2.1% in children and adolescents below the age of 18 years, and a prevalence of 16.9% in individuals ≥18 years of age (Fig 3).

HIV-infection was more prevalent in female (10.7%), compared to male participants (7.1%, chi-squared p = 0.003). Sixty-eight of the 968 adult individuals (7.0%) were infected with both pathogens and among the whole group of 2,104 individuals 69 co-infections (= 3.3%) were observed. The initial univariable analysis of the potential association of HIV with LF infection showed a higher prevalence of LF in HIV-positive (36.9%); compared to HIV-negative individuals (23.6%) (RR = 1.56, 95% CI = 1.26 to 1.94, p<0.001). But we already demonstrated that HIV and LF are both less common in children than in adults, which confounds this association. To further study the pattern of co-infection we analysed CFA positivity in HIV infected and uninfected individuals stratified by age (Fig 4); in adults (> = 18 years) only; and in log-link binomial multivariable regression adjusted for age and gender. None of these analyses showed a significant association of LF infection with HIV, neither within the single age strata nor overall in the multivariable regression model where the influence of age and gender were confirmed, but where the adjusted RR for HIV was only 1.04 (Table 2). When only analysing data from adults above 18, the CFA prevalence was 42.6% in the HIV-negative and 41.7% in the HIV-positive subgroup (univariable log-link regression RR = 0.98, 95% CI = 0.80 to 1.20; p = 0.84).

In order to compare antifilarial treatment success in the HIV-negative and positive subgroups we again performed two analysis: one for all tested individuals who participated in at least one survey (open cohort using chi-squared testing), and one only for the individuals who

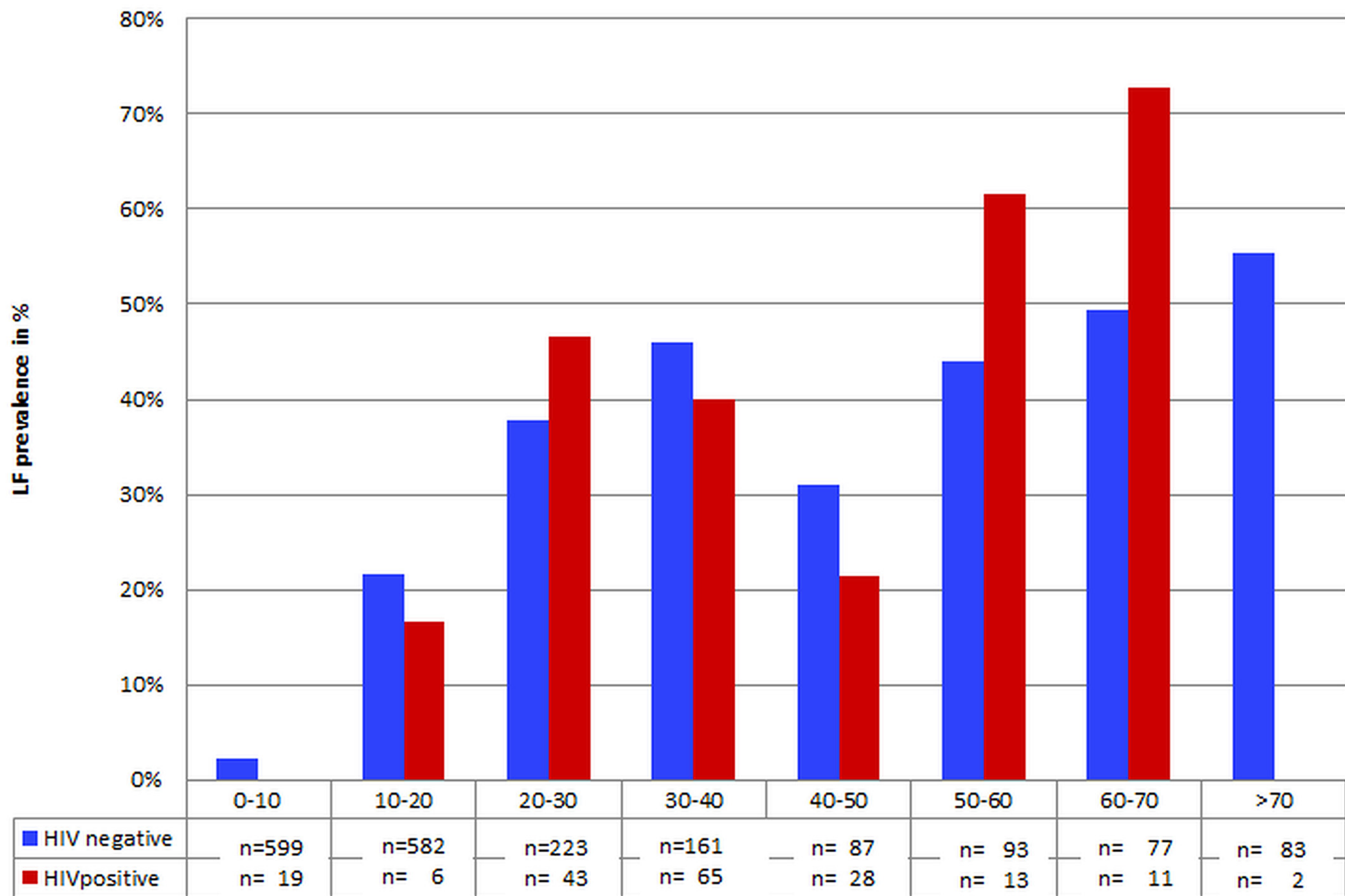


Fig 4. Baseline CFA prevalence by age for HIV-positive and HIV-negative participants. Prevalence of CFA positivity in HIV-negative (blue) and HIV-positive (red) participants. No HIV/LF co-infection was observed in participants below 10 years of age.

doi:10.1371/journal.pntd.0004618.g004

participated in both surveys before and after treatment (closed cohort, using McNemar´s exact test).

For the open cohort a CFA prevalence reduction from 23.6% to 18.9% (chi-squared p = 0.015, relative drop = 19.7%) was found in HIV-negative participants, and from 36.9% to 27.5% (chi-squared p = 0.023, relative drop = 25.4%) in HIV-positives. For the closed cohort we observed a drop in CFA positivity from 20.9% to 19.4% (McNemar´s exact p = 0.117, relative drop = 7.3%) in 723 HIV-negatives and from 27.5% to 21.7% (McNemar´s exact p = 0.125, relative drop = 21.1%) in 69 HIV-positive participants. The reason for this pronounced difference (7.3% vs. 21.1%) is a higher incidence of CFA positivity in the HIV negative participants where 15 (2.6%) of the 572 initially CFA negative participants turned CFA-positive, whereas none of the 50 HIV-positive participants who were initially CFA-negative turned CFA-positive (chi-squared p = 0.246). The proportion of initially CFA positives who turned CFA negative was very similar in HIV-negative (26/151 = 17.2%) and HIV-positive participants (4/19 = 21.1%, chi-squared p = 0.679). When combining this information about change in LF status in one outcome variable (-1 = turned CFA negative; 0 = no change in CFA status; 1 = turned CFA positive) univariable linear regression modelling resulted in a coefficient β for the HIV infected subgroup of -0.043 (95%CI = -0.102 to 0.016, p = 0.154).

Table 2. Uni- and Multivariable binomial log-link regression models showing associations of Age, Sex and HIV positivity with LF infection in 2092 individuals before antifilarial treatment commenced.

		Univariable				Multivariable		
All:	**n**	**LF pos (%)**	**RR**	**95% CI**	**P**	**RR**	**95% CI**	**P**
	2,092	24.8						
HIV								
neg	1,905	23.6	1	1.26 to 1.94	<0.001	1	0.85 to 1.27	0.689
pos	187	36.9	1.56			1.04		
Sex:								
female	1,067	23.2	1	0.99 to 1.32	0.064	1	1.11 to 1.44	<0.001
male	1,025	26.5	0.15			0.27		
Age (years)								
0-<18	1,124	9.6	1			1		
18-<50	689	39.2	4.01	3.27 to 5.08	<0.001	4.10	3.29 to 5.10	<0.001
> = 50	279	50.5	5.26	4.20 to 6.58	<0.001	4.16	4.32 to 6.70	<0.001

doi:10.1371/journal.pntd.0004618.t002

Analysing the prevalence reduction in the closed cohort for adults > = 18 years only, a drop from 42.7% to 40.3% (McNemar´s exact p = 0.248, relative drop = 5.6%) was noted for HIV-negatives, and from 32.7% to 25.5% (McNemar´s exact p = 0.125, relative drop = 22.0%) in the HIV-positive subgroup. Summarizing our results, we found more pronounced drops in prevalence among the HIV positive subgroup, compared with the HIV negative, no matter, whether all participants or only adults are analysed and also with both possible ways of evaluating the data (open cohort or closed cohort).

The measurement of CFA with the Trop Bio ELISA is semi-quantitative; with the OD of the plasma samples reflecting the participant's worm burden. Our findings for CFA intensities parallel those for CFA prevalence: geometric mean intensities before treatment were relatively similar between HIV-positives (157 units) and HIV-negatives (179 units, Wilcoxon rank sum p = 0.34), which is also true for the relative reduction of geometric mean intensity after treatment, which was 26% and 30% respectively (Wilcoxon rank sum p = 0.50)

Discussion

In our study we found a significant decrease in LF-prevalence after only 2 years of MDA in an area with high HIV co-infection in South-West Tanzania. This was in contrast to our expectations and previously published manuscripts [2, 6, 7, 16, 27]. Furthermore we did not find any evidence that HIV co-infection impairs the effectiveness of antifilarial treatment. On the contrary, our data show a more pronounced decrease in prevalence and CFA intensity among HIV-positive compared to HIV-negative participants. We tried to consider several factors which could have affected our analysis. The age distribution of LF and HIV infection had to be taken into account, but also the composition of the study population and potential changes during follow-up. However, an almost three-fold relative drop in LF prevalence was seen for HIV-positive compared to HIV-negative participants in the most stringent analysis which only considered individuals for whom we have data both before and after treatment. We do not want to overstate this result since the overall numbers of participants with HIV/LF-co-infection was low, despite an initially large cohort and accordingly the differences in cure and incidence rate between HIV-infected and uninfected participants are not significant. Moreover, we are unable to present an explanation for this finding. Reports about the LF prevalence in HIV-negative and HIV-positive individuals have been rare and conflicting in the past. Nielsen et al.

described a positive association of HIV and LF infection after adjusting for age and sex in a cross-sectional study from Northern Tanzania [20], even though a further evaluation of this group of individuals did not support an association between HIV and LF [19]. No difference regarding CFA levels was found in other cross sectional studies from India [18] and Malawi [28]. The latter findings are supported by the cross sectional analysis of our study participants in 2009. To date, no other longitudinal study has compared the effectiveness of antifilarial treatment in HIV-positive and negative subgroups.

One interesting finding is the higher pre-treatment CFA prevalence in the open compared to the closed cohort which demonstrates that LF-positive individuals were more likely to be lost to follow-up than LF-negative participants. This can at least partly be explained by the higher prevalence of LF in adults, who are more likely than younger participants to relocate (e.g. in search of a job), to harbour diseases that prevent them from further study participation (e.g. HIV-infection) and to die.

Most other studies analyse the treatment effectiveness after 6, 8 or even more years of treatment. Thus one limitation of our study is the short duration of follow-up, which is a consequence of funding restrictions and does not allow for a conclusive analysis of MDA effectiveness. Furthermore we are not able to identify individually who of our participants was treated and who was not: the local district medical officer and Neglected Tropical Diseases (NTD) coordinator, who supervised the drug distribution in Kyela district, reported coverages of 60.8% in October 2009 and 68.2% in 2010 (Mrs. Masawe, personal communication). However, very few participants of the SOLF study were aware of the treatment program against LF, when asked about their participation. Therefore, firm assumptions about efficacy, i.e. the effect of antifilarial treatment on CFA prevalence and intensity under ideal conditions cannot be made. Instead our data are better suited to describe the effectiveness of MDA under real-life conditions. Furthermore, information on CD4 count and antiretroviral treatment status of HIV infected individuals would have helped to refine our analysis, but this information was not collected.

The Og4C3 antibody is supposed to specifically recognise *Wuchereria bancrofti* antigen with no relevant cross-reaction to *Onchocerca volvulus*, *Brugia spp*., *Mansonella*, *Dracunculus medinensis*, *Ascaris lumbricoides* or *Strongyloides stercoralis*. In spite of this, cross reactivity with *Loa* microfilariae has been found in another test (Binax Now Filariasis Immunochromatographic Test, Alere, Scarborough, ME, USA), which detects the same antigen as the Trop Bio ELISA [29]. However, there is no significant reported disease burden of Loiasis in our study area [30].

Conclusion

In an area with high prevalence of and no previous treatment against LF we investigated the potential association of HIV and LF infection. When adjusting for age we found similar CFA prevalence and intensities in HIV-positive and negative participants. After two rounds of treatment a significant reduction in CFA prevalence and intensity was demonstrated, which was more pronounced in the HIV-positive compared to HIV-negative participants. Hence, HIV co-infection does not seem to negatively affect antifilarial treatment.

Acknowledgments

We thank all EMINI participants for their willingness to be part of the study, as well as the EMINI field- and laboratory-teams for their contribution. Special thanks to Mr. Weston Assisya for the coordination of the field work, practical advice and positive spirit.

Author Contributions

Conceived and designed the experiments: IK LMag PC WHM AHo UM MNM. Performed the experiments: IK AHa PM BP NM JM. Analyzed the data: IK ES DK. Contributed reagents/materials/analysis tools: LMag LMab TL MH. Wrote the paper: IK ES PC AHo WHM UM MNM MH.

References

1. Hotez PJ, Kamath A. Neglected tropical diseases in sub-saharan Africa: review of their prevalence, distribution, and disease burden. PLoS Negl Trop Dis. 2009; 3(8):e412. Epub 2009/08/27. doi: 10.1371/journal.pntd.0000412 PMID: 19707588; PubMed Central PMCID: PMC2727001.
2. Simonsen PE, Magesa SM, Dunyo SK, Malecela-Lazaro MN, Michael E. The effect of single dose ivermectin alone or in combination with albendazole on Wuchereria bancrofti infection in primary school children in Tanzania. Trans R Soc Trop Med Hyg. 2004; 98(8):462–72. doi: 10.1016/j.trstmh.2003.12.005 PMID: 15186934.
3. WHO. Global Programme to eliminate lymphatic filariasis: progress report on mass drug administration, 2010. Releve epidemiologique hebdomadaire / Section d'hygiene du Secretariat de la Societe des Nations = Weekly epidemiological record / Health Section of the Secretariat of the League of Nations. 2011; 86(35):377–88. PMID: 21887884.
4. Dreyer G, Addiss D, Noroes J, Amaral F, Rocha A, Coutinho A. Ultrasonographic assessment of the adulticidal efficacy of repeat high-dose ivermectin in bancroftian filariasis. Trop Med Int Health. 1996; 1 (4):427–32. PMID: 8765448.
5. Weil GJ, Ramzy RM, Chandrashekar R, Gad AM, Lowrie RC Jr, Faris R. Parasite antigenemia without microfilaremia in bancroftian filariasis. Am J Trop Med Hyg. 1996; 55(3):333–7. PMID: 8842125.
6. Dunyo SK, Nkrumah FK, Simonsen PE. Single-dose treatment of Wuchereria bancrofti infections with ivermectin and albendazole alone or in combination: evaluation of the potential for control at 12 months after treatment. Trans R Soc Trop Med Hyg. 2000; 94(4):437–43. PMID: 11127253.
7. Dunyo SK, Nkrumah FK, Simonsen PE. A randomized double-blind placebo-controlled field trial of ivermectin and albendazole alone and in combination for the treatment of lymphatic filariasis in Ghana. Trans R Soc Trop Med Hyg. 2000; 94(2):205–11. PMID: 10897370.
8. Simonsen PE, Pedersen EM, Rwegoshora RT, Malecela MN, Derua YA, Magesa SM. Lymphatic filariasis control in Tanzania: effect of repeated mass drug administration with ivermectin and albendazole on infection and transmission. PLoS Negl Trop Dis. 2010; 4(6):e696. doi: 10.1371/journal.pntd.0000696 PMID: 20532226; PubMed Central PMCID: PMC2879369.
9. Msisha WM, Kapiga SH, Earls FJ, Subramanian SV. Place matters: multilevel investigation of HIV distribution in Tanzania. AIDS. 2008; 22(6):741–8. doi: 10.1097/QAD.0b013e3282f3947f PMID: 18356604; PubMed Central PMCID: PMC2789284.
10. Tanzania NIMR-. Tanzania HIV/AIDS Malaria Indicator Survey (2011/2012) [01.02.1015].
11. Layer EH, Kennedy CE, Beckham SW, Mbwambo JK, Likindikoki S, Davis WW, et al. Multi-level factors affecting entry into and engagement in the HIV continuum of care in Iringa, Tanzania. PLoS One. 2014; 9(8):e104961. doi: 10.1371/journal.pone.0104961 PMID: 25119665; PubMed Central PMCID: PMCPMC4138017.
12. Layer EH, Brahmbhatt H, Beckham SW, Ntogwisangu J, Mwampashi A, Davis WW, et al. "I pray that they accept me without scolding:" experiences with disengagement and re-engagement in HIV care and treatment services in Tanzania. AIDS Patient Care STDS. 2014; 28(9):483–8. doi: 10.1089/apc.2014.0077 PMID: 25093247.
13. Hoerauf A, Pfarr K, Mand S, Debrah AY, Specht S. Filariasis in Africa—treatment challenges and prospects. Clin Microbiol Infect. 2011; 17(7):977–85. Epub 2011/07/05. doi: 10.1111/j.1469-0691.2011.03586.x PMID: 21722251.
14. Ramaiah KD, Ottesen EA. Progress and impact of 13 years of the global programme to eliminate lymphatic filariasis on reducing the burden of filarial disease. PLoS Negl Trop Dis. 2014; 8(11):e3319. doi: 10.1371/journal.pntd.0003319 PMID: 25412180; PubMed Central PMCID: PMC4239120.
15. Hotez PJ, Alvarado M, Basanez MG, Bolliger I, Bourne R, Boussinesq M, et al. The global burden of disease study 2010: interpretation and implications for the neglected tropical diseases. PLoS Negl Trop Dis. 2014; 8(7):e2865. doi: 10.1371/journal.pntd.0002865 PMID: 25058013; PubMed Central PMCID: PMC4109880.
16. Simonsen PE, Derua YA, Kisinza WN, Magesa SM, Malecela MN, Pedersen EM. Lymphatic filariasis control in Tanzania: effect of six rounds of mass drug administration with ivermectin and albendazole

on infection and transmission. BMC Infect Dis. 2013; 13:335. Epub 2013/07/23. 1471-2334-13-335 [pii] doi: 10.1186/1471-2334-13-335 PMID: 23870103; PubMed Central PMCID: PMC3723586.

17. Simonsen PE, Magesa SM, Derua YA, Rwegoshora RT, Malecela MN, Pedersen EM. Monitoring lymphatic filariasis control in Tanzania: effect of repeated mass drug administration on circulating filarial antigen prevalence in young schoolchildren. Int Health. 2011; 3(3):182–7. Epub 2011/09/01. j.inhe.2011.06.009 [pii] doi: 10.1016/j.inhe.2011.06.009 PMID: 24038368.
18. Talaat KR, Kumarasamy N, Swaminathan S, Gopinath R, Nutman TB. Filarial/human immunodeficiency virus coinfection in urban southern India. Am J Trop Med Hyg. 2008; 79(4):558–60. Epub 2008/10/09. 79/4/558 [pii]. PMID: 18840744; PubMed Central PMCID: PMC2596056.
19. Nielsen NO, Friis H, Magnussen P, Krarup H, Magesa S, Simonsen PE. Co-infection with subclinical HIV and Wuchereria bancrofti, and the role of malaria and hookworms, in adult Tanzanians: infection intensities, CD4/CD8 counts and cytokine responses. Trans R Soc Trop Med Hyg. 2007; 101(6):602–12. Epub 2007/03/31. S0035-9203(07)00034-X [pii] doi: 10.1016/j.trstmh.2007.02.009 PMID: 17395223.
20. Nielsen NO, Simonsen PE, Magnussen P, Magesa S, Friis H. Cross-sectional relationship between HIV, lymphatic filariasis and other parasitic infections in adults in coastal northeastern Tanzania. Trans R Soc Trop Med Hyg. 2006; 100(6):543–50. doi: 10.1016/j.trstmh.2005.08.016 PMID: 16324731.
21. Tafatatha T, Taegtmeyer M, Ngwira B, Phiri A, Kondowe M, Piston W, et al. Human Immunodeficiency Virus, Antiretroviral Therapy and Markers of Lymphatic Filariasis Infection: A Cross-sectional Study in Rural Northern Malawi. PLoS Negl Trop Dis. 2015; 9(6):e0003825. doi: 10.1371/journal.pntd.0003825 PMID: 26042839; PubMed Central PMCID: PMC4456405.
22. Talaat KR, Babu S, Menon P, Kumarasamy N, Sharma J, Arumugam J, et al. Treatment of W. bancrofti (Wb) in HIV/Wb coinfections in South India. PLoS Negl Trop Dis. 2015; 9(3):e0003622. doi: 10.1371/journal.pntd.0003622 PMID: 25793933; PubMed Central PMCID: PMC4368731.
23. Kroidl I, Clowes P, Mwalongo W, Maganga L, Maboko L, Kroidl AL, et al. Low specificity of determine HIV1/2 RDT using whole blood in south west Tanzania. PLoS One. 2012; 7(6):e39529. Epub 2012/07/07. doi: 10.1371/journal.pone.0039529 PONE-D-11-13142 [pii]. PMID: 22768086; PubMed Central PMCID: PMC3387183.
24. Rocha A, Addiss D, Ribeiro ME, Noroes J, Baliza M, Medeiros Z, et al. Evaluation of the Og4C3 ELISA in Wuchereria bancrofti infection: infected persons with undetectable or ultra-low microfilarial densities. Trop Med Int Health. 1996; 1(6):859–64. PMID: 8980602.
25. Chanteau S, Moulia-Pelat JP, Glaziou P, Nguyen NL, Luquiaud P, Plichart C, et al. Og4C3 circulating antigen: a marker of infection and adult worm burden in Wuchereria bancrofti filariasis. J Infect Dis. 1994; 170(1):247–50. PMID: 8014511.
26. Lammie PJ, Reiss MD, Dimock KA, Streit TG, Roberts JM, Eberhard ML. Longitudinal analysis of the development of filarial infection and antifilarial immunity in a cohort of Haitian children. Am J Trop Med Hyg. 1998; 59(2):217–21. PMID: 9715935.
27. Simonsen PE, Meyrowitsch DW, Mukoko DA, Pedersen EM, Malecela-Lazaro MN, Rwegoshora RT, et al. The effect of repeated half-yearly diethylcarbamazine mass treatment on Wuchereria bancrofti infection and transmission in two East African communities with different levels of endemicity. Am J Trop Med Hyg. 2004; 70(1):63–71. PMID: 14971700.
28. Tafatatha TT, Ngwira BM, Taegtmeyer M, Phiri AJ, Wilson TP, Banda LG, et al. Randomised controlled clinical trial of increased dose and frequency of albendazole and ivermectin on Wuchereria bancrofti microfilarial clearance in northern Malawi. Trans R Soc Trop Med Hyg. 2015; 109(6):393–9. doi: 10.1093/trstmh/trv027 PMID: 25877874.
29. Wanji S, Amvongo-Adjia N, Koudou B, Njouendou AJ, Chounna Ndongmo PW, Kengne-Ouafo JA, et al. Cross-Reactivity of Filariais ICT Cards in Areas of Contrasting Endemicity of Loa loa and Mansonella perstans in Cameroon: Implications for Shrinking of the Lymphatic Filariasis Map in the Central African Region. PLoS Negl Trop Dis. 2015; 9(11):e0004184. doi: 10.1371/journal.pntd.0004184 PMID: 26544042; PubMed Central PMCID: PMCPMC4636288.
30. Zoure HG, Wanji S, Noma M, Amazigo UV, Diggle PJ, Tekle AH, et al. The geographic distribution of Loa loa in Africa: results of large-scale implementation of the Rapid Assessment Procedure for Loiasis (RAPLOA). PLoS Negl Trop Dis. 2011; 5(6):e1210. doi: 10.1371/journal.pntd.0001210 PMID: 21738809; PubMed Central PMCID: PMCPMC3125145.

Ascaris lumbricoides Infection and Its Relation to Environmental Factors in the Mbeya Region of Tanzania, a Cross-Sectional, Population-Based Study

Steffen Andreas Schüle[1,2,3]*, Petra Clowes[4], Inge Kroidl[1,4], Dickens O. Kowuor[4], Anthony Nsojo[4], Chacha Mangu[4], Helene Riess[1,2], Christof Geldmacher[1,5], Rüdiger Paul Laubender[2], Seif Mhina[6], Leonard Maboko[4], Thomas Löscher[1], Michael Hoelscher[1,4,5], Elmar Saathoff[1,5]

1 Division of Infectious Diseases and Tropical Medicine, Medical Center of the University of Munich (LMU), Munich, Germany, **2** Institute for Medical Bioinformatics, Biometry, and Epidemiology, Ludwig-Maximilians-University, Munich, Germany, **3** Department of Social Epidemiology, Institute for Public Health and Nursing Research, University of Bremen, Bremen, Germany, **4** NIMR-Mbeya Medical Research Center (MMRC), Mbeya, Tanzania, **5** German Center for Infection Research (DZIF), partner site Munich, Munich, Germany, **6** Mbeya Regional Medical Office, Mbeya, Tanzania

Abstract

Background: With one quarter of the world population infected, the intestinal nematode *Ascaris lumbricoides* is one of the most common infectious agents, especially in the tropics and sub-tropics. Infection is caused by oral intake of eggs and can cause respiratory and gastrointestinal problems. To identify high risk areas for intervention, it is necessary to understand the effects of climatic, environmental and socio-demographic conditions on *A. lumbricoides* infection.

Methodology: Cross-sectional survey data of 6,366 study participants in the Mbeya region of South-Western Tanzania were used to analyze associations between remotely sensed environmental data and *A. lumbricoides* infection. Non-linear associations were accounted for by using fractional polynomial regression, and socio-demographic and sanitary data were included as potential confounders.

Principal Findings: The overall prevalence of *A. lumbricoides* infection was 6.8%. Our final multivariable model revealed a significant non-linear association between rainfall and *A. lumbricoides* infection with peak prevalences at 1740 mm of mean annual rainfall. Mean annual land surface temperature during the day was linearly modeled and negatively associated with *A. lumbricoides* infection (odds ratio (OR) = 0.87, 95% confidence interval (CI) = 0.78–0.97). Furthermore, age, which also showed a significant non-linear association (infection maximum at 7.7 years), socio-economic status (OR = 0.82, CI = 0.68–0.97), and latrine coverage around the house (OR = 0.80, CI = 0.67–0.96) remained in the final model.

Conclusions: *A. lumbricoides* infection was associated with environmental, socio-demographic and sanitary factors both in uni- and multivariable analysis. Non-linear analysis with fractional polynomials can improve model fit, resulting in a better understanding of the relationship between environmental conditions and helminth infection, and more precise predictions of high prevalence areas. However, socio-demographic determinants and sanitary conditions should also be considered, especially when planning public health interventions on a smaller scale, such as the community level.

Citation: Schüle SA, Clowes P, Kroidl I, Kowuor DO, Nsojo A, et al. (2014) *Ascaris lumbricoides* Infection and Its Relation to Environmental Factors in the Mbeya Region of Tanzania, a Cross-Sectional, Population-Based Study. PLoS ONE 9(3): e92032. doi:10.1371/journal.pone.0092032

Editor: Joseph A. Keating, Tulane University School of Public Health and Tropical Medicine, United States of America

Received November 18, 2013; **Accepted** February 17, 2014; **Published** March 18, 2014

Copyright: © 2014 Schüle et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The EMINI study was funded by the European Commission (SANTE/2004/078-545/130 & SANTE/2006/129-931). Helminth data collection was supported by the German Science foundation DFG (SA 1878/1-1) and the German Federal Ministry of Education and Research BMBF (01 KA 0904). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: steffenschuele@t-online.de

Introduction

The intestinal nematode *Ascaris lumbricoides* is one of the most common causes of infection among the soil-transmitted helminths (STH). Common in the tropics and sub-tropics, it is estimated that more than one quarter of the world population is infected with this helminth [1–3].

The highest morbidity is found in children, especially in those with a high worm burden. *A. lumbricoides* can lead to reduced physical fitness, growth retardation, and respiratory and gastrointestinal problems [3–6]. Evidence if *A. lumbricoides* infection has a negative impact on cognitive function and educational achievement in school children is controversially debated [7–10].

Infection occurs through the oral intake of eggs, usually contained in soil or food. Adult worms live in the lumen of the small intestine where the female lays unembryonated eggs which are excreted with the feces. In the open, the eggs have to go through three stages of development in order to become infectious; a time during which they are exposed to environmental conditions [5,11,12]. When embryonated eggs are swallowed by a human host, the larvae hatch in the small intestine, have a short migratory

phase (venous system, liver, lungs, trachea, esophagus) after which they return to the small intestine where they mature and mate [13,14].

Recently, remotely sensed environmental data have increasingly been used to get a better understanding of the epidemiology and spatial distribution of STH [15–22]. The use of environmental data in combination with geographic information systems (GIS) has become a powerful tool for mapping and predicting STH, with the main purpose to identify high risk areas for intervention [23–26].

However, there are still challenges in the statistical modeling of environmental data. One problem is the consideration of non-linear relationships between outcome and predictor variables. Although non-linear relationships between environmental data and STH infection are a recognized fact [16,22], this has rarely been taken into account in multivariable analysis. A further complication is the need to take care of potential confounders. Especially risk factors linked to transmission, such as poor sanitation facilities, crowding, and high population density [2,6,12,14,27–32], need to be considered when associations between environmental factors and STH infection are analyzed.

Therefore, the main objective of this study was to assess associations between remotely sensed environmental data and *A. lumbricoides* infection while considering potential non-linear relationships and confounders. A manuscript that examines associations of hookworm infection with environmental factors has recently been accepted [33], and manuscripts regarding *Trichuris trichiura* and schistosome infection are presently being prepared.

Methods

Ethics Statement

The study was approved by the ethics committee of the Tanzanian National Institute for Medical Research and conducted according to the principles expressed in the Declaration of Helsinki. All participants provided written informed consent before enrolment into the study; parents consented for their children below 18 years of age. Specifically, children who were old enough to understand the process were asked to participate in the consenting procedure, and children who were 12 years old or older were asked to sign/thumbprint the document in addition to their parent's signature/thumbprint.

Study Area and Epidemiological Data Collection

Data for this study were collected in nine study sites in the Mbeya Region in south-western Tanzania (Figure 1) from June 2008 until June 2009 during the third annual survey of the EMINI (Evaluating and monitoring the impact of new interventions) cohort study. The region is predominantly rural and most income generating activities are related to agriculture. During an initial population census in the nine sites, more than 42,000 households were identified and their geographical positions recorded, using handheld geographical positioning system (GPS) devices (Spor-Trak handheld GPS, Magellan Navigation Inc., Santa Clara, CA, USA). Geographically stratified random selection was used to choose 10% (4,283) of these households to participate in the main EMINI cohort study. During each annual survey these households were visited once to collect biological specimen and interview data. All participants provided written informed consent before inclusion into the study, with parents or care takers consenting for their minors.

The collection of stool samples started in 2008. Due to logistic constraints, households were randomized into two groups of equal size of which only one was annually sampled for stool.

Interviews to characterize the socio-economic status (SES) of each household were conducted with the household head and included questions regarding infrastructure of the household, ownership of livestock, the availability of certain household assets, and materials that were used to build the house(s) in each homestead. Data on socio-demographic status (sex, age, marital status, religious denomination, education, occupation etc.), relevant behavior, knowledge and practices regarding various diseases were collected in interviews with each individual household member or – for children below 12 years – with their caretaker. All interviews and medical examinations were performed at the household and conducted in Kiswahili language.

Before stool collection started in the third survey round, intestinal nematodes were neither diagnosed nor treated as part of this study, and to our knowledge no other treatment programs had been conducted in the region. Stool samples were collected in pre-labeled screw-top containers, refrigerated at 4 °C directly after collection, using mobile refrigerators (WAECO CoolFreeze CF-50, WAECO, Emsdetten, Germany) and kept cool until slide preparation in the laboratory within two days of collection. The *A. lumbricoides* infection status of participants was established by Kato-Katz examination [34] of two sub-samples (41.7 mg each) from a single stool specimen, which was thoroughly mixed before slide preparation. Kato-Katz slides were examined for *A. lumbricoides* eggs by experienced staff within two days after slide preparation. *A. lumbricoides* infection was defined as the presence of at least one *A. lumbricoides* egg in any of the two slides and infection intensity was classified according to Montresor et al. [35]. To assure the quality of our lab results all Kato-Katz slides were archived and a sample of randomly selected slides were reexamined after at least one month by different lab staff.

Helminth infected participants were offered treatment with albendazole (for *A. lumbricoides* and other intestinal nematode infections) and/or praziquantel (for schistosome infections), according to their respective diagnosis.

Environmental Data

The following remotely sensed environmental data were considered for this analysis: Elevation was obtained using the NASA Shuttle Radar Topography Mission (SRTM) global digital elevation model (DEM) version 2.1 [36]. These elevation data were also used to calculate the slope. Mean annual rainfall and ambient temperature were downloaded from the WorldClim – Global Climate Data website [37]. Mean annual land surface temperature during day and night (LST-day and LST-night) and vegetation cover (Enhanced vegetation index (EVI)) which had been collected during NASA's Moderate-Resolution Imaging Spectroradiometer (MODIS) Terra mission, were downloaded from the Land Processes Distributed Active Archive Center (LP DAAC) [38,39].

Household positions and inhabitant numbers from the initial population census were used to calculate population density around the household. Population density, ambient temperature, elevation, rainfall, LST, EVI, and slope were averaged for a buffer area within a 1,000 meter radius around each homestead in order to characterize the environmental situation around the household. This approach was preferred to using the respective spot values at the homestead position because spot data are more prone to random error than averages for a wider area. Latrine coverage in the surroundings of each household was calculated as the inverse distance weighted percentage of households with their own latrine within one kilometer around the household.

Initial processing of remotely sensed data was done in Idrisi GIS software v.32 (Clark Labs, Worcester, MA, USA). The GIS

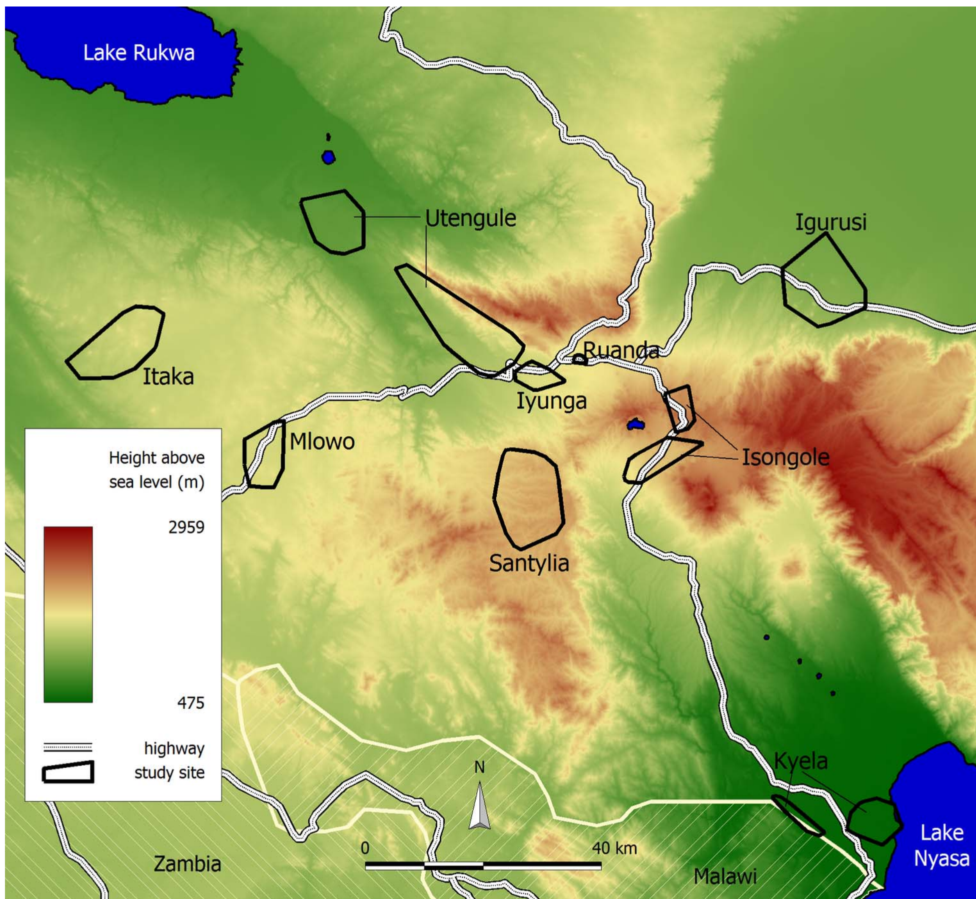


Figure 1. Location and altitude of the EMINI study sites. The large altitude range results in very diverse environmental conditions regarding temperature, vegetation, slope etc.
doi:10.1371/journal.pone.0092032.g001

program Manifold System 8.0 Professional Edition (Manifold Net Ltd, Carson City, NV) was used to combine household positions and environmental data.

Socio-economic Status and Other Confounding Variables

Household income and expenditure data in developing countries, especially in rural areas, are often unreliable because many people do not have a regular cash income. To overcome this problem we employed a modification of a method initially proposed by Filmer and Pritchett (2001) that uses principal component analysis to generate an SES score using proxy variables [40–42]. The following proxy variables were used: Household assets (clock or watch, radio, television, mobile telephone, refrigerator, hand cart, bicycle, motor cycle, car, savings account), construction materials for the house, and sources of household fuels and drinking water. In addition to the above described SES score, age, sex, population density, latrine coverage around the household, and presence of a latrine in the household were considered as potential confounders.

Statistical Analysis

All statistical analyses were performed using Stata/SE (Version 11.2, StataCorp LP, College Station, TX). Because our environmental variables showed a high degree of correlation, multicollinearity was assessed using the variance inflation factor (VIF) ($VIF_i = 1/T_i$) calculated with the tolerance (T) ($T_i = 1 - R_i^2$). R_i^2 is the calculated variance of each covariate associated with the rest of the other independent variables. A VIF higher than 10 indicates a serious problem of multicollinearity [43–45].

After multicollinearity analysis we performed univariable linear logistic regressions for each considered independent variable. All variables with a univariable Wald's $p < 0.2$ were included in the multivariable analysis.

In our study design individual observations were clustered in households and these were clustered within study sites. Therefore, household clustering was accounted for by calculation of robust standard errors using Huber/White variance estimates [46–48] and the nine study sites were taken into account as dummy variables.

Multivariable logistic regression with the inclusion of fractional polynomials which is a flexible parametric approach for modeling continuous factors was applied to analyze non-linear associations between *A. lumbricoides* infection and environmental variables [49]. The power transformations x^p are found with a predefined set of powers $S = -2, -1, -0.5, 0, 0.5, 1, 2, 3$ where x^0 is defined as $\ln x$. A fractional polynomial model with one degree (FP1) takes the form$\beta_0 + \beta_1 x^p * \ln x$, with two degrees (FP2)$\beta_0 + \beta_1 x^p + \beta_2 x^p * \ln x$. The restriction of powers and the consideration of polynomials with degree 1 and 2 provide enough flexibility for statistical modeling [50,51].

Multivariable fractional polynomial (MFP) models, an extended algorithm introduced by Royston and Sauerbrei, were used to detect non-linear associations. The MFP algorithm contains a

function selection procedure which compares null, linear, and FP1 sub models for each covariate with an FP2 model based on the deviance [52]. A detailed description of the MFP algorithm is found in Ambler and Royston (2001) and in Sauerbrei and Royston (2008) [53,54]. For the function selection procedure a lower p-value than 0.05 is recommended to avoid over fitting [50]. Therefore, a p-value of 0.01 was chosen as cut off when non-linear sub models were compared.

Our final model was calculated by removing variables with a p-value above 0.05. Changes of the Akaike Information Criterion (AIC) [55] and the Bayesian Information Criterion (BIC) [56], measuring the relative goodness of fit, were simultaneously considered. In order to asses spatial autocorrelation in the raw *A. lumbricoides* infection data and in the deviance residuals of our final logistic model the Stata module "spatcorr" was used to calculate Moran's I [57].

Results

Descriptive Results

The overall prevalence of *A. lumbricoides* infection in the study population was 6.8% (n = 433/6,366). Most infections were of low intensity, moderate and high intensity infections were rare. The highest prevalences were found in Kyela (25.2%) and Isongole (16.9%). sites. Figure 2 demonstrates that *A. lumbricoides* infection was clustered both between and within sites.

Men (47%) and women (53%) were almost equally represented in the study and the mean age was 23.6 years. Thus the majority of the study population were children and adolescents and the peak of *A. lumbricoides* infection occurred before the age of ten years (Figure 3). Nearly all households (97.5%) had their own latrine, which was a pit latrine in most cases (Table 1). The prevalence of *A. lumbricoides* infection was similar in female (6.57%) and male (7.11%) participants.

Univariable Logistic Regression and Multicollinearity Analysis

In univariable analysis, all considered environmental variables were significantly associated with *A. lumbricoides* infection (Table 2). Elevation, LST-day and slope showed an inverse association, whereas all other environmental variables were positively associated. Therefore, all environmental variables apart from elevation and ambient temperature were included in the multivariable analysis. Sex, household size, and population density were excluded because their p-values were above 0.2, the threshold which was chosen for the inclusion in multivariable analysis.

Due to high multicollinearity of the variables elevation (VIF = 116.65), ambient temperature (VIF = 71.05) and LST-night (VIF = 17.19), elevation and ambient temperature were excluded from multivariable analysis, since their VIFs by far exceeded the threshold of 10. We decided to include LST-night not only because of its lower VIF, but more importantly, because soil temperature appears to be more directly linked to helminth egg development than elevation or ambient temperature, as eggs develop in the soil or at the soil surface.

Multivariable Logistic Regression with Fractional Polynomials

In MFP analysis we found a non-linear relationship of rainfall and age with *A. lumbricoides* infection. In the full and in the final reduced model a fractional polynomial transformation with two degrees (FP2) was implemented.

For the other variables the linear assumption was retained and odds ratios (ORs) were calculated. The full and the final reduced multivariable regression models are shown in Table 3. Rainfall and LST-day were kept as significant environmental variables in the final model. LST-day showed an inverse association with *A. lumbricoides* infection. With every degree Celsius increase in LST-day the odds of being infected with *A. lumbricoides* decreased by about 13%.

The calculated beta coefficients for the non-linear functions of rainfall and age are not directly interpretable as ORs. For rainfall, the odds of *A. lumbricoides* infection are increasing until a rainfall maximum of 1,740 mm and are decreasing again at higher values (Figure 4). For age there is a steep increase until an infection maximum at 7.7 years, after which the odds are decreasing (curve not shown). This is in agreement with the unadjusted age prevalence curve shown in figure 2.

Except for latrine ownership in the household, all included confounding variables (age, SES score and latrine coverage) were significantly negatively associated with *A. lumbricoides* infection and therefore retained in the final multivariable model.

In order to check our final model for plausible interactions we calculated interaction terms between each beta of rainfall and LST-day and between SES and latrine coverage. However, none of these interactions were significant (data not shown).

The raw *A. lumbricoides* infection data show strong positive spatial autocorrelation within separation distances of up to 8 km (Figure 5). The lower values for Moran's I in the deviance residuals of our final model indicate that the variables in the model account for a large part of this autocorrelation.

Discussion

Our univariable results show that *A. lumbricoides* infection is significantly associated with several environmental factors. Of these, mean annual rainfall and mean annual LST-day remained significant in the multivariable model. LST-day had a linear negative association with *A. lumbricoides* infection, whereas the association of rainfall and age was non-linear with maximum infection odds at 1,740 mm of mean annual rainfall and at an age of 7.7 years. SES and latrine coverage around the household showed significant negative associations with *A. lumbricoides* infection, both in univariable and multivariable analyses.

Our results concerning LST-day are in line with the published literature [21,23–25]. Two studies from Cameroon and Southeast Asia found that a higher LST was significantly associated with a lower risk of *A. lumbricoides* infection because high soil temperatures reduce humidity and thus lead to desiccation of *Ascaris* eggs [23,24]. These studies considered mean, minimum and maximum LST, not LST for day and night as in our analysis. However, in both studies minimum LST was excluded from multivariable analysis which is in parallel to the exclusion of our LST-night variable. Maximum LST was significantly negatively associated with infection which is in agreement with the significant LST-day variable in our final model. One study from Uganda found that *A. lumbricoides* prevalence is $<5\%$ where maximum LST exceeds 36–37°C [21] and a study conducted in 20 schools in the Chad predicted no *A. lumbricoides* prevalence in areas where mean LST exceeds 37 °C [25].

Denser vegetation, as indicated by a higher EVI, showed a strong positive association with *A. lumbricoides* infection in univariable analysis, which, however, turned non-significant when including other variables in the multivariable model. Our significant univariable result for EVI is in line with multivariable results from former studies [23,24,58]. The non-significance of EVI in multivariable analysis is likely due to differences in local

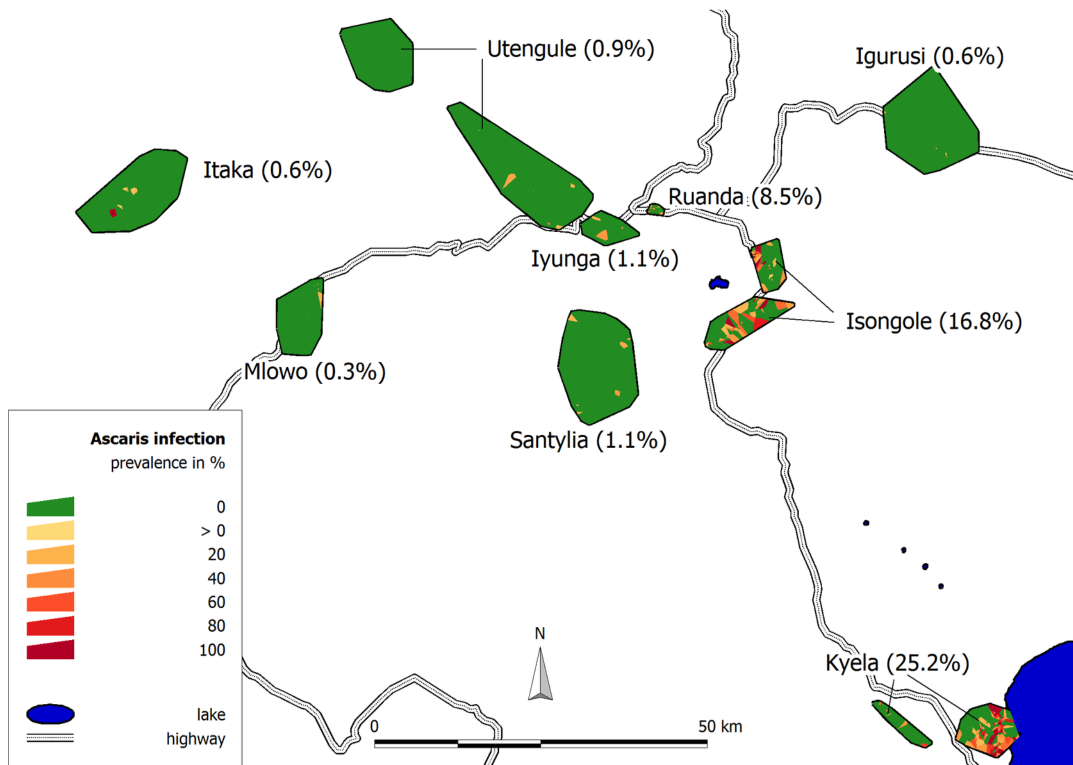


Figure 2. *A. lumbricoides* prevalence in the study sites. Color coding indicates household prevalence, labels indicate site name and site prevalence. *A. lumbricoides* infection is strongly clustered both between and within sites.
doi:10.1371/journal.pone.0092032.g002

conditions compared to the former studies where EVI showed a significant association.

The non-linear relationship of rainfall with infection can be explained based on the life cycle of *A. lumbricoides*. Rainfall is an important determinant of larval development because humidity,

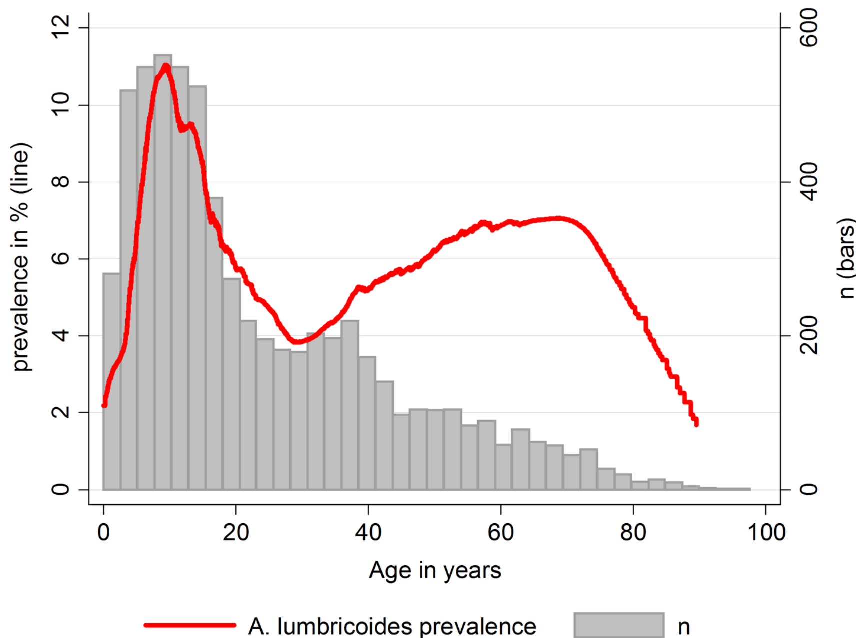


Figure 3. Lowess smoothed plot of unadjusted *A. lumbricoides* prevalence over age. The main prevalence peak in childhood is in accordance with the age of maximum infection intensity mentioned in the literature [14,67]. The second rise above the age of 30 with a less pronounced peak in older age seems less common.
doi:10.1371/journal.pone.0092032.g003

Table 1. Description of variables.

Variables	N	Mean or percentage [a)]	Std. Dev.	Min	Max
Ascaris infected	6,366	6.8%			
Ascaris infection intensity [b)]:					
No infection (0 EPG)	5,933	93.2%			
Low intensity (1–1,999)	317	4.98%			
Moderate intensity (2,000–3,999 EPG)	66	1.04%			
Heavy intensity (≥4,000 EPG)	50	0.97%			
Elevation [m]	6,366	1455	486	479	2313
Mean annual ambient temperature [°C]	6,366	19.8	2.8	14.7	25.0
Mean annual rainfall [mm]	6,366	1437	378	1013	2342
Mean annual LST-day [°C]	6,366	32.4	2.5	22.5	38.6
Mean annual LST-night [°C]	6,366	14.5	3.4	9.2	21.4
Mean annual EVI	6,366	0.288	0.058	0.151	0.472
Slope [°]	6,366	3.03	2.21	0.35	13.64
Age [years]	6,366	23.6	19.2	0	97.7
Male gender	6,317	47.0%			
Household size [persons]	6,363	6.5	3.6	1	30
Population density [persons/km^2]	6,366	1875	3179	10	13133
SES	6,363	−0.52	1.17	−2.82	4.08
Households with latrine	6,363	97.5%			
Latrine coverage in surroundings [%] [c)]	6,366	95.8	8.2	29.6	100

N = number of observations; Std. Dev. = standard deviation; EPG = eggs per gram of feces; LST = land surface temperature; EVI = enhanced vegetation index; SES = socio-economic score.
[a)]Mean for continuous and % for categorical variables.
[b)]According to Montresor, 1998 [35].
[c)]Percentage of households with a latrine within one kilometer around the participant's household.
doi:10.1371/journal.pone.0092032.t001

Table 2. Univariable association of environmental and socio-demographic factors with *A. lumbricoides* infection [a)].

Variables	OR (95% CI)	p-value
Elevation, per 100 meters	0.88 (0.84 to 0.91)	<0.001
Mean annual ambient temperature, per 1°C	1.23 (1.14 to 1.32)	<0.001
Mean annual rainfall, per 1000 mm	7.93 (5.85 to 10.75)	<0.001
Mean annual LST-day, per 1°C	0.73 (0.70 to 0.76)	<0.001
Mean annual LST-night, per 1°C	1.20 (1.13 to 1.27)	<0.001
Mean annual EVI, per 0.1 units	5.91 (4.10 to 8.50)	<0.001
Slope, per 1 °	0.72 (0.63 to 0.83)	<0.001
Age, per 10 years	0.95 (0.90 to 1.00)	0.060
Sex	1.09 (0.90 to 1.32)	0.389
Household size	1.01 (0.96 to 1.07)	0.603
Population density, per 1000/km^2	0.98 (0.94 to 1.03)	0.379
SES, per 1 unit	0.59 (0.48 to 0.73)	<0.001
Latrine in household (yes/no)	0.24 (0.13 to 0.46)	<0.001
Latrine coverage, per 10%[b)]	0.57 (0.50 to 0.65)	<0.001

OR = odds ratio; 95% CI = confidence interval; LST = land surface temperature; EVI = enhanced vegetation index; SES = socio-economic score.
[a)]Results of logistic regression models adjusted for household clustering using Huber/White variance estimates.
[b)]Percentage of households with a latrine within one kilometer around the participant's household.
doi:10.1371/journal.pone.0092032.t002

soil moisture, and land surface temperature have a strong impact on embryonation [22]. Laboratory studies showed that higher humidity facilitates larval development [59,60]. A multiple regression analysis of rainfall, number of wet days and ambient temperature in Sri Lanka found a significant association between increased number of wet days per month and higher rates of *A. lumbricoides* infections [61]. These findings are in line with the first part of our predicted non-linear curve showing higher infection odds when rainfall increases. However, another laboratory study showed that the development of eggs located on an extremely wet soil surface was delayed due to evaporation and the resulting low temperatures [59]. Since, in contrast to hookworm larvae, *A. lumbricoides* eggs are non-motile [12], they are directly exposed to rainfall and LST on the soil surface. Crompton states that eggs may be washed away by rainfall, too [5]. Increased rainfall can lead to a leaching effect and eggs are washed to deeper regions of the soil [62]. All these findings indicate that up to a certain amount, increasing rainfall supports larval development but that too much rain can delay larval development and thus reduce transmission.

Regarding the examined possible confounding variables, our univariable and multivariable results indicate that low SES and bad sanitary conditions in and around the household are risk factors for *A. lumbricoides* infection which is in line with former publications [12,14,27,28,30,32]. The non-linear association between age and *A. lumbricoides* infection describing a higher risk in children and an infection peak in later childhood, is in line with former epidemiological studies [3–6]. Although none of these variables substantially confounded the associations of *A. lumbricoides*

Table 3. Multivariable association of environmental and socio-demographic factors with *A. lumbricoides* infection using logistic regression with fractional polynomials (n = 6,363).

	Full Model [a]		Final Model after Backward Elimination [a]	
Covariables	**β/OR (95% CI)**	**p**	**β/OR (95% CI)**	**p**
Mean annual rainfall, per 1000 mm	$\beta_1 = 1.92$ (0.33 to 3.51)	0.018	$\beta_1 = 1.58$ (0.14 to 3.02)	0.032
(FP2 polynomial transformation[b])	$\beta_2 = -2.08$ (−3.63 to −0.52)	0.009	$\beta_2 = -1.78$ (−3.19 to −0.37)	0.013
Mean annual LST-day, per 1 °C	OR = 0.82 (0.73 to 0.93)	0.002	OR = 0.87 (0.78 to 0.97)	0.012
Mean annual LST-night, per 1 °C	OR = 1.24 (0.81 to 1.90)	0.315	.	.
Mean annual EVI, per 0.1 units	OR = 0.79 (0.42 to 1.50)	0.471	.	.
Slope, per 1 °	OR = 0.90 (0.72 to 1.12)	0.324	.	.
Age, per 10 years	$\beta_1 = 2.57$ (1.38 to 3.76)	<0.001	$\beta_1 = 2.56$ (1.38 to 3.74)	<0.001
(FP2 polynomial transformation[c])	$\beta_2 = 1.13$ (0.57 to 1.70)	<0.001	$\beta_2 = 1.13$ (0.56 to 1.69)	<0.001
SES, per 1 unit	OR = 0.84 (0.70 to 1.00)	0.052	OR = 0.82 (0.68 to 0.97)	0.024
Latrine in household (yes/no)	OR = 0.68 (0.32 to 1.43)	0.310	.	.
Latrine coverage, per 10%[d]	OR = 0.82 (0.68 to 0.99)	0.043	OR = 0.80 (0.67 to 0.96)	0.018
AIC	2178		2178	
BIC	2313		2287	

β = beta coefficient; OR = odds ratio; 95% CI = confidence interval; LST = land surface temperature; EVI = enhanced vegetation index; SES = socio economic score, AIC = Akaike information criterion; BIC = Bayesian information criterion.
[a]Adjusted for household clustering using Huber/White/Sandwich variance estimates and for study sites.
[b]Fractional polynomial transformation with two degrees and powers p = 3: $\beta_1 x^p + \beta_2 x^p * \ln x$.
[c]Fractional polynomial transformation with two degrees and powers p = −0.5: $\beta_1 x^p + \beta_2 x^p * \ln x$.
[d]Percentage of households with a latrine within one kilometer around the participant's household.
doi:10.1371/journal.pone.0092032.t003

infection with environmental variables, it is important to consider such factors when planning interventions or further studies on a smaller scale, e.g. the community level.

In the literature, non-linear associations between environmental variables and STH infection have rarely been analyzed in a multivariable context. Brooker et al. [21] predict the prevalence of various STH infections with generalized additive models in a spatial analysis in Uganda and found that the predicted prevalence of *A. lumbricoides* infection showed non-linear relationships with LST and rainfall. A case study in Cameroon observed non-linear

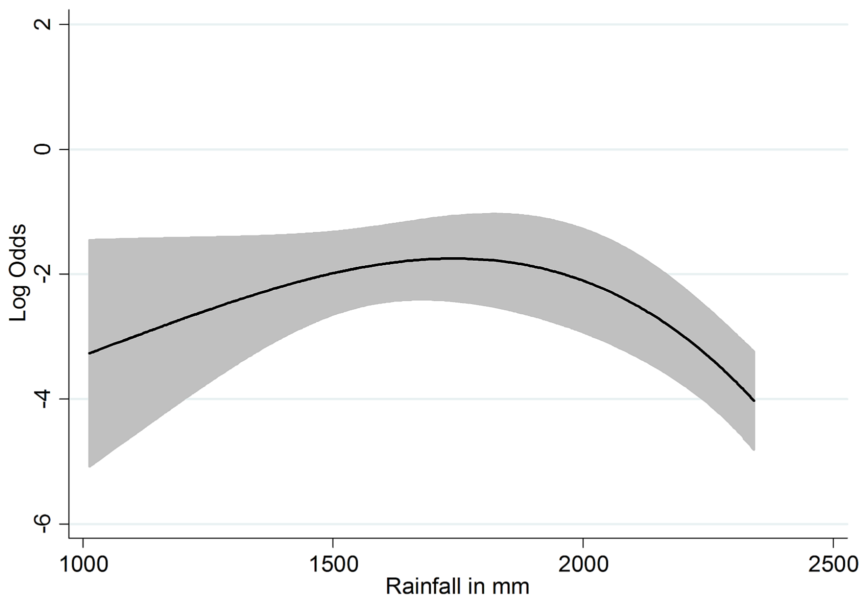


Figure 4. Non-linear partial prediction of the log odds of *A. lumbricoides* infection by annual rainfall. The partial predicted curve is adjusted for LST-day, slope, SES, age, latrine coverage and the nine study sites. The maximum is at 1740 mm of mean annual rainfall. Grey shadings indicate 95% confidence band.
doi:10.1371/journal.pone.0092032.g004

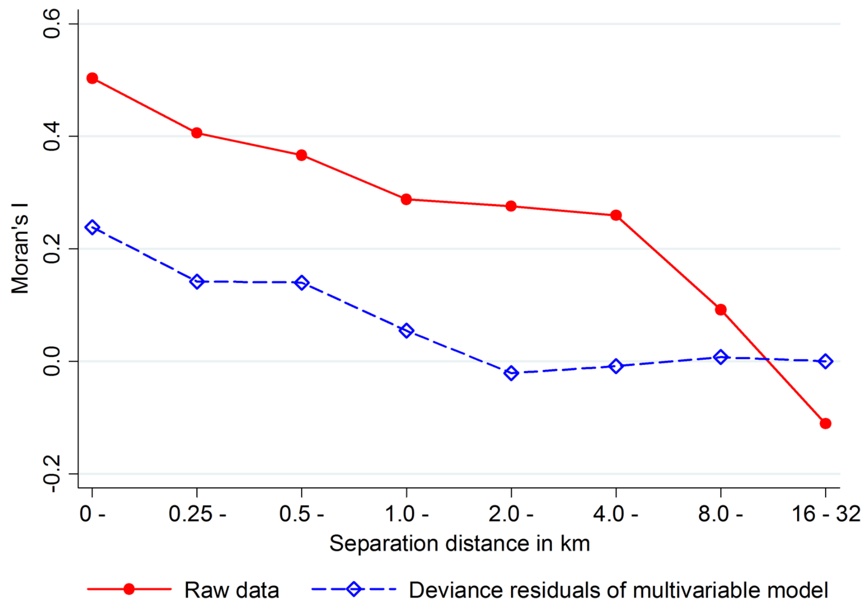


Figure 5. Spatial autocorrelation of *A. lumbricoides* infection within sites. Moran's I for spatial autocorrelation of *A. lumbricoides* infection in the raw data and in the deviance residuals of the final multivariable model. Values above 0 indicate positive, values below 0 negative spatial autocorrelation. The figure only considers autocorrelation between households within the same sites.
doi:10.1371/journal.pone.0092032.g005

associations between environmental variables (LST, rainfall and vegetation) and *A. lumbricoides* infection in scatterplots of prevalence and environmental data [22]. Our results suggest that the MFP procedure can be effectively used as a multivariable parametric approach to detect non-linear associations between environmental data and *A. lumbricoides* infection. Especially when a turning point within a non-linear prediction is detected, such as for rainfall in our study, the MFP procedure can provide new insights into the relationship between environmental conditions and STH infection. A more precise understanding of such relationships could play an important role in the future prediction of high prevalence areas to be targeted for interventions.

However, non-linear analysis also has its limitations. Transformed variables are not directly interpretable, and thus hard to generalize and compare between studies. Moreover, fractional polynomials are very sensitive to outliers and over fitting, and transformations can often be due to extreme observations. To avoid this, it is very important to analyze transformed variables for outliers and define lower p-values for the function selection procedure when non-linear models are compared. Besides that, it is recommended to analyze non-linearity only if prior knowledge for non-linear relationships exists [54].

One problem when assessing STH infection by Kato-Katz (and most other microscopy based techniques) is the low sensitivity of the method. This is best compensated by the examination of more than one stool specimen, which was logistically impossible in our study. Instead, we examined two separate Kato-Katz slides from the same sample. Although this should have increased sensitivity, we have inevitably missed some of the lighter infections. However, the Kato-Katz examination of a single stool specimen shows a better sensitivity for the detection of *A. lumbricoides* infection than for other STH infections [63,64].

Another limitation of our study is the lack of information concerning soil types in our study sites, which could be important in the context of rainfall and the survival of *Ascaris* eggs. Beaver states that *A. lumbricoides* infections were more common in regions with clayey soils [65,66]. Sandy soils are more permeable and are unable to keep moisture. Moreover, in sandy soils eggs are more likely to be washed down to deeper soil strata. Furthermore we are unable to account for seasonal aspects of rainfall or the intensity of rainfall within a short time. Gunawardena et al. [61] found out that the number of wet-days per month were more significantly associated with *A. lumbricoides* infection than the total amount of rainfall. In this context they state that heavy rains facilitate the scattering of eggs both vertically and horizontally, whereas steady rainfall helps to maintain soil moisture.

Although our final model was able to account for most of the spatial autocorrelation in the raw *A. lumbricoides* infection data (Figure 5), the figure also shows some remaining positive spatial autocorrelation in the deviance residuals of our final model. This might have influenced the variance estimates of the model and in turn might have led to spuriously low p-values for some of our environmental variables. Therefore, these p-values should be interpreted with caution.

To conclude, *A. lumbricoides* infection was associated with several environmental, socio-demographic and sanitary factors in univariable analyses. Of these, mean annual rainfall, mean annual LST-day, age, SES and latrine coverage remained significant in multivariable analysis. MFP models can be used as an effective statistical tool to get a better understanding of the – often non-linear - relationship between environmental factors and *A. lumbricoides* infection. Future studies should therefore consider potential non-linear relationships between environmental factors and STH in a multivariable context to yield more precise predictions. However, if data are available, socio-demographic and sanitary conditions should also be considered, especially when planning interventions.

Supporting Information

Checklist S1 STROBE checklist.
(DOC)

Acknowledgments

We thank the participants of the EMINI study for their participation and continued support, and the field and laboratory teams at MMRP for their hard work.

Author Contributions

Conceived and designed the experiments: MH LM SM IK TL ES. Performed the experiments: PC IK AN CM SM LM CG ES. Analyzed the data: SAS HR RPL ES. Contributed reagents/materials/analysis tools: DOK ES. Wrote the paper: SAS ES.

References

1. Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, et al. (2006) Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet 367: 1521–1532.
2. de Silva NR, Brooker S, Hotez PJ, Montresor A, Engels D, et al. (2003) Soil-transmitted helminth infections: updating the global picture. Trends Parasitol 19: 547–551.
3. de Silva NR, Chan MS, Bundy DA (1997) Morbidity and mortality due to ascariasis: re-estimation and sensitivity analysis of global numbers at risk. Trop Med Int Health 2: 519–528.
4. de Silva NR, Guyatt HL, Bundy DA (1997) Worm burden in intestinal obstruction caused by Ascaris lumbricoides. Trop Med Int Health 2: 189–190.
5. Crompton DWT, Pawlowski ZS (1985) Life history and development of Ascaris lumbricoides and the persistence of human ascariasis. In: Crompton DWT, Nesheim MC, Pawlowski ZS, editors. Ascariasis and its public health significance: a volume based on the agenda and discussions of the 1984 Banff conference, organized by WHO Parasitic Diseases Programme and Division of Nutritional Sciences, Cornell University, New York. London; Philadelphia: Taylor & Francis.pp. 9–23.
6. Scott ME (2008) Ascaris lumbricoides: A review of Its Epidemiology and Relationship to Other Infections. Annales Nestlé 66: 7–22.
7. Drake LJ, Jukes MCH, Sternberg RJ, Bundy DAP (2000) Geohelminth Infections (Ascariasis, Trichuriasis, and Hookworm): Cognitive and Developmental Impacts. Seminars in Pediatric Infectious Diseases 11: 245–251.
8. Abidin SAN, Hadidjaja P (2003) The effect of soil-transmitted helminth infection on the cognitive function of schoolchildren. In: Crompton DWT, Montresor A, Nesheim MC, Savioli L, editors. Controlling disease due to helminth infections: World Health Organization. pp. 67–71.
9. Dickson R, Awasthi S, Williamson P, Demellweek C, Garner P (2000) Effects of treatment for intestinal helminth infection on growth and cognitive performance in children: systematic review of randomised trials. BMJ 320: 1697–1701.
10. Taylor-Robinson DC, Maayan N, Soares-Weiser K, Donegan S, Garner P (2012) Deworming drugs for soil-transmitted intestinal worms in children: effects on nutritional indicators, haemoglobin and school performance. Cochrane Database Syst Rev 7: CD000371.
11. Anderson RM (1982) The population dynamics and control of hookworm and roundworm infections. In: Anderson RM, editor. The Population dynamics of infectious diseases: theory and applications. London; New York: Chapman and Hall.pp. 68–108.
12. Crompton DWT, Savioli L (2007) Handbook of helminthiasis for public health. Boca Raton: CRC/Taylor & Francis. 362 p.p.
13. Crompton DW (2001) Ascaris and ascariasis. Adv Parasitol 48: 285–375.
14. O'Lorcain P, Holland CV (2000) The public health importance of Ascaris lumbricoides. Parasitology 121 Suppl: S51–71.
15. Brooker S, Clements AC, Bundy DA (2006) Global epidemiology, ecology and control of soil-transmitted helminth infections. Adv Parasitol 62: 221–261.
16. Magalhaes RJ, Clements AC, Patil AP, Gething PW, Brooker S (2011) The applications of model-based geostatistics in helminth epidemiology and control. Adv Parasitol 74: 267–296.
17. Brooker S, Hay SI, Bundy DA (2002) Tools from ecology: useful for evaluating infection risk models? Trends Parasitol 18: 70-74.
18. Brooker S, Clements AC (2009) Spatial heterogeneity of parasite co–infection: Determinants and geostatistical prediction at regional scales. Int J Parasitol 39: 591–597.
19. Brooker S, Rowlands M, Haller L, Savioli L, Bundy DA (2000) Towards an atlas of human helminth infection in sub-Saharan Africa: the use of geographical information systems (GIS). Parasitol Today 16: 303–307.
20. Brooker S, Kabatereine NB, Smith JL, Mupfasoni D, Mwanje MT, et al. (2009) An updated atlas of human helminth infections: the example of East Africa. Int J Health Geogr 8: 42.
21. Brooker S, Kabatereine NB, Tukahebwa EM, Kazibwe F (2004) Spatial analysis of the distribution of intestinal nematode infections in Uganda. Epidemiol Infect 132: 1065–1071.
22. Brooker S, Michael E (2000) The potential of geographical information systems and remote sensing in the epidemiology and control of human helminth infections. Adv Parasitol 47: 245–288.
23. Brooker S, Hay SI, Tchuente LAT, Ratard R (2002) Using NOAA-AVHRR data to model human helminth distributions in planning disease control in Cameroon, West Africa. Photogramm Eng Rem S 68: 175–179.
24. Brooker S, Singhasivanon P, Waikagul J, Supavej S, Kojima S, et al. (2003) Mapping soil-transmitted helminths in Southeast Asia and implications for parasite control. Southeast Asian J Trop Med Public Health 34: 24–36.
25. Brooker S, Beasley M, Ndinaromtan M, Madjiouroum EM, Baboguel M, et al. (2002) Use of remote sensing and a geographical information system in a national helminth control programme in Chad. Bull World Health Organ 80: 783–789.
26. Pullan RL, Gething PW, Smith JL, Mwandawiro CS, Sturrock HJ, et al. (2011) Spatial modelling of soil-transmitted helminth infections in Kenya: a disease control planning tool. PLoS Negl Trop Dis 5: e958.
27. Kan SP (1985) Ascaris lumbricoides in Malaysia. In: Crompton DWT, Nesheim MC, Pawlowski ZS, editors. Ascariasis and its public health significance: a volume based on the agenda and discussions of the 1984 Banff conference, organized by WHO Parasitic Diseases Programme and Division of Nutritional Sciences, Cornell University, New York. London; Philadelphia: Taylor & Francis.pp. 69–82.
28. Stephenson LS (1985) Factors affecting the prevalence and control of Ascaris lumbricoides infection in Kenya. In: Crompton DWT, Nesheim MC, Pawlowski ZS, editors. Ascariasis and its public health significance: a volume based on the agenda and discussions of the 1984 Banff conference, organized by WHO Parasitic Diseases Programme and Division of Nutritional Sciences, Cornell University, New York. London; Philadelphia: Taylor & Francis.pp. 113–127.
29. Tshikuka JG, Scott ME, Gray-Donald K (1995) Ascaris lumbricoides infection and environmental risk factors in an urban African setting. Ann Trop Med Parasitol 89: 505–514.
30. Walker M, Hall A, Basanez MG (2011) Individual predisposition, household clustering and risk factors for human infection with Ascaris lumbricoides: new epidemiological insights. PLoS Negl Trop Dis 5: e1047.
31. Forrester JE, Scott ME, Bundy DA, Golden MH (1988) Clustering of *Ascaris lumbricoides* and *Trichuris trichiura* infections within households. Trans R Soc Trop Med Hyg 82: 282–288.
32. Carneiro FF, Cifuentes E, Tellez-Rojo MM, Romieu I (2002) The risk of Ascaris lumbricoides infection in children as an environmental health indicator to guide preventive activities in Caparao and Alto Caparao, Brazil. Bull World Health Organ 80: 40–46.
33. Riess H, Clowes P, Kroidl I, Kowuor DO, Nsojo A, et al. (2013) Hookworm infection and environmental factors in mbeya region, Tanzania: a cross-sectional, population-based study. PLoS Negl Trop Dis 7: e2408.
34. WHO (1991) Basic laboratory methods in medical parasitology. Geneva: World Health Organization. viii, 114 p.p.
35. Montresor A, Crompton DW, Bundy DAP, Hall A, Savioli L (1998) Guidelines for the evaluation of soil-transmitted helminthiasis and schistosomiasis at community level. Geneva: WHO. 45 p.
36. Farr TG, Rosen PA, Caro E, Crippen R, Duren R, et al. (2007) The shuttle radar topography mission. Reviews of Geophysics 45.
37. Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A (2005) Very high resolution interpolated climate surfaces for global land areas. Int J Climatol 25: 1965–1978.
38. NASA Land Processes Distributed Active Archive Center (LP DAAC) (2001) MODIS 11A2. USGS/Earth Resources Observation and Science (EROS) Center, Sioux Falls, South Dakota.
39. NASA Land Processes Distributed Active Archive Center (LP DAAC) (2001) MODIS 13Q1. Resources Observation and Science (EROS) Center, Sioux Falls, South Dakota.
40. Kolenikov S, Angeles G (2009) Socioeconomic Status Measurement with Discrete Proxy Variables: Is Principal Component Analysis a Reliable Answer? Rev Income Wealth 55: 128–165.
41. Filmer D, Pritchett LH (2001) Estimating wealth effects without expenditure data—or tears: an application to educational enrollments in states of India. Demography 38: 115–132.
42. Vyas S, Kumaranayake L (2006) Constructing socio-economic status indices: how to use principal components analysis. Health Policy Plan 21: 459–468.
43. Alin A (2010) Multicollinearity. WIREs Computational Statistics 2: 370–374.
44. Harrell FE (2001) Regression modeling strategies: with applications to linear models, logistic regression, and survival analysis. New York: Springer. xxii, 568 p. p.
45. Menard SW (2002) Applied logistic regression analysis. Thousand Oaks, Calif.: Sage Publications. viii, 111 p. p.
46. Huber PJ (1967) The behavior of maximum likelihood estimates under nonstandard conditions. Proceedings of the Fifth Berkeley Symposium on Mathematical Statistics and Probability 1: 221–223.
47. White H (1980) A heteroskedasticity-consistent covariance matrix estimator and a direct test for heteroskedasticity. Econometrica 48: 817–830.
48. Rogers WH (1993) Regression standard errors in clustered samples. Stata Technical Bulletin 3: 19–23.
49. Royston P, Altman DG (1994) Regression Using Fractional Polynomials of Continuous Covariates - Parsimonious Parametric Modeling. Appl Stat-J Roy St C 43: 429–467.

50. Royston P, Ambler G, Sauerbrei W (1999) The use of fractional polynomials to model continuous risk variables in epidemiology. Int J Epidemiol 28: 964–974.
51. Royston P, Altman DG (1997) Approximating statistical functions by using fractional polynomial regression. Statistician 46: 411–422.
52. Sauerbrei W, Meier-Hirmer C, Benner A, Royston P (2006) Multivariable regression model building by using fractional polynomials: Description of SAS, STATA and R programs. Comput Stat Data An 50: 3464–3485.
53. Ambler G, Royston P (2001) Fractional polynomial model selection procedures: Investigation of type I error rate. J Stat Comput Sim 69: 89–108.
54. Royston P, Sauerbrei W (2008) Multivariable model-building: a pragmatic approach to regression analysis based on fractional polynomials for modelling continuous variables. Chichester, England; Hoboken, NJ: John Wiley. xvii, 303 p. p.
55. Akaike H (1974) A new look at the statistical model identification. IEEE Transactions on Automatic Control 19: 716–723.
56. Schwarz G (1978) Estimating the dimension of a model. Annals of Statistics 6: 461–464.
57. Pisati M (2001) Tools for spatial data analysis. Stata Technical Bulletin 60: 21–37.
58. Saathoff E, Olsen A, Kvalsvig JD, Appleton CC, Sharp B, et al. (2005) Ecological covariates of *Ascaris lumbricoides* infection in schoolchildren from rural KwaZulu-Natal, South Africa. Trop Med Int Health 10: 412–422.
59. Otto GF (1929) A study of the moisture requirements of the eggs of the horse, the dog, human and pig ascarids. Am J Hyg 10: 0497–0520.
60. Spindler LA (1929) The relation of moisture to the distribution of human trichuris and ascaris. Am J Hyg 10: 0476–0496.
61. Gunawardena GS, Karunaweera ND, Ismail MM (2004) Wet-days: are they better indicators of Ascaris infection levels? J Helminthol 78: 305–310.
62. Storey GW, Phillips RA (1985) The survival of parasite eggs throughout the soil profile. Parasitology 91 (Pt3): 585–590.
63. Tarafder MR, Carabin H, Joseph L, Balolong E Jr, Olveda R, et al. (2010) Estimating the sensitivity and specificity of Kato-Katz stool examination technique for detection of hookworms, Ascaris lumbricoides and Trichuris trichiura infections in humans in the absence of a 'gold standard'. Int J Parasitol 40: 399–404.
64. Knopp S, Mgeni AF, Khamis IS, Steinmann P, Stothard JR, et al. (2008) Diagnosis of soil-transmitted helminths in the era of preventive chemotherapy: effect of multiple stool sampling and use of different diagnostic techniques. PLoS Negl Trop Dis 2: e331.
65. Beaver PC (1975) Biology of Soil-Transmitted Helminths - Massive Infection. Health Lab Sci 12: 116–125.
66. Beaver PC (1952) Observation on the epidemiology of ascariasis in a region of high hookworm endemicity. Journal of Parasitology 38: 445–453.
67. Elkins DB, Haswell-Elkins M, Anderson RM (1986) The epidemiology and control of intestinal helminths in the Pulicat Lake region of Southern India. I. Study design and pre- and post-treatment observations on Ascaris lumbricoides infection. Trans R Soc Trop Med Hyg 80: 774–792.

Hookworm Infection and Environmental Factors in Mbeya Region, Tanzania: A Cross-Sectional, Population-Based Study

Helene Riess[1,2], Petra Clowes[3], Inge Kroidl[1,3], Dickens O. Kowuor[3], Anthony Nsojo[3], Chacha Mangu[3], Steffen A. Schüle[1,2], Ulrich Mansmann[2], Christof Geldmacher[1,4], Seif Mhina[5], Leonard Maboko[3], Michael Hoelscher[1,3,4], Elmar Saathoff[1,4]*

1 Division of Infectious Diseases and Tropical Medicine, Medical Center of the University of Munich, Munich, Germany, **2** Institute for Medical Bioinformatics, Biometry, and Epidemiology, Ludwig-Maximilians-University, Munich, Germany, **3** NIMR-Mbeya Medical Research Center, Mbeya, Tanzania, **4** German Centre for Infection Research (DZIF), Partner Site Munich, Munich, Germany, **5** Mbeya Regional Medical Office, Mbeya, Tanzania

Abstract

Background: Hookworm disease is one of the most common infections and cause of a high disease burden in the tropics and subtropics. Remotely sensed ecological data and model-based geostatistics have been used recently to identify areas in need for hookworm control.

Methodology: Cross-sectional interview data and stool samples from 6,375 participants from nine different sites in Mbeya region, south-western Tanzania, were collected as part of a cohort study. Hookworm infection was assessed by microscopy of duplicate Kato-Katz thick smears from one stool sample from each participant. A geographic information system was used to obtain remotely sensed environmental data such as land surface temperature (LST), vegetation cover, rainfall, and elevation, and combine them with hookworm infection data and with socio-demographic and behavioral data. Uni- and multivariable logistic regression was performed on sites separately and on the pooled dataset.

Principal Findings: Univariable analyses yielded significant associations for all ecological variables. Five ecological variables stayed significant in the final multivariable model: population density (odds ratio (OR) = 0.68; 95% confidence interval (CI) = 0.63–0.73), mean annual vegetation density (OR = 0.11; 95% CI = 0.06–0.18), mean annual LST during the day (OR = 0.81; 95% CI = 0.75–0.88), mean annual LST during the night (OR = 1.54; 95% CI = 1.44–1.64), and latrine coverage in household surroundings (OR = 1.02; 95% CI = 1.01–1.04). Interaction terms revealed substantial differences in associations of hookworm infection with population density, mean annual enhanced vegetation index, and latrine coverage between the two sites with the highest prevalence of infection.

Conclusion/Significance: This study supports previous findings that remotely sensed data such as vegetation indices, LST, and elevation are strongly associated with hookworm prevalence. However, the results indicate that the influence of environmental conditions can differ substantially within a relatively small geographic area. The use of large-scale associations as a predictive tool on smaller scales is therefore problematic and should be handled with care.

Citation: Riess H, Clowes P, Kroidl I, Kowuor DO, Nsojo A, et al. (2013) Hookworm Infection and Environmental Factors in Mbeya Region, Tanzania: A Cross-Sectional, Population-Based Study. PLoS Negl Trop Dis 7(9): e2408. doi:10.1371/journal.pntd.0002408

Editor: Aaron R. Jex, University of Melbourne, Australia

Received February 13, 2013; **Accepted** July 24, 2013; **Published** September 5, 2013

Copyright: © 2013 Riess et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was funded by the European Union (SANTE/2004/078-545 and SANTE/2006/129-931) and by the German Research Foundation (DFG, grant SA 1878/1-1). The funders had no role in study design, data collection and analysis, preparation of the manuscript or decision to publish.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: saathoff@lrz.uni-muenchen.de

Introduction

Hookworm disease caused by *Ancylostoma duodenale* and *Necator americanus* is among the most common infections in sub-Saharan Africa (SSA) and affects up to 198 million people in this region [1–3]. It causes iron deficiency anemia and protein malnutrition, and has been shown to potentially cause growth retardation as well as intellectual and cognitive impairments in children [2–4]. Although hookworm disease causes only limited mortality, it ranks 49th in terms of years lost due to disability globally and between 30 and 49 in SSA countries [5]. The educational, economic, and public-health importance of hookworm infection necessitates comprehensive control strategies. To assure the effectiveness of control programs, financial as well as human resources have to be targeted to areas of greatest need. This warrants reliable estimates of hookworm distribution and of population numbers requiring intervention [6]. As hookworms do not replicate inside the human body and larvae become infective only under favorable conditions once excreted, environmental factors are crucial to hookworm development and therefore to possible transmission to humans. In recent years, the use of remotely sensed data has helped to enhance the understanding of the epidemiology and spatial

Author Summary

Hookworm disease, caused by the nematodes *Ancylostoma duodenale* and *Necator americanus*, is an important cause of maternal and child morbidity in the developing countries of the tropics and subtropics. In children, hookworm disease has been shown to potentially result in growth retardation as well as intellectual and cognitive impairments. In a cross-sectional survey in Mbeya region, Tanzania, we assessed the effects of possible risk factors for hookworm infection with a focus on remotely sensed ecological factors such as elevation, vegetation density, land surface temperature, and rainfall. We found that several ecological variables were significantly associated with hookworm infection. However, differing effects for these factors were estimated when performing the analyses separately for the two sites with the highest hookworm prevalence. Our study shows that effects are scale-dependent and that prediction at smaller scales using large-scale data and *vice versa* should be handled with caution, because regional variation can substantially influence the presence of hookworm infection.

distribution of hookworm infection [7,8]. Model-based geostatistics have been used to map helminth infection prevalence and to predict prevalence at unsampled locations at national, provincial, and regional levels [6,9–11], however, under the assumption that the estimated associations are the same at all levels and not modified by regional characteristics.

This study aimed to investigate the relationship between hookworm infection and remotely sensed ecological factors, such as elevation, vegetation density, land surface temperature (LST), and rainfall, at an individual level in a cross-sectional survey of the "Evaluating and Monitoring the Impact of New Interventions" (EMINI - http://www.mmrp.org/projects/cohort-studies/emini.html) cohort in Mbeya region in south-western Tanzania. Furthermore, we analyzed the influence of potential confounders, such as age, sex and socio-economic status (SES), on these associations. The main focus was on the investigation of site-specific effects and their comparison to effects in the pooled data set to ascertain if associations between ecological factors and hookworm infection found on a larger scale can equally be applied at smaller scales. Additional articles pointing to *Ascaris lumbricoides*, *Trichuris trichiura*, *Schistosoma mansoni*, and *Schistosoma haematobium* infection are in preparation.

Methods

Ethics Statement

The study was approved by the ethics committee of the Tanzanian National Institute for Medical Research and conducted according to the principles expressed in the Declaration of Helsinki. All participants provided written informed consent before enrolment into the study; parents consented for their minor children.

Study Area and Data Collection

Mbeya region is situated in south-western Tanzania. The region is predominantly rural and most income-generating activities are related to agriculture. Data for this study were collected from June 2008 to June 2009 as part of the third annual survey of the EMINI cohort study. In preparation for EMINI, a complete census was undertaken in nine distinct sites of Mbeya region. Over 42,000 households were identified and their locations were georeferenced using hand-held global positioning system (GPS) receivers (SporTrak handheld GPS, Magellan Navigation Inc., Santa Clara, CA, United States of America). A geographically stratified random sample of approximately 10% of these households was selected to participate in the cohort study. During the first two EMINI surveys only blood (for HIV and *Plasmodium falciparum* malaria testing), urine (for *S. haematobium* diagnosis), and sputum samples (from participants with persistent cough for tuberculosis diagnosis) were collected. Interventions during this time included HIV and tuberculosis counseling and referral, treatment of malaria (with artemether/lumefantrine) and *S. haematobium* infections (with praziquantel).

Stool collection only started at the third annual survey, and only included inhabitants of a 50% random sample of the EMINI households. Before this survey, intestinal nematodes were neither diagnosed nor treated as part of this study, and to our knowledge no other treatment programs had been conducted in the region. Stool samples were collected in pre-labeled screw-top containers, refrigerated at 4°C directly after collection using mobile refrigerators (WAECO CoolFreeze CF-50, WAECO, Emsdetten, Germany) and kept cool until examined in the laboratory within two days of collection. The hookworm infection status of participants was established by Kato-Katz examination of two sub-samples (41.7 mg each) from a single stool specimen which was thoroughly mixed before slide preparation. Kato-Katz slides were examined for hookworm eggs by experienced staff within one hour and for other helminth eggs within two days after slide preparation. Hookworm infection was defined as the presence of at least one hookworm egg in any of the two slides. Helminth-infected participants were offered treatment with albendazole (for hookworm and other intestinal nematode infections) and/or praziquantel (for schistosome infections), according to their respective diagnoses.

Interviews were conducted to collect socio-demographic information. Age, sex, latrine type, and previous worm treatment were included as potential confounders to be adjusted for during analyses. In order to adjust for possible socio-economic confounding, we constructed an SES score using polychoric principal component analysis (PCA) [12,13] to characterize the socio-economic situation of each household. This score combines information on the availability of certain items in the household (radio, TV, mobile telephone, refrigerator, hand cart, bicycle, motor cycle, car, savings account); sources of energy and drinking water; quality of materials used to build the main house; and number of persons per room in the household.

Ecological Data

Information on elevation was retrieved using the NASA Shuttle Radar Topography Mission (SRTM) global digital elevation model (DEM) version 2.1 with a nominal resolution of 90 m [14]. Rainfall and ambient temperature interpolated surfaces with 1 km spatial resolution [15] were downloaded from the WorldClim – Global Climate Data website (http://www.worldclim.org/).

LST during the day (LSTday) and during the night (LST-night), and vegetation density (EVI = enhanced vegetation index) were retrieved from data collected during NASA's Moderate-Resolution Imaging Spectroradiometer (MODIS) mission and were acquired from the Land Processes Distributed Active Archive Center (LP DAAC), located at the U.S. Geological Survey (USGS) Earth Resources Observation and Science (EROS) Center [16]. LST data (version MOD11A2) have 8 days temporal and ~1 km spatial resolution. Vegetation data (version MOD13Q1) have 16 days temporal and 250 m spatial resolution [17].

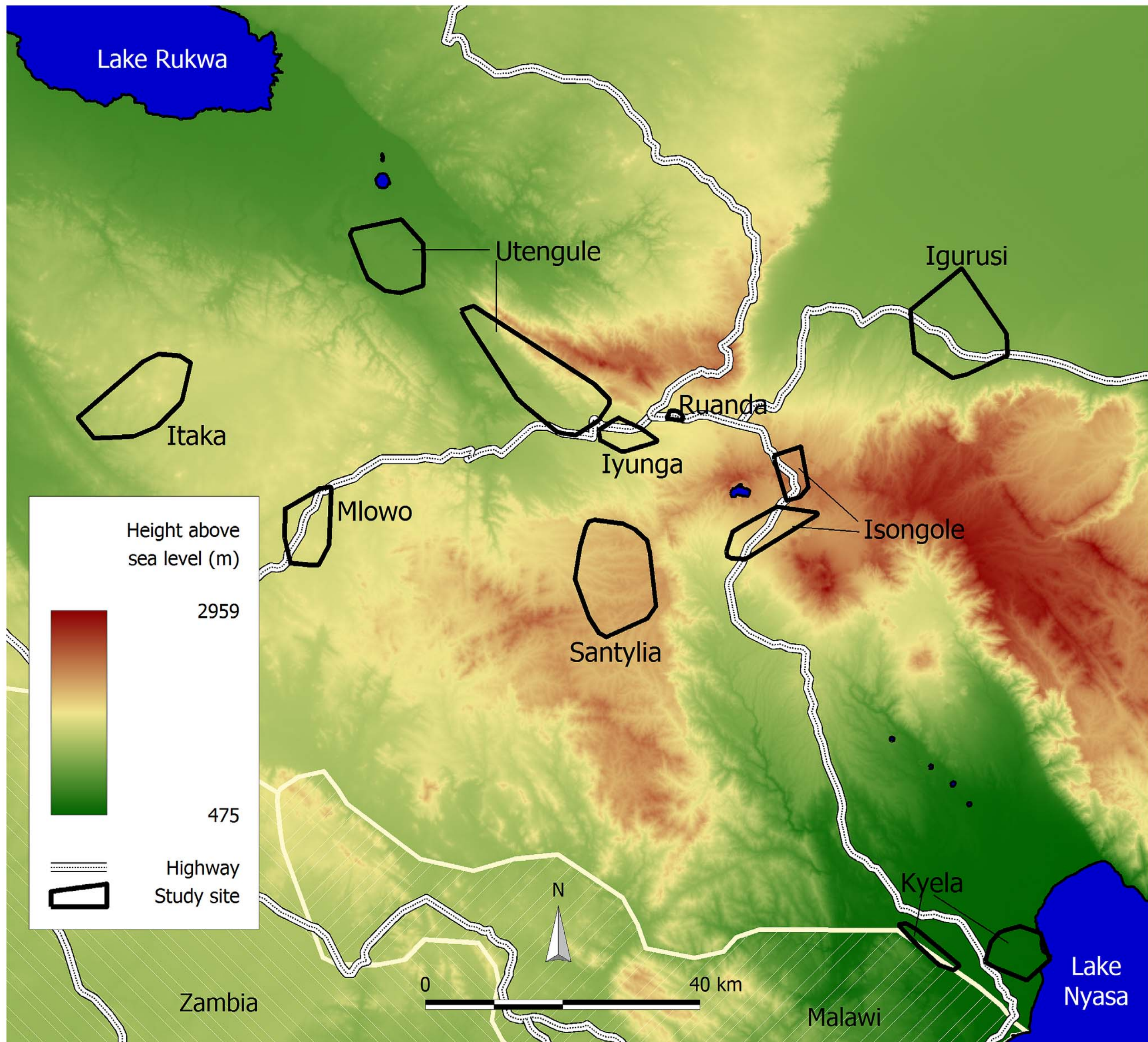


Figure 1. Location and altitude of the nine EMINI study sites (Mbeya region, Tanzania, 2008/2009). Elevation of the participating households ranges from 480 to 2,300 m above sea-level, resulting in large ranges also for the other environmental parameters that were examined.
doi:10.1371/journal.pntd.0002408.g001

Both, LST and vegetation data were processed in the following way to produce long-term averages: data surfaces for every 8-day period (LST) and every 16-day period (EVI) for the years 2003 to 2008 were imported into Idrisi GIS software v.32 (Clark Labs, Worcester, MA, United States of America). In Idrisi, long-term averages of day- and night-LST and EVI were calculated utilizing only those pixels that were "good data" according to the quality assessment layers that are distributed together with the actual data. Then LST was converted to °C and EVI was converted back to its native range between -1 and $+1$. Population and household densities, ambient temperature, rainfall, LST, EVI, and elevation variables were averaged for a buffer area within a 1000 m radius around each household in order to characterize the ecological situation around the household.

Statistical Analyses

Stata statistics software (version 11, StataCorp, College Station, TX, United States of America) was used for all statistical analyses. Some of the variables were transformed in order to yield interpretable results. Reported odds ratios (OR) for continuous variables correspond to an increase of 10 years for age, 100 m for elevation, 10 mm for mean annual rainfall, 1,000 people/km^2 for population density, and 0.1 units for EVI.

Univariable logistic regression was performed with each variable, adjusting for within-household clustering using Huber/White/Sandwich variance estimates [18–20]. Variables that either had a Wald's p-value<0.2 or were considered to be causally linked to hookworm infection were included in the following selection process.

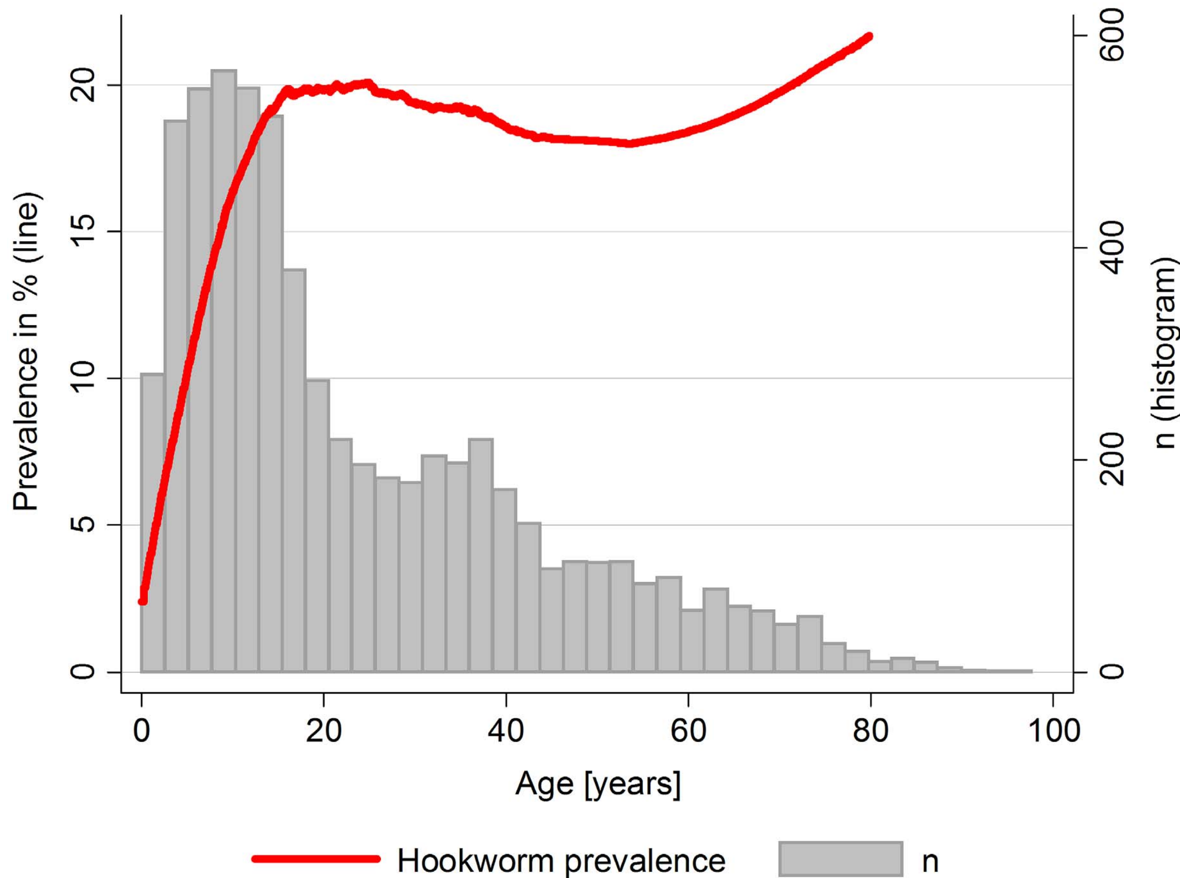


Figure 2. Lowess smoothed plot of hookworm prevalence over age in EMINI participants (Mbeya region, Tanzania 2008/2009). Prevalence rises sharply from birth to adolescence and reaches a plateau in early adulthood after which it stays relatively stable.
doi:10.1371/journal.pntd.0002408.g002

This study mainly focused on ecological data which by their nature are prone to be correlated. To avoid problems in effect estimation such as variance inflation, all variables of interest were tested for multicollinearity by calculating the variance inflation factor (VIF) [18]. A VIF above 10 was considered as an indicator for serious multicollinearity [19,20] and the respective variables were removed from further analyses.

Subsequently, two separate logistic models were developed: the first contained solely variables collected on an individual level, i.e., age, sex, and previous worm treatment variables; the second model grouped together variables that were collected at the household level. These included environmental variables as well as the SES score, latrine coverage, and latrine type. Model selection was based on a 5% significance level, i.e., removal of variables that had $p>0.05$, and on the contribution to the goodness of model fit according to the Bayesian information criterion (BIC). When the removal of a variable whose effect estimate did not reach statistical significance resulted in a major increase of the BIC, the variable was not excluded from the model. Remaining variables from the separate models were merged into a final model where variables with $p>0.05$ were removed by hand. The resulting model was then run on a reduced dataset restricted to the two sites with the highest hookworm prevalence. To detect differences in effects on a site level, a moderated multiple regression was performed by introducing a site dummy variable as the moderator and interactions of this moderator with each environmental variables. Furthermore, the final model was applied to each of these two sites separately and compared to the results of the moderated model.

Results

Descriptive Statistics

Of the 6,375 subjects (from 1,617 households) participating in this study, 17% (1,080 participants) were tested positive for hookworm infection. Most infected participants had low intensity infections (1,061), whereas medium (14) and high intensity infections (5) were rare [21]. The diverse environmental conditions in the study area are indicated by large ranges for elevation (Figure 1) and other environmental variables (Table 1). The study population included slightly more female than male participants. The median age of 16.6 years indicates that the majority of study subjects were children and adolescents. The prevalence of hookworm infection rose sharply from birth to adolescence and reached a plateau in early adulthood, after which it stayed relatively constant (Figure 2). Most households had simple or improved ventilated pit latrines, whereas water flush toilets were uncommon.

Site-specific hookworm prevalences ranged from less than 2% in Iyunga to more than 50% in Itaka (Figure 3). With 931 participants, Kyela was the biggest site and Iyunga with 444 participants the smallest. Due to exceptionally high hookworm prevalences, Itaka (53.1%) and Kyela (40.8%) were selected for the site-specific analyses.

Table 1. Characteristics of the study participants and environmental conditions at their places of residence.

Variable	N	Median or proportion (%)*	IQR	Min	Max
Hookworm infection binary	6,375	16.9%			
Hookworm infection intensity					
Median EPG (infected only)	1,080	132	228	12	6,187
Grouped†:					
No infection (0 EPG)	5,295	83.1%			
Low intensity (1–1,999 EPG)	1,061	16.6%			
Moderate intensity (2,000–3,999 EPG)	14	0.2%			
Heavy intensity (≥4,000 EPG)	5	0.1%			
Ecological variables					
Elevation [m]	6,375	1,574	510	479	2,313
Mean annual EVI	6,375	0.287	0.071	0.151	0.472
Mean annual LST-day [°C]	6,375	33.2	3.4	22.5	38.6
Mean annual LST-night [°C]	6,375	14.0	4.2	9.2	21.4
Mean annual ambient temperature [°C]	6,375	19.5	3.7	14.7	25.0
Mean annual rainfall [mm]	6,375	1,254	444	1,013	2,342
Slope [°]	6,375	2.34	2.76	0.35	13.64
Population density [persons/km^2]	6,375	415	1,745	10	13,133
Adjustment variables					
Age [years]	6,375	16.6	26.5	0	97.7
Sex [0 = female/1 = male]	6,326	47.0%			
SES score	6,372	−0.092	1.202	−1.892	3.997
Anthelmintic treatment in past year [0 = n/1 = y]	5,829	7.1%			
Latrine coverage in surroundings [%]	6,375	99.3	5.7	36.15	100
Latrine type in household	6,372				
None	160	2.5%			
Pit latrine simple	5,860	92.0%			
Ventilated improved pit latrine	230	3.6%			
Water flush toilet	122	1.9%			

EPG = eggs per gram of feces; EVI = enhanced vegetation index; IQR = inter-quartile range; LST = land surface temperature; N = number of observations; SES = socio-economic status.

*Median for continuous and proportion in percent for binary variables; IQR, minimum and maximum values only shown for continuous variables.

†According to Montresor, 1998 [21].

doi:10.1371/journal.pntd.0002408.t001

Univariable Logistic Regression and Multicollinearity Analysis

In univariable logistic regression analyses of the complete dataset which included all nine sites (Table 2) the estimates for all considered variables had p-values below 0.2, which was chosen as the cut-off for inclusion into further analyses. Population density, elevation and slope were inversely associated with hookworm infection, whereas the other ecological variables showed positive associations. SES, previous anthelmintic treatment, and latrine coverage were again inversely related to hookworm infection. Multicollinearity analysis revealed a VIF above 10 for the variables LST-night (VIF = 15.77), elevation (VIF = 75.18), and mean ambient temperature (VIF = 46.84). Elevation and mean annual ambient temperature were therefore excluded from subsequent analyses, and LST-night included since soil temperature seems more directly related to the development of hookworm larvae than ambient temperature and elevation. Removal of these two variables reduced the VIF for LST-night to 2.5.

Model Selection

In the multivariable regression model including only household-level data (not shown), the p-values for mean annual rainfall, slope, and latrine type exceeded the 5% threshold and were excluded from the model. When including individual-level data into the model, only sex yielded a p-value above 0.05 and was excluded, whereas age and previous anthelmintic treatment remained significantly associated with hookworm infection. Compared to univariable regression results, the direction of the effect in the multivariable models changed for several variables: the ORs for EVI and LST-day switched from above to below unity; the negative univariable association of latrine coverage changed to positive in multivariable analysis.

In the multivariable model combining household-level and individual-level variables (Table 3, "All sites") all included variables yielded significant p-values. No qualitative changes in the ORs compared to the separate models for household-level and individual-level variables (data not shown) were observed. Equally,

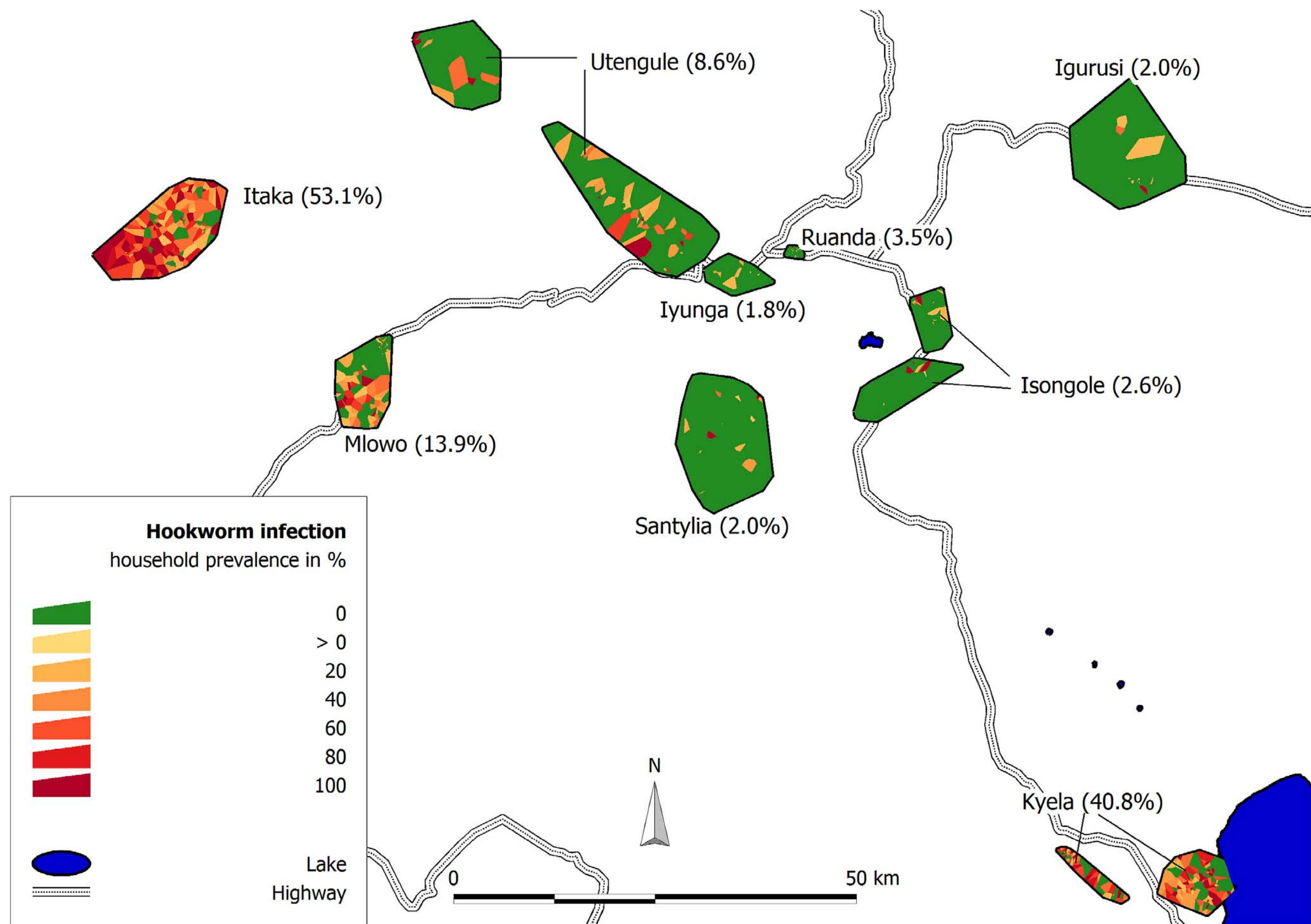


Figure 3. Hookworm prevalence in the EMINI study sites (Mbeya region, Tanzania, 2008/2009). Color shading of Voronoi polygons drawn around each household indicates household prevalence, labels indicate site name and site prevalence.
doi:10.1371/journal.pntd.0002408.g003

the magnitude of effects in the combined model is comparable to those of the separate models, indicating that the effects of both sets of variables are independent of each other.

Running a model with the same variables on data from Kyela (Table 3, "Kyela site") only yielded statistically significant ORs for LST-day (OR = 1.38; p = 0.005), SES score (OR = 0.68; p = 0.015), and age (OR = 1.12; p = 0.002). While the magnitude of ORs for SES score and age differed only marginally from the all-sites model, a qualitative difference was observed for LST-day where the association with hookworm infection switched from negative in the all sites model to positive in the Kyela site model. Site-specific analysis for Itaka (Table 3, "Itaka site") resulted in significant ORs for population density (OR = 0.08; p = 0.008), LST-night (OR = 1.56; p = 0.010), latrine coverage (OR = 0.94; p = 0.022), age (OR = 1.16; p = 0.001), and prior anthelmintic treatment (OR = 0.42; p = 0.042), of which only latrine coverage differed qualitatively from the all-sites model.

To test the presence of site-specific effects, we introduced site-interaction terms for environmental variables. In the moderated model, which was estimated on a data set restricted to observations from Itaka and Kyela sites, only the interaction term for population density yielded a significant p-value (p = 0.008). The p-values for the interaction terms for EVI and latrine coverage slightly exceeded the 5% threshold (p = 0.052 and 0.086, respectively) and were therefore also considered as relevant. The main effects of the moderated model represent the effects in Itaka, i.e., site = 0, whereas the effects for Kyela (site = 1) can be calculated by multiplying the main effect with the respective effect of the interaction term.

Keeping all variables at their average value, infection odds did not vary significantly between Kyela and Itaka (OR = 3.26; p = 0.592). However, the effect of population density, EVI and latrine coverage on infection odds was strongly dependent on the site. The data in Table 4 summarize the conditions in Kyela and Itaka, and the site-specific predictions of hookworm infection probability in Figure 4 demonstrate that a qualitative difference between the two sites was present for the association of population density and EVI with hookworm infection, whereas the association of latrine coverage differed only quantitatively between Itaka and Kyela sites.

Discussion

Our results demonstrate that hookworm infection in the study population is strongly associated with ecological factors. The univariable analyses further show that infection is favored when these factors entail more tropical conditions. This is in agreement with the literature, where similar associations of infection with elevation, temperature, rainfall, and vegetation (as an indicator of soil humidity and shade) are reported [22–24]. It also concurs with laboratory studies which show that hookworm larvae require warm and moist conditions in order to survive [25,26], a fact that

Table 2. Univariable associations of considered variables with hookworm infection*.

Variable	OR	95% CI	p-value
Ecological variables			
Elevation [per 100 m]	0.87	0.85–0.89	<0.001
Mean annual EVI [per 0.1 units]	1.37	1.11–1.69	0.003
Mean annual LST-day [per 1°C]	1.19	1.14–1.24	<0.001
Mean annual LST-night [per 1°C]	1.24	1.21–1.27	<0.001
Mean annual ambient temperature [per 1°C]	1.37	1.32–1.41	<0.001
Mean annual rainfall [per 10 mm]	1.005	1.001–1.008	0.005
Slope [per 1°]	0.86	0.82–0.91	<0.001
Population density [per 1,000 persons/km^2]	0.76	0.71–0.82	<0.001
Adjustment variables			
Age [per 10 years]	1.08	1.05–1.12	<0.001
Sex [0 = female/1 = male]	1.11	0.97–1.26	0.131
SES score [per 1 unit]	0.63	0.56–0.71	<0.001
Anthelmintic treatment in past year			
No	1.00		
Yes	0.44	0.30–0.64	<0.001
Missing	1.47	1.05–2.04	0.025
Latrine coverage [per 1%]	0.97	0.96–0.98	<0.001
Latrine type in household			
None	1		
Pit latrine simple	0.57	0.36–0.92	0.020
Ventilated improved pit latrine	0.15	0.05–0.44	0.001
Water flush toilet	0.12	0.03–0.48	0.003

CI = confidence interval; EVI = enhanced vegetation index; LST = land surface temperature; OR = odds ratio; SES = socio-economic status.
*From separate logistic regression models which only include one covariate, adjusted for household clustering using robust variance estimates.
doi:10.1371/journal.pntd.0002408.t002

is also demonstrated by the absence of hookworm infection in more temperate climates world-wide [27] and very low prevalences in the high-altitude sites within our study area.

However, our data also show that some of these associations switch direction in multivariable analysis. The associations of EVI and LSTday with hookworm infection change from positive in univariable analysis to negative in the all-sites multivariable model, whereas the association of latrine coverage changes from negative to positive. These switches in direction are mainly due to the inclusion of LST-night, which appears to be the best predictor of hookworm infection among the environmental variables. When excluding LST-night from the all-sites multivariable model shown in Table 3, both EVI and LST-day maintain the significant positive association with infection (data not shown) that they have in univariable analysis (Table 2) and latrine coverage maintains its negative association, although this is no longer significant. For LST-day this makes sense in an area including high altitude sites with rather low temperatures. In this setting, the minimum temperature (for which LST-night is a better proxy than LST-day) is the main limiting factor for the survival of hookworm larvae. Therefore, in the complete model that includes both LST-night and LST-day, LST-night explains most of the variation that is due to unsuitably low minimum temperatures, whereas the role of LST-day in this model is limited to explain the variation that is due to unsuitably high maximum temperatures. In our study area, LST-day ranges from 22 to 39°C. Thus our finding corresponds with experimental study results suggesting that development of hookworm larvae reaches its peak between 20 and 30°C and ceases at around 40°C [25]. The switches in direction of the associations of EVI and latrine coverage with hookworm infection in multivariable analysis are harder to explain but are most likely based on similar effects. Contrary to the above described differences between univariable and multivariable models, population density, SES, age, and previous deworming show similar associations with hookworm infection in uni- and multivariable analyses. The negative associations of SES and deworming with infection are highly plausible and have also been reported in other studies [1,28]. This also applies to the positive association of age with infection [29,30].

Regarding the relationship of population density with infection, the interpretation is more complicated. While higher population densities increase the chance of hookworm larvae to find a host and could thus favor transmission, in our study area, they are also an indicator of more urban and thus more developed conditions, which would reduce transmission. Thus, the negative association with hookworm infection found in this study is also plausible, and accordingly both negative and positive associations have been reported in the literature [10,24].

Another interesting phenomenon are the differences in association of several factors, when comparing the two site specific multivariable models for Kyela and Itaka (columns "Kyela site" and "Itaka site" in Table 3) with each other and when comparing each of them with the all-sites model ("All sites" in Table 3). When comparing Tables 1 and 4, it is obvious that ecological variables in the all-sites model cover a much wider range of conditions than in each site-specific model, which is a plausible reason for the differences of the site-specific models *versus* the all-sites model.

Figure 4 demonstrates that the above reasoning may also apply to the contradictory results when comparing the two site-specific models with each other: population density and latrine coverage show far more variation in Kyela than in Itaka, and the EVI ranges for both sites do not show any overlap, with much lower vegetation cover in Itaka. Thus, the different conditions in the two sites are a likely explanation for the different associations of these factors in the two sites. However, it is also possible that these differences in association are a consequence of one or more unobserved factors that our analysis is unable to account for.

Strengths of this study include the large number of participants of all age groups and the detailed information that we have for each individual, including the place of residence. This allows for a detailed assessment of individual exposure to environmental factors. In contrast, most other studies into the spatial epidemiology of hookworm and other soil-transmitted helminth infections are school-based [9–11,24,31–33]. Thus, they do not examine hookworm infection in adults and rely on the geographical position of the school to quantify participant's exposure to environmental factors [34].

However, our study also has some limitations. The use of only one stool specimen for the determination of hookworm infection status is known to lack sensitivity due to the intra-specimen and day-to-day variation in hookworm egg output [35,36]. Although we prepared two Kato-Katz thick smears from each stool specimen to increase sensitivity, it is likely that we missed some of the lighter infections. Unfortunately the Kato-Katz examination of stool is unable to differentiate between *N. americanus and A. duodenale*. However, previous studies indicate that *N. americanus* is the predominant species in East Africa [32], and stool-PCR data

Table 3. Multivariable associations of selected ecological and adjustment variables with hookworm infection status*.

	All sites†			Kyela site			Itaka site			Moderated model‡		
	N = 6,372			N = 931			N = 846			N = 1,777		
Variables	OR	95% CI	p	OR	95% CI	p	OR	95% CI	p	OR	95% CI	p
Mean annual EVI [per 0.1 units]	0.11	0.06–0.18	<0.001	2.10	0.84–5.26	0.114	0.21	0.02–1.74	0.147	0.21	0.03–1.73	0.147
Mean annual LST-day [per 1°C]	0.81	0.75–0.88	<0.001	1.38	1.10–1.73	0.005	1.19	0.61–2.33	0.611	1.18	0.61–2.28	0.633
Mean annual LST-night [per 1°C]	1.54	1.44–1.64	<0.001	0.57	0.15–2.16	0.408	1.56	1.11–2.19	0.010	1.54	1.10–2.16	0.012
Population density [per 1,000 persons/km^2]	0.68	0.63–0.73	<0.001	1.08	0.78–1.50	0.627	0.08	0.01–0.51	0.008	0.08	0.01–0.53	0.008
Age [per 10 years]	1.10	1.06–1.14	<0.001	1.12	1.04–1.20	0.002	1.16	1.06–1.28	0.001	1.14	1.07–1.20	0.000
SES score [per 1 unit]	0.82	0.73–0.93	0.002	0.68	0.50–0.93	0.015	0.79	0.58–1.09	0.158	0.79	0.57–1.08	0.143
Anthelmintic treatment [0 = n/1 = y]§	0.45	0.30–0.69	<0.001	0.73	0.36–1.46	0.376	0.42	0.18–0.97	0.042	0.52	0.29–0.94	0.030
Latrine coverage [per 1%]	1.02	1.01–1.04	0.002	0.99	0.96–1.02	0.484	0.94	0.89–0.99	0.022	0.94	0.89–0.99	0.022
Site [0 = Itaka/1 = Kyela]										3.26	0.04–243	0.592
EVI* Site										9.82	0.98–98.5	0.052
LST-day* Site										1.17	0.58–2.36	0.655
LST-night* Site										0.35	0.09–1.41	0.140
Population density* Site										12.79	1.97–83.2	0.008
SES score* Site										0.87	0.56–1.36	0.542
Latrine coverage* Site										1.05	0.99–1.12	0.086

CI = confidence interval; EVI = enhanced vegetation index; LST = land surface temperature; OR = odds ratio; SES = socio-economic status.
*Results of logistic regression adjusted for within-household clustering with robust variance estimates with each model containing only those variables for which data are shown in the Table.
†Performed on pooled dataset combining all nine sites.
‡Moderated model for Kyela and Itaka sites with site-interaction terms for environmental variables.
§Missing stratum not shown.
doi:10.1371/journal.pntd.0002408.t003

from our own ongoing WHIS study, where we only find *N. americanus* infections, seem to indicate that this is also the case for our study area. Thus it is likely that most or all of the hookworm infections in our study population were caused by *N. americanus*, although we cannot completely exclude that *A. duodenale* is also present.

Unfortunately, we are also lacking information about soil composition in the study area which has been shown to strongly influence hookworm infection [24,31]. Motility of the hookworm larvae is crucial to avoid adverse environmental conditions and is thus important for their survival. The porosity of sandy soils facilitates larval movement deeper into the soil to escape desiccation and upwards movement to avoid rising water levels after heavy rainfall. Soils with high clay content are less porous and thus inhibit larval motility [23,37–39].

Furthermore, apart from previous worm treatment which was assessed by interview, our study does not account for behavioral factors which also can strongly influence hookworm transmission and prevalence. However, although soil composition and behavior are both important determinants of hookworm infection which would likely have improved our models if included, data on these factors are rarely available in tropical developing countries where hookworm is most prevalent. Thus, their potential to predict infection in order to plan helminth control is limited, especially in those regions where control is urgently needed.

This study and many others have shown that remotely sensed data such as vegetation indices, LST, and elevation are strongly associated with hookworm prevalence [2,8]. However, our study also shows that these associations are scale-dependent and that predictions using these data should be handled with care. On a large scale, they can provide powerful tools to identify regions that warrant control and intervention programs, their big advantage being public availability and global coverage.

Nevertheless, when making predictions of hookworm infection on a smaller scale, regional characteristics, such as seasonal flooding, dry spells, etc., have to be taken into account. As our study has shown, even within a relatively small geographic area the effects of environmental conditions can differ to a large extent. Thus, large-scale findings cannot necessarily be used for prediction on smaller scales and *vice versa*.

Supporting Information

Checklist S1 STROBE checklist.
(DOC)

Acknowledgments

We would like to thank the study participants and the EMINI field, laboratory, and coordination teams for their support throughout the study.

Author Contributions

Conceived and designed the experiments: MH LM ES. Performed the experiments: PC IK AN CM SM LM CG ES. Analyzed the data: HR ES SAS UM. Wrote the paper: HR ES. Acquired funding: MH LM SM. Acquired and managed the data: DOK ES. Critically revised the manuscript and approved the final version: HR PC IK DOK AN CM SAS UM CG SM LM MH ES.

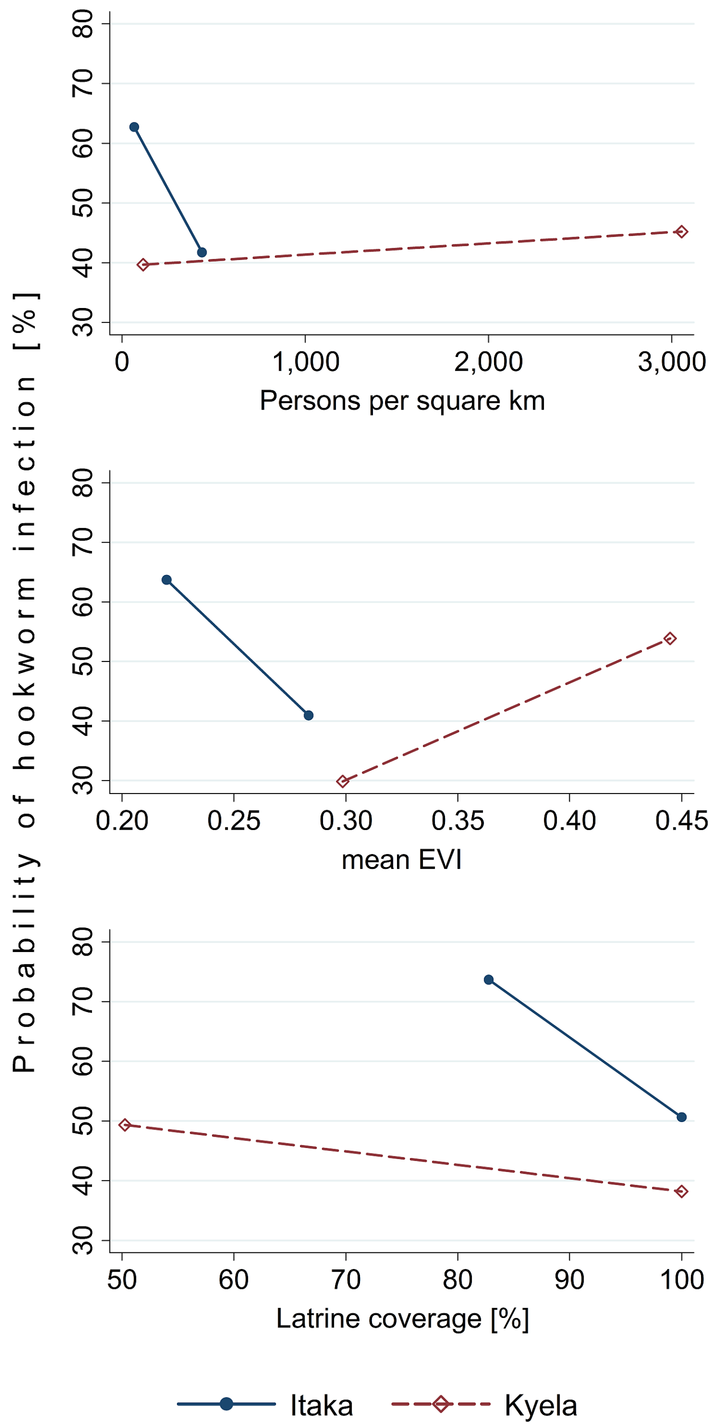


Figure 4. Linear predictions of hookworm infection probabilities for population density, EVI, and latrine coverage. According to the site-specific models for Kyela and Itaka, adjusted for all variables shown in the site-specific models in Table 3.
doi:10.1371/journal.pntd.0002408.g004

Table 4. Characteristics of study participants and environmental conditions at their places of residence in Kyela and Itaka site.

	Kyela site					Itaka site				
Variable	**N**	**Median or proportion (%)***	**IQR**	**Min**	**Max**	**N**	**Median or proportion (%)***	**IQR**	**Min**	**Max**
Hookworm infection binary	931	40.8%				846	53.1%			
Hookworm infection intensity										
Median EPG (infected only)	380	144	276	12	5,012	449	144	228	12	4,772
Grouped†:										
No infection (0 EPG)	551	59.2%				397	46.9%			
Low (1–1,999 EPG)	375	40.3%				440	52.0%			
Moderate (2,000–3,999 EPG)	2	0.2%				8	1.0%			
Heavy (≥4,000 EPG)	3	0.3%				1	0.1%			
Ecological variables										
Mean annual EVI	931	0.373	0.060	0.299	0.445	846	0.249	0.016	0.220	0.283
Mean annual LST-day [°C]	931	32.1	3.2	27.8	35.0	846	34.4	0.6	33.1	35.4
Mean annual LST-night [°C]	931	21.1	0.3	20.6	21.4	846	15.0	1.0	12.9	16.2
Population density [persons/km^2]	931	441	325	116	3053	846	214	167	68	437
Adjustment variables										
Age [years]	931	16.2	25.1	0.0	90.1	846	15.8	22.7	0.5	93.6
SES score	931	−0.59	1.16	−1.85	2.82	846	−0.18	0.84	−1.53	2.78
Anthelmintic treatment in past year	612	6.0%				840	5.5%			
Latrine coverage in surroundings [%]	931	89.5	15.5	50.3	100	846	100	0.0	82.8	100

IQR = inter-quartile range; EPG = eggs per gram of feces; EVI = enhanced vegetation index; LST = land surface temperature; N = number of observations; SES = socio-economic status.
*Median for continuous and proportion in percent for binary variables; IQR, minimum and maximum values are not given for binary variables.
†According to Montresor 1998 [21].
doi:10.1371/journal.pntd.0002408.t004

References

1. de Silva NR, Brooker S, Hotez PJ, Montresor A, Engels D, et al. (2003) Soil-transmitted helminth infections: updating the global picture. Trends Parasitol 19: 547–551.
2. Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, et al. (2006) Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet 367: 1521–1532.
3. Hotez PJ, Kamath A (2009) Neglected tropical diseases in sub-Saharan Africa: review of their prevalence, distribution, and disease burden. PLoS Negl Trop Dis 3: e412.
4. Hotez PJ, Bethony J, Bottazzi ME, Brooker S, Buss P (2005) Hookworm: "the great infection of mankind". PLoS Med 2: e67.
5. Murray CJL, Vos T, Lozano R, Naghavi M, Flaxman AD, et al. (2012) Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 380: 2197–2223.
6. Soares Magalhães RJ, Clements ACA, Patil AP, Gething PW, Brooker S (2011) The applications of model-based geostatistics in helminth epidemiology and control. Adv Parasitol 74: 267–296.
7. Brooker S, Rowlands M, Haller L, Savioli L, Bundy DAP (2000) Towards an atlas of human helminth infection in sub-Saharan Africa: the use of geographical information systems (GIS). Parasitol Today 16: 303–307.
8. Brooker S, Clements ACA, Bundy DAP (2006) Global epidemiology, ecology and control of soil-transmitted helminth infections. Adv Parasitol 62: 221–261.
9. Raso G, Vounatsou P, McManus DP, Utzinger J (2007) Bayesian risk maps for *Schistosoma mansoni* and hookworm mono-infections in a setting where both parasites co-exist. Geospat Health 2: 85–96.
10. Koroma JB, Peterson J, Gbakima AA, Nylander FE, Sahr F, et al. (2010) Geographical distribution of intestinal schistosomiasis and soil-transmitted helminthiasis and preventive chemotherapy strategies in Sierra Leone. PLoS Negl Trop Dis 4: e891.
11. Soares Magalhães RJ, Biritwum NK, Gyapong JO, Brooker S, Zhang Y, et al. (2011) Mapping helminth co-infection and co-intensity: geostatistical prediction in Ghana. PLoS Negl Trop Dis 5: e1200.
12. Filmer D, Pritchett LH (2001) Estimating wealth effects without expenditure data - or tears: an application to educational enrollments in states of India. Demography 38: 115–132.
13. Kolenikov S, Angeles G (2009) Socioeconomic status measurement with discrete proxy variables: is principal component analysis a reliable answer? Rev Income Wealth 55: 128–165.
14. Farr TG, Rosen PA, Caro E, Crippen R, Duren R, et al. (2007) The shuttle radar topography mission. Rev Geophys 45: RG2004.
15. Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A (2005) Very high resolution interpolated climate surfaces for global land areas. Int J Climatol 25: 1965–1978.
16. NASA Land Processes Distributed Active Archive Center (LP DAAC) (2001) MODIS 11A2 & MODIS 13Q1. USGS/Earth Resources Observation and Science (EROS) Center, Sioux Falls, South Dakota.
17. Huete A, Didan K, Miura T, Rodriguez EP, Gao X, et al. (2002) Overview of the radiometric and biophysical performance of the MODIS vegetation indices. Remote Sens Environ 83: 195–213.
18. O'Brien RM (2007) A caution regarding rules of thumb for variance inflation factors. Qual Quant 41: 673–690.
19. Kennedy P (2003) A guide to econometrics Cambridge, MA: The MIT Press.
20. Marquardt DW (1970) Generalized inverses, ridge regression, biased linear estimation, and nonlinear estimation. Technometrics 12: 591–612.
21. Montresor A, Crompton DWT, Bundy DAP, Hall A, Savioli L (1998) Guidelines for the evaluation of soil-transmitted helminthiasis and schistosomiasis at community level. Geneva: World Health Organization.
22. Brooker S, Michael E (2000) The potential of geographical information systems and remote sensing in the epidemiology and control of human helminth infections. Adv Parasit 47: 245–288.
23. WHO (1964) Soil-transmitted helminths—report of a WHO expert committee on helminthiasis. WHO Tech Rep Ser 277: 19–23.
24. Saathoff E, Olsen A, Sharp B, Kvalsig JD, Appleton CC, et al. (2005) Ecologic covariates of hookworm infection and reinfection in rural Kwazulu-Natal/South

Africa: a geographic information system-based study. Am J Trop Med Hyg 72: 384–391.
25. Udonsi JK, Atata G (1987) *Necator americanus*: temperature, pH, light, and larval development, longevity, and desiccation tolerance. Exp Parasitol 63: 136–142.
26. Smith G, Schad GA (1989) *Ancylostoma duodenale* and *Necator americanus*: effect of temperature on egg development and mortality. Parasitology 99: 127–132.
27. WHO (2010) Working to overcome the global impact of neglected tropical diseases. Geneva: World Health Organization.
28. Hotez PJ, Molyneux DH, Fenwick A, Kumaresan J, Ehrlich Sachs S, et al. (2007) Control of neglected tropical diseases. N Engl J Med 357: 1018–1027.
29. Gandhi NS, Jizhang C, Khoshnood K, Fuying X, Shanwen L, et al. (2001) Epidemiology of *Necator americanus* hookworm infections in Xiulongkan village, Hainan province, China: high prevalence and intensity among middle-aged and elderly residents. J Parasitol 87: 739–743.
30. Jardim-Botelho A, Brooker S, Geiger SM, Fleming F, Souza Lopes AC, et al. (2008) Age patterns in undernutrition and helminth infection in a rural area of Brazil: associations with ascariasis and hookworm. Trop Med Int Health 13: 458–467.
31. Mabaso ML, Appleton CC, Hughes JC, Gouws E (2003) The effect of soil type and climate on hookworm (*Necator americanus*) distribution in KwaZulu-Natal, South Africa. Trop Med Int Health 8: 722–727.
32. Brooker S, Clements ACA (2009) Spatial heterogeneity of parasite co-infection: determinants and geostatistical prediction at regional scales. Int J Parasitol 39: 591–597.
33. Raso G, Vounatsou P, Gosoniu L, Tanner M, N'Goran EK, et al. (2006) Risk factors and spatial patterns of hookworm infection among schoolchildren in a rural area of western Côte d'Ivoire. Int J Parasitol 36: 201–210.
34. Anderson RM, Truscott JE, Pullan RL, Brooker S, Deirdre Hollingsworth T (2013) How effective is school-based deworming for the community-wide control of soil-transmitted helminths? PLoS Negl Trop Dis 7: e2027.
35. Booth M, Vounatsou P, N'Goran EK, Tanner M, Utzinger J (2003) The influence of sampling effort and the performance of the Kato-Katz technique in diagnosing *Schistosoma mansoni* and hookworm co-infections in rural Côte d'Ivoire. Parasitology 127: 525–531.
36. Krauth SJ, Coulibaly JT, Knopp S, Traoré M, N'Goran EK, et al. (2012) An in-depth analysis of a piece of shit: distribution of *Schistosoma mansoni* and hookworm eggs in human stool. PLoS Negl Trop Dis 6: e1969.
37. Vinayak VK, Chitkara NL, Chhuttani PN (1979) Soil dynamics of hookworm larvae. Indian J Med Res 70: 609–614.
38. Augustine DL (1922) Investigations on the control of hookworm disease. VIII. Experiments on the migration of hookworm larvae in soils. Am J Hyg 2: 162–187.
39. Beaver PC (1975) Biology of soil-transmitted helminths: the massive infection. Health Lab Sci 12: 116–125.

Low Specificity of Determine HIV1/2 RDT Using Whole Blood in South West Tanzania

Inge Kroidl[1]*[9], Petra Clowes[2][9], Wolfram Mwalongo[2], Lucas Maganga[2], Leonard Maboko[2], Arne L. Kroidl[1,2,3], Christof Geldmacher[1,3], Harun Machibya[4], Michael Hoelscher[1,3], Elmar Saathoff[1,3]

1 Division of Infectious Diseases and Tropical Medicine, Medical Center of the University of Munich (LMU), Munich, Germany, **2** NIMR-Mbeya Medical Research Programme, Mbeya, Tanzania, **3** German Centre for Infection Research, LMU, Munich, Germany, **4** Mbeya Regional Medical Office, Mbeya, Tanzania

Abstract

Objective: To evaluate the diagnostic performance of two rapid detection tests (RDTs) for HIV 1/2 in plasma and in whole blood samples.

Methods: More than 15,000 study subjects above the age of two years participated in two rounds of a cohort study to determine the prevalence of HIV. HIV testing was performed using the Determine HIV 1/2 test (Abbott) in the first (2006/2007) and the HIV 1/2 STAT-PAK Dipstick Assay (Chembio) in the second round (2007/2008) of the survey. Positive results were classified into faint and strong bands depending on the visual appearance of the test strip and confirmed by ELISA and Western blot.

Results: The sensitivity and specificity of the Determine RDT were 100% (95% confidence interval =86.8 to 100%) and 96.8% (95.9 to 97.6%) in whole blood and 100% (99.7 to 100%) and 97.9% (97.6 to 98.1%) in plasma respectively. Specificity was highly dependent on the tested sample type: when using whole blood, 67.1% of positive results were false positive, as opposed to 17.4% in plasma. Test strips with only faint positive bands were more often false positive than strips showing strong bands and were more common in whole blood than in plasma. Evaluation of the STAT-PAK RDT in plasma during the second year resulted in a sensitivity of 99.7% (99.1 to 99.9%) and a specificity of 99.3% (99.1 to 99.4%) with 6.9% of the positive results being false.

Conclusions: Our study shows that the Determine HIV 1/2 strip test with its high sensitivity is an excellent tool to screen for HIV infection, but that – at least in our setting – it can not be recommended as a confirmatory test in VCT campaigns where whole blood is used.

Citation: Kroidl I, Clowes P, Mwalongo W, Maganga L, Maboko L, et al. (2012) Low Specificity of Determine HIV1/2 RDT Using Whole Blood in South West Tanzania. PLoS ONE 7(6): e39529. doi:10.1371/journal.pone.0039529

Editor: Patricia Kissinger, Tulane University, United States of America

Received July 11, 2011; **Accepted** May 23, 2012; **Published** June 29, 2012

Copyright: © 2012 Kroidl et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was funded by the European Commission as part of EuropAid (SANTE/2004/078-545 and SANTE/2006/129-931). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ikroidl@lrz.uni-muenchen.de

[9] These authors contributed equally to this work.

Introduction

The World Health Organisation (WHO) Global Programme on AIDS first recommended the use of simple rapid detection tests (RDTs) for HIV in 1992 [1]. Since then RDTs have been widely used throughout Africa and the developing world for blood safety screening, surveillance and in prenatal or voluntary counselling and testing (VCT) centres [2]. Until recently the National guidelines for VCT in Tanzania recommended using the SD Bioline HIV 1/2 3.0 (Standard Diagnostics, Kyonggi-do, Korea) as a screening test, with initial reactive samples re-tested using the Determine HIV 1/2 test (Abbott Laboratories, Abbott Park, IL). The Uni-Gold^{TM} HIV-1/2 (Trinity Biotech, Bray Ireland) is used as a final test if results are discrepant [3]. A fourth HIV rapid test, HIV 1/2 STAT-PAK (Chembio Diagnostics Systems, Medford, NY, USA) is used in neighbouring countries mainly as a confirmatory test. All four RDTs are less expensive than ELISA or Western Blot tests, require little or no equipment, can be stored at room temperature and are easy to use and to read. Because they allow for real-time, point-of-care HIV testing, individuals can receive their test result during a single clinic visit which is likely to increase the uptake of VCT [4,5], especially in Sub Saharan Africa, where in some settings more than two weeks may be needed for laboratory results to become available [6]. However, it seems that the diagnosis of HIV by RDT is challenged by confounding regional factors. Especially the Determine HIV 1/2 test received very diverse reports regarding its specificity which ranged from 91.7% to above 99% [7,8,9,10,11,12].

Here we evaluate the diagnostic test performance for HIV testing of the Determine HIV 1/2 and the HIV 1/2 STAT-PAK (Chembio Diagnostics Systems, Medford, NY, USA) RDTs in a general population cohort in South West Tanzania where, in contrast to previous studies, children and adults of both genders were included. The two tests are referred to below as the

"Determine" RDT and the "STAT-PAK" RDT, in order to improve the readability of this article. The manufacturer's information applies to the time of the study; today both tests are manufactured and sold by Alere, formerly named Inverness Medical Professional Diagnostics, Princeton, NJ.

Methods

Ethical Considerations

The study was approved by the National Ethical Committee/Medical Research Coordinating Committee of the National Institute for Medical Research, Tanzania. All adult participants provided written informed consent prior to enrolment into the study. Parents gave the permission for their children, if they were below 18 years of age. In addition children above the age of 12 years signed their own consent form together with their parents. The study, including the consent forms for adults and children, was additionally approved by the Mbeya Medical Research and Ethics Committee.

Data Collection

Data for this study were collected between May 2006 and June 2008 in the Mbeya Region in Tanzania. The EMINI project (Establishment of the infrastructure to **E**valuate and **M**onitor the **I**mpact of **N**ew **I**nterventions), a population based cohort study, investigated the prevalence and incidence of HIV in 9 selected communities with a total population of ~171,000 people. The urban, semi-urban and rural study sites were carefully chosen in order to reflect the geographic diversity of the Region, from altitudes of about 475 meters above sea level near Lake Nyassa, to over 2300 meters. Subsequently, 10% of all households in the study sites were randomly selected, which resulted in a cohort of approximately 18,000 individuals of all age groups and both sexes. Study participants were visited annually for specimen and data collection, including an HIV rapid test. Collected data further included socio-economic characteristics of the study participants, previous and current medical history, as well as individual knowledge regarding different diseases.

During the first survey (2006/2007) the Determine RDT was used to screen for HIV-1 infection. 2.7 ml of blood were collected by venae puncture from participants above the age of 6 years. However, venae puncture was not possible in all participants, and especially problematic in younger children. In these cases, 300 µl of blood were collected after finger prick, using microvettes (Sarstedt, Nürnbrecht, Germany). In most cases this smaller amount of blood did not supply sufficient plasma, so that HIV testing in children below 6 years was mostly done using whole blood. During the second and third annual survey round, where the STAT-PAK RDT was used for HIV screening, we collected 2.7 ml of blood from all participants above 2 years of age, thus all our STAT-PAK RDT results were obtained from plasma samples. Results of the third survey are not reported here, they were only used when needed to verify results from the two previous surveys. For both RDTs our results only include data from participants above the age of 2 years in order to exclude false test-positivity due to the presence of maternal antibodies.

According to the manufacturer's instructions for both assays, any test result with visible lines in both test control areas, regardless of intensity, were considered reactive [13,14]. However, we recorded variations of intensity of the strip test. Any assay with an apparent positive band which was noticeably lighter than the control band on the test card was regarded as "faint".

Additional testing to verify the initial RDT results was only done if.

i. the RDT result was the first HIV positive result obtained from this participant (true for positive results from survey 1 and newly positive results from survey 2), or if
ii. the RDT result differed from the result of the previous or the following survey.

In these cases the sample was retested using an ELISA HIV test (Enzygnost Anti HIV 1/2 Plus, DADE-Behring, Marburg, Germany) (Figures 1 and 2). Samples where the RDT and the ELISA had discordant results were tested again, using a second ELISA (rLAV, Biorad Laboratories, Redmond WA, USA). If both ELISAs were in agreement, the ELISA result was used as the reference-standard. Samples with discordant ELISA results were retested by Western Blot (MPD HIV Blot 2.2, MP Biomedicals, and Geneva, Switzerland) to determine the HIV status. If the Western Blot result was indeterminate, the results for this sample were excluded from statistical analysis. In addition, samples with discordant results were repeat-tested with the respective RDT to reconfirm the initial RDT results and to exclude a technical error.

Retesting of whole blood results was not possible due to lack of stored plasma. Instead we regarded these results as true positive if the result in the next survey was also positive and as false positive if the participant had a negative test result in the subsequent survey, which was confirmed using the above reference algorithm. Whole blood results from participants not participating in at least one of the two following surveys were excluded from our analysis because their true HIV status could not be determined.

Negative RDTs from the first or second annual surveys which were confirmed by another negative RDT in the following survey and were not in conflict with earlier positive results, were regarded as confirmed and not further tested. For all HIV incident cases, which were found newly positive in surveys 2 or 3, the negative result of the previous round was confirmed by the above described testing reference algorithm.

All participants from the nine different study sites were visited at their home. The collected specimens were brought to the central laboratory in Mbeya, where the RDTs were performed in order to ascertain comparable test quality across sites and field teams. In addition to HIV other concomitant infections and influences were examined. *Plasmodium falciparum* infection was assessed using Rapid Malaria Tests (ICT Diagnostics, Cape Town, South Africa) and *Schistosoma haematobium* infection was assessed by urine microscopy for *S. haematobium* eggs.

Information on elevation was retrieved using data from the NASA Shuttle Radar Topography Mission (SRTM) global digital elevation model (DEM) version 2.1 with a nominal resolution of 90 meters. Annual ambient temperature interpolated surfaces with 1 km spatial resolution were downloaded from the WorldClim – Global Climate Data website (http://www.worldclim.org/) [15,16]. These data and the participant household's positions which had been collected using handheld GPS receivers were combined in a geographic information system in order to calculate elevation and mean annual ambient temperature at the household position.

Statistical Analysis

All statistical analyses were performed using Stata version 10 (Stata Corp., College Station, TX). Diagnostic test performance (sensitivity, specificity, predictive values and diagnostic likelihood ratios) was calculated only for participants where a definitive HIV diagnosis (reference-standard) was available, using the "diagt" Stata component. Because predictive values are affected by the

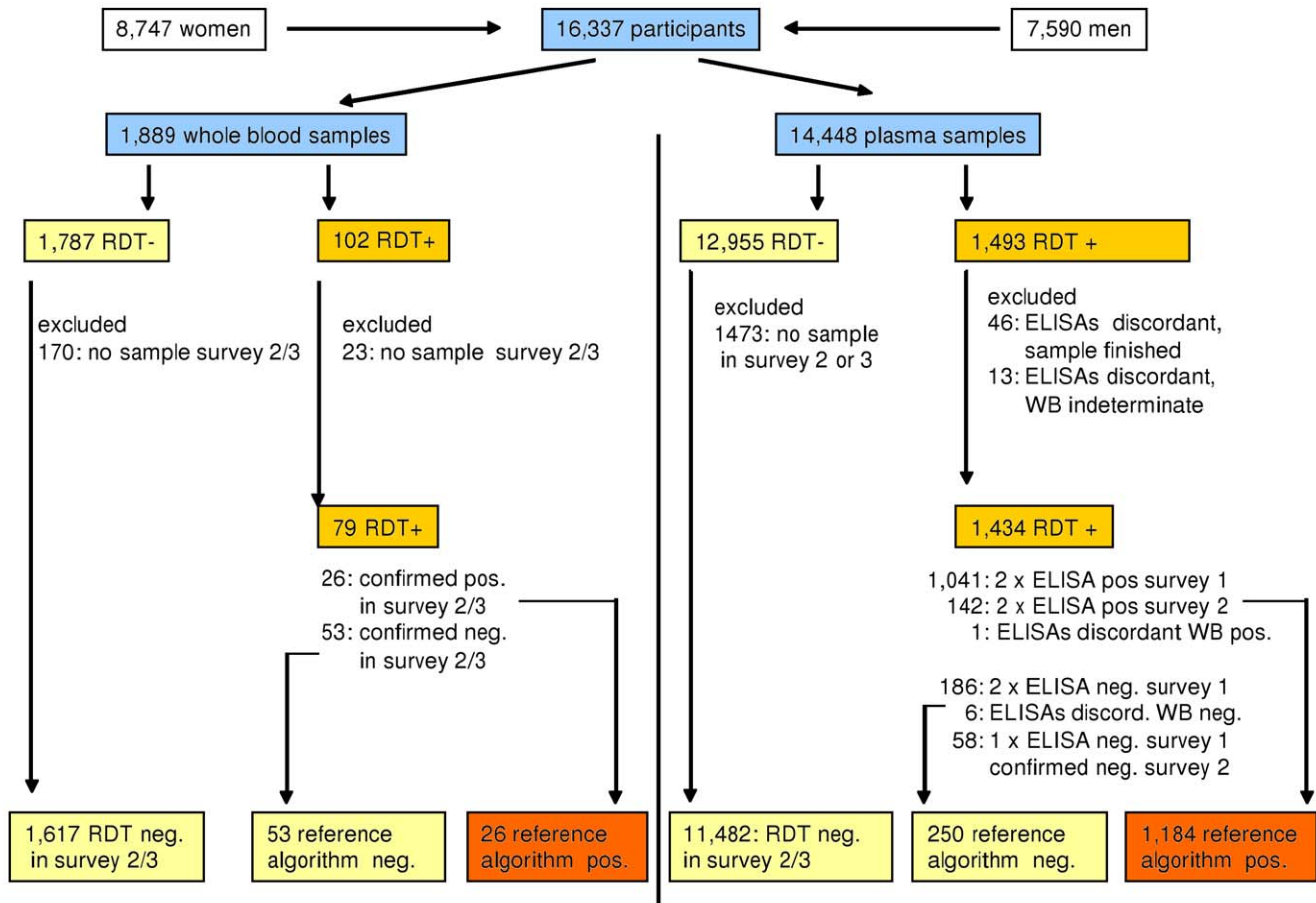


Figure 1. HIV-Testing Algorithm and Exclusion criteria Survey 1. In Survey 1 we tested 1,889 whole blood specimen and 14,448 plasma specimen with the Determine HIV1/2 RDT. The confirmation algorithm included two different ELISAs and one Western Blot. If both ELISAs were in agreement their result was used as the reference standard result. Samples with discordant ELISA results were retested by Western Blot and the Western Blot result used. Samples with indeterminate Western Blot results were excluded from analysis. Negative RDT results were not directly confirmed, but regarded as true negative if the result of the following survey was also negative. Whole blood results where confirmation by ELISA testing was impossible due to lack of plasma, were regarded as true positive if the result in the next survey was confirmed positive and as false positive if a negative test result in the next survey was confirmed using the above reference algorithm. Results where the true HIV status could not be verified according to the reference algorithm were excluded from this analysis.
doi:10.1371/journal.pone.0039529.g001

prevalence of disease in the tested population, we also report the positive and negative diagnostic likelihood ratios (DLRs), which are less affected by prevalence [17]. DLRs incorporate both the sensitivity and specificity of a test and provide a direct estimate of how much a test result will change the odds of having or not having a disease. DLR calculation uses the following formulae:

$$\text{positive DLR} = \text{sensitivity}/(1-\text{specificity})$$

$$\text{negative DLR} = (1-\text{sensitivity})/\text{specificity}.$$

Positive DLRs can thus range from unity to infinity, negative DLRs from unity to 0 with better tests having DLRs further away from unity.

Uni-variable and multi-variable binomial regression with a log link adjusted for within household clustering was used to calculate risk ratios for possible associations of false positive RDT results with participant age, sex, *P. falciparum* and *S. haematobium* infection status, and elevation and annual mean ambient temperature at the participant's residence.

Results

During the first survey we obtained Determine RDT results from 8,747 female and 7,590 male participants between 2 and 97 years of age with a median age of 19.8 years (inter-quartile-range [IQR] = 10.2 to 35.3) and 16.8 years (IQR = 9.2 to 34.1) respectively. Of these RDT results 1,889 were obtained using whole blood samples (median age = 4.1 years, range 2 to 87, IQR = 2.9 to 5.3 years) and 14,448 using plasma (median age = 21.1 years, range 2 to 97, IQR = 12.1 to 36.9). Samples that could not be verified according to the above reference algorithm were excluded. Therefore, the below analysis was done with 1,696 and 12,916 Determine RDT results, obtained from testing whole blood and plasma samples respectively (Figure 1).

During the second survey the STAT-PAK RDT was used instead of the Determine RDT and we collected 2.7 ml of blood by venae puncture from all participants above the age of 2 years, so that testing with whole blood was no longer necessary. We obtained STAT-PAK RDT results from 8,574 female and 7,383 male participants with a median age of 19.1 (range = 2 to 98, IQR = 9.9 to 35.6) and 16.1 (range = 2 to 96, IQR = 8.8 to 34.0)

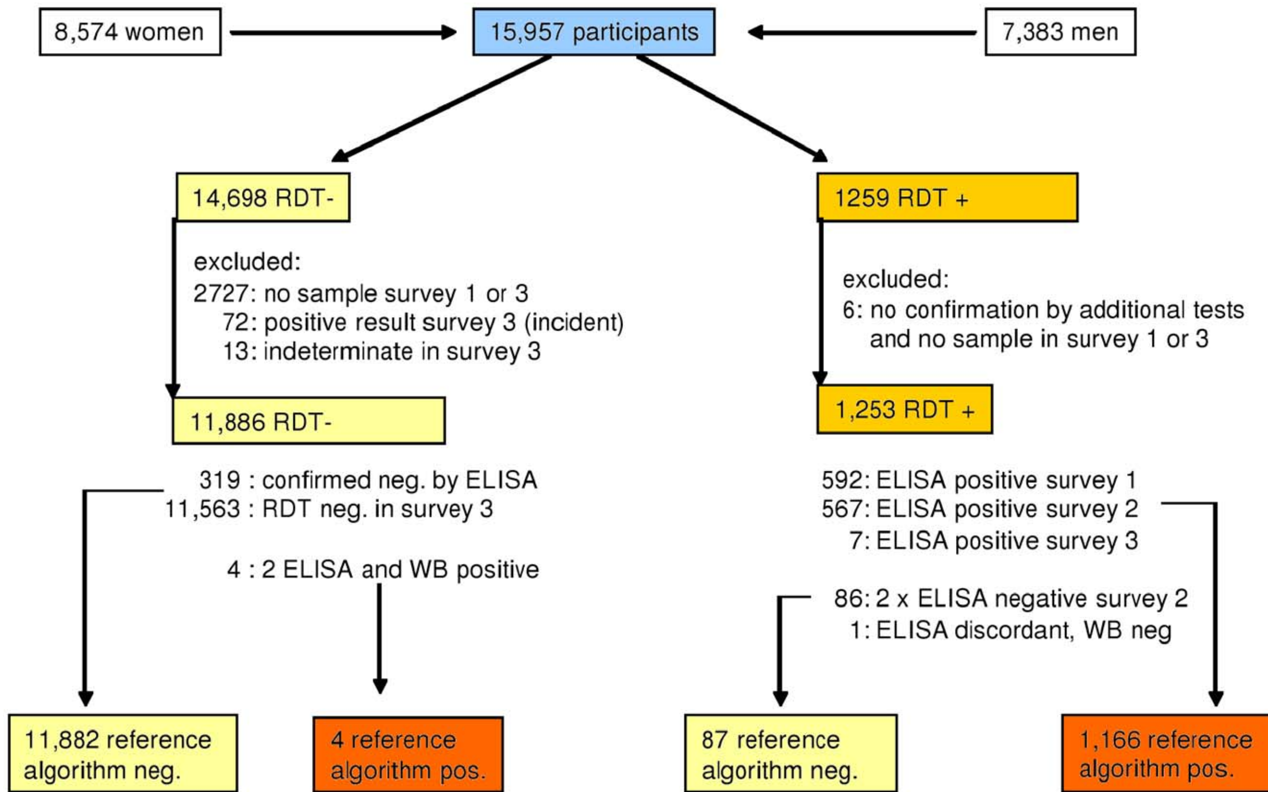


Figure 2. HIV-Testing Algorithm and Exclusion criteria Survey 2. In Survey 2 we tested only plasma samples from 15,957 participants using the STAT-PAK-HIV1/2 RDT. The confirmation algorithm was essentially the same as in Survey 1.It included two different ELISAs and one Western Blot for RDT positive samples. Participants, who's positive RDT result had been confirmed in Survey 1 already were not re-tested. 319 negative RDT results were directly confirmed by Behring ELISA, 11563 were regarded as true negative because the result of the following survey was also negative.
doi:10.1371/journal.pone.0039529.g002

years respectively. Again we excluded all samples where verification of the result was not possible which resulted in performing the analysis with 13,139 STAT-PAK RDT results, all derived from plasma samples (Figure 2).

Table 1. Diagnostic Test performance of Determine and STAT-PAK RDT numbers of positive and negative results.

HIV RDT:	Determine		STAT-PAK
tested sample:	**whole blood**	**plasma**	**plasma**
N included	1696	12916	13139
Reference standard positives	26	1184	1170
Reference standard negatives	1670	11732	11969
True positives, all	26	1184	1166
True positives, faint bands	1	17	55
True positives, strong band	25	1167	1111
False positives, all	53	250	87
False positives, faint band	49	207	66
False positives, strong band	4	43	21
True negatives	1617	11482	11882
False negatives	0	0	4

doi:10.1371/journal.pone.0039529.t001

Determine RDT

The sensitivity and specificity of the Determine RDT were 100% and 96.8% in whole blood and 100% and 97.9% in plasma respectively. We did not encounter any false negative results with this test (tables 1 and 2). However, the positive predictive value was low in plasma (82.6%) and very low in whole blood (32.9%), meaning that 67.1% of the positive results were incorrect in whole blood and 17.4% in plasma. This high proportion of false positive tests was mainly due to the appearance of faint bands. Only 2% of whole blood samples with faint bands were truly HIV-1 positive according to the reference-standard. In plasma the proportion of true positive faint bands was 7.6%. For those whole blood and plasma samples where strong bands were recorded the concordance with the reference-standard was 86.2% and 96.4% respectively.

STAT-PAK RDT

The sensitivity and specificity of the STAT-PAK RDT were 99.7% and 99.3% respectively; the positive and negative predictive values were 93.1% and 99.97%. Of the 11,886 samples with a negative STAT-PAK RDT result, four were positive according to ELISA and Western Blot testing. All four tests had a positive result in the previous and the following survey but were repeatedly negative when retested using the STAT-PAK RDT. Of the 1,253 positive STAT-PAK RDT results 9.7% showed a faint band, and again the proportion of true positives was lower in faint-banded (45.5%) than in strong-banded tests (98.1%). Of the positive RDT results, 1166 (93.1%) were confirmed with the reference algorithm.

Table 2. Diagnostic Test performance of Determine and STAT-PAK RDT sensitivity, specificity, predictive values and likelihood ratios.

	Determine RDT in whole blood		Determine RDT in blood plasma		STAT-PAK RDT in blood plasma	
	Result	**95%CI**	**Result**	**95%CI**	**Result**	**95%CI**
HIV prevalence according to reference standard[a] (%)	1.53	1.00 to 2.24	9.17	8.67 to 9.68	8.90	8.42 to 9.40
Sensitivity (%)	100.00	86.77 to 100.00	100.00	99.69 to 100.00	99.66	99.13 to 99.91
Specificity (%)	96.83	95.87 to 97.61	97.87	97.59 to 98.12	99.27	99.10 to 99.42
Positive predictive value (%)	32.91	22.75 to 44.40	82.57	80.50 to 84.50	93.06	91.51 to 94.40
Negative predictive value (%)	100.00	99.77 to 100.00	100.00	99.97 to 100.00	99.97	99.91 to 99.99
ROC area	0.984	0.980 to 0.988	0.989	0.988 to 0.991	0.995	0.993 to 0.996
Positive likelihood ratio	31.51	24.18 to 41.07	46.93	41.51 to 53.05	137.10	111.20 to 169.04
Negative likelihood ratio[b]	0.00	– to –	0.00	– to –	<0.01	0.00 to 0.01

[a]Prevalences differ from total population prevalence due to exclusion of participants.
[b]No confidence interval can be calculated if sensitivity = 100%.
doi:10.1371/journal.pone.0039529.t002

Comparison of Determine and STAT-PAK Performance

The 250 false positive plasma samples from survey 1 were retested with the Determine RDT in order to exclude technical errors. The Determine RDT was repeatedly false positive in all samples. To compare the performance of the two RDTs in false positive samples, we later re-tested 25 (10%) of these false positive plasma samples from survey 1 with the STAT-PAK RDT. None of these samples was positive using the STAT-PAK RDT. The 250 participants with false positive plasma samples in Survey 1 were followed closely in the consecutive surveys. Thirty-six did not join the surveillance in survey 2, of the remaining 214 participants only 2 had a false positive test result again using the STAT-PAK RDT. However, in other survey 2 participants the STAT PAK RDT was actually false positive, demonstrating a different pattern of false positivity for both tests. In order to investigate whether false-positivity might be a transient problem or a feature associated with the Determine RDT, samples of 19 participants who had a false positive result in survey 1 were re-tested in survey 2 both with the STAT PAK and the Determine RDT. It appeared that the false positive result of the Determine RDT was not transient, but persisted in 10 out of 19 cases (52.6%) whereas STAT-PAK RDTs performed on the same samples were negative in all these cases.

Comparing the Determine RDT results which were obtained in plasma, with those of the STAT-PAK RDT, we found a higher positive predictive value and a higher specificity of the STAT-PAK RDT. Four false negative results however lead to a lower sensitivity of the latter test. Comparing the overall performance of both tests we found a positive DLR of 46.9 for the Determine RDT and of 137.1 for the STAT-PAK RDT, when using plasma for both tests. This means that in our study population the initial likelihood to be HIV-infected increases 46.9 times with a positive Determine RDT and 137.1 times after receiving a positive result with the STAT-PAK RDT when these tests are done with plasma samples.

Possible Influence of Concomitant Infections

We hypothesized that false positive results for both RDTs might be related to exposure to infectious agents other than HIV. Therefore we examined the associations of co-infection with *S. haematobium (urinary* schistosomiasis) and *P. falciparum (*malaria tropica), participant age, sex, altitude and ambient temperature at the site of the participant's home with false positivity (coded as 0 = RDT true negative; 1 = RDT false positive) as the binary outcome, using only results obtained in plasma samples. The binomial regression models (tables 3 and 4) showed significant associations of false-positivity with age, both for the Determine RDT (probability ratio (PR) per 10 years increase in age = 1.09; 95% confidence interval (95%CI) = 1.03 to 1.15) and for the STAT-PAK RDT (PR = 1.10; 95%CI = 1.00 to 1.21), indicating that the probability of a false positive result in both tests increases by about 10% with a 10 years increase in age. Furthermore, false positive results of the Determine RDT were associated with low altitude (PR per 100 m increase = 0.78; 95%CI = 0.66 to 0.93). Associations of Determine RDT false positivity with ambient temperature and *P. falciparum* co-infection, which were significant in uni-variable regression, both became non-significant in the multi-variable analysis when altitude of residence was included into the model.

As opposed to the Determine RDT, false positive STAT PAK results were also associated with participant sex, with males having a significantly lower proportion of false positive results than females (PR = 0.63, 95%CI = 0.42 to 0.98).

Discussion

An essential requirement of all HIV testing is the accuracy of the test results. HIV RDTs are usually designed to be used as screening tests with a positive result being confirmed by more specific methods. Therefore they are geared towards high sensitivity. This is reflected in the results of both RDTs that we evaluated: when read according to manufacturer's instructions, the Determine RDT was 100% sensitive and the sensitivity of the STAT-PAK RDT was very close to this optimum. However, both tests had problems regarding specificity, which – for the Determine RDT – was worse when using whole blood instead of plasma for testing.

According to a WHO publication from 2002, the sensitivity and specificity of the Determine RDT in whole blood was 100% and 99.7% respectively [18]. Studies from the Ivory Coast [10], the Central African Republic [11], Cameroon [19], Tanzania [20,21] and the Netherlands [12] reported slightly lower sensitivities, but similar specificity when using plasma [10,11,12] or whole blood [19,20,21]. However, two recent publications from Uganda demonstrated lower than expected specificities of 94% [7] and 91.7% [8] for the Determine RDT.

Table 3. Association of various factors with false positive Determine RDT results in plasma; uni- and multi-variable log-link binomial regression results adjusted for clustering within household (N = 11732).

	uni-variable			multi-variable*		
Covariate stratum	**PR**	**95%CI**	**P**	**PR**	**95%CI**	**P**
Age						
per 10 years	1.10	(1.04 to 1.16)	0.001	1.09	(1.03 to 1.15)	0.002
Elevation						
per 100 meters	0.88	(0.86 to 0.90)	<0.001	0.78	(0.66 to 0.93)	0.006
Ambient temperature						
per °C	1.26	(1.20 to 1.32)	<0.001	0.81	(0.59 to 1.10)	0.180
***P. falciparum* infection**						
negative	1	-	-	1	-	-
positive	1.93	(1.07 to 3.47)	0.028	1.47	(0.81 to 2.68)	0.210
Gender						
female	1	-	-			
male	1.00	(0.79 to 1.27)	0.992			
***S. haematobium* infection**						
negative	1	-	-			
positive	0.99	(0.58 to 1.69)	0.980			

PR = probability ratio for a false positive result; p = p-value; 95% CI = 95% confidence interval.
*covariates were only included in multi-variable model if uni-variable p-value <0.2.
doi:10.1371/journal.pone.0039529.t003

Table 4. Association of various factors with false positive STAT-PAK RDT results in plasma; uni- and multi-variable log-link binomial regression results adjusted for clustering within household (N = 11969).

	uni-variable			multi-variable*		
Covariate stratum	**PR**	**95%CI**	**P**	**PR**	**95%CI**	**p**
Age						
per 10 years	1.11	(1.01 to 1.21)	0.035	1.10	(1.00 to 1.21)	0.045
Gender						
female	1	-	-	1	-	-
male	0.63	(0.41 to 0.96)	0.031	0.64	(0.42 to 0.98)	0.038
Elevation						
per 100 meters	1.02	(0.97 to 1.06)	0.444			
Ambient temperature						
per °C	0.99	(0.92 to 1.07)	0.808			
***S. haematobium* infection**						
negative	1	-	-			
positive	0.62	(0.15 to 2.52)	0.502			

PR = probability ratio for a false positive result; p = p-value; 95% CI = 95% confidence interval.
P. falciparum infection not included due to empty cells (no false positive results in *P. falciparum* infected participants because of low *P. falciparum* prevalence in survey 2).
*variables were only retained in multi-variable model if uni-variable p-value <0.2.
doi:10.1371/journal.pone.0039529.t004

One possible reason for these diverse reports could be the subjective interpretation of test results [22]. A recent study from Uganda suggests that the difference in reported specificity of the Determine RDT could be caused by different interpretation of faint positive bands [7]. The authors found a specificity of 94.1% for the Determine RDT in plasma, which improved to 99.6% after exclusion of faint positive bands. Our data confirm these findings in a much larger population and show that the problem is aggravated when using whole blood instead of plasma. The interpretation of faint positive bands as negative might thus be one reason for the differences in sensitivity and specificity of the Determine RDT that are reported in some of the previous studies.

Most studies that examine the diagnostic performance of HIV RDTs use plasma or serum for testing. However, another study from Uganda that used whole blood found a specificity of the Determine RDT of only 96.2% which is very similar to our results [23].

Because the STAT-PAK RDT results were exclusively derived from plasma samples we can only compare them to the plasma results of the Determine RDT. The STAT-PAK RDT was considerably more specific than the Determine RDT. However, still 6.9% of all positive test results were false and again the proportion of true positives was lower in faint-banded (45.5%) than in strong-banded tests (98.1%).

It is noteworthy that half of our participants with false positive Determine RDT results were still false positive when re-tested with the Determine RDT one year later. This shows that for these participants the cause of the false positive result remains present over at least 12 month. It has been suggested that the laboratory diagnosis of HIV might be confounded by regional factors, for example cross reaction with antibodies against other diseases such as schistosomiasis [24]. We demonstrated that in our study area with an altitude range from 475 to over 2300 meters, false positive results of the Determine RDT, but not the STAT-PAK RDT, are more common in the lower parts with higher ambient temperatures that favour co-infections with various infective agents [25,26]. In contrast to the published data regarding the Murex HIV Ag/Ab Combination EIA [24] we did not find an association with concurrent schistosomiasis infection for any of the rapid tests. Results in the uni-variable analyses show an association of Determine RDT false positivity with *P. falciparum* infection with nearly twice as many false positive Determine results in participants who were co-infected with *P. falciparum*. However, in multi-variable analysis, which included altitude of residence as an independent variable, this association became non-significant, suggesting that the cause of the false positive result was not *P. falciparum* infection. Similarly ambient temperature became non-significant when adjusted for altitude. It might be that in low altitude regions of our study area, where several other infections are common [27], humoral immune responses against concomitant infections interfere with the Determine RDT result. These potential cross-reactions could explain some of the differences between previous reports regarding the diagnostic performance of the Determine and other RDTs.

Interestingly we found no such association for temperature, altitude or co-infection with *P. falciparum* for the Stat-Pak RDT false positivity. The gender difference showing less false positive STAT PAK results in men than in women was not reported previously. This might be due to the fact that most studies focus on either women (microbicide trials) or men (circumcision trials). But we presently do not have an explanation for this finding.

Since age is a proxy for lifetime exposure to different diseases, cross reactions with persisting antibodies to other diseases should occur more frequently in older people, which is compatible with the rise of false positivity with age which is apparent in our data.

The relatively high number of false positive results that we found for both tests may not be a problem in settings where every positive screening result is confirmed by more specific, reliable and expensive methods. It is however problematic in resource constrained VCT settings, where testing is done on whole blood and where positive RDT screening results are usually confirmed only by additional RDTs, which might be susceptible to the same characteristics that caused the initial false positive result.

Since most of our false positive results showed faint bands, it is an important question how to deal with these. Interpreting them as negative is problematic as it would decrease sensitivity (e.g. from 100% to 98.6% for Determine RDT testing of plasma in our study) and classify some HIV-infected patients as uninfected with all the negative consequences this would have for transmission and the management of their infection. Interpreting them as positive would also be problematic as it would further decrease specificity of the test and cause unnecessary anxiety in some patients. We therefore believe that the best option in a screening setting is to record the strength of the band when testing and to consider this information once the standard testing reference algorithm for a positive sample is finalised. Positive results with faint bands should therefore be considered for additional testing in one of the Care and Treatment Centres, which have access to more specific methods, such as ELISA, Western Blot or PCR testing.

To avoid too many questionable results it would be best to chose a testing strategy which leaves as few open questions as possible. Thus it seems more sensible to use the Determine RDT with its high sensitivity but low specificity during initial screening and not as a confirmatory test as it was practiced until recently. For confirmation, a more specific test such as the Stat-Pak RDT should be used.

A strength of this study is its huge sample size and the fact that it includes male and female participants of all age groups, apart from infants. To our knowledge it is the first study to date to confirm the findings from Uganda showing that faint bands pose a problem when using the Determine RDT with plasma [7]. It is the only study which shows that this problem also occurs when using the STAT-PAK RDT and is aggravated when using whole blood. The two main limitations of this study concern the lack of direct confirmation (i.e. retesting of the same sample by other means) of negative results in whole blood and in plasma and of positive results obtained with whole blood. As mentioned above, RDT-negative samples were only retested if the RDT-result of the previous or the next survey was in disagreement (= positive) and regarded as reference-standard-negative if the result in the following survey was also negative. This could have led us to overestimate the sensitivity of both tests because we can not exclude that both the Determine and the STAT-PAK RDT are repeatedly false-negative in the same person. However, we believe that this would occur only very rarely, if at all. This is supported by the fact that none of the Determine-negative plasma samples that needed to be retested due to a positive RDT result in the next survey were regarded as positive according to our testing reference algorithm.

Because retesting of positive Determine RDT results obtained from whole blood samples was impossible due to lack of plasma, these were regarded as true positive if the result of the following survey was confirmed positive, as false positive if the result of the following survey was confirmed negative and excluded from this analysis if no HIV results from later surveys were available. This could have increased our specificity estimates for the Determine RDT in whole blood if initially HIV negative participants with a false positive Determine RDT result got HIV infected before the following survey. Equally it could have decreased our specificity estimates if an initial positive Determine RDT result was due to technical error. Although both is possible, it is again unlikely that this has happened in more than a few cases which would only marginally change the numbers presented here. Furthermore the specificity of the Determine RDT in whole blood is so low that a slight change in this diagnostic parameter would not make any difference in practice.

To summarize the above: Due to small shortcomings in our reference standard we can not completely exclude that the true sensitivities for both tests are slightly lower than reported in this article and that the true specificity of the Determine in whole blood may slightly deviate from the result reported here. However, both is unlikely, and with the large number of tests that were done, the very few cases where the reference standard might be incorrect would not substantially change the presented results.

Conclusion

The Determine RDT strip test can not be recommended as a confirmatory test for prevalence studies and in VCT settings, due to its low specificity and low positive predictive value. The specificity of the test is even lower when using whole blood instead of plasma. However, with its optimal sensitivity of 100%, the Determine RDT is an excellent screening test, if another more specific RDT is used for confirmation of positive test results. Since our study did not apply the HIV-testing algorithm as stipulated in the National guidelines for voluntary counselling and testing in Tanzania, we are unable to judge the diagnostic performance of this algorithm. Nevertheless, the practice of using the Determine RDT as confirmatory assay in voluntary counselling and testing settings, where whole blood is used, should be reconsidered. The HIV 1/2 STAT-PAK RDT performed much better regarding specificity and was only slightly less sensitive. However, the problem of faint positive bands with their low positive predictive value could also be demonstrated for the STAT-PAK RDT, although to a far lesser extent.

Acknowledgments

We thank all EMINI participants for their willingness to be part of the study, as well as the EMINI field- and laboratory-teams for their contribution. Special thanks to Mr. Weston Assisya for the coordination of the field work, practical advice and positive spirit.

Author Contributions

Conceived and designed the experiments: MH PC IK. Performed the experiments: IK PC WM. Analyzed the data: IK ES. Wrote the paper: IK PC ES. Performed the statistical analysis: IK ES. Designed the EMINI study, obtained funding, and supervised its conduct: MH. Involved in the implementation of the study: PC L. Maganga L. Maboko MH ALK CG HM.

References

1. Anonymous (1992) Global programme on AIDS. Recommendations for the selection and use of HIV antibody tests. Wkly Epidemiol Rec 67: 145–149.
2. Anonymous (1998) The importance of simple/rapid assays in HIV testing. Wkly Epidemiol Rec 73: 321–326.

3. National AIDS Control Program website. Standard Operating Procedures for HIV testing and counselling (HTC) services. Dar es Salaam: Ministry of Health and Social Welfare. 40 p. Available: http://www.nacp.go.tz/modules/doc_sm/admin/docs/HTC%20A4%20final.pdf Accessed 2011 Mar 11.
4. McKenna SL, Muyinda GK, Roth D, Mwali M, Ng'andu N, et al. (1997) Rapid HIV testing and counseling for voluntary testing centers in Africa. AIDS 11 Suppl 1: S103–110.
5. Hutchinson PL, Mahlalela X (2006) Utilization of voluntary counseling and testing services in the Eastern Cape, South Africa. AIDS Care 18: 446–455.
6. Respess RA, Rayfield MA, Dondero TJ (2001) Laboratory testing and rapid HIV assays: applications for HIV surveillance in hard-to-reach populations. AIDS 15 Suppl 3: S49–59.
7. Gray RH, Makumbi F, Serwadda D, Lutalo T, Nalugoda F, et al. (2007) Limitations of rapid HIV-1 tests during screening for trials in Uganda: diagnostic test accuracy study. BMJ 335: 188.
8. Singer DE, Kiwanuka N, Serwadda D, Nalugoda F, Hird L, et al. (2005) Use of stored serum from Uganda for development and evaluation of a human immunodeficiency virus type 1 testing algorithm involving multiple rapid immunoassays. J Clin Microbiol 43: 5312–5315.
9. Claassen M, van Zyl GU, Korsman SN, Smit L, Cotton MF, et al. (2006) Pitfalls with rapid HIV antibody testing in HIV-infected children in the Western Cape, South Africa. J Clin Virol 37: 68–71.
10. Koblavi-Deme S, Maurice C, Yavo D, Sibailly TS, N'Guessan K, et al. (2001) Sensitivity and specificity of human immunodeficiency virus rapid serologic assays and testing algorithms in an antenatal clinic in Abidjan, Ivory Coast. J Clin Microbiol 39: 1808–1812.
11. Menard D, Mairo A, Mandeng MJ, Doyemet P, Koyazegbe T, et al. (2005) Evaluation of rapid HIV testing strategies in under equipped laboratories in the Central African Republic. J Virol Methods 126: 75–80.
12. van den Berk GE, Frissen PH, Regez RM, Rietra PJ (2003) Evaluation of the rapid immunoassay determine HIV 1/2 for detection of antibodies to human immunodeficiency virus types 1 and 2. J Clin Microbiol 41: 3868–3869.
13. Anonymous (2010) Clearview HIV1/2 STAT-PAK, product brochure. Louisville, CO: inverness medical professional diagnostics. Available: http://www.invernessmedicalpd.com/point_of_care/hiv%E2%81%84stds/clearview%C2%AE_hiv_stat-pak.aspx. Accessed 2012 Dec 01.
14. Anonymous (2009) Determine HIV-1/2, product brochure. Stockford, UK: inverness medical professional diagnostics. Available: http://www.determinetest.com/pdf/240373R2%20HIV1&2%20PI%20for%20CE.pdf. Accessed 2010 Dec 01.
15. Farr TG, Rosen PA, Caro E, Crippen R, Duren R, et al. (2007) The Shuttle Radar Topography Mission. Rev Geophys 45: RG2004.
16. Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A (2005) Very high resolution interpolated climate surfaces for global land areas. International Journal of Climatology 25: 1965–1978.
17. Halkin A, Reichman J, Schwaber M, Paltiel O, Brezis M (1998) Likelihood ratios: getting diagnostic testing into perspective. QJM 91: 247–258.
18. World Health Organization, Dept. of essential health technologies (2002) HIV Assays: operational characteristics, Report 12: World Health Organization.
19. Granade TC, Parekh BS, Tih PM, Welty T, Welty E, et al. (2005) Evaluation of rapid prenatal human immunodeficiency virus testing in rural cameroon. Clin Diagn Lab Immunol 12: 855–860.
20. Mayhood MK, Afwamba IA, Odhiambo CO, Ndanu E, Thielman NM, et al. (2008) Validation, performance under field conditions, and cost-effectiveness of Capillus HIV-1/HIV-2 and determine HIV-1/2 rapid human immunodeficiency virus antibody assays using sequential and parallel testing algorithms in Tanzania. J Clin Microbiol 46: 3946–3951.
21. Lyamuya EF, Aboud S, Urassa WK, Sufi J, Mbwana J, et al. (2009) Evaluation of simple rapid HIV assays and development of national rapid HIV test algorithms in Dar es Salaam, Tanzania. BMC Infect Dis 9: 19.
22. Dax EM, O'Connell R (1999) Standardisation of subjectively scored HIV immunoassays: developing a quality assurance program to assist in reproducible interpretation of results using an anti-HIV particle agglutination assay as a model. J Virol Methods 82: 113–118.
23. Eller LA, Eller MA, Ouma BJ, Kataaha P, Bagaya BS, et al. (2007) Large-scale human immunodeficiency virus rapid test evaluation in a low-prevalence ugandan blood bank population. J Clin Microbiol 45: 3281–3285.
24. Everett DB, Baisely KJ, McNerney R, Hambleton I, Chirwa T, et al. (2010) Association of schistosomiasis with false-positive HIV test results in an African adolescent population. J Clin Microbiol 48: 1570–1577.
25. Sellers RF (1980) Weather, host and vector–their interplay in the spread of insect-borne animal virus diseases. J Hyg (Lond) 85: 65–102.
26. Gould EA, Higgs S (2009) Impact of climate change and other factors on emerging arbovirus diseases. Trans R Soc Trop Med Hyg 103: 109–121.
27. Heinrich N, Saathoff E, Weller N, Clowes P, Kroidl I, et al. (2012) High seroprevalence of rift valley Fever and evidence for endemic circulation in mbeya region, Tanzania, in a cross-sectional study. PLoS Negl Trop Dis 6: e1557.

ORIGINAL ARTICLE

Prospective Study of Acute HIV-1 Infection in Adults in East Africa and Thailand

Merlin L. Robb, M.D., Leigh A. Eller, Ph.D., Hannah Kibuuka, M.B., Ch.B., Kathleen Rono, M.B., Ch.B., Lucas Maganga, M.B., Ch.B., Sorachai Nitayaphan, M.D., Eugene Kroon, M.D., Fred K. Sawe, M.B., Ch.B., Samuel Sinei, M.B., Ch.B., Somchai Sriplienchan, M.D., Linda L. Jagodzinski, Ph.D., Jennifer Malia, Dr.Ph., Mark Manak, Ph.D., Mark S. de Souza, Ph.D., Sodsai Tovanabutra, Ph.D., Eric Sanders-Buell, B.S., Morgane Rolland, Ph.D., Julie Dorsey-Spitz, B.S., Michael A. Eller, Ph.D., Mark Milazzo, B.A., Qun Li, M.Sc., Andrew Lewandowski, Ph.D., Hao Wu, Ph.D., Edith Swann, Ph.D., Robert J. O'Connell, M.D., Sheila Peel, Ph.D., Peter Dawson, Ph.D., Jerome H. Kim, M.D., and Nelson L. Michael, M.D., Ph.D., for the RV 217 Study Team*

ABSTRACT

BACKGROUND

Acute human immunodeficiency virus type 1 (HIV-1) infection is a major contributor to transmission of HIV-1. An understanding of acute HIV-1 infection may be important in the development of treatment strategies to eradicate HIV-1 or achieve a functional cure.

METHODS

We performed twice-weekly qualitative plasma HIV-1 RNA nucleic acid testing in 2276 volunteers who were at high risk for HIV-1 infection. For participants in whom acute HIV-1 infection was detected, clinical observations, quantitative measurements of plasma HIV-1 RNA levels (to assess viremia) and HIV antibodies, and results of immunophenotyping of lymphocytes were obtained twice weekly.

RESULTS

Fifty of 112 volunteers with acute HIV-1 infection had two or more blood samples collected before HIV-1 antibodies were detected. The median peak viremia (6.7 $\log_{10}$ copies per milliliter) occurred 13 days after the first sample showed reactivity on nucleic acid testing. Reactivity on an enzyme immunoassay occurred at a median of 14 days. The nadir of viremia (4.3 $\log_{10}$ copies per milliliter) occurred at a median of 31 days and was nearly equivalent to the viral-load set point, the steady-state viremia that persists durably after resolution of acute viremia (median plasma HIV-1 RNA level, 4.4 $\log_{10}$ copies per milliliter). The peak viremia and downslope were correlated with the viral-load set point. Clinical manifestations of acute HIV-1 infection were most common just before and at the time of peak viremia. A median of one symptom of acute HIV-1 infection was recorded at a median of two study visits, and a median of one sign of acute HIV-1 infection was recorded at a median of three visits.

CONCLUSIONS

The viral-load set point occurred at a median of 31 days after the first detection of plasma viremia and correlated with peak viremia. Few symptoms and signs were observed during acute HIV-1 infection, and they were most common before peak viremia. (Funded by the Department of Defense and the National Institute of Allergy and Infectious Diseases.)

From the U.S. Military HIV Research Program, Walter Reed Army Institute of Research, Silver Spring (M.L.R., L.A.E., L.L.J., J.M., M. Manak, S.T., E.S.-B., M.R., J.D.-S., M.A.E., M. Milazzo, Q.L., S.P., J.H.K., N.L.M.), Henry M. Jackson Foundation for the Advancement of Military Medicine (M.L.R., L.A.E., M. Manak, S.T., E.S.-B., M.R., J.D.-S., M.A.E., M. Milazzo, Q.L.), the Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (E.S.), Bethesda, and Emmes, Rockville (A.L., H.W., P.D.) — all in Maryland; Makerere University Walter Reed Project, Kampala, Uganda (H.K.); Walter Reed Project, Kericho, Kenya (K.R., F.K.S., S. Sinei); Mbeya Medical Research Centre, Mbeya, Tanzania (L.M.); the Armed Forces Research Institute of Medical Sciences (S.N., E.K., S. Sriplienchan, M.S.S., R.J.O.) and SEARCH, Thai Red Cross AIDS Research Center (E.K., M.S.S.) — both in Bangkok, Thailand; and the International Vaccine Institute, Seoul, South Korea (J.H.K.). Address reprint requests to Dr. Robb at the U.S. Military HIV Research Program, 6720A Rockledge Dr., Bethesda, MD 20817, or at mrobb@hivresearch.org.

*A complete list of the members of the RV 217 Study Team is provided in the Supplementary Appendix, available at NEJM.org.

This article was published on May 18, 2016, at NEJM.org.

N Engl J Med 2016;374:2120-30.
DOI: 10.1056/NEJMoa1508952
Copyright © 2016 Massachusetts Medical Society.

The New England Journal of Medicine
Downloaded from nejm.org on December 21, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

Events during acute human immunodeficiency type 1 (HIV-1) infection may modulate the long-term course of HIV-1 disease.[1-4] Acute and early HIV-1 infection is a major contributor to the epidemic spread of HIV-1,[5-7] and limiting this spread through "test and treat" strategies may require treatment of persons during the acute phase of infection.[8-10] The HIV-1 reservoir, which confounds efforts to cure infection,[11] may be more responsive to antiviral therapy during acute HIV-1 infection than during chronic infection.[12-14] Intervention during this stage of infection could dramatically reduce epidemic spread,[15] reduce the size of the HIV-1 reservoir, and potentially achieve long-term control of plasma viremia without the use of long-term antiviral treatment.[16]

Studies of the clinical presentation and kinetics of viremia in persons with acute HIV-1 infection and of the role of these factors in predicting long-term outcomes show conflicting results. Initial descriptions of acute HIV-1 infection were based on cohorts of persons who were identified on the basis of symptoms that were often characterized as those of seronegative mononucleosis.[1,17-21] The use of pooled nucleic acid testing has permitted broader identification of acute HIV-1 infection, and classification systems for the staging of acute HIV-1 infection have been developed on the basis of the sequential reactivity of nucleic acid testing, the presence of the p24 antigen in plasma, and results of antibody testing.[22,23]

We performed a study involving volunteers who were at high risk for HIV-1 infection. Plasma nucleic acid testing was performed twice weekly, and a systematic analysis of the clinical, virologic, and immunologic characteristics of the earliest stage of HIV-1 infection was conducted.

METHODS

STUDY DESIGN AND POPULATION

RV 217 is a prospective natural-history study conducted at the Makerere University Walter Reed Project, Kampala, Uganda; the Walter Reed Project, Kericho, Kenya; the Mbeya Medical Research Centre, Mbeya, Tanzania; and the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. The protocol (available with the full text of this article at NEJM.org) was approved by the local ethics review boards and the Walter Reed Army Institute of Research. Written informed consent was obtained from all participants.

Participants were recruited from bars, clubs, and other locations associated with transactional sex. Men and women, 18 to 50 years of age, who were at high risk for HIV-1 infection were identified with the use of an audio computer-assisted self-interview. To be eligible for study entry, participants had to meet at least one of the following four criteria within the previous 3 months: had exchanged goods for sex, had unprotected sex with a known HIV-positive partner, had unprotected sex with three or more partners, and had symptoms of a sexually transmitted infection. In the first part of the study, which involved surveillance of participants who were not infected, volunteers who had at least one of these high-risk criteria underwent small-volume blood collections by fingerstick measurement twice weekly and large-volume blood collections of 26 to 67 ml every 6 months. Small-volume blood samples were tested for HIV-1 RNA within 24 to 48 hours after collection.

Volunteers in whom tests for HIV-1 RNA were reactive entered the second part of the surveillance phase, during which large-volume blood samples were obtained and a structured medical evaluation was performed twice weekly for 4 weeks. Volunteers with confirmed HIV-1 infection were enrolled in the long-term follow-up phase. Full details of the study design and statistical analysis plan are provided in the protocol.

MEDICAL MANAGEMENT

Volunteers with HIV-1 infection were referred to a local care provider for treatment, including antiretroviral therapy. Counseling regarding HIV risk reduction was provided every 3 months and informally during small-volume blood collections. Condoms and lubricants were provided to participants at the study sites. The study team encouraged care providers to initiate treatment promptly if the volunteers had clinically significant symptoms of acute retroviral syndrome, were pregnant, or met national guidelines for the initiation of antiretroviral therapy.

NUCLEIC ACID TESTING

Approximately 600 μl of whole blood measured with a fingerstick device was collected into a BD Microtainer (Becton Dickinson) containing EDTA. Whole blood was centrifuged at 9000 $\times g$ for 3 minutes and plasma was separated into aliquots for same-day or next-day testing. Plasma was diluted (in a 1:5 ratio) in phosphate-buffered

The New England Journal of Medicine
Downloaded from nejm.org on December 21, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

saline (pH, 7.0 to 7.5) and tested for HIV-1 RNA with the use of the Aptima HIV-1 RNA Qualitative Assay (Hologic).

HIV Serologic Testing, Measurement of Viral RNA, and Flow Cytometry

HIV serologic testing with the use of standard diagnostic methods was performed at screening, every 6 months, and during the second part of the surveillance phase (see the Supplementary Appendix, available at NEJM.org). Plasma HIV-1 RNA levels were measured in batches with the use of the RealTime HIV-1 Assay (m2000 RealTime System, Abbott Molecular). EDTA-anticoagulated samples of whole blood were analyzed with the use of the BD Multitest on an FACSCalibur flow cytometer (Becton Dickinson). HIV-1 subtyping was performed as described previously (see the Supplementary Methods section in the Supplementary Appendix).

Data Analysis

Day 0 was defined as the day on which the first blood sample was reactive for HIV-1 RNA. Viral RNA levels below the lower limit of quantitation were imputed by dividing the limit of quantitation by two. The viral upslope was calculated from the date of the last negative sample to the peak viral load, excluding data from participants for whom the period between the last negative sample and the first sample that was positive for HIV-1 RNA was more than 10 days. The early nadir in the HIV-1 RNA viral load was defined as the lowest viral load after the peak viral load through day 42. Viral downslope was calculated from the peak viral load to the early nadir viral load. The viral-load set point was defined as the average viral load of all samples collected before antiretroviral therapy was administered between days 42 and 365 among participants in whom at least two viral-load values were measured during this period.

Results of physical examinations and reported clinical symptoms are described according to the study visit and per patient. Data on participants were censored at the initiation of antiretroviral therapy.

Statistical Analysis

We performed an exploratory analysis of viral-load dynamics in acute HIV-1 infection without prespecified hypotheses. Correlations between viral load and immune factors were assessed with the use of Spearman's rank-correlation coefficients (rho). Regional differences in viral loads in East Africa and Thailand were evaluated with the use of Wilcoxon rank-sum tests. We used Wilcoxon signed-rank test to assess changes from baseline. Log-transformed viral RNA dynamics during the first year were assessed with the use of regression splines with participant-specific intercepts and slopes. Lymphocyte data were assessed with the use of repeated-measures models with adjustment for region and study visit. (Details of the statistical analysis are provided in the Supplementary Methods section in the Supplementary Appendix.)

Clinical signs (abnormal physical findings on examination) and symptoms were described primarily with comparisons between geographic regions for individual findings with the use of Fisher's exact test. All analyses were performed with the use of SAS software, version 9.3 (SAS Institute) and GraphPad Prism, version 6.0a (GraphPad Software).

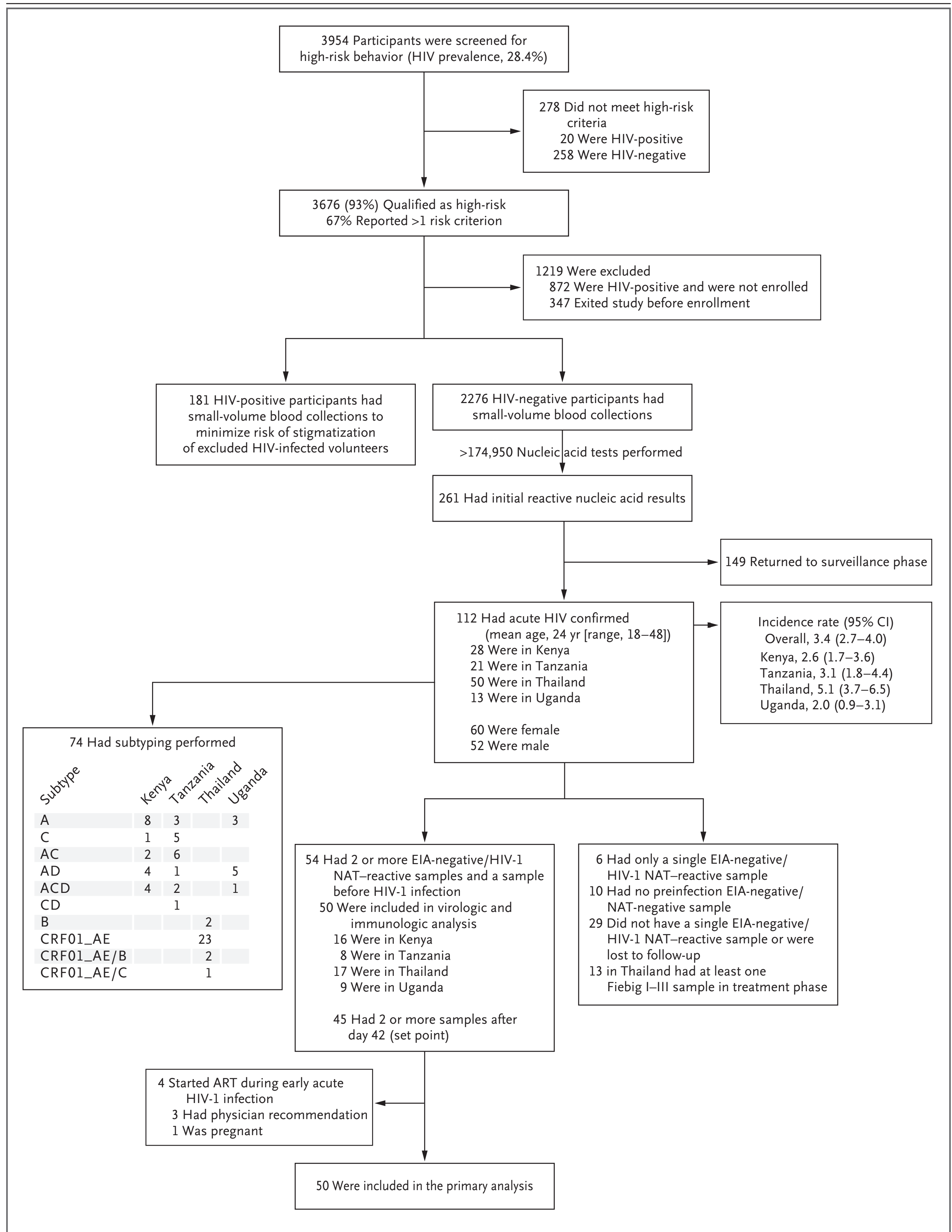
Figure 1 (facing page). Enrollment and Outcomes.
Single false reactive results on qualitative nucleic acid testing were common, but acute HIV infection was confirmed in all participants who had two consecutive plasma samples that were reactive for HIV-1 RNA on qualitative nucleic acid testing. Among 112 participants with acute infection, peak viremia could be accurately defined in 54 participants who had at least two samples in which testing for HIV-1 RNA was reactive and enzyme immunoassay was nonreactive. Four of these participants were excluded from virologic and immunologic analyses because they received early antiretroviral therapy (ART). Most infections were of the subtype HIV-1 CRF01_AE in Thailand and of the HIV-1 subtype A and recombinant form of the A, C, and D strains in East Africa. Fiebig stages range from I through VI, with higher stages indicating a more mature stage of antibody response to HIV. CI denotes confidence interval, EIA enzyme immunoassay, and NAT nucleic acid test.

Results

Study Participants

From June 2009 through June 2015, a total of 3954 volunteers were screened (Fig. 1) and 2276 of 3676 high-risk participants (61.9%) with negative results on an enzyme immunoassay for HIV antibodies entered the surveillance phase.

The New England Journal of Medicine
Downloaded from nejm.org on December 21, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.



Subtype	Kenya	Tanzania	Thailand	Uganda
A	8	3		3
C	1	5		
AC	2	6		
AD	4	1		5
ACD	4	2		1
CD		1		
B			2	
CRF01_AE			23	
CRF01_AE/B			2	
CRF01_AE/C			1	

The New England Journal of Medicine
Downloaded from nejm.org on December 21, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

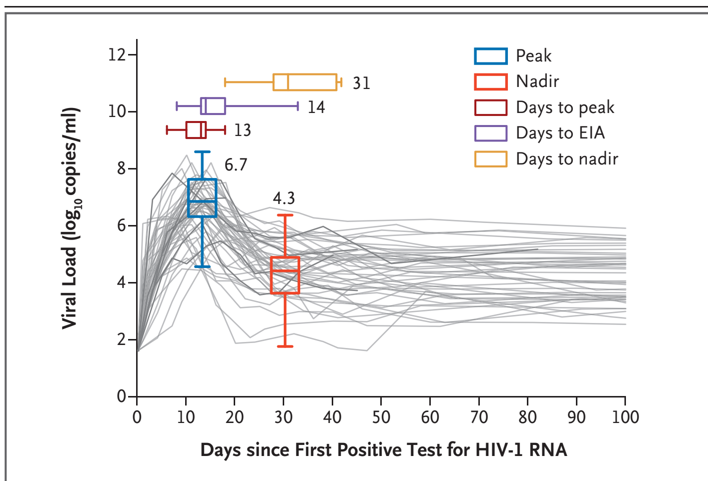


Figure 2. **Viral Loads over the First 100 Days of HIV-1 Infection in 50 Participants.**
Longitudinal viral-load values are plotted against the number of days since the first blood sample was reactive for HIV-1 RNA in 33 participants from East Africa and 17 from Thailand who had two or more blood samples that were nonreactive on enzyme immunoassay (EIA) and were reactive on nucleic acid testing. Day 0 is the day of the first positive nucleic acid test. The box-and-whisker plots show the median, interquartile range, and range for each variable. The vertical box plots show peak and nadir viral loads, and the horizontal box plots show the number of days from the first reactive result on nucleic acid testing to the peak viral load, to reactivity on the EIA, and to the nadir viral load. Median values are shown for each variable.

Figure 3 (facing page). **Viral-Load Associations.**
Spearman's correlations of peak (Panel A) and nadir (Panel B) viremia with the viral-load set point in participants from Thailand (black dots) and East Africa (red dots) are shown. Five participants were excluded because of missing viral-load data or the initiation of antiretroviral therapy. The early viremic nadir and viral-load set point were highly correlated (Panel B) and did not differ significantly (Panel C). A significant positive Spearman's correlation of viral-load downslope with the viral-load set point was observed in 44 participants with acute HIV-1 infection (Panel D). Viral-load upslope did not show significant Spearman's correlation with the viral-load set point in the overall sample (Panel E). Seven of 45 participants were excluded from the upslope analysis because the interval between the last test that was negative for HIV-1 RNA and the first test that was reactive for HIV-1 RNA was longer than 10 days. Comparisons with the use of a Wilcoxon rank-sum test did not show regional differences between East Africa and Thailand with respect to peak viral load (Panel F); however, the viral-load set point was higher in participants in Thailand, with a narrower range of values (Panel G). Sex, race or ethnic group, and HIV-1 subtypes also differed between the two groups.

The majority of participants reported receiving goods for sex (64%), having symptoms of a sexually transmitted infection (61%), or both (Table S1 in the Supplementary Appendix). Most participants with acute HIV-1 infection in the three African sites were heterosexual women, whereas most participants with acute infection from Thailand were homosexual men or transgender women.

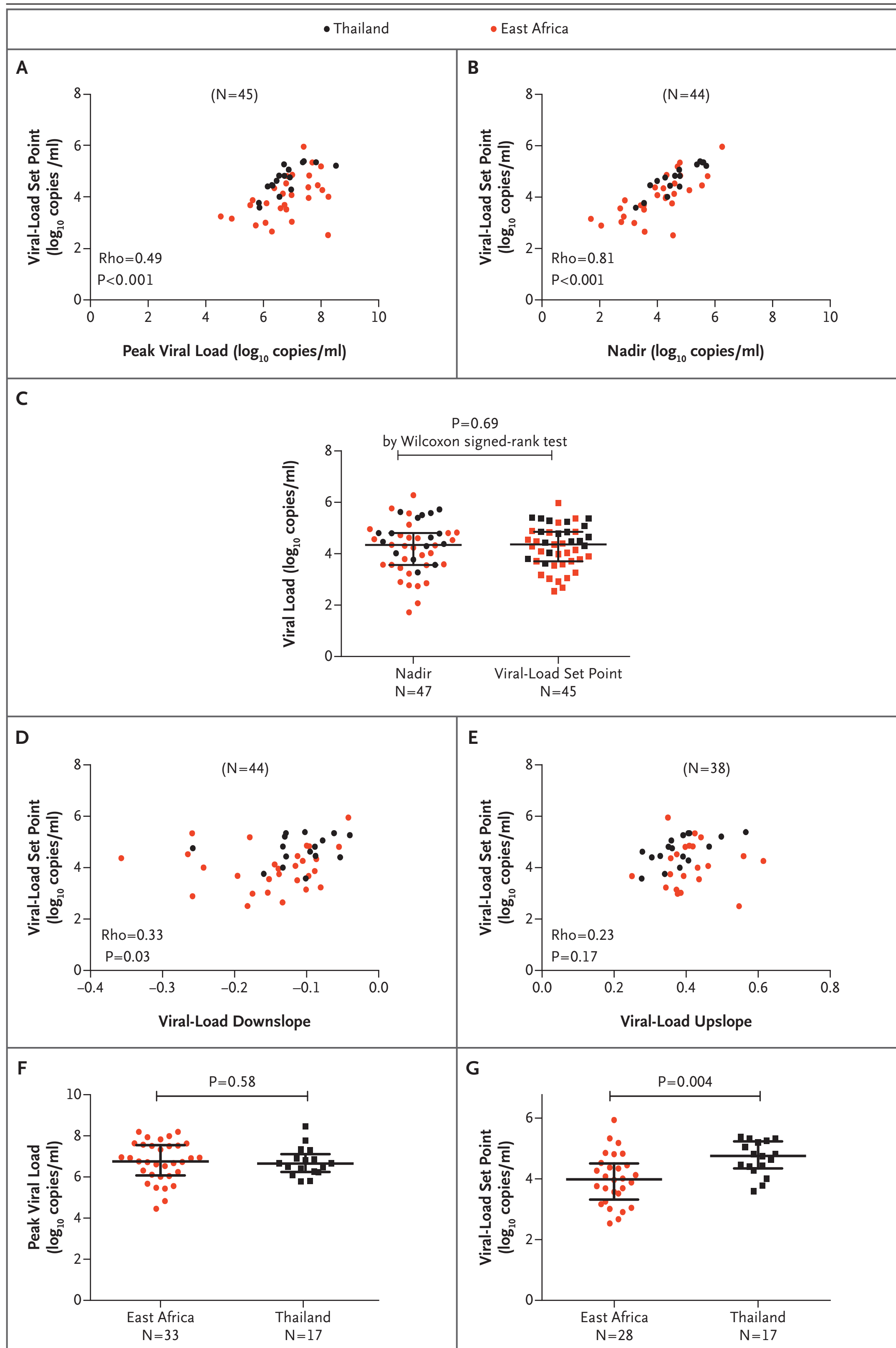
To accurately define peak viremia, we restricted the analysis to the 50 participants in whom at least two large-volume blood samples showed detectable HIV-1 RNA and a nonreactive enzyme immunoassay, who had had at least one study visit before detection of viral RNA, and who had quantitative HIV-1 RNA data. Analysis of the viral-load set point in 45 participants who had not received antiretroviral treatment required two blood samples obtained after day 42. In these participants, a median of 4 days (range, 2 to 162 days) occurred between the last negative sample and the first sample that was reactive for HIV-1 RNA.

HIV-1 VIRAL DYNAMICS

Figure 2 shows HIV-1 viremia during the first 100 days of HIV-1 infection, including the median days to the peak viral load, enzyme immunoassay reactivity, and early nadir viral load. The median initial viral RNA level was 4.0 log_{10} copies per milliliter (range, 1.3 to 7.3), and a median peak of 6.7 log_{10} copies per milliliter (range, 4.5 to 8.5) was reached 13 days (range, 6 to 18) after the first sample showed reactivity for RNA on nucleic acid testing. A third-generation enzyme immunoassay was reactive at a median of 14 days (range, 8 to 48). The median early nadir viral RNA level, 4.3 log_{10} copies per milliliter (range, 1.7 to 6.3), occurred at a median of 31 days (range, 18 to 42). The median viral-load RNA set point was 4.4 log_{10} copies per milliliter (range, 2.5 to 6.0) (Tables S3 and S4 in the Supplementary Appendix).

The spline models showed a significant interaction between viremia and geographic region and indicated differences in viral RNA dynamics among regions. The models also showed that the viral-load set point was established at the conclusion of acute viremia and remained stable subsequently (Fig. S1 in the Supplementary Appendix). Peak viremia was positively correlated

The New England Journal of Medicine
Downloaded from nejm.org on December 21, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.



The New England Journal of Medicine
Downloaded from nejm.org on December 21, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

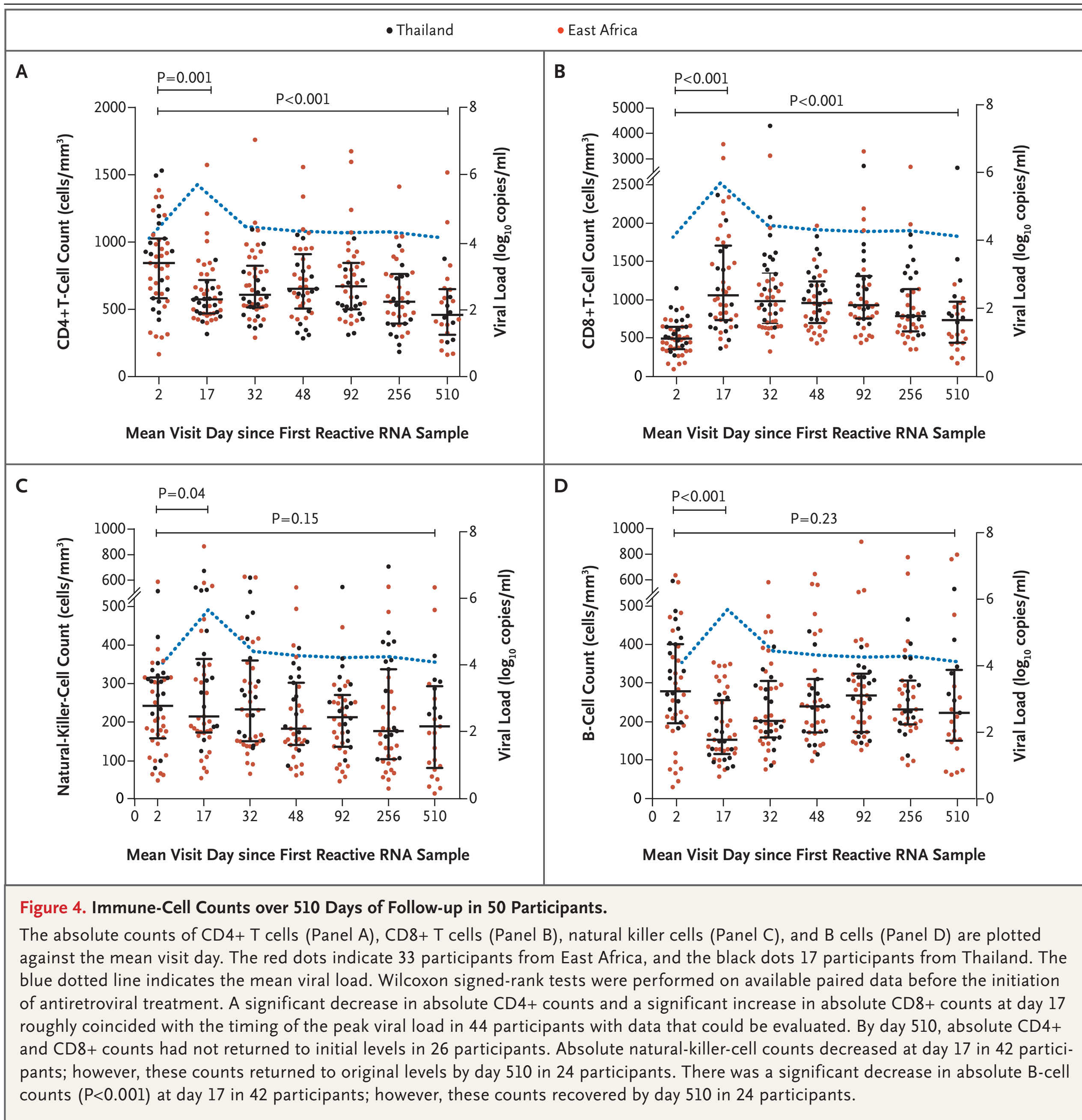


Figure 4. **Immune-Cell Counts over 510 Days of Follow-up in 50 Participants.**

The absolute counts of CD4+ T cells (Panel A), CD8+ T cells (Panel B), natural killer cells (Panel C), and B cells (Panel D) are plotted against the mean visit day. The red dots indicate 33 participants from East Africa, and the black dots 17 participants from Thailand. The blue dotted line indicates the mean viral load. Wilcoxon signed-rank tests were performed on available paired data before the initiation of antiretroviral treatment. A significant decrease in absolute CD4+ counts and a significant increase in absolute CD8+ counts at day 17 roughly coincided with the timing of the peak viral load in 44 participants with data that could be evaluated. By day 510, absolute CD4+ and CD8+ counts had not returned to initial levels in 26 participants. Absolute natural-killer-cell counts decreased at day 17 in 42 participants; however, these counts returned to original levels by day 510 in 24 participants. There was a significant decrease in absolute B-cell counts (P<0.001) at day 17 in 42 participants; however, these counts recovered by day 510 in 24 participants.

with the viral-load RNA set point in the total cohort (rho=0.49, P<0.001) (Fig. 3A) and independently in each geographic region (Fig. S2A in the Supplementary Appendix). There was a strong correlation between an early viral RNA nadir at the end of acute HIV-1 infection and the viral-load set point (rho=0.81, P<0.001) (Fig. 3B); this correlation remained significant within each region (Fig. S2B in the Supplementary Appendix). The values of the early nadir and set point did not differ significantly (Fig. 3C); this shows that the viral-load RNA set point was established within the first 42 days after viremia was detectable.

The downslope of viral RNA was correlated with the viral-load RNA set point (rho=0.33, P=0.03) (Fig. 3D). The upslope of viral RNA was not correlated with the viral-load set point in the overall cohort (Fig. 3E); it was strongly correlated in Thailand only (rho=0.66, P=0.004) (Fig.

The New England Journal of Medicine
Downloaded from nejm.org on December 21, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

S2D in the Supplementary Appendix). Although the peak viral RNA level was nearly the same in Thailand and East Africa (Fig. 3F), the viral-load set point differed significantly between the two regions (Fig. 3G).

IMMUNOPHENOTYPE

Immunophenotyping of lymphocytes showed no change or a minimal variation from normal values at the first study visit after the onset of plasma viremia (Fig. 4A through 4D, and Fig. S3 in the Supplementary Appendix). Subsequently, levels of both B cells and CD4+ T cells decreased sharply at the time of peak viremia, while levels of CD8+ T cells increased significantly. Changes in levels of natural killer cells during acute infection were variable. After peak viremia, levels of CD4+ T cells increased and levels of CD8+ T cells decreased, but these levels never returned to a normal range.

Levels of CD4+ T cells and B cells were inversely correlated with contemporaneous viral RNA levels, whereas the increase in the number of CD8+ T cells was correlated directly with contemporaneous viral RNA levels in a model adjusted for geographic region and study visit. After adjustment for other cell counts, region, and visit, a 100-cell increase in the CD4+ T-cell count was associated with an average decrease of 0.1 in $\log_{10}$ viral RNA across visits. The nadir CD4+ T-cell count was correlated with the CD4+ T-cell count at 12 months (rho=0.59, P<0.001).

CLINICAL PRESENTATION

A structured history was obtained and a physical examination was performed at study entry, every 6 months, and every 3 or 4 days throughout the period of acute infection. Symptoms and signs were identified at least once during observation in 94% of the participants with acute HIV-1 infection (88% reported at least one symptom and 78% reported at least one sign). However, during the period of acute infection, in 367 of 518 visits in which participants underwent a physical examination (71%), participants reported no symptoms, and 50% of these participants had neither symptoms nor signs.

Fever, headache, and malaise were the most common symptoms, and tachycardia, lymphadenopathy, and other head and neck findings were the most common signs (Table S5 in the Supplementary Appendix). The greatest number of symptoms was reported at the study visit before the peak viral RNA level (median, 1; range, 0 to 15) and was reported at a median of two visits (Fig. 5A). Observed signs on physical examination peaked at the visit before the peak viremia (median, 1; range, 0 to 3) and were recorded for a median duration of three visits (Fig. 5B). Heat maps enumerating each volunteer's symptoms or signs at each visit show that most findings

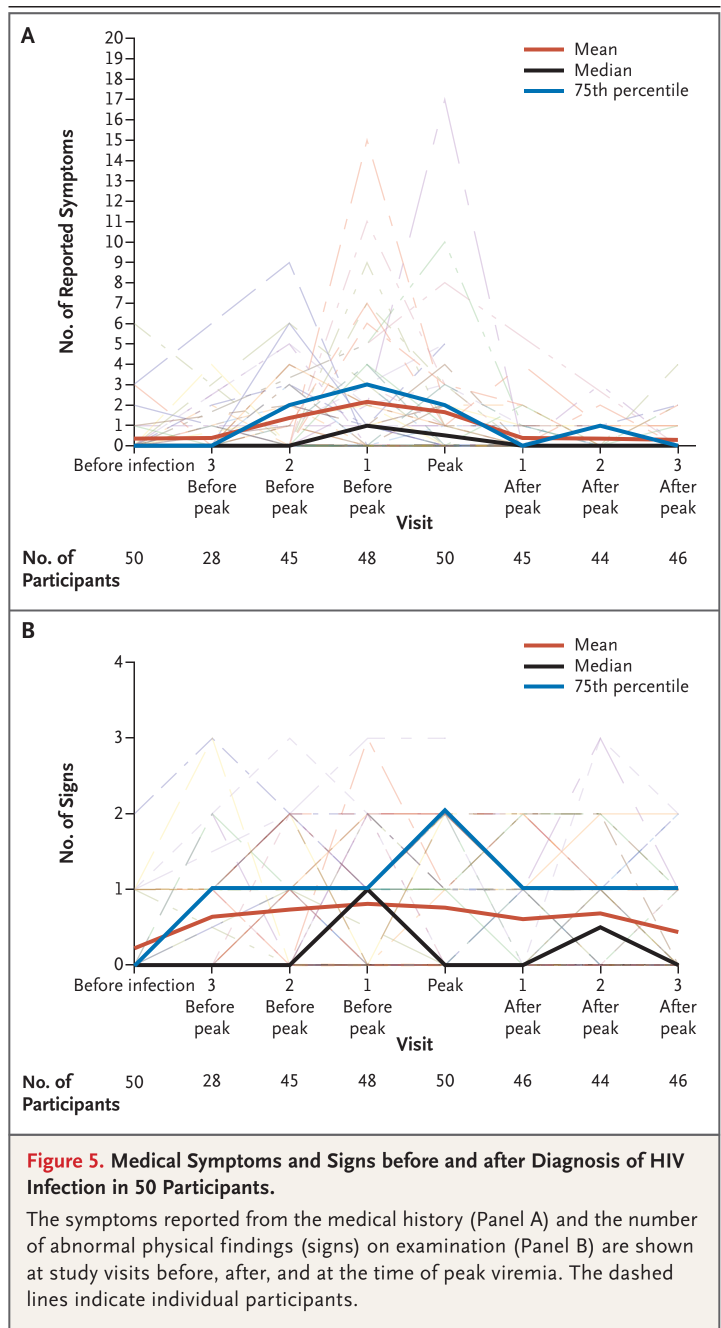


Figure 5. Medical Symptoms and Signs before and after Diagnosis of HIV Infection in 50 Participants.

The symptoms reported from the medical history (Panel A) and the number of abnormal physical findings (signs) on examination (Panel B) are shown at study visits before, after, and at the time of peak viremia. The dashed lines indicate individual participants.

The New England Journal of Medicine
Downloaded from nejm.org on December 21, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

occurred before and at the time of peak viremia but waned quickly thereafter (Fig. S4A and S4B in the Supplementary Appendix). Although fever was the most common reported symptom, only six volunteers were found to be febrile on physical examination.

Lymphadenopathy was more common among participants in Thailand than among those in East Africa (P<0.001) (Table S5 in the Supplementary Appendix). The magnitude of the lymph-node enlargement was minimal; the maximal lymph-node diameter was greater than 2 cm in only five volunteers.

Disease Progression

The CD4+ T-cell count at 12 months after the diagnosis of HIV-1 infection and the last available CD4+ T-cell count were used to evaluate the disease course. The start of antiretroviral therapy was not used as an end point because it was most frequently initiated because of pregnancy.

After exclusion of five participants without a CD4+ T-cell count within 120 days before or after the 12-month time point, the CD4+ T-cell count was inversely correlated with the viral-load RNA set point (rho = −0.65, P<0.001). Similarly, the last available CD4+ T-cell count was highly and inversely correlated with the viral-load RNA set point (rho = −0.50, P = 0.004).

A surrogate clinical end point was the number of days to two consecutive visits during which a CD4+ T-cell count of less than 350 cells per cubic millimeter was recorded. After exclusion of volunteers who were pregnant or who had started to receive antiretroviral therapy for reasons other than a CD4 T-cell count below 350 cells per cubic millimeter, 15 of 50 participants reached this surrogate clinical end point in a median of 306 days (range, 7 to 1083).

There was no difference in follow-up time between participants who reached the end point and those who did not (P = 0.68). The peak viral RNA level did not differ significantly between participants who reached the end point and those who did not; however, the viral-load RNA set point was significantly higher among those who reached the end point than among those who did not (median, 4.83 $\log_{10}$ copies per milliliter vs. 4.02 $\log_{10}$ copies per milliliter, P = 0.002), absolute CD4+ T-cell counts at 1 year were lower (median, 356 cells per cubic millimeter vs. 639.5 cells per cubic millimeter, P = 0.002), and CD8+ T-cell peaks were higher (median, 1661 cells per cubic millimeter vs. 1202 cells per cubic millimeter, P = 0.06).

Discussion

In contrast to previous studies of acute HIV-1 infection in which volunteers were evaluated less often, we evaluated high-risk volunteers twice weekly in order to systematically describe both the clinical disease and host–virus interactions, with precise determination of the onset and dynamics of acute plasma viremia. The upslope, peak, and downslope of viremia in acute infection were defined with precision and at a high frequency among observed cases of HIV-1 infection. These variables were significantly associated with the viral-load set point; this association underscored the crucial role of the very earliest interactions between the host and virus in determining the long-term course of the disease. Our study showed that the viral-load RNA set point was established within 42 days after detectable viremia, was steady over the period of observation, and was associated with the early clinical outcome as measured by the CD4+ T-cell count 12 months after infection and a CD4+ T-cell count below 350 cells per cubic millimeter.

The peak viremia reported here was of a higher magnitude (median, $\log_{10}$ 6.7; range, $\log_{10}$ 4.5 to 8.5) than that which is commonly reported,[24-28] probably because of the frequency of assessment. Although the peak viremia was nearly equivalent in East Africa and Thailand, there was a significant difference of 0.8 $\log_{10}$ copies per milliliter in the viral-load RNA set point. Differences in viral-load RNA set points according to sex have been reported; set points in men are generally approximately three times as high as set points in women who have infections of the same viral subtype.[29] Because of multiple confounding variables, including host genetic factors, viral subtype, endemic disease, and risk characteristics, a mechanistic explanation for this regional variation in the viral set point remains undefined.

This study showed the alterations in cell phenotype before and during peak viremia. At the onset of plasma viremia, immunophenotypes were largely normal, but subsequently, CD4+ T-cell counts decreased and CD8+ T-cell counts increased around the time of the peak viral RNA level, were highly correlated with the viral RNA level during acute HIV-1 infection, and did not

The New England Journal of Medicine
Downloaded from nejm.org on December 21, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

fully recover as the viral RNA level decreased to the set point. Ndhlovu et al. recently described an association between the timing and magnitude of CD8+ T-cell activation and the viral-load set point in 11 cases of acute infection.[30] The observed, dramatic loss of B cells has been reported in simian immunodeficiency virus,[31-33] but data on the role of this loss in acute HIV-1 infection in humans are lacking.[34]

In many studies of HIV-1, patients had symptoms before an evaluation for the diagnosis of acute infection was initiated.[35,36] As in other studies of acute HIV-1 infection, most persons in our study (94%) had clinical manifestations sometime during acute infection. However, nonspecific symptoms and signs were most common, severe manifestations were not observed, volunteers reported symptoms in only 29% of visits, and on any given visit day the likelihood of observing a symptom or sign was only 50%. The frequency of clinical manifestations of disease clustered before the peak viremia and at the time of peak viremia and resolved quickly, but the median number of symptoms and signs was only 1.

Since the study scheduled visits throughout the period of acute HIV-1 infection, the proportion of participants who would have sought medical care is unknown. Sullivan et al. reported symptoms and signs of acute infection among discordant couples who were prospectively followed every 3 months and found that a majority of patients with incident HIV-1 infection could not recall an illness and did not pursue medical care.[21] Thus, systematic identification of acute HIV-1 infection may be challenging and will probably require nucleic acid testing with a rapid turnaround and frequent evaluation of high-risk groups rather than clinical presentation in a health care setting.

The contribution of acute HIV-1 infection to HIV-1 transmission may be substantial.[5-7,37,38] Presumably, a high viral load plays a part, but the biologic characteristics of transmitted founder viruses, the homogeneity of viral sequence during acute HIV-1 infection, and the incomplete or immature host immune response may create a transmission diathesis. HIV-1 may evolve to maintain efficient replication in the host and lose characteristics that are favorable for transmission. Recent data show that transmitting viruses are qualitatively distinct from those emerging under host immune pressure.[39] Viral RNA is a dominant factor in the risk of transmission in various patient groups (e.g., among infants who may become infected through perinatal transmission and among heterosexuals), and if a high level of viral RNA in acute HIV-1 infection is the primary variable contributing to increased infectiousness in early and acute HIV-1 infection, the duration of this risk appears to be brief because the viral-load RNA set point is achieved within a few weeks after peak viremia. This observation may have important consequences and may limit the effect of test-and-treat strategies on overall rates of transmission.

The data reported here emphasize the importance of acute infection to our understanding of the pathogenesis of HIV-1. They also provide evidence that during the acute phase, identification of cases of HIV-1 on the basis of clinical criteria may prove to be difficult.

The views expressed are those of the authors and should not be construed to represent the positions of the Departments of the Army or Defense or the National Institutes of Health (NIH).

Supported by the Department of Defense through cooperative agreements (W81XWH-07-2-0067 and W81XWH-11-0174) with the Henry M. Jackson Foundation for the Advancement of Military Medicine and by the National Institute of Allergy and Infectious Diseases, NIH, through an interagency agreement with the U.S. Army (Y1-AI-2642-17).

No potential conflict of interest relevant to this article was reported.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

We thank the RV 217 study volunteers and study teams in Uganda, Kenya, Tanzania, and Thailand for their contribution and commitment to HIV research; Margaret Johnston, Ph.D., and Bonnie Mathieson, Ph.D., of the National Institutes of Health for their guidance and advocacy from the beginning of the study design; and Jintanat Ananworanich, M.D., for expert review of an earlier version of the manuscript.

REFERENCES

1. Kelley CF, Barbour JD, Hecht FM. The relation between symptoms, viral load, and viral load set point in primary HIV infection. J Acquir Immune Defic Syndr 2007;45:445-8.

2. Lavreys L, Baeten JM, Chohan V, et al. Higher set point plasma viral load and more-severe acute HIV type 1 (HIV-1) illness predict mortality among high-risk HIV-1-infected African women. Clin Infect Dis 2006;42:1333-9.

3. Lefrère JJ, Roudot-Thoraval F, Mariotti M, et al. The risk of disease progression is determined during the first year of human immunodeficiency virus type 1 infection. J Infect Dis 1998;177:1541-8.

4. Lindbäck S, Karlsson AC, Mittler J, et al. Viral dynamics in primary HIV-1 infection. AIDS 2000;14:2283-91.

5. Brenner BG, Roger M, Routy JP, et al. High rates of forward transmission events after acute/early HIV-1 infection. J Infect Dis 2007;195:951-9.

6. Pilcher CD, Tien HC, Eron JJ Jr, et al.

The New England Journal of Medicine
Downloaded from nejm.org on December 21, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

Brief but efficient: acute HIV infection and the sexual transmission of HIV. J Infect Dis 2004;189:1785-92.
7. Wawer MJ, Gray RH, Sewankambo NK, et al. Rates of HIV-1 transmission per coital act, by stage of HIV-1 infection, in Rakai, Uganda. J Infect Dis 2005;191:1403-9.
8. Cohen MS, Chen YQ, McCauley M, et al. Prevention of HIV-1 infection with early antiretroviral therapy. N Engl J Med 2011;365:493-505.
9. Cohen MS, Dye C, Fraser C, Miller WC, Powers KA, Williams BG. HIV treatment as prevention: debate and commentary — will early infection compromise treatment-as-prevention strategies? PLoS Med 2012;9(7):e1001232.
10. Powers KA, Ghani AC, Miller WC, et al. The role of acute and early HIV infection in the spread of HIV and implications for transmission prevention strategies in Lilongwe, Malawi: a modelling study. Lancet 2011;378:256-68.
11. Finzi D, Blankson J, Siliciano JD, et al. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat Med 1999;5:512-7.
12. Ananworanich J, Schuetz A, Vandergeeten C, et al. Impact of multi-targeted antiretroviral treatment on gut T cell depletion and HIV reservoir seeding during acute HIV infection. PLoS One 2012;7(3):e33948.
13. Jain V, Hartogensis W, Bacchetti P, et al. Antiretroviral therapy initiated within 6 months of HIV infection is associated with lower T-cell activation and smaller HIV reservoir size. J Infect Dis 2013;208:1202-11.
14. Sáez-Cirión A, Bacchus C, Hocqueloux L, et al. Post-treatment HIV-1 controllers with a long-term virological remission after the interruption of early initiated antiretroviral therapy ANRS VISCONTI Study. PLoS Pathog 2013;9(3):e1003211.
15. Cohen MS, Smith MK, Muessig KE, Hallett TB, Powers KA, Kashuba AD. Antiretroviral treatment of HIV-1 prevents transmission of HIV-1: where do we go from here? Lancet 2013;382:1515-24.
16. Hill AL, Rosenbloom DI, Fu F, Nowak MA, Siliciano RF. Predicting the outcomes of treatment to eradicate the latent reservoir for HIV-1. Proc Natl Acad Sci U S A 2014;111:13475-80.
17. Schacker T, Collier AC, Hughes J, Shea T, Corey L. Clinical and epidemiologic features of primary HIV infection. Ann Intern Med 1996;125:257-64.
18. Gay C, Dibben O, Anderson JA, et al. Cross-sectional detection of acute HIV infection: timing of transmission, inflammation and antiretroviral therapy. PLoS One 2011;6(5):e19617.
19. Hecht FM, Busch MP, Rawal B, et al. Use of laboratory tests and clinical symptoms for identification of primary HIV infection. AIDS 2002;16:1119-29.
20. Pilcher CD, Joaki G, Hoffman IF, et al. Amplified transmission of HIV-1: comparison of HIV-1 concentrations in semen and blood during acute and chronic infection. AIDS 2007;21:1723-30.
21. Sullivan PS, Fideli U, Wall KM, et al. Prevalence of seroconversion symptoms and relationship to set-point viral load: findings from a subtype C epidemic, 1995-2009. AIDS 2012;26:175-84.
22. Ananworanich J, Fletcher JL, Pinyakorn S, et al. A novel acute HIV infection staging system based on 4th generation immunoassay. Retrovirology 2013;10:56.
23. Fiebig EW, Wright DJ, Rawal BD, et al. Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. AIDS 2003;17:1871-9.
24. Henrard DR, Daar E, Farzadegan H, et al. Virologic and immunologic characterization of symptomatic and asymptomatic primary HIV-1 infection. J Acquir Immune Defic Syndr Hum Retrovirol 1995;9:305-10.
25. Kaufmann GR, Cunningham P, Kelleher AD, et al. Patterns of viral dynamics during primary human immunodeficiency virus type 1 infection. J Infect Dis 1998;178:1812-5.
26. Kaufmann GR, Cunningham P, Zaunders J, et al. Impact of early HIV-1 RNA and T-lymphocyte dynamics during primary HIV-1 infection on the subsequent course of HIV-1 RNA levels and CD4+ T-lymphocyte counts in the first year of HIV-1 infection. J Acquir Immune Defic Syndr 1999;22:437-44.
27. Novitsky V, Woldegabriel E, Kebaabetswe L, et al. Viral load and CD4+ T-cell dynamics in primary HIV-1 subtype C infection. J Acquir Immune Defic Syndr 2009;50:65-76.
28. Richardson BA, Mbori-Ngacha D, Lavreys L, et al. Comparison of human immunodeficiency virus type 1 viral loads in Kenyan women, men, and infants during primary and early infection. J Virol 2003;77:7120-3.
29. Sterling TR, Vlahov D, Astemborski J, Hoover DR, Margolick JB, Quinn TC. Initial plasma HIV-1 RNA levels and progression to AIDS in women and men. N Engl J Med 2001;344:720-5.
30. Ndhlovu ZM, Kamya P, Mewalal N, et al. Magnitude and kinetics of CD8+ T cell activation during hyperacute HIV infection impact viral set point. Immunity 2015;43:591-604.
31. Dykhuizen M, Mitchen JL, Montefiori DC, et al. Determinants of disease in the simian immunodeficiency virus-infected rhesus macaque: characterizing animals with low antibody responses and rapid progression. J Gen Virol 1998;79:2461-7.
32. Kuhrt D, Faith SA, Leone A, et al. Evidence of early B-cell dysregulation in simian immunodeficiency virus infection: rapid depletion of naïve and memory B-cell subsets with delayed reconstitution of the naïve B-cell population. J Virol 2010;84:2466-76.
33. Mattapallil JJ, Letvin NL, Roederer M. T-cell dynamics during acute SIV infection. AIDS 2004;18:13-23.
34. Levesque MC, Moody MA, Hwang KK, et al. Polyclonal B cell differentiation and loss of gastrointestinal tract germinal centers in the earliest stages of HIV-1 infection. PLoS Med 2009;6(7):e1000107.
35. Bebell LM, Pilcher CD, Dorsey G, et al. Acute HIV-1 infection is highly prevalent in Ugandan adults with suspected malaria. AIDS 2010;24:1945-52.
36. Sanders EJ, Mugo P, Prins HA, et al. Acute HIV-1 infection is as common as malaria in young febrile adults seeking care in coastal Kenya. AIDS 2014;28:1357-63.
37. Goodreau SM, Carnegie NB, Vittinghoff E, et al. What drives the US and Peruvian HIV epidemics in men who have sex with men (MSM)? PLoS One 2012;7(11):e50522.
38. Hollingsworth TD, Pilcher CD, Hecht FM, Deeks SG, Fraser C. High transmissibility during early HIV infection among men who have sex with men — San Francisco, California. J Infect Dis 2015;211:1757-60.
39. Parrish NF, Gao F, Li H, et al. Phenotypic properties of transmitted founder HIV-1. Proc Natl Acad Sci U S A 2013;110:6626-33.

Copyright © 2016 Massachusetts Medical Society.

RECEIVE IMMEDIATE NOTIFICATION WHEN AN ARTICLE IS PUBLISHED ONLINE FIRST

To be notified by e-mail when *Journal* articles are published Online First, sign up at NEJM.org.

The New England Journal of Medicine
Downloaded from nejm.org on December 21, 2016. For personal use only. No other uses without permission.
Copyright © 2016 Massachusetts Medical Society. All rights reserved.

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Robb ML, Eller LA, Kibuuka H, et al. Prospective study of acute HIV-1 infection in adults in East Africa and Thailand. N Engl J Med. DOI: 10.1056/NEJMoa1508952

Table of contents:

RV217 Study Team and Affiliations **2**

Additional Study Details **3**

Supplementary methods **4**

Table S1: Description of screening, surveillance and acute infection cohorts **5**

Table S2: Acute cohort: Analysis and excluded cohorts **6**

Table S3: Summary quantitative viral RNA statistics **7**

Figure S1: Viral Load Regional Spline Models **8**

Table S4: Individual Viral Load Data **9**

Figure S2: Regional viral load correlations **10**

Figure S3: Immune cell counts over 510 days of follow up by region **11**

Table S5: Frequency of clinical symptoms and signs overall and by region **12**

Figure S4A: Heat map-Symptoms reported in medical history **13**

Figure S4B: Heat map-Physical Exam findings **14**

References: **15**

RV217 Study Team and Affiliations

Elizabeth M. Adams[1,2], Siriwat Akapirat[3], Fatim Cham[1,4,5], Agnes Chenine[1,4], Angela Eser[6], Alan Fix[7], Christof Geldmacher[8,9], Michael Hoelscher[8,9], Matthew Johnston[1,3,4], Gustavo H. Kijak[1,4], Shelly J. Krebs[1,4], Arne Kroidl[8,9], Inge Kroidl[6,8,9], Anjali Kunz[10], Cornelia Lueer[6], Leonard Maboko[6], Susan Mason[1,4], Monica Millard[4,5], Joseph Oundo[11], Victoria R. Polonis[4], Erica Sanga[6], Alexandra Schuetz[1,3,4], Arthur Sekiziyivu[12], Doug N. Shaffer[11*], Kibet Shikuku[11], Bonnie H. Slike[1,4], Hendrik Streeck[4,13,], Don Stablein[14], Ali Taylor[1,4], Rapee Trichavaroj[3], Aimee Valenzuela[15], Amy Weintrob[16]

Affiliations

[1]Henry M. Jackson Foundation for the Advancement of Military Medicine, Bethesda, MD,
[2]Division of AIDS, NIAID, NIH, Bethesda, MD,
[3]Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand,
[4]U.S. Military HIV Research Program, Walter Reed Army Institute of Research, Silver Spring, MD,
[5]Makere University Walter Reed Project, Kampala, Uganda,
[6]National Institute for Medical Research-Mbeya Medical Research Centre, Mbeya, Tanzania,
[7]PATH, Washington, DC,
[8]German Center for Infection Research (DZIF), partner site Munich, Germany,
[9]Division of Infectious Diseases and Tropical Medicine, Medical Center of the University of Munich (LMU), Munich, Germany,
[10] Madigan Army Medical Center, JBLM, Tacoma, WA
[11]Walter Reed Project, Kericho, Kenya,
[12]Infectious Diseases Institute-Makerere University College of Health Sciences, Kampala Uganda,
[13]Institute for HIV Research, University Hospital Essen, University Duisburg-Essen, Essen, Germany,
[14]EMMES Corporation, Rockville, MD,
[15]Mathematica Policy Research, Washington, DC,
[16] George Washington University, Washington, DC and Veterans Affairs Medical Center, Washington, DC,

[*] Current Affiliation-Office of the Global AIDS Coordinator, Department of State, Washington, DC

Additional Study Details: RV 217 "HIV-1 Prevalence, Incidence, Cohort Retention, and Host Genetics and Viral Diversity in Cohorts in East Africa and Thailand"

Design Details:

Phase Ia: Non-randomized, cohort, prospective, 24-month observational study to be conducted in two parts. The study conducted a screening visit and initial follow-up in all enrolled volunteers. This included both HIV negative and HIV prevalent cases. Prevalence was estimated to be high in these populations and as many as 1000-1500 volunteers will be enrolled and evaluated in the first two visits with an enrollment target of 500 HIV negative, high-risk volunteers per site. Subsequently, only HIV negative volunteers were followed for a period of 24 months, except for a small number of HIV positive individuals for masking to minimize risk of stigmatization. After the initial two visits, volunteers were seen at the research clinic approximately every 3 months. In addition, at the research clinic or other locations that were convenient to the volunteers, small blood volume samples were collected using microvettes twice weekly. These samples were analyzed and results returned to the site within 48 hours to permit new HIV positive participants to be identified while they remain antibody negative, HIV nucleic acid test positive. Any participant with a reactive nucleic acid test was entered into phase Ib.

Phase Ib: Participants had repeat medical encounters twice weekly for a total of 9 visits to include HIV-1 nucleic acid testing, HIV antibody testing and larger blood collections to characterize host immune responses and a structured history and physical. The purpose of this intensive analysis was to establish HIV diagnosis and characterize the viral-host interaction during acute HIV infection. All newly infected participants were referred to phase II of the study.

Phase II: study of HIV incident cases arising within phase I. HIV incident volunteers were asked to consent to participate in phase II to intensively characterize viral dynamics, host immune response and potential impact of host genetics over a period of at least 50 months from the time of entering the AHI phase..

Participants

The study population will include men and women, aged 18-50 years old, who may be members of the following high risk groups: female and male sex workers (SWs), barworkers (BWs), sexually transmitted infection clinic attendees (STIAs), motorcycle passenger transporters (Boda-Boda), transgenders (TG), and men who have sex with men (MSM). Although these populations are known to be among "Most at-Risk Populations" or MARPs, not all members of these occupational groups engage in equivalent risk behavior. Enrollment will therefore engage members of these populations whose behavior places them at higher risk as documented through an Audio-Computer Assisted Self-Interview. Only participants identifying at least one of four risk factors were enrolled (see table S1).

Supplementary methods:

Informed Consent: Written informed consent was obtained in the native language of the participant. Standard procedures for enrolling illiterate volunteers were observed.

HIV serological diagnostics:
HIV serology was performed at screening, every 6 months and during phase IB using the Genetic Systems HIV-1/HIV-2 Plus O EIA (Bio-Rad Laboratories, Inc; Redmond, WA). Reactive samples were repeated in duplicate then confirmed by the Genetic Systems HIV-1 Western Blot (Bio-Rad Laboratories).

HIV subtyping: HIV-1 near full-length genomes or two overlapping half genomes were sequenced from plasma RNA using a single genome amplification strategy.[1] The PCR amplification and sequencing methods were as described.[2] HIV-1 subtype was assigned using the HIV-1 Genotyping Tool at the National Center for Biotechnology Information and confirmed by Maximum Likelihood phylogenetic analysis and *jumping profile hidden Markov model* (*jpHMM*).[3,4]

Spline statistical methods:
The spine was defined using the truncated power function basis with degree 2. The number of spline knots were selected by minimizing the Akaike information criteria (AIC). Region-specific spline effects were were added to the base model after being tested for significance using the AIC. Subject-specific splines were also considered but did not improve model fit or the AIC. Splines models were fit in PROC GLIMMIX.

Flow Cytometry Analysis:
Lymphocyte analysis focused on visits where more than 30 observations were available. Models were fit using restricted maximum likelihood estimation accounting for within subject covariance using unstructured covariance matrices for univariable and multivariable models. All models were adjusted for region and visit.

Study Conduct and Analysis: The study was designed by Merlin Robb, Leigh Anne Eller, Sheila Peel, Robert O'Connell, Mark deSouza, and Nelson Michael. Data were gathered by Hannah Kibuuka, Kathleen Rono, Lucas Maganga, Sorachai Nitayaphan, Eugene Kroon, Somchai Sriplienchan, Samuel Sinei, Fred Sawe, Linda Jagodzinski, Mark Manak, Sodsai Tovanabutra, and Mike Eller. Data analysis was performed by Merlin Robb, Leigh Anne Eller, Andrew Lewandowski, Hao Wu, and Peter Dawson. Merlin Robb, Leigh Anne Eller, Mark Millazo, and Peter Dawson vouch for the data. The paper was written by Merlin Robb, Leigh Anne Eller, Peter Dawson, Jerome Kim and Nelson Michael. The decision to publish the paper was made by Merlin Robb and Nelson Michael.

Table S1: Description of screening, surveillance and acute infection cohorts

	All Sites	Kenya	Tanzania	Uganda overall	Uganda-Female	Uganda MSM	Thailand Overall	Thailand FSW	Thailand MSM	Thailand TG
SCREENING POPULATION										
Volunteers screened	3954	1046	1010	870	681	187	1028	165	444	418
HIV prevalence at screening (%)	28.4	23.0	40.3	32.2	37.0	14.2	19.5	1.3	28.2	17.7
Entry risk criteria for high risk pop										
Received goods for sex (%)	64	80	18	68	70	61	87	88	87	88
Unprot. Sex w/ known HIV+ (%)	17	4	29	14	11	22	19	9	24	18
Unprot Sec w/ 3 or more (%)	50	50	34	66	61	88	51	27	58	53
Reported STI symptoms (%)	61	36	86	69	71	58	54	76	51	49
Lost to follow up once in SBV (%)	30.0	28.4	34.0	29.5	35.1	14.1	29.2	30.5	28.6	30.3
SBV compliance (%)	86.8	95.4	84.7	89.7	90.2	87.9	77.2	81.8	77.6	74.1
ACUTE INFECTIONS										
Total # acute cases	112	28	21	13	9	4	50	2	32	16
Incidence rate (95% CI)	3.3 (2.7-3.95)	2.6 (1.7-3.6)	3.1 (1.8-4.4)	2.0 (0.9-3.1)	1.7 (0.6-2.8)	3.6 (0.7-7.1)	5.1 (3.7-6.5)	0.8 (0.3-2.0)	8.4 (5.5-11.3)	4.5 (2.3-6.7)
Person-Years Follow Up	3362	1060	684	640.3	529	111	977.7	239.8	380.2	357.7
Median age incident cases (range)	23 (18-48)	24 (18-34)	23 (18-34)	24 (18-33)	26 (18-33)	21 (18-24)	23 (18-48)	40 (32-48)	23 (18-35)	22 (18-29)
Entry high risk criteria										
Received goods for sex (%)	77	89	24	85	78	100	90	100	94	81
Unprot. Sex w/ known HIV+ (%)	21	4	29	8	11	0	30	50	31	25
Unprot Sec w/ 3 or more (%)	54	61	43	85	78	100	46	50	47	44
Reported STI symptoms (%)	58	46	91	46	67	0	54	50	59	44

[1]Participants were enrolled if they reported one of the following: 1) vaginal or anal intercourse in exchange for money, goods or services 2) unprotected vaginal or anal intercourse with one or more known HIV-positive partners 3) unprotected vaginal or anal intercourse with three or more partners of known or unknown HIV status 4) new sexually transmitted disease.
STI=Sexually Transmitted Infection; SBV=Small blood volume (twice weekly collections)

Table S2: Acute cohort: Analysis and excluded cohorts

	All cases (112)	n=50 included in primary analysis	n=62 excluded from primary analysis	p value
# Gender (%)	Male: 52 (46) Female: 60 (54)	Male: 19(38) Female: 31(62)	Male: 33(53.2) Female: 29(46.8)	0.13
Median Age (IQR)	23(20-27)	24(20-25)	23(20-27)	0.72
# per site (%)	Kenya: 28 (25) Uganda: 13 (11.6) Tanzania: 21 (18.8) Thailand: 50 (44.6)	Kenya: 16(32) Uganda: 9(18) Tanzania: 8(16) Thailand: 17(34)	Kenya: 12(19.4) Uganda: 4(6.5) Tanzania: 13(21) Thailand: 33(53.2)	0.057
Entry high risk criteria (%)	Received goods for sex: 77 Unprot. Sex w/ known HIV+: 21 Unprot Sec w/ 3 or more: 54 Reported STI symptoms: 58	Received goods for sex: 80 Unprot. Sex w/ known HIV+: 12 Unprot Sec w/ 3 or more: 58 Reported STI symptoms: 50	Received goods for sex: 74 Unprot. Sex w/ known HIV+: 27 Unprot Sec w/ 3 or more: 50 Reported STI symptoms: 65	0.51 0.060 0.45 0.13
Median Viral Load set point ($\log_{10}$ copies/ml)	4.31 (3.80-4.87), n=78	4.35 (3.70-4.83), n=45	4.31 (4.09-4.92), n=33	0.73

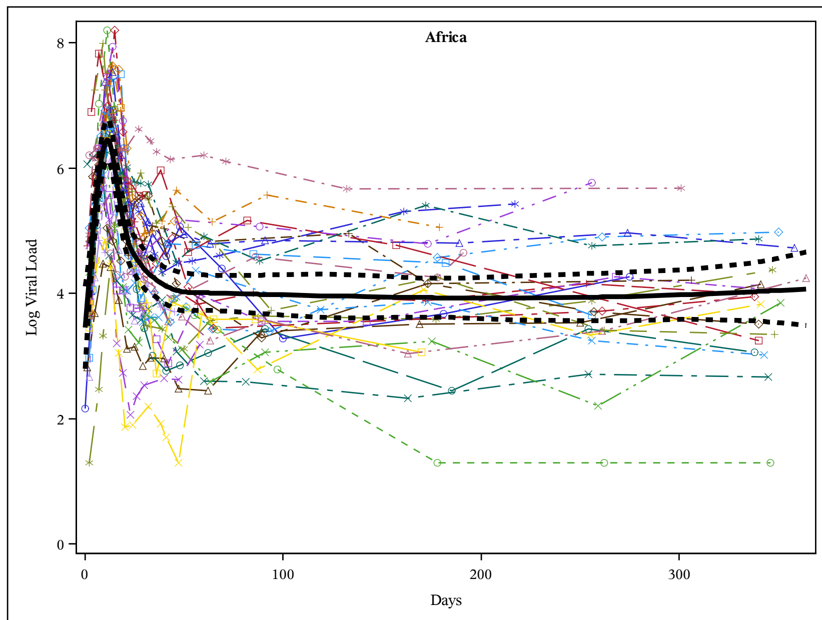
Table S3: Summary quantitative viral RNA statistics

	Mean, Median (IQR)		
	All	**Africa**	**Thailand**
Peak VL (n=50)	6.75, 6.74 (6.24, 7.50)	6.76, 6.76 (6.12, 7.53)	6.73, 6.66 (6.26, 6.91)
Peak Day (n=50)	12.5, 13 (10, 14)	12.2, 13 (10, 14)	13.1, 14 (11, 15)
Nadir VL(n=47)[1]	4.24, 4.33 (3.55, 4.79)	4.03, 4.22 (3.43, 4.71)	4.65, 4.70 (4.14, 5.44)
Nadir Day(n=47) [1]	32.7, 31 (28, 41)	32.1, 31 (27, 38)	33.9, 33 (28, 42)
Set-Point VL (n=45) [2]	4.26, 4.35 (3.7, 4.83)	4.00, 3.99 (3.39,4.50)	4.69, 4.77 (4.41, 5.22)
EIA Reactive Day (n=43) [3]	15.4, 14 (13,18)	15.1, 14 (13-18)	15.4, 14 (13,18)
Up Slope (n=43) [4]	0.40, 0.39 (0.35, 0.43)	0.41, 0.40 (0.36, 0.44)	0.38, 0.38 (0.34, 0.41)
Down Slope (n=49)[5]	-0.14, -0.13 (-0.16, -0.09)	-0.15, -0.14 (-0.18, -0.10)	-0.11, -0.10 (-0.13, -0.08)

[1]2 cases not included due to start of ART and 1 case not included due to missing visits
[2]5 cases not included due to start of ART
[3]7 cases not included due to missing data
[4]7 cases not included due to interval >10 days between last negative and first positive
[5]1 case not included due to missing visit

Figure S1: Viral Load Regional Spline Models

A.



B.

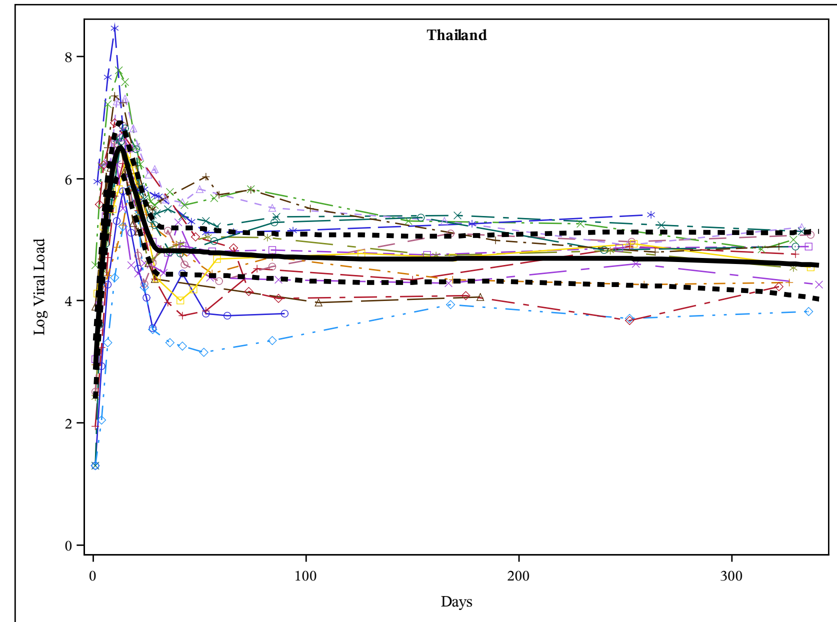


Figure S1: Regression splines with subject-specific slopes and intercepts were fit using the first year of viral load data for each region (panel B and C). Day 0 is defined as the first reactive APTIMA RNA test. The spline was defined using the truncated power function basis with degree 2. The number of spline knots were selected by minimizing the Akaike information criteria (AIC). Region-specific spline effects were added to the base model after being tested for significance using the AIC. Subject-specific splines were also considered, but did not improve model fit or the AIC. Splines models were fit in PROC GLIMMIX of SAS version 9.3.

Table S4: Individual Viral Load Data

Region	peak day	peak vl $\log_{10}$ copies/ml	nadir day $\log_{10}$ copies/ml	nadir vl $\log_{10}$ copies/ml	set point vl $\log_{10}$ copies/ml	Day of last negative	VL Downslope (Rate of Change Peak to Nadir)	VL Upslope (Rate of Change Last Negative to Peak)	Last known Absolute CD4 count (cells/ul)	Absolute CD4 Count 1 year (cells/ul)
Africa	11	7.51	34	4.29	3.97	-21	-0.14		556	771
Africa	12	5.57	42	2.88	3.89	-28	-0.09		594	594
Africa	18	5.49	39	3.43	3.69	-4	-0.10	0.25	618	1035
Africa	10	6.92	35	4.01	4.08	-5	-0.12	0.46	290	514
Africa	14	6.74	42	3.55	3.52	-14	-0.11		746	746
Africa	13	6.62	27	4.59	4.13	-14	-0.15		625	554
Africa	6	6.32	30	4.22	4.35	-162	-0.09		453	
Africa	14	7.63	31	4.94		-4	-0.16	0.42	442	
Africa	14	7.94	32	4.71	5.20	-4	-0.18	0.44	323	323
Africa	9	7.99	36	5.12	4.28	-4	-0.11	0.61	1543	1412
Africa	8	6.25	28	3.57	2.66	-32	-0.13		376	579
Africa	11	8.2	31	4.55	2.52	-4	-0.18	0.55	814	873
Africa	13	6.68	29	3.54	3.70	-4	-0.20	0.39	345	347
Africa	14	7.64	25	4.79	5.35	-4	-0.26	0.42	495	265
Africa	11	6.02	27	3.21	3.01	-5	-0.18	0.38	368	760
Africa	13	6.12	25	3.56		-4	-0.21	0.36	676	
Africa	11	6.54	36	2.72	3.57	-4	-0.15	0.44	788	
Africa	13	6.95	39	4.33	4.87	-4	-0.10	0.41	186	186
Africa	15	8.2	31	4.31	4.02	-4	-0.24	0.43	725	890
Africa	14	6.93	41	2.76	3.04	-4	-0.15	0.39	534	661
Africa	14	6.73	22	4.61	4.54	-4	-0.27	0.37	249	257
Africa	9	5.68	23	2.06	2.90	-21	-0.26		465	
Africa	9	4.47	29	2.84	3.25	-4	-0.08	0.34	669	1041
Africa	17	5.45	38	3.78		-7	-0.08	0.23	935	935
Africa	12	7.57				-3		0.50	509	
Africa	18	7.5	28	3.93	4.38	-3	-0.36	0.36	427	618
Africa	14	6.76	32	5.75	4.83	-3	-0.06	0.40	382	454
Africa	11	7.34	36	6.26	5.96	-10	-0.04	0.35	474	474
Africa	10	4.85	41	1.7	3.16	-3	-0.10	0.37	393	685
Africa	7	6.05	18	4.52	3.77	-10	-0.14	0.36	259	463
Africa	14	7.53	42	4.81	4.85	-4	-0.10	0.42	379	379
Africa	7	7.83	27	5.56	4.46	-7	-0.11	0.56	1018	1021
Africa	18	6.96				-3		0.33	583	
Thailand	15	7.31	42	5.61	5.35	-3	-0.06	0.41	360	421
Thailand	14	5.79	28	3.55	3.78	-3	-0.16	0.34	307	
Thailand	16	6.4	41	4	4.64	-7	-0.10	0.28	180	627
Thailand	10	8.46	31	5.71	5.22	-7	-0.13	0.50	180	180
Thailand	14	6.24	42	3.76	4.47	-5	-0.09	0.33	249	868
Thailand	11	6.66	42	5.38	5.28	-6	-0.04	0.39	283	464
Thailand	13	6.5	29	4.36	4.02	-4	-0.13	0.38	695	
Thailand	14	6.67	35	4.79	4.83	-5	-0.09	0.35	491	462
Thailand	10	7.35	28	5.49	5.39	-3	-0.10	0.57	189	264
Thailand	12	6.49	26	4.62	4.83	-2	-0.13	0.46	343	358
Thailand	17	5.81	42	3.26	3.60	-4	-0.10	0.28	490	433
Thailand	14	6.86	24	4.28	4.77	-5	-0.26	0.36	225	391
Thailand	18	6.09	42	4.78	4.41	-2	-0.05	0.30	257	603
Thailand	10	6.91			4.30	-7		0.41	781	971
Thailand	7	6.26	21	4.45	4.45	-9	-0.13	0.39	431	525
Thailand	15	6.82	41	4.77	5.07	-4	-0.08	0.36	154	356
Thailand	12	7.77	29	5.57	5.35	-7	-0.13	0.41	81	344

Figure S2: Regional viral load correlations

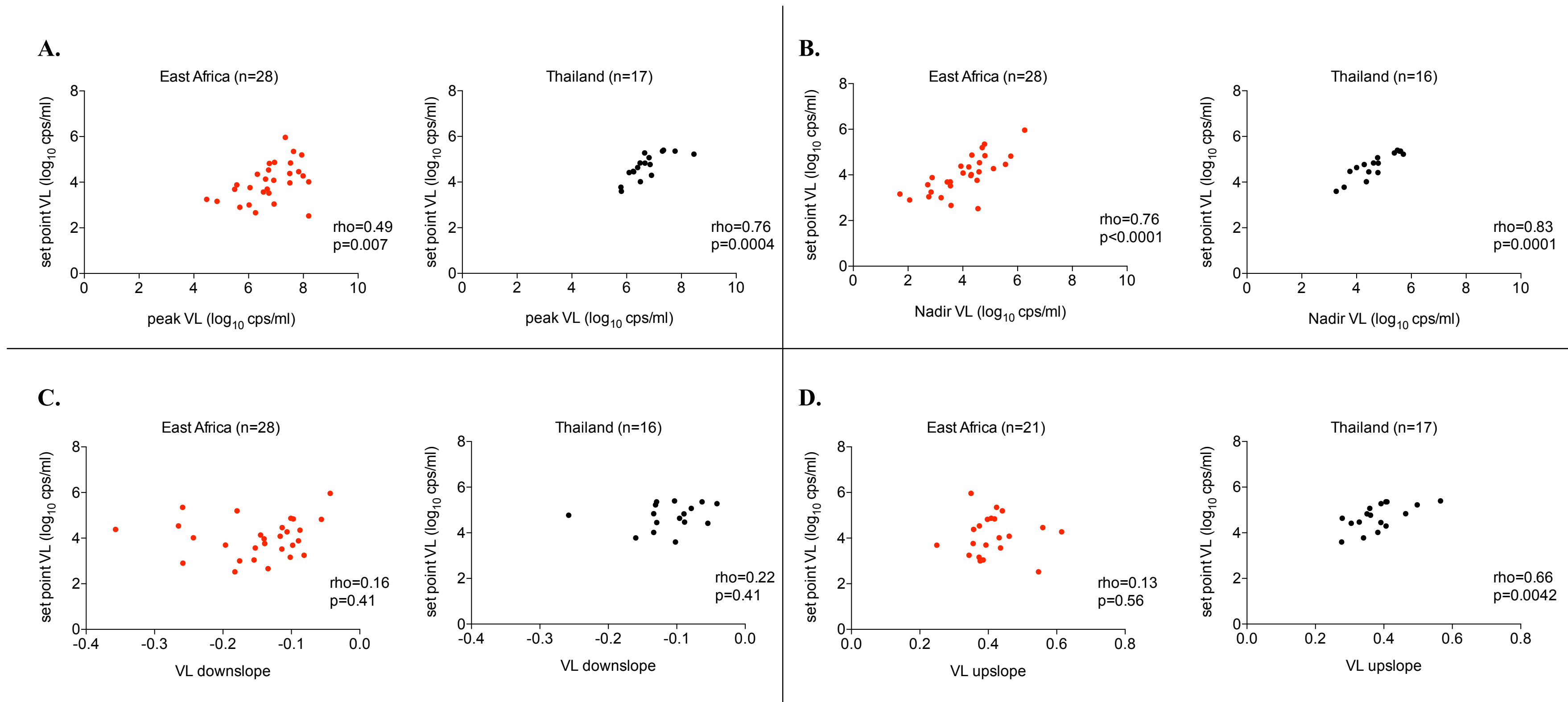


Figure S2: Peak vial load correlated to set point viral load in East Africa (EA) and Thailand (TH) (Panel A). Nadir and set point viral load were strongly correlated in both regions (Panel B). Although downslope correlated to set point in the combined group (Figure 3D, main paper), when separated by region, the correlation was not present in East Africa (EA) or Thailand (Panel C). The correlation of viral load upslope to set point viral load was only significant in Thailand (Panel D).

Figure S3: Immune cell counts over 510 days of follow up by region

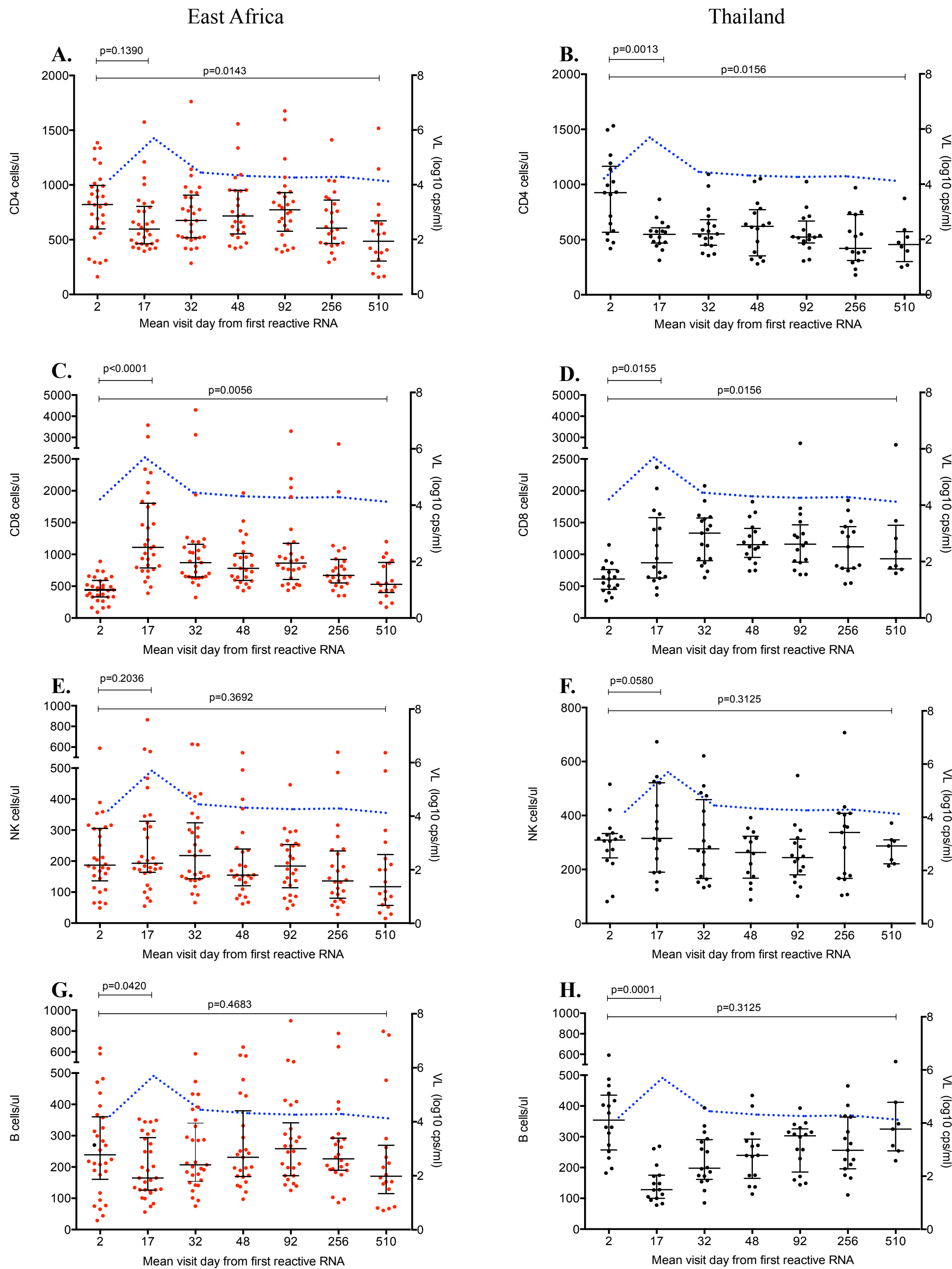


Figure S3: The absolute counts for CD4 (Panel A,B), CD8 (Panel C,D), NK cell (Panel E,F) and B cell (Panel G,H) for 50 subjects were plotted by the mean visit day on the x-axis. African participants (n=32) are shown on the left and Thailand participants (n=18) on the right. The mean viral load at each time point is plotted in blue on the right Y-axis. Wilcoxon Signed-Rank tests were performed on available paired data prior to start of ARV treatment.

Table S5: Frequency of clinical symptoms and signs overall and by region

	Africa (n=31)	Thailand (n=17)	All (n=48)
Symptom:	N(%)	N(%)	N(%)
Fever	18 (55%)	7 (41%)	25 (50%)
Headache	17 (52%)	6 (35%)	23 (46%)
Feeling of Illness	14 (42%)	5 (29%)	19 (38%)
Coughing	10 (30%)	9 (53%)	19 (38%)
Abnormality:			
HEENT**	6 (18%)	16 (94%)	22 (44%)
Lymph*	3 (9%)	16 (94%)	19 (38%)
Tachycardia	11 (33%)	5 (29%)	16 (32%)

***Fisher's Exact p-value < 0.001**
****Fisher's Exact p-value <0.001**

Figure S4A: Heat map-Symptoms reported in medical history

Region	Pre-Infection	Visit 5 Pre-Peak	Visit 4 Pre-Peak	Visit 3 Pre-Peak	Visit 2 Pre-Peak	Visit 1 Pre-Peak	Peak	Visit 1 After Peak	Visit 2 After Peak	Visit 3 After Peak	Visit 4 After Peak	Visit 5 After Peak	Total # Visits Post-infection w/ Symptoms	Total Reported Symptoms Post-infection
Africa	0	.	.	0	0	0	0	0	0	0	0	0	**0**	**0**
Africa	0	.	0	0	0	0	0	0	.	0	0	0	**0**	**0**
Africa	0	.	.	.	0	0	0	0	0	0	0	0	**0**	**0**
Africa	0	.	.	.	0	0	0	0	0	0	0	0	**0**	**0**
Africa	0	.	.	0	0	0	0	0	0	0	0	0	**0**	**0**
Thailand	0	.	0	0	0	0	0	0	0	0	0	0	**0**	**0**
Africa	0	.	.	.	6	.	0	0	0	0	0	0	**1**	**6**
Africa	0	.	.	.	.	3	0	0	0	0	0	0	**1**	**3**
Africa	0	.	.	0	0	.	0	0	0	0	0	4	**1**	**4**
Africa	0	.	.	0	3	0	0	0	0	0	0	0	**1**	**3**
Africa	0	.	.	0	3	0	0	0	0	0	0	0	**1**	**3**
Africa	0	.	.	0	1	0	0	.	0	0	0	0	**1**	**1**
Africa	0	.	.	.	0	1	0	0	0	0	0	0	**1**	**1**
Africa	0	.	0	4	0	0	0	0	0	0	0	0	**1**	**4**
Africa	0	.	.	.	.	0	0	0	0	4	0	0	**1**	**4**
Thailand	0	.	.	.	.	2	0	0	0	0	0	0	**1**	**2**
Africa	0	.	.	0	6	1	0	0	.	0	0	0	**2**	**7**
Africa	0	.	.	.	1	0	2	0	0	0	0	.	**2**	**3**
Africa	1	.	.	.	1	1	0	0	0	0	0	0	**2**	**2**
Africa	3	.	.	.	9	0	0	0	0	0	0	1	**2**	**10**
Africa	0	.	.	.	0	9	1	0	0	0	0	0	**2**	**10**
Africa	0	.	.	0	0	11	3	0	0	0	0	0	**2**	**14**
Africa	0	1	0	1	0	0	0	0	0	0	.	0	**2**	**2**
Africa	0	.	.	.	.	5	8	.	.	0	0	0	**2**	**13**
Africa	0	.	4	.	2	0	0	.	.	.	.	.	**2**	**6**
Thailand	0	.	0	0	0	1	1	0	0	0	0	0	**2**	**2**
Thailand	2	.	.	.	0	1	2	0	0	0	0	0	**2**	**3**
Thailand	0	.	.	0	0	0	3	1	0	0	0	0	**2**	**4**
Thailand	6	.	.	.	0	7	3	0	0	.	0	0	**2**	**10**
Thailand	0	.	.	0	0	0	1	1	0	0	0	0	**2**	**2**
Africa	0	.	.	.	0	2	17	0	1	0	0	0	**3**	**20**
Africa	0	.	.	0	2	1	5	.	.	.	.	.	**3**	**8**
Africa	0	.	.	0	1	4	0	0	0	0	0	7	**3**	**12**
Thailand	0	.	.	.	1	3	5	.	.	.	.	.	**3**	**9**
Africa	0	.	.	.	5	0	0	0	3	0	1	0	**3**	**9**
Africa	0	.	.	2	0	1	2	2	0	0	0	0	**4**	**7**
Africa	3	.	.	0	0	15	2	0	2	0	1	0	**4**	**20**
Africa	1	.	.	.	2	1	4	0	1	0	0	0	**4**	**8**
Thailand	1	.	2	0	0	4	1	0	0	1	0	0	**4**	**8**
Thailand	0	3	2	1	0	0	0	2	0	0	0	0	**4**	**8**
Thailand	0	.	0	0	0	6	3	2	0	2	0	0	**4**	**13**
Thailand	0	.	.	0	4	2	1	1	1	0	0	0	**5**	**9**
Thailand	0	1	0	0	2	3	1	0	1	0	0	0	**5**	**8**
Africa	0	.	.	.	.	5	10	1	0	2	0	1	**5**	**19**
Africa	0	.	.	.	5	2	3	1	1	0	2	0	**6**	**14**
Africa	0	.	.	.	0	1	2	1	1	0	1	1	**6**	**7**
Thailand	0	.	1	1	0	1	1	1	1	0	1	0	**7**	**7**
Thailand	0	.	1	0	4	2	1	0	1	1	1	0	**7**	**11**
Thailand	0	.	2	2	3	1	0	0	1	2	1	1	**8**	**13**
Thailand	0	.	.	0	1	7	1	4	1	1	1	1	**8**	**17**
Average # Symptoms per Visit	**0.34**	**1.7**	**0.92**	**0.39**	**1.4**	**2.1**	**1.7**	**0.38**	**0.34**	**0.28**	**0.2**	**0.35**		

Figure S4B: Heat map-Physical Exam findings

Region	Pre-Infection	Visit 5 Pre-Peak	Visit 4 Pre-Peak	Visit 3 Pre-Peak	Visit 2 Pre-Peak	Visit 1 Pre-Peak	Peak	Visit 1 After Peak	Visit 2 After Peak	Visit 3 After Peak	Visit 4 After Peak	Visit 5 After Peak	Total # Visits Post-infection w/ Physical Abnormalities	Total Physical Abnormalities Post-Infection
Africa	0	.	.	0	0	0	0	0	0	0	0	0	0	0
Africa	0	.	0	0	0	0	0	0	.	0	0	0	0	0
Africa	0	.	.	.	0	0	0	0	0	0	0	.	0	0
Africa	0	.	.	.	0	0	0	0	0	0	0	0	0	0
Africa	0	.	.	.	.	0	0	0	0	0	0	0	0	0
Africa	0	.	.	0	0	.	0	0	0	0	0	0	0	0
Africa	0	.	.	.	0	0	0	0	0	0	0	0	0	0
Africa	0	.	.	.	.	0	0	0	0	0	0	0	0	0
Africa	0	.	.	0	0	0	0	0	0	0	0	0	0	0
Africa	0	.	.	.	.	0	0	.	.	0	0	0	0	0
Africa	0	.	0	.	0	0	0	.	.	.	.	.	0	0
Africa	0	.	.	0	1	0	0	0	.	0	0	0	1	1
Africa	0	.	.	.	1	.	0	0	0	0	0	0	1	1
Africa	0	.	.	.	0	0	0	1	0	0	0	0	1	1
Africa	0	.	.	.	0	0	0	0	1	0	0	0	1	1
Africa	1	.	0	3	0	0	0	0	0	0	0	0	1	3
Africa	0	.	.	0	0	1	0	0	0	0	0	0	1	1
Thailand	0	.	0	0	1	0	0	0	0	0	0	0	1	1
Africa	0	.	.	0	0	0	1	1	0	0	0	0	2	2
Africa	0	.	.	1	1	0	0	0	0	0	0	0	2	2
Africa	0	.	.	1	0	2	0	0	0	0	0	0	2	3
Africa	0	.	.	0	0	1	1	.	.	.	.	.	2	2
Africa	0	0	0	1	0	0	0	0	0	0	.	1	2	2
Africa	0	.	.	.	0	0	0	0	1	0	1	0	2	2
Africa	1	.	.	.	.	1	2	0	0	1	0	0	3	4
Africa	0	.	.	.	1	1	0	1	0	0	0	0	3	3
Africa	1	.	.	.	0	2	1	1	0	0	0	0	3	4
Thailand	1	.	.	.	2	3	3	.	.	.	.	.	3	8
Africa	0	.	.	0	1	0	0	0	1	1	0	0	3	3
Africa	0	.	.	.	2	0	2	1	1	0	0	0	4	6
Thailand	0	.	1	0	0	1	1	0	1	0	0	0	4	4
Thailand	0	.	1	0	0	2	2	0	0	0	0	1	4	6
Thailand	0	1	0	0	2	1	0	0	0	1	0	0	4	5
Africa	0	.	.	0	0	3	1	2	1	0	1	0	5	8
Thailand	0	.	1	0	0	1	2	2	1	0	0	1	6	8
Thailand	1	.	.	.	1	1	2	1	1	.	1	0	6	7
Africa	1	.	.	.	3	2	0	0	3	2	1	1	6	12
Africa	0	.	.	.	2	1	2	1	3	1	0	0	6	10
Africa	0	.	.	.	2	0	2	1	1	1	1	2	7	10
Thailand	0	.	.	.	2	1	2	1	1	1	0	1	7	9
Thailand	1	.	.	.	.	1	1	1	1	1	1	1	7	7
Africa	0	.	.	1	1	2	1	2	2	0	2	2	8	13
Thailand	0	.	0	0	1	2	2	1	2	2	2	1	8	13
Thailand	0	.	0	2	0	2	2	2	1	1	2	1	8	13
Thailand	1	.	.	1	2	1	2	2	2	1	1	1	9	13
Thailand	0	.	.	1	1	1	1	1	1	1	1	1	9	9
Thailand	1	.	.	1	2	2	2	2	1	1	1	2	9	14
Thailand	0	.	.	1	1	1	1	1	1	2	1	1	9	10
Thailand	2	.	1	3	2	2	1	1	1	2	2	2	10	17
Thailand	0	2	2	2	1	1	1	2	2	1	1	1	11	16
Average # of Abnormalities per visit	0.22	1	0.46	0.64	0.73	0.81	0.76	0.61	0.68	0.43	0.41	0.43		

Figure S4A and S4B: The number of symptoms reported in the medical history (Figure S4A) and physical findings on exam (Figure S4B) at each visit is shown for each participant. The visit for each participant where peak viral load occurred is shown at the center of the table and the visits preceding peak viral load are to the left and visits subsequent to peak viral load are on the right. The Pre column indicates the number of reported symptoms for each participant at a pre-infection visit. The total number of findings and visits with findings are shown on the right for each participant.

References:

1. Salazar-Gonzalez JF, Bailes E, Pham KT, et al. Deciphering human immunodeficiency virus type 1 transmission and early envelope diversification by single-genome amplification and sequencing. Journal of virology 2008;82:3952-70.
2. Heipertz RA, Jr., Sanders-Buell E, Kijak G, et al. Molecular epidemiology of early and acute HIV type 1 infections in the United States Navy and Marine Corps, 2005-2010. AIDS research and human retroviruses 2013;29:1310-20.
3. Rozanov M, Plikat U, Chappey C, Kochergin A, Tatusova T. A web-based genotyping resource for viral sequences. Nucleic acids research 2004;32:W654-9.
4. Schultz AK, Zhang M, Bulla I, et al. jpHMM: improving the reliability of recombination prediction in HIV-1. Nucleic acids research 2009;37:W647-51.

Performance of urine lipoarabinomannan assays for paediatric tuberculosis in Tanzania

Inge Kroidl[1,3,11], Petra Clowes[3,11], Klaus Reither[4,5], Bariki Mtafya[3], Gabriel Rojas-Ponce[3], Elias N. Ntinginya[3], Mariam Kalomo[6], Lilian T. Minja[3,4,5,7], Dickens Kowuor[3], Elmar Saathoff[1,2], Arne Kroidl[1,2,3], Norbert Heinrich[1,2], Leonard Maboko[3], Matthew Bates[8,9,10], Justin O'Grady[8,9,10], Alimuddin Zumla[8,9,10], Michael Hoelscher[1,2,3] and Andrea Rachow[1,2,3]

Affiliations: [1]Division of Infectious Diseases and Tropical Medicine, Medical Centre of the University of Munich (LMU), Germany. [2]German Centre for Infection Research (DZIF), partner site Munich, Germany. [3]National Institute for Medical Research–Mbeya Medical Research Centre, Mbeya, Tanzania. [4]Medical Services and Diagnostic, Swiss Tropical and Public Health Institute, Basel, Switzerland. [5]Medical Services and Diagnostic (Swiss TPH), University of Basel, Basel, Switzerland. [6]Dept for Paediatrics and Child Health, Mbeya Referral Hospital, Mbeya, Tanzania. [7]Ifakara Health Institute, Bagamoyo, Tanzania. [8]University of Zambia-University College London Medical School Research and Training Project, University Teaching Hospital, Lusaka, Zambia. [9]Division of Infection and Immunity, University College London, London, UK. [10]NIHR Biomedical Research Centre, University College London Hospitals, London, UK. [11]Both authors contributed equally.

Correspondence: Inge Kroidl, Division of Infectious Diseases and Tropical Medicine, Medical Centre of the University of Munich (LMU), Germany. E-mail: ikroidl@lrz.uni-muenchen.de

ABSTRACT We evaluated the diagnostic performance of two tests based on the release of lipoarabinomannan (LAM) into the urine, the MTB-LAM-ELISA assay and the Determine TB-LAM-strip assay, in children with suspected tuberculosis (TB) in a high TB/HIV-prevalence setting.

In a prospective study, 132 children with suspected active TB were assigned to diagnostic subgroups. Urine samples were subjected to testing by both assays to ascertain sensitivity and specificity. Host factors associated with positive LAM results were investigated and LAM excretion monitored after antituberculous treatment initiation.

18 (13.6%) children had culture-confirmed pulmonary TB. The assays' sensitivity was higher in HIV-positive *versus* HIV-negative children: 70% (95% confidence interval 35–93%) *versus* 13% (0–53%) for MTB-LAM-ELISA and 50% (19–81%) *versus* 0% (0–37%) for Determine TB-LAM. In 35 (27%) children with excluded active TB, both assays showed a specificity of 97.1% (85–100%). Proteinuria and low body mass index were independently associated with LAM positivity. In most patients, LAM excretion declined to zero during or at conclusion of antituberculous treatment.

HIV/TB co-infected children might benefit from LAM-based tests to aid early TB diagnosis and subsequent positive impact on morbidity and mortality. Using LAM as a rule-in and treatment-monitoring tool may also show further potential.

@ERSpublications
Urine lipoarabinomannan assays show reasonable sensitivity in HIV+ but not HIV− TB-infected children http://ow.ly/N56aG

This article has supplementary data available from erj.ersjournals.com

Earn CME accreditation by answering questions about this article. You will find these at erj.ersjournals.com/misc/cmeinfo.xhtml

Received: Jan 09 2015 | Accepted after revision: April 09 2015 | First published online: June 25 2015

Support statement: This study was supported by Bundesministerium für Bildung und Forschung and the European Commission as part of EuropAid. Funding information for this article has been deposited with FundRef.

Conflict of interest: None declared.

Copyright ©ERS 2015

Introduction

The diagnosis of tuberculosis (TB) is usually established by detecting mycobacteria, either through smear microscopy or culturing methods, which is considered the current gold standard. However, due to the paucibacillary nature of TB in children, the microbiological diagnosis is extremely difficult. Smear microscopy is positive in <15% [1–3] and mycobacterial culture is positive in 20–80% of children with presumed TB [1, 2, 4]. Paediatric TB diagnosis is therefore mainly based on clinical assessments, scoring systems and radiological findings, which can be erroneous. In an autopsy study from Zambia, TB accounted for 18% of deaths in HIV co-infected and 26% of deaths in HIV-negative children with respiratory illnesses [5]. In contrast, during a recent review of the global burden of disease in children [6, 7], TB was not listed among the most common causes of paediatric deaths, demonstrating the low number of microbiologically confirmed cases.

For TB cases in clinical settings, the inconsistency between pathological and epidemiological data highlights the need for new and more accurate methods to diagnose TB in children, using non-invasive clinical samples. Lipoarabinomannan (LAM) detection in urine for TB diagnosis was first investigated in the late 1990s [8–10]. LAM is a 19 kD (±8.5 kD) lipopolysaccharide, specific to the cell wall of members of the *Mycobacterium* genus and is released from metabolically active or degrading bacterial cells [11, 12]. It can subsequently be detected in urine and other body fluids. Advantages of urine LAM diagnosis include the ease of specimen collection, short bench-time, low cost and relatively low training and set up requirements [13, 14]. Ideally the LAM-strip assay can be even performed as a point-of-care test in remote settings. The reported sensitivity of the different LAM diagnostic tests in adults ranges between 13% [15] and 67% [16], with best performance in patients with advanced HIV disease [15–18]. Data on children are scarce with only one study published previously on performance of LAM diagnostic tests in children [19].

The two diagnostic assays evaluated here, the MTB-LAM-ELISA (Chemogen, Portland, OR, USA) and the Determine TB-LAM (Alere, Waltham, MA, USA), have been studied recently in South African children, for whom they performed poorly [19]. We evaluated the diagnostic performance of both assays in Tanzanian children with presumed TB and a high HIV co-infection rate. We also investigated host factors that might be related to LAM performance, as well as the change in LAM excretion during the course of anti-TB treatment.

Methods

Study design and setting

We undertook a prospective observational study in children presenting with suspected TB at the outpatient department of the Mbeya Zonal Referral Hospital (Mbeya, Tanzania). The study was coordinated by the National Institute for Medical Research (NIMR) Mbeya Medical Research Centre (MMRC), in close collaboration with the Mbeya Zonal Referral Hospital. The study was approved by the ethics committee of the Tanzania National Institute for Medical Research and the local Mbeya Medical Research and Ethics Committee. Written informed consent for all children was obtained from an accompanying parent or a legal guardian. In addition, children aged ⩾9 years signed an assent form.

Clinical study procedures

From May 2008 till November 2010, we approached all children with suspected TB attending the outpatient clinic and invited them to take part in the study. Inclusion criteria were 6 weeks to 14 years of age, and at least one of the following symptoms: persistent unremitting cough for >21 days; repeated episodes of fever within the last 21 days; weight loss or failure to thrive within the previous 3 months. Children who had received antituberculous treatment within the last 3 months were excluded from the study. Recruitment procedures, baseline diagnostics, physical assessment and clinical treatment of this cohort have previously been described [20]. Follow-up visits were scheduled at 1, 3, 6 and 12 months after enrolment or after antituberculous treatment initiation. For this evaluation, children were retrospectively assigned to distinct diagnostic subgroups, based on the recently proposed classification by Graham *et al.* [21] (fig. 1).

A decision on antituberculous treatment initiation was made in liaison with the paediatric department of the Mbeya Zonal Referral Hospital and the District TB and Leprosy Coordinators and was based on microbiological and clinical findings (including tuberculin skin test and chest radiography), and previous medical history. Antituberculous treatment was administered following Tanzanian National Guidelines and patients diagnosed with HIV infection were referred for further staging and treatment to the relevant HIV Care and Treatment Centres.

Sample collection and laboratory procedures

Up to three induced sputum samples were collected from each child at baseline and processed for smear microscopy and *Mycobacterium tuberculosis* (MTB) culture [20]. Additionally, the Xpert MTB/rifampicin

 DOI: 10.1183/09031936.00003315

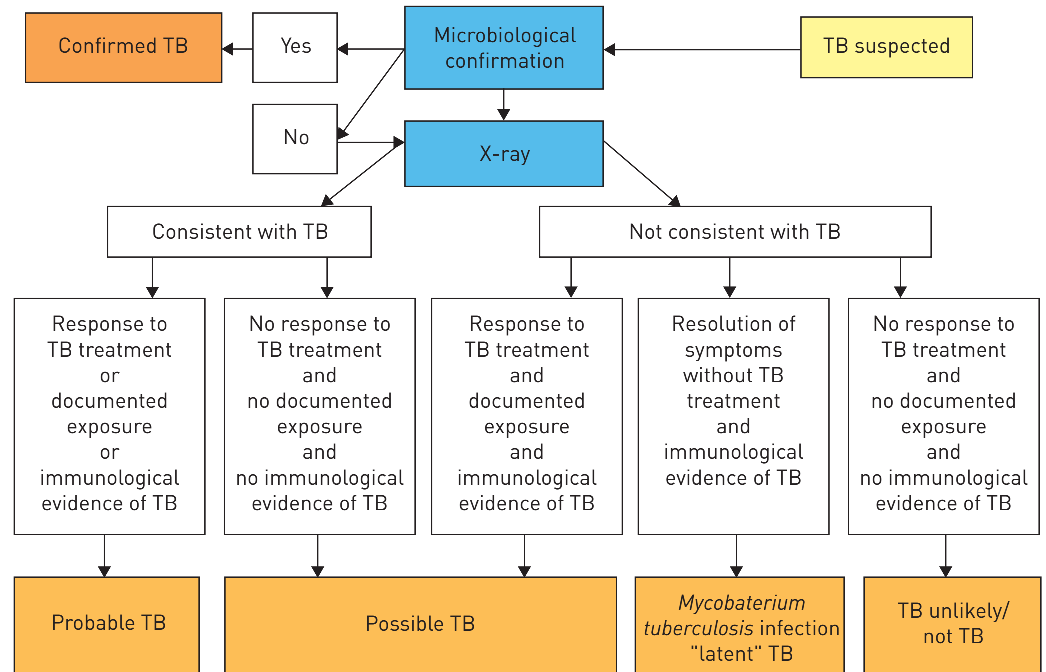


FIGURE 1 Algorithm for diagnostic classification. Diagnostic classification for children presenting with suspected pulmonary/intrathoracic tuberculosis (TB). In total, 379 sputum samples were analysed, 122 children gave three sputa, six children only two and three children only one sputum sample. One child classified as "No TB", gave no sputum sample. In five children with signs of extrapulmonary TB, one pleural fluid sample, one ascites sample and three lymph node aspirates were analysed. *Mycobacterium tuberculosis* was confirmed in the pleural fluid and in one lymph node aspirate. Both children were additionally diagnosed with sputum culture confirmed TB. All 132 children received chest radiography examination which was assessed by a radiologist and two clinical study investigators who were blinded to clinical and microbiological data. In case of discrepant readings a consensus radiography diagnosis was made after discussion among the investigators. Clinical follow-up of all analysed children allowed assessment of treatment response in those who received antituberculous treatment.

(RIF) assay (Cepheid, Sunnyvale, CA, USA) was performed on stored, frozen sputum samples as reported previously [20]. The results were not included in the diagnostic algorithm as all children were enrolled before Xpert endorsement by the World Health Organization (WHO). At each study visit, a urine sample was collected for LAM testing, either midstream urine when possible or with a collection bag in younger children. Within 8 h of collection, all urine was boiled at 95–100°C for 30 min, centrifuged and the supernatants frozen at −20°C. For the execution of the MTB-LAM-ELISA assay, the thawed urine was processed in duplicate, according to the manufacturer's instructions as described previously [18]. For the Determine TB-LAM assay, 60 µL of pre-treated urine was applied onto the sample pad of the LAM strip. After 25 min, the test band colour intensity was compared to the colour intensity of a series of bands on a paper reference card supplied by the manufacturer. Grade 1 colour intensity and above were defined as positive results, consistent with the manufacturer's instructions at the time of the study [22]. According to the manufacturer (personal communication) and our own data, results for the Determine TB-LAM assay do not differ between pre-treated (boiled and centrifuged) or native urine samples. All lab staff performing LAM tests was blinded to culture results and clinical data. Furthermore, all urine samples underwent testing with urine dipsticks (Combur-Test; Roche, Basel, Switzerland) for detection of protein, glucose, leukocytes and erythrocytes.

All participants were screened for HIV, using the HIV1/2 STAT-PAK RDT (Chembio Diagnostics Systems, Medford, NY, USA) and following the manufacturer's instructions. RDT results were confirmed with a third generation ELISA (Biorad Laboratories, Redmond WA, USA) and, in case of discordance, retested by Western Blot (MPD HIV Blot 2.2, MP Biomedicals, Geneva, Switzerland). For children below the age of 2 years a PCR (Roche Amplicor) was performed instead of ELISA. In HIV-positive participants CD4 count and HIV viral load were determined by flow cytometry and PCR (Amplicor; Roche). HIV-positive children were classified according to the WHO classification of HIV-associated immunodeficiency in infants and children (see the online supplementary data) [23].

Statistical analysis

In contrast to the classification in the original study, all children were re-classified into five different diagnostic groups in order to comply with the recently published proposed consensus of paediatric clinical

case definition by Graham *et al.* [21]. Statistical analyses were performed using Stata statistics software (version 12; Stata Corp., College Station, TX, USA). The sensitivity, specificity and their respective confidence intervals were calculated using the "diagt" command in Stata. Pearson's Chi-squared test was used to compare binominal variables between groups (confirmed TB *versus* no-TB or HIV-positive *versus* HIV-negative) and the non-parametric Wilcoxon rank sum test was used to compare selected baseline characteristics of continuous variables, since none of the continuous variables was normally distributed. The correlation of optical density values and grading of MTB-LAM ELISA and Determine TB-LAM assays were compared by Spearman rank correlation. Univariable and multivariable log link binomial regression analyses, using robust variance estimates, were performed to examine the influence of potentially important factors on LAM positivity.

Results

Between May 2008 and November 2010, 180 children with presumed intrathoracic TB were enrolled into the study. Due to an incomplete data set, 48 study subjects were excluded from this analysis. In the majority of excluded children, 37 (77%) out of 48, no urine sample was collected at baseline. As depicted in figure 2, all diagnostic groups were equally affected by this exclusion criterion. The remaining 132 children were assigned to one of five distinct diagnostic classification groups in line with the definitions by Graham *et al.* [21] (figs. 1 and 2). Antituberculous treatment was offered to all children with confirmed and probable TB and to more than half of the children with possible TB, depending on their clinical and radiological presentation. Five children initially received antituberculous treatment, but a different diagnosis was later established. 14 (10.5%) children who demonstrated immunological evidence of TB at baseline, but improved without antituberculous treatment, were classified with MTB infection. Overall, a decision for antituberculous treatment was made for 80 (61%) of the 132 children. 69 of all recruited

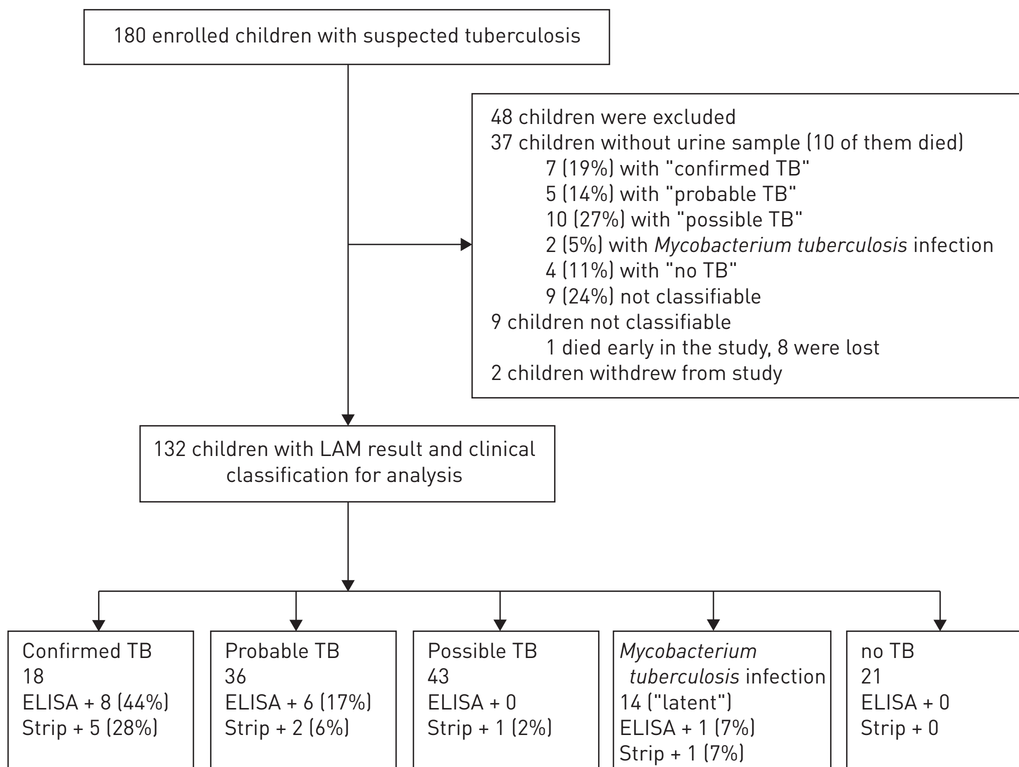


FIGURE 2 Study flow chart with diagnostic classification of participants. The number of *Mycobacterium tuberculosis*-lipoarabinomannan (LAM)-ELISA positive urines (ELISA +) and the number of Determine TB LAM-Strip positive urines (STRIP +) is given for each clinical diagnostic group. Confirmed TB: 18 children had microbiological confirmation of *M. tuberculosis* infection. Probable TB: tuberculosis could not be microbiologically confirmed, but was highly probable in 36 children and antituberculous treatment was offered to all of them. Possible TB: tuberculosis could not be reliably excluded in 43 children, but they did not fulfil criteria for any other group 24 (56%) of the children with possible tuberculosis received tuberculosis treatment. MTB-infection: 14 children demonstrated immunologic evidence of TB, but improved without antituberculous treatment. None of them died. No TB: In 21 children, tuberculosis was retrospectively reliably excluded. Five children initially received antituberculous treatment, but subsequently an alternative diagnosis was established. The tuberculin skin test was non-reactive in all children, all recovered during the follow-up.

DOI: 10.1183/09031936.00003315

children were hospitalised at or during enrolment, 42 of whom were included in this analysis. Demographical, radiological and clinical data of all children and differences in parameters per group at the time of enrolment are displayed in table 1. Apart from the clinical and radiological data presented in table 1, no further data on extra-thoracic TB disease spectrum were systematically collected.

Out of the group of culture-confirmed TB, eight TB-cases were identified by the MTB-LAM-ELISA and five by the Determine TB-LAM-strip assay, resulting in a sensitivity of 44% (95% CI 22–69%) and 28% (95% CI 10–54%), respectively (table 2). Among the children with a strong clinical suspicion of TB, six and two (additional) children were flagged by the MTB-LAM-ELISA and the Determine TB-LAM-strip assay, respectively. In a combined approach, including children of both groups, the sensitivity was 26% (95% CI 15–40%) and 13% (95% CI 5–25%), respectively. Concerning specificity, none of the LAM-assays was positive in the group of children where TB was reliably excluded, resulting in a specificity of 100% (95% CI 84–100%) at baseline evaluation. One child with MTB infection had positive LAM diagnostic tests. If this diagnostic group is included in the calculation, the overall specificity was 97.1% (95% CI 85–100%) for both tests. In the direct comparison of both LAM-assays, the MTB-LAM-ELISA detected 14 of the 54 children with confirmed or probable TB, whereas the Determine TB-LAM-strip assay only identified 7 of those children.

The overall HIV prevalence in our study cohort was 51%. In children with confirmed TB, the sensitivity of both LAM diagnostic tests was significantly higher in HIV-positive compared with HIV-negative children: 70% (95% CI 35–93%) *versus* 13% (95% CI 0–53%) were detected by the MTB-LAM-ELISA and 50% (95% CI 19–81%) *versus* 0% (95% CI 0–37%) by the Determine TB-LAM assay (table 2). The comparison of the performance of both LAM-assays in children with advanced or severe immunosuppression *versus* those with mild or no immunosuppression does however not suggest a higher sensitivity of LAM diagnostics in those with advanced HIV infection.

Employing binominal regression analysis, we found an independent and strong association between proteinuria and LAM positivity for the MTB-LAM-ELISA (table 3). No other urine-associated factors such as haematuria, leukocyturia, specific weight or glycosuria could be linked with a positive LAM result (data not shown). Testing the influence of additional host factors, we found that a positive MTB-LAM-ELISA was independently associated with a low body mass index (BMI) for age and with higher mortality (table 3).

Similarly, a significant association of the Determine TB-LAM with low BMI was found. Trends for increased risk were demonstrated for concomitant HIV infection and proteinuria, but did not reach significance (table 4). No significant influence of age or sex on test positivity could be demonstrated for either assay.

In the per-sample analysis of the 16 urine samples identified as positive by one of the assays at baseline, the quantitative readouts of the Determine TB-LAM (grade 1–5) and the LAM ELISA optical density measurements showed a good correlation, reflected by a Spearman rank correlation of rho=0.79, p=0.0003 (data not shown). Seven out of eight positive Determine TB-LAM tests were graded 2 or above, the remaining test was grade 1, which was a positive result at the time of testing.

In 13 out of 14 children with confirmed or probable TB and a positive LAM result at baseline, both LAM assays were performed at follow up visits. Figure 3 shows the general decline of signal positivity for the MTB-LAM-ELISA at different time points after starting antituberculous treatment. Overall, signal intensity reached zero after 3 months of antituberculous treatment in six participants with MTB-LAM-ELISA-positive urine samples at baseline. Only one child excreted measurable LAM more than 7 month after antituberculous treatment started. Clinically, all children responded well to treatment and were considered cured after 6 months of therapy. The same trend towards a major decline of signal positivity during antituberculous treatment was seen for the Determine TB LAM assay (data not shown).

In the HIV-infected subgroup with confirmed TB diagnosis, both LAM-diagnostic tests demonstrated a better sensitivity than smear microscopy, which detected only 30% of all HIV-positive confirmed TB-cases (fig. 4). The combination of smear microscopy and Determine TB LAM strip led to a combined sensitivity of 60% (fig. 4) and smear microscopy plus MTB-LAM-ELISA amounted to a sensitivity of 80%. Combining the Xpert MTB/RIF assay and any of the LAM assays led to an overall sensitivity of 90% amongst these children, as both LAM-tests detected one confirmed TB case which was negative in the Xpert MTB/RIF-assay (fig. 4).

Discussion

We evaluated the diagnostic performance of the MTB-LAM-ELISA and the new and easier-to-use Determine TB-LAM strip test in a paediatric cohort from a resource limited setting in Tanzania with high TB and HIV burden. In line with studies on adults [15, 16, 18, 24], both assays showed a poor sensitivity when compared to MTB culture, which increased significantly in children with HIV co-infection. However, contrary to reports on adults [15, 16, 25], a positive correlation between advanced immunosuppression and increased sensitivity of the LAM-tests could not be confirmed by our paediatric data. Interestingly, Lawn *et al.* [26] reported that

TABLE 1 Baseline characteristics of children in different diagnostic classes

	Included children	Excluded children[#]	Confirmed TB	Probable TB	Possible TB	MTB infection	No TB	p-value[¶]
Subjects n	132	45	18	36	43	14	21	
Sex male[+]	70 (53)	22 (50)	7 (39)	19 (53)	22 (51)	8 (57)	14 (67)	0.083
Age years	6.8 (3.9–9.5)	2.1 (0.7–5.5)	7.3 (4.8–11.5)	6.8 (3.9–9.4)	7.2 (3.9–10.0)	5.2 (2.6–8–8)	5.9 (3.9–10.1)	0.383
BMI for age z-score	−0.44 (−1.5–0.4)	−1.35 (−3.1–0.1)	−1.21 (−2.6–0.1)	−0.11 (−1.2–0.6)	−0.96 (−2.0–0.2)	0.56 (−0.3–1.1)	−0.49 (−1.2–0.4)	0.105
TST reactive	44 (36)	11 (31)	10 (59)	18 (55)	5 (12)	11 (92)	0	<0.001
Proteinuria >30 mg[§]	13 (10)	3 (20)	4 (22)	4 (11)	4 (10)	0	1 (5)	0.104
Mortality	11 (8)	11 (24)	0	3 (8)	8 (19)	0	0	
Days to treatment	21 (7–58)	15 (5–40)	9 (6–25)	18 (7–44)	31 (13–76)	N/A	40 (6–61)	0.307
HIV-positive	67 (51)	19 (53)	10 (56)	18 (50)	22 (51)	4 (29)	13 (62)	0.688
In HIV-positive children:								
ART at baseline	17 (25)	3 (16)	1 (10)	5 (28)	7 (32)	0	4 (31)	0.231
No significant immunosuppression[f]	18 (27)	1 (6)	1 (10)	5 (28)	5 (24)	1 (25)	6 (46)	0.062
Mild immunosuppression[f]	5 (8)	1 (6)	2 (20)	0	2 (10)	0	1 (8)	0.385
Advanced immunosuppression[f]	8 (12)	0	3 (30)	2 (11)	1 (5%)	1 (25)	1 (8)	0.162
Severe immunosuppression[f]	35 (53)	15 (88)	4 (40)	11 (61)	13 (62)	2 (50)	5 (38)	0.940

Data are presented as n (%) or median (interquartile range), unless otherwise stated. Site of tuberculosis for children with confirmed tuberculosis (TB): two perihilar infiltrate, nine hilar lymphadenopathy, three tuberculous bronchopneumonia, four tuberculous pleural effusion. Site of tuberculosis for children with probable TB: nine perihilar infiltrate, nine hilar lymphadenopathy, seven tuberculous bronchopneumonia, three tuberculous pleural effusion, three military TB, one cavitating pulmonary TB. MTB: *Mycobacterium tuberculosis*; BMI: body mass index; TST, tuberculin skin test; ART: antiretroviral therapy. **#**: for three of the 48 excluded children no further clinical data were available; ¶: p-value for comparison between children with confirmed TB and no TB using Pearson's Chi-squared test for binary and the Wilcoxon rank-sum test for continuous variables; **+:** for one (excluded) child no sex information was available; **§**: for two included children no urine dipstick result was available; *f*: for one included child no CD4 count was measured and the level of immunosuppression could not be calculated.

DOI: 10.1183/09031936.00003315

TABLE 2 Diagnostic performance of both lipoarabinomannan (LAM) assays

	Sensitivity#			Specificity¶	
	Confirmed TB	**Probable TB**	**Combined TB diagnosis**	**TB excluded**	**Combined TB excluded and TB infection**
All children					
ELISA	8/18 (44)	6/36 (17)	14/54 (26)	21/21 (100)	34/35 (97)
Determine strip	5/18 (28)	2/36 (6)	7/54 (13)	21/21 (100)	34/35 (97)
HIV-negative children					
ELISA	1/8 (13)	2/18 (11)	3/26 (12)	8/8 (100)	18/18 (100)
Determine strip	0/8 (0)	1/18 (6)	1/26 (4)	8/8 (100)	18/18 (100)
HIV-positive children					
ELISA	7/10 (70)	4/18 (22)	11/28 (39)	13/13 (100)	16/17 (94)
Determine strip	5/10 (50)	1/18 (6)	6/28 (21)	13/13 (100)	16/17 (94)
Immunosuppression					
Mild or not significant					
ELISA	3/3 (100)	2/5 (40)	5/8 (63)	7/7 (100)	8/8 (100)
Determine strip	2/3 (67)	1/5 (20)	3/8 (38)	7/7 (100)	8/8 (100)
Advanced or severe					
ELISA	4/7 (57)	2/13 (15)	6/20 (30)	6/6 (100)	8/9 (89)
Determine strip	3/7 (43)	0/13 (0)	3/20 (15)	6/6 (100)	8/9 (89)

The diagnostic performance of both LAM assays is shown for HIV-negative and HIV-positive children. For HIV-positive children, two subgroups were analysed. The children with no significant or mild immune suppression were compared with thechildren with advanced or severe immune suppression. The Pearson's Chi-squared test was used to compare LAM positivity between HIV-positive *versus* HIV-negative children. The difference of MTB-LAM positivity in confirmed and probable TB HIV-negative *versus* HIV-positive was p=0.020. The difference of Determine TB LAM strip positivity in confirmed and probable TB HIV-negative *versus* HIV-positive was p=0.055. #: data presented as test positive/total n children in diagnostic class (%); ¶: data presented as test negative/total n TB negative children (%)

TABLE 3 Association of host factors with MTB-LAM-ELISA positivity in confirmed and probable TB cases

	Subjects	LAM	RR (95% CI)	p-value
Age	54	14 (26)	1.07 (0.93–1.22)	0.371
Sex				
Female	28	8 (29)	1	
Male	26	6 (23)	0.81 (0.32–2.03)	0.650
HIV status				
Negative	26	3 (12)	1	
Positive	28	11 (39)	3.40 (1.06–11.0)	**0.040**
Immunosuppression				
None or mild	8	5 (63)	1	
Advanced or severe	20	6 (30)	0.48 (0.20–1.15)	0.100
Proteinuria				
No	39	7 (18)	1	
Yes	14	7 (50)	2.79 (1.18–6.58)	**0.019**
BMI for age				
Z score	54	14 (26)	0.76 (0.60–0.96)	**0.020**
Died				
No	51	12 (24)	1	
Yes	3	2 (67)	2.83 (1.09–7.32)	**0.032**
TB classification				
Confirmed TB	18	8 (44)	1	
Probable TB	36	6 (17)	0.38 (0.15–0.93)	**0.033**

Data are presented as n or n (%), unless otherwise specified. Results from separate univariable binomial log link regression models for each of the above variables. A significantly increased risk for a positive *Mycobacterium tuberculosis* (MTB)- lipoarabinomannan (LAM)-ELISA result was found in participants with confirmed tuberculosis (TB) diagnosis, concomitant HIV infection, proteinuria, low body mass index (BMI) and participants who died during the course of the trial. In a multivariable model, which only included HIV, BMI and proteinuria, the risk ratios (RRs) and p-values remained similar, demonstrating an independent association of these variables with LAM-positivity.

TABLE 4 Association of host factors with Determine TB LAM positivity in confirmed and probable TB cases

	Subjects	LAM	RR (95% CI)	p-value
Age	54	7 (13)	1.23 (0.96–1.58)	0.100
Sex				
Female	28	5 (18)	1	
Male	26	2 (8)	0.43 (0.09–2.06)	0.292
HIV status				
Negative	26	1 (4)	1	
Positive	28	6 (21)	5.57 (0.70–44.1)	0.104
Immunosuppression				
None or mild	8	3 (38)	1	
Advanced or severe	20	3 (15)	0.40 (0.09–1.62)	0.199
Proteinuria				
No	39	4 (10)	1	
Yes	14	3 (21)	2.09 (0.53–8.30)	0.295
BMI for age				
Z score	54	7 (13)	0.67 (0.48–0.92)	**0.012**
Died				
No	51	6 (12)	1	
Yes	3	1 (33)	2.83 (0.48–16.9)	0.253
TB classification				
Confirmed TB	18	5 (28)	1	
Probable TB	36	2 (6)	0.20 (0.04–0.95)	**0.042**

Data are presented as n or n (%), unless otherwise stated. Results from separate univariable binomial log link regression models for each of the above variables. A significantly increased risk for a positive Determine TB LAM was demonstrated for confirmed tuberculosis (TB) diagnosis and low body mass index (BMI) for age, similar to the association demonstrated for the *Mycobacterium tuberculosis* (MTB)-lipoarabinomannan (LAM)-ELISA. Trends for increased risk were demonstrated for concomitant HIV infection and proteinuria, but did not reach significance level.

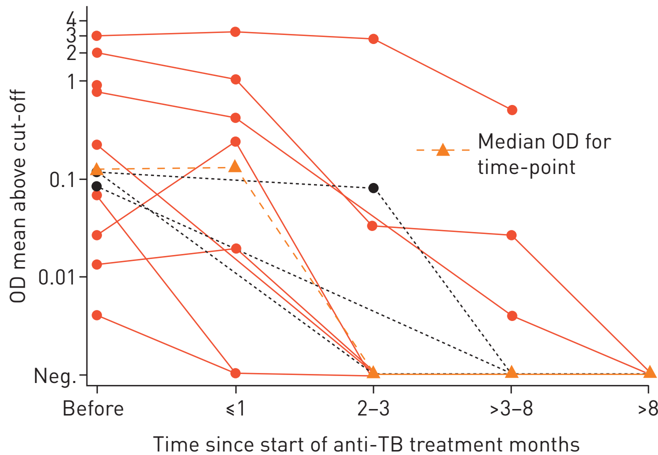


FIGURE 3 Signal intensity of *Mycobacterium tuberculosis* (MTB)- lipoarabinomannan (LAM)-ELISA at different time points after initiation of tuberculosis (TB) treatment. A drop of signal intensity of the MTB-LAM-ELISA for children with confirmed and probable TB during the course of antituberculous treatment was observed. Optical density (OD) results for HIV-positive participants are shown in red solid lines, results for HIV-negative participants as black dashed lines. The median OD at baseline was higher for HIV-positive children compared with HIV-negative children (0.221 *versus* 0.118, respectively). The signal intensity reached 0 after 3 month of treatment in six participants. One child excreted measurable LAM more than 7 months after TB therapy started. Y-axis shows logarithmic scale for mean OD. Neg.: negative.

especially those (adult) individuals with advanced disease and poor outcome were detected by urine-based TB diagnostic assays such as the LAM-test. The fact that, in our study, LAM positivity was positively and independently associated with culture-confirmed TB, a low BMI-z-score and death might further support these findings. Furthermore, and equally to findings from adult studies [18], we found a correlation between

DOI: 10.1183/09031936.00003315

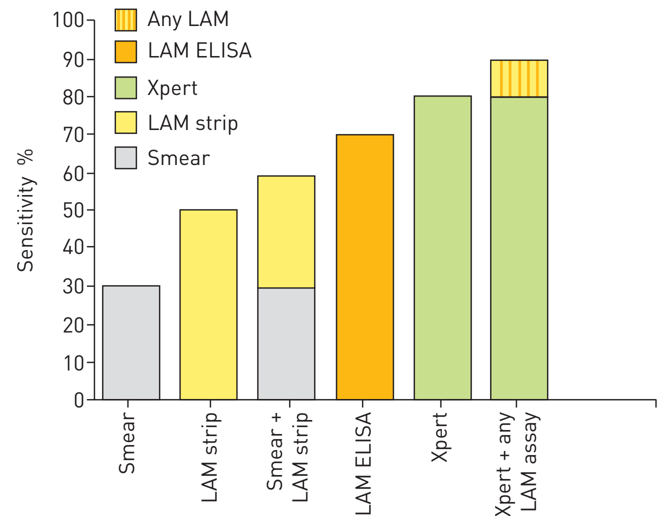


FIGURE 4 Sensitivity of single and combined tuberculosis (TB) diagnostic tests in HIV-infected children. This graph shows the sensitivity of each diagnostic test alone and the additional gain when combining lipoarabinomannan (LAM) diagnostic tests with either smear microscopy or Xpert MTB/RIF-assay in HIV-postive individuals with confirmed TB. LAM-Strip: Determine-TB-LAM; LAM ELISA: MTB LAM-ELISA; Xpert: GeneXpert.

proteinuria and LAM-positivity, indicating that the excretion of LAM might also depend on the condition of the kidney membrane. This and other risk factors for LAM-positivity require further investigation in order to better define the potential paediatric target group for LAM-based diagnostics in the future.

Our data regarding the overall sensitivity of the Determine TB-LAM strip test, were comparable with those previously published for a paediatric cohort from Cape Town [19]. However, the Cape Town study was unable to demonstrate an improved LAM sensitivity in TB/HIV co-infected children. Unfortunately, an in-depth comparison of our findings with those of Nicol *et al.* [19] is hampered by the extremely low sensitivity of both LAM tests, as well as the poor correlation between the MTB-LAM-ELISA and the Determine TB-LAM strip test in that study.

Contrary to our previously published data from the same setting in Tanzania [18, 27] and the data published by Nicol *et al.* [19], the specificity of both LAM assays was high in our cohort. Depending on the hygienic standards and the procedures during sample collection, false-positive LAM results had been observed previously, most likely due to contamination of the sample with environmental mycobacteria or other bacteria [27]. In this study, we collected urine samples with great precautions, including washing instructions and the use of clean containers, in order to avoid false positive results. However, especially in HIV-positive children response to antituberculous treatment should be closely monitored as LAM-based urine tests cross react with other pathogenic mycobacteria (*e.g. Mycobacterium avium* complex) and might influence results. Although the sensitivity of both LAM assays was unsatisfactory, and the requirements for correct sample collection are high, our data indicate that the use of urine LAM-based tests as rule-in test could still be advantageous for children in certain settings where sophisticated TB diagnostics are not available but strict urine collection criteria can be adhered to.

Furthermore, the fact that a decline of LAM-excretion could be measured during treatment may open up a possibility to monitor antituberculous treatment success in LAM positive children. Although LAM-based TB diagnosis has the disadvantage that it does not include information on drug resistance, it could be hypothesised that ongoing excretion of urine LAM during treatment might provide information on insufficiently treated drug resistant TB. Larger studies with a long clinical follow up are needed to further scrutinise this hypothesis.

One weakness of our study is the relatively low number of 18 confirmed TB cases, which prevented a definite conclusion of the performance of LAM assays in certain subgroups, such as HIV-negative children or in children with HIV co-infection and different levels of immunosuppression. Furthermore, the exclusion of 37 children from the analysis because no urine sample or LAM result was available at baseline, may have introduced a selection bias. Urine collection was more cumbersome especially in younger and sicker children as it requires both the child's and the caregiver's cooperation and may be affected by medical causes such as dehydration. However, gathering a urine specimen was not a priority when the main study was designed, and we are confident that the proportion of children with an available

sample would be higher if staff could be trained accordingly. We hypothesise that the exclusion of young children with advanced disease might have led rather to an underestimation of LAM-sensitivity, as data from our analysis indicate.

In conclusion, both LAM tests demonstrated a reasonable sensitivity in HIV-positive TB-infected children, whereas for HIV-negative children the sensitivity was extremely poor. The combination of LAM tests with other rapid TB diagnostics could substantially improve the detection of TB in HIV co-infected children. This holds promise for earlier TB-diagnosis in children, which might in turn have an impact on childhood morbidity and mortality associated with TB. Additionally, clean sample collection methods to achieve a high specificity have to be defined in more detail.

Acknowledgements

We thank the staff at the Active Detection of Active Tuberculosis (ADAT) paediatric TB clinic, the laboratories at NIMR-MMRC (Mbeya, Tanzania) and staff from the paediatric wards at the Mbeya Zonal Referral Hospital for their dedicated work, as well as the children and their parents or guardians who agreed to participate in this study. Also we want to thank Alere (Waltham, MA, USA) for providing the Determine TB-LAM Strips and some MTB-LAM-ELISA.

References

1 Marais BJ, Pai M. Recent advances in the diagnosis of childhood tuberculosis. *Arch Dis Child* 2007; 92: 446–452.
2 Starke JR. Pediatric tuberculosis: time for a new approach. *Tuberculosis (Edinb)* 2003; 83: 208–212.
3 Zar HJ, Hanslo D, Apolles P, *et al.* Induced sputum versus gastric lavage for microbiological confirmation of pulmonary tuberculosis in infants and young children: a prospective study. *Lancet* 2005; 365: 130–134.
4 Marais BJ, Hesseling AC, Gie RP, *et al.* The bacteriologic yield in children with intrathoracic tuberculosis. *Clin Infect Dis* 2006; 42: e69–e71.
5 Chintu C, Mudenda V, Lucas S, *et al.* Lung diseases at necropsy in African children dying from respiratory illnesses: a descriptive necropsy study. *Lancet* 2002; 360: 985–990.
6 Liu L, Johnson HL, Cousens S, *et al.* Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. *Lancet* 2012; 379: 2151–2161.
7 Liu L, Oza S, Hogan D, *et al.* Global, regional, and national causes of child mortality in 2000-13, with projections to inform post-2015 priorities: an updated systematic analysis. *Lancet* 2015; 385: 430–440.
8 Hamasur B, Bruchfeld J, Haile M, *et al.* Rapid diagnosis of tuberculosis by detection of mycobacterial lipoarabinomannan in urine. *J Microbiol Methods* 2001; 45: 41–52.
9 Hamasur B, Kallenius G, Svenson SB. A new rapid and simple method for large-scale purification of mycobacterial lipoarabinomannan. *FEMS Immunol Med Microbiol* 1999; 24: 11–17.
10 Tessema TA, Hamasur B, Bjun G, *et al.* Diagnostic evaluation of urinary lipoarabinomannan at an Ethiopian tuberculosis centre. *Scand J Infect Dis* 2001; 33: 279–284.
11 Boehme C, Molokova E, Minja F, *et al.* Detection of mycobacterial lipoarabinomannan with an antigen-capture ELISA in unprocessed urine of Tanzanian patients with suspected tuberculosis. *Trans R Soc Trop Med Hyg* 2005; 99: 893–900.
12 Chan J, Fan XD, Hunter SW, *et al.* Lipoarabinomannan, a possible virulence factor involved in persistence of Mycobacterium tuberculosis within macrophages. *Infect Immun* 1991; 59: 1755–1761.
13 Peter JG, Cashmore TJ, Meldau R, *et al.* Diagnostic accuracy of induced sputum LAM ELISA for tuberculosis diagnosis in sputum-scarce patients. *Int J Tuberc Lung Dis* 2012; 16: 1108–1112.
14 Peter JG, Theron G, Dheda K. Can Point-of-Care Urine LAM Strip Testing for Tuberculosis Add Value to Clinical Decision Making in Hospitalised HIV-Infected Persons? *PLoS One* 2013; 8: e54875.
15 Dheda K, Davids V, Lenders L, *et al.* Clinical utility of a commercial LAM-ELISA assay for TB diagnosis in HIV-infected patients using urine and sputum samples. *PLoS One* 2010; 5: e9848.
16 Shah M, Variava E, Holmes CB, *et al.* Diagnostic accuracy of a urine lipoarabinomannan test for tuberculosis in hospitalized patients in a High HIV prevalence setting. *J Acquir Immune Defic Syndr* 2009; 52: 145–151.
17 Mutetwa R, Boehme C, Dimairo M, *et al.* Diagnostic accuracy of commercial urinary lipoarabinomannan detection in African tuberculosis suspects and patients. *Int J Tuberc Lung Dis* 2009; 13: 1253–1259.
18 Reither K, Saathoff E, Jung J, *et al.* Low sensitivity of a urine LAM-ELISA in the diagnosis of pulmonary tuberculosis. *BMC Infect Dis* 2009; 9: 141.
19 Nicol MP, Allen V, Workman L, *et al.* Urine lipoarabinomannan testing for diagnosis of pulmonary tuberculosis in children: a prospective study. *Lancet global health* 2014; 2: e278–e284.
20 Rachow A, Clowes P, Saathoff E, *et al.* Increased and expedited case detection by Xpert MTB/RIF assay in childhood tuberculosis: a prospective cohort study. *Clin Infect Dis* 2012; 54: 1388–1396.
21 Graham SM, Ahmed T, Amanullah F, *et al.* Evaluation of tuberculosis diagnostics in children: 1. Proposed clinical case definitions for classification of intrathoracic tuberculosis disease. Consensus from an expert panel. *J Infect Dis* 2012; 205:Suppl. 2, S199–208.
22 Alere. http://alerehiv.com/hiv-comorbidities/procedure/ Date last updated: 2012. Date last accessed: Jan 9, 2015.
23 WHO. WHO Immunological Classification of HIV-related diseases in adults and children. http://wwwwhoint/hiv/pub/guidelines/HIVstaging150307pdf Date last updated: 2007. Date last accessed: Jan 15, 2015.
24 Wood R, Racow K, Bekker LG, *et al.* Lipoarabinomannan in urine during tuberculosis treatment: association with host and pathogen factors and mycobacteriuria. *BMC Infect Dis* 2012; 12: 47.
25 Lawn SD, Kerkhoff AD, Vogt M, *et al.* Diagnostic accuracy of a low-cost, urine antigen, point-of-care screening assay for HIV-associated pulmonary tuberculosis before antiretroviral therapy: a descriptive study. *Lancet Infect Dis* 2012; 12: 201–209.
26 Lawn SD, Kerkhoff AD, Vogt M, *et al.* HIV-associated tuberculosis: relationship between disease severity and the sensitivity of new sputum-based and urine-based diagnostic assays. *BMC Med* 2013; 11: 231.
27 Kroidl I, Clowes P, Mwakyelu J, *et al.* Reasons for false-positive lipoarabinomannan ELISA results in a Tanzanian population. *Scand J Infect Dis* 2014; 46: 144–148.

 DOI: 10.1183/09031936.00003315

For Peer Review

Reasons for false positive Lipoarabinomannan-ELISA results in a Tanzanian population

Journal:	*Scandinavian Journal of Infectious Diseases*
Manuscript ID:	SINF-2013-0475.R2
Manuscript Type:	Short Communication
Date Submitted by the Author:	n/a
Complete List of Authors:	Kroidl, Inge; University of Munich (LMU), Infectious Diseases and Tropical Medicine; NIMR-Mbeya Medical Research Centre (MMRC), Clowes, Petra; NIMR-Mbeya Medical Research Centre (MMRC), Mwakyelu, Joshua; NIMR-Mbeya Medical Research Centre (MMRC), Maboko, Leonard; NIMR-Mbeya Medical Research Centre (MMRC), Kiangi, Abubakary; Mbeya Referral Hospital, Laboratory Rachow, Andrea; University of Munich (LMU), Infectious Diseases and Tropical Medicine; NIMR-Mbeya Medical Research Centre (MMRC), ; DZIF German Centre for Infection Research, Reither, Klaus; Swiss Tropical and Public Health Institute, ; University of Basel , Saathoff, Elmar; University of Munich (LMU), Infectious Diseases and Tropical Medicine; DZIF German Centre for Infection Research, Jung, Jutta; NIMR-Mbeya Medical Research Centre (MMRC), Hoelscher, Michael; University of Munich (LMU), Infectious Diseases and Tropical Medicine; NIMR-Mbeya Medical Research Centre (MMRC), ; DZIF German Centre for Infection Research,
Keywords:	Tuberculosis, Diagnostic, Lipoarabinomannan, Contamination

SCHOLARONE™
Manuscripts

Lipoarabinomannan (LAM), a cell wall component of mycobacteria, can be detected in the urine of tuberculosis (TB) patients. Advantages of this include the ease of sample collection and test conduction. However, as with most new TB diagnostics, the LAM tests were evaluated in well controlled laboratory settings and subsequently need assessment under real working conditions. Our experience has shown that the diagnosis of TB, using the detection of LAM in urine under field conditions, is prone to false positive results due to contamination. Dust and soil, but also stool seem to lead to increased OD values and thus false positive results of the enzyme-linked immunosorbent assay (*ELISA*) for ~~MTB~~ LAM ~~ELISA~~, whereas contamination with blood, as well as bacterial or fungal organisms had no influence. The collection of urine for the detection of LAM should therefore follow strict collection criteria and avoid contamination.

Formatted: English (U.K.)

For Peer Review Only

Worldwide, tuberculosis (TB) causes significant morbidity and mortality, especially among people with human immunodeficiency virus (HIV) infection. Early diagnosis is a high priority for ~~tuberculosis~~TB control and prevention of mortality. The use of lipoarabinomannan (LAM) detection in urine for TB diagnosis was first investigated in the late 1990s (1-3) but to date has not been introduced as a standard TB diagnostic. LAM is a 19 kilo Dalton (± 8.5 kD) sized lipopolysaccharide, specific to the cell wall in the genus *Mycobacterium,* and is released from metabolically active or degrading bacterial cells. The lipopolysaccharide is then filtered by the kidneys and can subsequently be detected in the urine. Advantages of TB diagnosis using LAM include the ease of specimen collection and test conduction, and higher sensitivity in patients with immunodeficiency (4-6).

Since 2002, several trials conducted at the Mbeya Medical Research Centre in Tanzania evaluated the performance of the TB-LAM-ELISA. *Boehme et al*. assessed the non-commercial first product generation of an ELISA for LAM (MTB-LAM-ELISA, Chemogen, Portland USA) in 231 TB suspects and 103 healthy volunteers and found high sensitivity and specificity in both HIV infected and uninfected individuals (7). The encouraging results of that study led to additional evaluations of the LAM-ELISA at our research centre.

In a study on 291 TB suspects tested with the commercially available second generation MTB-LAM-ELISA from Chemogen, which utilizes a different polyclonal antibody compared to the test used by *Boehme et al.* (7), *Reither et al*. (4) demonstrated an overall sensitivity of only 50.7%, which was much lower than the previous results from *Boehme*, who reported a sensitivity of 81%. Surprisingly, the study showed a higher sensitivity, but lower specificity in female compared to male study subjects (66.7% versus 38.5% for sensitivity, 83.7 versus 93.9% for specificity, respectively), which had not been reported previously (4). However, higher sensitivity in HIV-positive (62.0%), compared to HIV-negative subjects (21.1%), was in concordance with other publications.

In an attempt to evaluate the second generation MTB-LAM-ELISA (Chemogen, Portland, USA) as a TB screening tool in a large cohort, it was utilised in a general population study, which was conducted between 2006 and 2011 in the Mbeya Region in Tanzania. The EMINI project (**E**valuate and **M**onitor the **I**mpact of **N**ew **I**nterventions), a population based cohort study, investigated the prevalence and incidence of HIV, TB

For Peer Review Only

and malaria in 9 selected communities with more than 170,000 community members. Geographically stratified random selection was used to select 10% of all households, which resulted in a cohort of more than 17,000 individuals of all age groups and both genders. From 2006 to 2008 study participants were visited annually for specimen and data collection, including blood for HIV testing which was performed using the Determine HIV 1/2 test (Abbott) rapid diagnostic test (RDT) in the first (2006/2007) and the HIV 1/2 STAT-PAK Dipstick Assay (Chembio) in the second round (2007/2008) of the survey. All positive results were confirmed using an ELISA HIV test (Enzygnost Anti HIV 1/2 Plus, DADE-Behring, Marburg, Germany) and subsequent Western blot in discordant cases. Negative RDTs from the first annual surveys which were confirmed by another negative RDT in the following survey were regarded as confirmed and not further tested. For all HIV incident cases in survey 2, the negative result of the previous round was confirmed by the above described testing algorithm (8).

Sample collection during the survey further included morning and spot urine to perform the LAM-ELISA for the diagnosis of TB and in selected cases with positive symptom screening or clinical evidence of active ~~tuberculosis~~TB , sputum was collected on three consecutive days.

In the first year of the EMINI study, clean urine collection containers with separate lids were provided but no specific collection instructions were given. In the second survey round urine containers with closed lids were supplied, as well as instructions to provide a mid stream urine sample directly into the sample container. Immediately after collection in the households, the urine samples were stored at -20°C in portable freezers. The MTB-LAM-ELISA was performed on thawed samples and according to the manufacturer's instruction two to three weeks after collection in the field.

In the first year of the survey, only 275 participants reported to be coughing during symptom screening or had clinical evidence of active ~~tuberculosis~~TB . Smear microscopy (Ziehl-Neelsen-staining) was performed on collected sputum samples and only four participants had a positive smear. For 3 of these 4 TB cases, the urine test for LAM-ELISA was positive. All three were co-infected with HIV, whereas the smear positive case which was missed by the LAM-ELISA was HIV negative. However, with these low numbers, the sensitivity of the LAM-ELISA could not be reliably calculated.

For Peer Review Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

In 2006 - the first year of our annual EMINI surveys - we found positive LAM screening results in 2,918 of 17,369 analysed urine samples (16.8%), which by far exceeded all reasonable expectations of detectable TB cases. The TB & Leprosy coordinator for the Mbeya region had reported 3,600 confirmed TB cases in an area with 2,000,000 inhabitants in 2006, which would translate to 31 culture-confirmed TB cases in a population of 17,369 participants. Even when adjusting this number for extra-pulmonary and smear negative TB cases, our results were beyond any expectations. No difference between HIV positive (16.6%) and HIV negative participants (16.8%) was seen but similar to *Reither et al* (4) we found a significant difference in LAM-positivity between female and male study subjects (Table 1), with 23.3% versus 9.4% LAM positive participants, respectively (Prevalence Ratio (PR) = 2.49, 95%CI = 2.31 to 2.69, $p<0.001$). In addition, we found significantly less LAM positive urine samples in adults than in children, and lower numbers of LAM positive results were recorded in the rainy compared to the dry season. Suspecting dust as a possible contaminator, soil samples were taken from the area around the clinic in Mbeya. Two out of four soil samples were positive for non-tuberculous mycobacteria in smear or culture. Subsequently we concluded that dust contamination could explain the higher numbers of LAM positive results in the dry compared to the rainy season. Changes in sample collection procedures between the first and second round of the survey lead to significant reduction of positive LAM results (Table 1). However, despite the decline from 16.8% to 10.3% positive results, the results were still beyond reasonable expectations. During the second year we continued to find no differences between HIV positive (10.7%) and HIV negative individuals (10.3%), but strong differences in LAM positivity between women (13.6%) and men (4.1%) which were consistent with the first survey and the previously reported results from *Reither et al.* (4). Fungal and bacterial urinary tract infections, which are more common in women than in men (9), as well as contamination with menstrual blood could be potential causes for these differences. In order to systematically evaluate these hypotheses, the following investigations were conducted:

Whole blood was added to previously LAM negative urine samples in a series of dilutions, from 1:1 up to 1:2,048. This led to insignificant changes in the optical density (OD) of the LAM result, but never reached the threshold for positivity.

For Peer Review Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Ten to 10 million units of *Candida albicans* were diluted in one ml of previously LAM negative urine samples. The maximum change of the OD was 0.03 above the uncontaminated urine, never reaching the cut-off for a positive result.

One hundred and two participants with clinical symptoms of urinary tract infection (UTI) were examined to determine the effect of a bacterial UTI on the LAM result. Urine samples collected before and after antibiotic treatment were subjected to microbial testing and LAM-ELISA. Although 11 cases of UTI could be confirmed, with *E. coli, K. pneumoniae* and *Proteus species* as the underlying causes, the LAM ELISA was negative in 10 of 11 cases. One case of UTI due to *Proteus* species was initially LAM positive; however this urine sample was heavily contaminated with stool. Repeat testing on a clear urine sample prior to antibiotic treatment showed a negative LAM result. In concordance with these finding are the results of *Boehme et al* (7) who had already investigated possible cross-reactivity of LAM with different pathogens in the urinary tract and found no influence on the ELISA results.

In order to explore the significance of stool as a contaminating factor, LAM negative urine samples of 13 individuals living in Mbeya, Tanzania with no signs or clinical suspicion of TB were mixed with 80 mg of stool from the respective individual. The solution was centrifuged and the supernatant diluted in a series of 1:1 up to 1:8, causing a significant increase of OD in 11 out of 13 individuals (Figure 1). The experiment was repeated using urine and stool samples from 12 individuals living in Munich, Germany. Again a significant increase of OD could be demonstrated. The presence of non-TB-mycobacteria in stool specimen was already reported in 1990 by *Colebunders et al*. in a study conducted in Kinshasa, DRC (10). As the LAM ELISA will react positive with several mycobacteria, including *M. fortuitum, M. kansasii, M. phlei* and *M. gordonae,* as shown by *Boehme et .al.* (7), contamination of urine samples with stool containing any of the above mycobacteria could have contributed to the high number of positive test results for the MTB-LAM_ELISA within our study cohort.

There are several limitations to our study. The EMINI survey was planned before data about the preferential use of the ELISA in patients with immune suppression became available (6, 11). The described specificity of 99% in the study by *Boehme et al. (7)*, led to the idea of testing the LAM-ELISA in a field study, however with more data about the specific indication of the LAM-ELISA in immuno-compromised individuals it is

For Peer Review Only

clear that a healthy general population is not the ideal target cohort for the use of this specific test. Furthermore, the study *by Boehme et al.*(7) was conducted with the non-commercial version of the LAM-ELISA, whereas the commercial version of the LAM-ELISA from Chemogen was used in the EMINI survey. Therefore, comparisons of the results are difficult to make. The TB screening method used in the EMINI project was too insensitive to find the potentially "real positive" TB cases among the LAM-positive results Lastly, no culture was performed on the stool-contaminated urine samples, therefore our conclusion that contamination with environmental mycobacteria could cause false positive results remains hypothetical as it is impossible to say which factor in the stool specimen lead to the documented increase of ODs.

Conclusion

The specificity of the MTB-LAM-ELISA was previously described as excellent (1, 6) with values above 97.5%. However, other publications reported lower specificities such as 88% to 94% (4-5). We found the performance of the commercially available MTB-LAM-ELISA significantly influenced by different environmental factors. Dust and soil, but also stool seem to lead to increased OD values and thus false positive results of the MTB-LAM-ELISA, whereas contamination with blood, as well as bacterial or fungal organisms had no influence. As non-tuberculous mycobacteria such as *M. gordonae* and others are present worldwide in tap water and soil and have been described in human stool (10), it is possible that NTMs are the causative agent for false positive results. If urine is collected under field conditions, where unclean genital areas or additional collection devices could add stool particles or soil to the urine, false positive results due to contamination are likely. This can explain the varying results from different studies. The collection of urine for the detection of LAM should therefore follow strict collection criteria and avoid contamination. Nevertheless, under controlled sampling conditions and addressing the correct target population, LAM diagnosis remains a promising method to detect *Mycobacterium tuberculosis.*

Ethical considerations:

The study was approved by the National Ethical Committee/Medical Research Coordinating Committee of the National Institute for Medical Research, Tanzania. All

For Peer Review Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

participants provided written informed consent prior to enrolment into the study, parents consented for their minors.

References

1. Hamasur B, Bruchfeld J, Haile M, Pawlowski A, Bjorvatn B, Kallenius G, et al. Rapid diagnosis of tuberculosis by detection of mycobacterial lipoarabinomannan in urine. J Microbiol Methods. 2001 May;45(1):41-52.
2. Hamasur B, Kallenius G, Svenson SB. A new rapid and simple method for large-scale purification of mycobacterial lipoarabinomannan. FEMS Immunol Med Microbiol. 1999 May;24(1):11-7.
3. Tessema TA, Hamasur B, Bjun G, Svenson S, Bjorvatn B. Diagnostic evaluation of urinary lipoarabinomannan at an Ethiopian tuberculosis centre. Scand J Infect Dis. 2001;33(4):279-84.
4. Reither K, Saathoff E, Jung J, Minja LT, Kroidl I, Saad E, et al. Low sensitivity of a urine LAM-ELISA in the diagnosis of pulmonary tuberculosis. BMC Infect Dis. 2009;9:141.
5. Mutetwa R, Boehme C, Dimairo M, Bandason T, Munyati SS, Mangwanya D, et al. Diagnostic accuracy of commercial urinary lipoarabinomannan detection in African tuberculosis suspects and patients. Int J Tuberc Lung Dis. 2009 Oct;13(10):1253-9.
6. Dheda K, Davids V, Lenders L, Roberts T, Meldau R, Ling D, et al. Clinical utility of a commercial LAM-ELISA assay for TB diagnosis in HIV-infected patients using urine and sputum samples. PLoS One. 2010;5(3):e9848.
7. Boehme C, Molokova E, Minja F, Geis S, Loscher T, Maboko L, et al. Detection of mycobacterial lipoarabinomannan with an antigen-capture ELISA in unprocessed urine of Tanzanian patients with suspected tuberculosis. Trans R Soc Trop Med Hyg. 2005 Dec;99(12):893-900.
8. Kroidl I, Clowes P, Mwalongo W, Maganga L, Maboko L, Kroidl AL, et al. Low specificity of determine HIV1/2 RDT using whole blood in south west Tanzania. PLoS One. 2012;7(6):e39529.
9. Foxman B. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. Am J Med. 2002 Jul 8;113 Suppl 1A:5S-13S.
10. Colebunders R, Nembunzu M, Portaels F, Lusakumundu K, Kapita B, Piot P. Isolation of mycobacteria from stools and intestinal biopsies from HIV seropositive and HIV seronegative patients with and without diarrhea in Kinshasa, Zaire. Ann Soc belge Méd trop. 1990;70:S303-9.
11. Peter J, Green C, Hoelscher M, Mwaba P, Zumla A, Dheda K. Urine for the diagnosis of tuberculosis: current approaches, clinical applicability, and new developments. Curr Opin Pulm Med. 2010 May;16(3):262-70.

For Peer Review Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

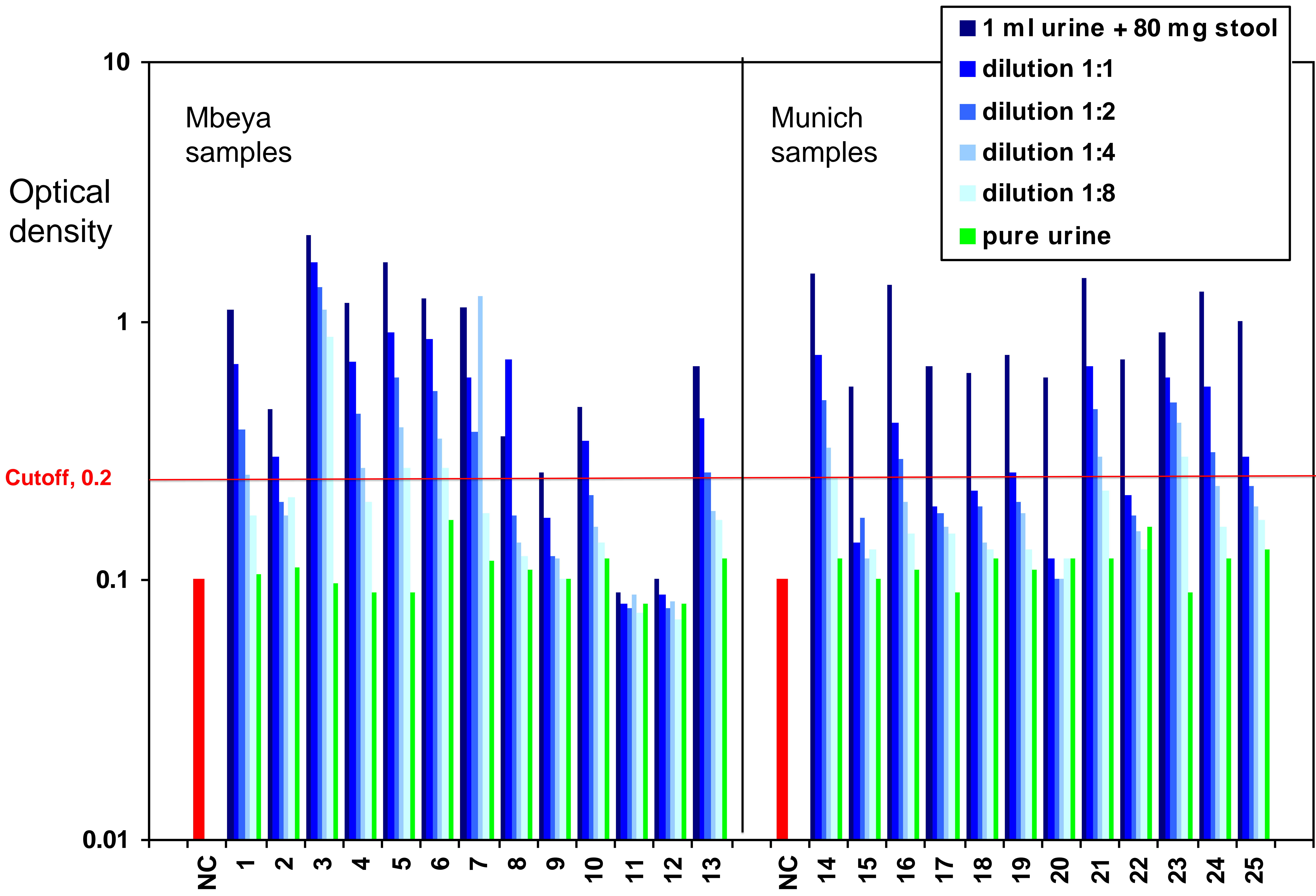
Acknowledgement:
We want to thank all EMINI participants for their willingness to be part of the study, as well as the EMINI field- and laboratory-teams for their contribution. Special thanks to Mr. Weston Assisya for the coordination of the field work and practical advice.

This study was funded by the European Union (SANTE/2004/078-545 and SANTE/2006/129-931).

Author contribution: IK wrote the initial draft of this manuscript, supervised the laboratory work for the EMINI study including the MTB-LAM-ELISA and performed the additional tests for possible contamination. MH designed the EMINI study, obtained funding and supervised its conduct. PC and LM were involved in the implementation of the EMINI study. PC was responsible for the overall conduct of the EMINI study and PC, JM and IK supervised the analysis of patients with urinary tract infections, for which AK performed the microbiology tests. KR, AR, AN and JJ were responsible for tuberculosis diagnosis. IK and ES did the statistical analysis. All authors read and critically revised the manuscript and approved the final version.

Conflicts of Interest and Source of Funding:
None of the authors has a conflict of interest regarding the contents of this manuscript.

For Peer Review Only


1 ml urine + 80 mg stool
dilution 1:1
dilution 1:2
dilution 1:4
dilution 1:8
pure urine
Mbeya samples
Munich samples
Optical density
10
1
0.1
0.01
Cutoff, 0.2
NC
1
2
3
4
5
6
7
8
9
10
11
12
13
NC
14
15
16
17
18
19
20
21
22
23
24
25

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 1: LAM-ELISA results from 25 different subjects, with and without addition of stool in different concentrations

Urine of 13 participants from Mbeya, Tanzania (samples 1-13), and urine of 12 individuals from Munich, Germany (sample 14-25) was tested for the presence of LAM. Mean optical density (OD) for the negative control (NC) was 0.1 for both groups; cut-off for LAM positivity was 0.2 (NC+0.1). None of the samples was tested LAM-positive, when testing the original urine sample (yellow bars). For the urine-stool-mix, eighty mg stool of each participant was mixed with 1ml urine of the respective individual and then diluted up to 1:16 (blue bars).

For Peer Review Only

Table 1: Diagnostic test performance of LAM-ELISA by age and gender
LAM-positivity defined as a positive result for at least one out of two urine samples

	n	**LAM + prevalence**	**Prevalence Ratio (95% CI) LAM+**	**HIV prevalence**
Survey 2006	17,369	16.8%		7.1%
Male	8,106	9.4%		6.2%
Female	9,263	23.3%	2.49 (2.31-2.69)	7.9%
Participants > 5 years	15,211	15.5%		7.8%
Children < 5 years	2,158	26.3%	1.70 (1.57-1.84)	2.3%
HIV neg participants	16,136	16.8%		
HIV pos participants	1,233	16.6%	0.98 (0.86-1.12)	
Survey 2007 (dust protection)	12,668	10.3%		6.6%
Male (all ages)	5,833	5.3%		5.8%
Female (all ages)	6,835	14.6%	2.77 (2.45-3.14)	7.2%
Participants > 5 years	10,965	9.3%		7.4%
Children < 5 years	1,703	16.9%	1.82 (1.62-2.06)	1.4%
HIV neg participants	11,837	10.3%		
HIV pos participants	831	10.7%	1.04 (0.85-1.28)	

For Peer Review Only

QuantiFERON®-TB Gold In-Tube Performance for Diagnosing Active Tuberculosis in Children and Adults in a High Burden Setting

Michala V. Rose[1,2]*, Godfather Kimaro[3], Thomas N. Nissen[2], Inge Kroidl[4,5], Michael Hoelscher[5], Ib C. Bygbjerg[1], Sayoki G. Mfinanga[3], Pernille Ravn[6]

1 Department of International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark, **2** Clinical Research Centre, University Hospital Hvidovre, Hvidovre, Denmark, **3** Muhimbili Medical Research Centre, National Institute for Medical Research, Dar es Salaam, Tanzania, **4** NIMR-Mbeya Medical Research Programme, Mbeya, Tanzania, **5** Department of Infectious Diseases and Tropical Medicine, Klinikum of the University of Munich, Munich, Germany, **6** Department for Infectious Diseases, University Hospital Odense, Odense, Denmark

Abstract

Aim: To determine whether QuantiFERON®-TB Gold In-Tube (QFT) can contribute to the diagnosis of active tuberculosis (TB) in children in a high-burden setting and to assess the performance of QFT and tuberculin skin test (TST) in a prospective cohort of TB suspect children compared to adults with confirmed TB in Tanzania.

Methods: Sensitivity and specificity of QFT and TST for diagnosing active TB as well as indeterminate QFT rates and IFN-γ levels were assessed in 211 TB suspect children in a Tanzanian district hospital and contrasted in 90 adults with confirmed pulmonary TB.

Results: Sensitivity of QFT and TST in children with confirmed TB was 19% (5/27) and 6% (2/31) respectively. In adults sensitivity of QFT and TST was 84% (73/87) and 85% (63/74). The QFT indeterminate rate in children and adults was 27% and 3%. Median levels of IFN-γ were lower in children than adults, particularly children <2 years and HIV infected. An indeterminate result was associated with age <2 years but not malnutrition or HIV status. Overall childhood mortality was 19% and associated with an indeterminate QFT result at baseline.

Conclusion: QFT and TST showed poor performance and a surprisingly low sensitivity in children. In contrast the performance in Tanzanian adults was good and comparable to performance in high-income countries. Indeterminate results in children were associated with young age and increased mortality. Neither test can be recommended for diagnosing active TB in children with immature or impaired immunity in a high-burden setting.

Citation: Rose MV, Kimaro G, Nissen TN, Kroidl I, Hoelscher M, et al. (2012) QuantiFERON®-TB Gold In-Tube Performance for Diagnosing Active Tuberculosis in Children and Adults in a High Burden Setting. PLoS ONE 7(7): e37851. doi:10.1371/journal.pone.0037851

Editor: Lorenz von Seidlein, Menzies School of Health Research, Australia

Received January 15, 2012; **Accepted** April 27, 2012; **Published** July 12, 2012

Copyright: © 2012 Rose et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The study was financially supported by the KNCV Tuberculosis Foundation, Reinholdt Jorck's Fund, Cluster in International Health, Justesen's Fund, Danish Research Council and Danish International Development Assistance (74-08-UHH DANIDA). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have read the journal's policy and have the following conflicts to declare: Pernille Ravn has been an invited speaker by Cellestis and has received QFT-IT kits at a reduced price for nonprofit research. Hvidovre Hospital has filed patents on the use of IP-10 as a marker for infection with Mycobacterium tuberculosis. Pernille Ravn is one of the registered co-inventors. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

* E-mail: michala.rose@gmail.com

Introduction

The aim of this study was to investigate the potential of the interferon-γ release assay (IGRA) QuantiFERON®-TB Gold In-Tube (QFT) (Cellestis Limited Chadstone, Australia) as a reliable diagnostic tool for physicians in Tanzania, a high TB burden country, with considerable prevalence of HIV and malnutrition, for diagnosing active TB in children.

TB is a major contributor to childhood morbidity and mortality, with children <5 years at highest risk and 40–50% of infected infants developing disease within 1–2 years [1,2]. However childhood TB is notoriously difficult to confirm, due to its pauci-bacillary nature, and existing tools have limited performance [3].

In TB endemic countries, diagnosis is usually a clinical diagnosis, relying on recognition of clinical features, suggestive chest x-ray (CXR) and if available a positive tuberculin skin test (TST) [4]. However it can be difficult to distinguish between the signs and symptoms of TB, HIV and malnutrition, and CXRs and TST results can be difficult to interpret [5,6].

QFT relies on *M.tuberculosis* (MTB) specific T-cell responses, measuring levels of interferon-gamma (IFN-γ) released in whole blood in response to stimulation with the MTB specific antigens: ESAT-6, CFP-10 and TB7.7, indicating past or present infection.

IGRAs are generally more specific than TST, since they do not cross-react with the BCG vaccine, *M.avium* or most other non-tuberculous mycobacteria (NTM). IGRAs are increasingly being

used worldwide as an alternative to, or in conjunction with, TST [7] for diagnosing latent TB and although not directly diagnostic for active TB, IGRAs are often used by clinicians as an additional tool in the diagnosis of active TB [8]. However there is a lack of evidence of the performance in children, especially in young children in a high burden setting [9].

In this study the performance of QFT was assessed in terms of sensitivity, specificity, indeterminate rates, median IFN-γ levels and risk factor analysis for positive and indeterminate results. Performance of TST for diagnosing active TB was included for comparison. Considering the diagnostic challenges in children and difficulties in assuring a gold standard of confirmed TB, adults with confirmed active TB, from the same location, were included as a measure of contrast.

Methods

Ethics Statement

The study protocol was approved by the Tanzanian Medical Research Coordinating Committee (NIMR/HQ/R.8a/Vol IX/584) and was evaluated by the Danish Central Ethical Committee without any objections. Written informed consent was obtained from the immediate caretaker, or next of kin, prior to inclusion, on behalf of children participating in the study. The standards for reporting diagnostic accuracy studies (STARD) criteria were followed in reporting the results.

Study Setting and Population

The study participants were recruited prospectively at Muheza designated district hospital, Tanga, Tanzania. Muheza is a rural district with a population of 209.480, where 90% are engaged in peasantry, fishery and small-scale business [10]. The TB notification rate in the district in 2009 was 431/100.000 [11].

Children <15 years with TB suspect signs and symptoms were included consecutively from the paediatric ward and outpatients departments, including the HIV, TB, and mother-child clinics. Adults with active TB were included consecutively from the TB clinic. Inclusion criteria are listed in Box S1.

A standardized questionnaire was used to record demographic and clinical details of the participants, including age, weight, height, TB specific signs and symptoms and presence of BCG scar. For the children additional information was collected about history of BCG vaccine, TB exposure, response to prior antibiotic treatment and history of health facility visits.

All the study participants provided blood for QFT and HIV testing. Chest x-rays and TST were performed and either sputum or gastric wash samples were sent for microscopy and culture examination. Follow-up of the children was conducted 2 and 6 months after inclusion, involving a standardised clinical examination and questionnaire concerning health status since last examination. Children who did not return for follow-up were traced within 7–12 months of inclusion.

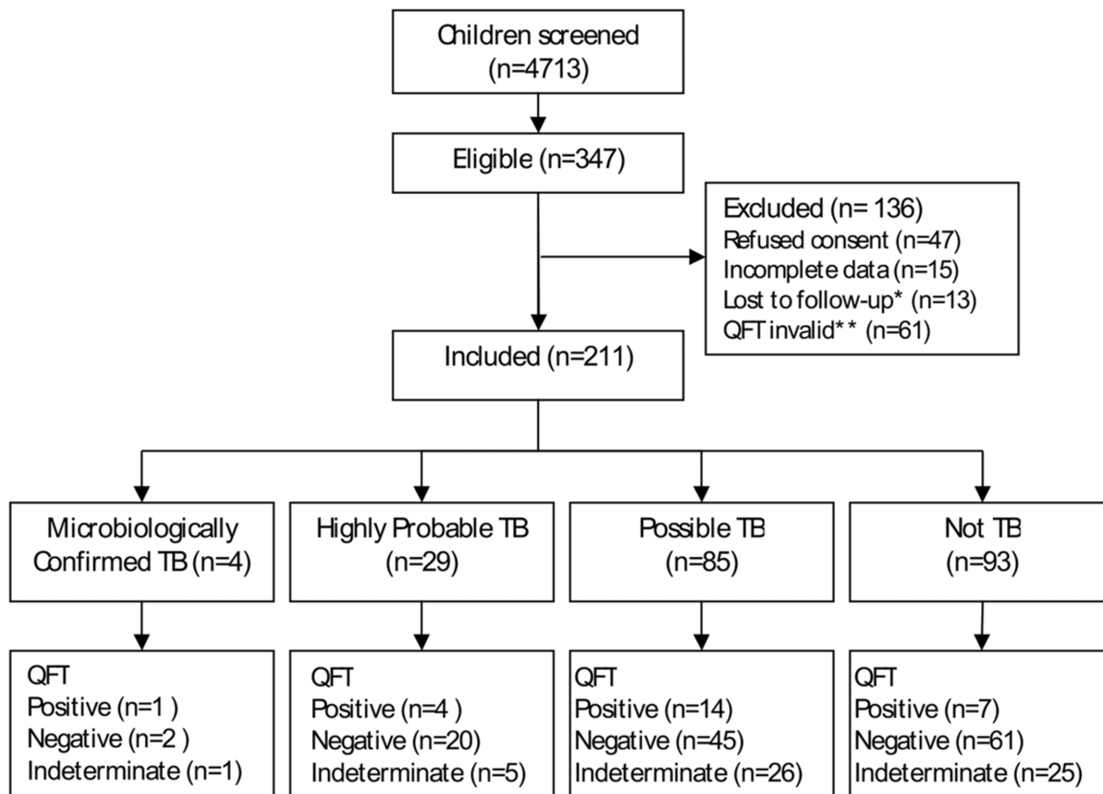


Figure 1. Summary of recruitment and diagnostic classification of children. * Children without follow-up data were excluded since they could not be classified according to the TB classifications. However one child had culture confirmed TB and could therefore be classified without follow-up data. ** The first 61 QFT results were excluded, when the initial QFT analysis showed very poor response in all the QFT tubes, including the mitogen. This was attributed to incorrect storage in a room reaching temperatures above 30°C. Subsequent tubes were all stored at 5–10°C.
doi:10.1371/journal.pone.0037851.g001

Table 1. Characteristics of study population.

	Children all		Confirmed		Possible		Not TB		Adults	
	N = 211		**N = 33**		**N = 85**		**N = 93**		**N = 90**	
Age mean yrs (SD)	**4.4**	**(3.8)**	4.7	(3.7)	4.1	(3.9)	4.5	(3.7)	**39.5**	**(14.9)**
Age groups n (%)										
<2 yrs	**77**	**(37)**	7	(21)	36	(42)	34	(37)	–	–
2–4.9 yrs	**53**	**(25)**	13	(40)	21	(25)	19	(20)	–	–
5–9.9 yrs	**51**	**(24)**	8	(24)	15	(18)	28	(30)	–	–
>10 yrs	**30**	**(14)**	5	(15)	13	(15)	12	(13)	–	–
Male n (%)	**124**	**(59)**	24	(73)	47	(55)	53	(57)	**72**	**(80)**
HIV Positive † n (%)	**78**	**(37)**	18	(55)	38	(45)	22	(24)	**25**	**(28)**
Z-score ≤ -2 † n (%)	**109**	**(58)**	15	(47)	45	(63)	49	(58)	–	–
BMI <18.5 n (%)	–	–	–	–	–	–	–	–	**57**	**(63)**
History contact † n (%)	**72**	**(35)**	13	(39)	30	(36)	29	(32)	–	–
BCG history n (%)	**196**	**(93)**	30	(91)	81	(95)	85	(91)	–	–
BCG scar n (%)	**192**	**(91)**	28	(85)	75	(88)	89	(96)	**70**	**(78)**
Clinical TB * n (%)	**76**	**(34)**	33	(100)	43	(51)	0	0	**90**	**(100)**
Follow-up status† n (%)										
Healthy	**152**	**(72)**	31	(97)	28	(33)	93	(100)	–	–
Still ill	**19**	**(9)**	–	–	19	(22)	0	0	–	–
Dead	**39**	**(19)**	1	(3)	38	(45)	0	0	–	–

†HIV test result available for 209 children, weight-for-age Z-score data available for 188 children, history of contact data available for 208 children (reported contact to case of TB in last 2 years), 1 child with confirmed TB did not have follow-up data (follow-up status defined as health status of child 6 months after inclusion into study).
*Clinical TB in children defined as active TB diagnosed by local physician based on clinical examination, CXR and TST. Clinical TB in adults defined as active TB diagnosed by local physician, all included adults had positive Ziehl-Neelsen smear microscopy as well as either positive culture and/or positive fluorescence microscopy.
doi:10.1371/journal.pone.0037851.t001

Interferon Gamma Release Assay (IGRA)

Venous blood was collected in a syringe and immediately dispensed into the QFT tubes, Nil (negative control coated with saline), TB-Ag (coated with MTB specific antigens) and Mitogen (positive control coated with phytohaemagglutin), according to the manufacturer's instructions (Cellestis Ltd). The tubes were taken to the laboratory within 4 hours and incubated at 37°C for 16–24 hours.

Immediately after incubation the samples were centrifuged and the supernatants stored at minus 70°C, until IFN-γ was measured using the QFT ELISA at the NIMR-Mbeya Medical Research Programme laboratory, Tanzania. The results were reported as positive, negative or indeterminate according to the manufacturer's instructions. In addition the raw quantitative results were recorded.

The results of the first 61 QFT tests revealed that 82% of the results were indeterminate with poor response in all the QFT tubes. Thorough quality assurance of all the test procedures indicated that the tubes had been stored under conditions with temperatures reaching above 30°C. This was considered to be the most likely the reason for the high number of indeterminate results. All the tubes were replaced and new tubes were stored at 5–10°C, until the time they were used.

Tuberculin Skin Testing (TST)

Two units of purified protein derivate RT23 from Staten Serum Institute, Denmark, were administered intradermally using the mantoux technique recommended by the manufacturer. The transverse diameter of the induration was recorded in millimetres after 48–72 hours. An induration of ≥5 mm was considered positive in HIV positive children and adults whilst an induration of ≥10 mm was considered positive for all others.

Chest X-Ray (CXR)

The children were chest x-rayed at inclusion. The CXRs were read by three experts; the hospital radiologist and two independent senior radiologists, who were unaware of the clinical status of the child. Using a standardised recording form, the CXRs were classified as either certain TB, highly suggestive TB, uncertain or not TB, based on TB specific or suggestive features. CXRs from 202 children were included.

Microbiology

Sputum or gastric wash samples were collected from the children on three consecutive mornings. Gastric wash samples were collected in containers containing 100 mg sodium carbonate. Ascites samples were included for 2 patients, whilst no cerebrospinal fluid or lymph node samples were included. The adults were initially diagnosed with smear positive TB at the Muheza district hospital laboratory using the Ziehl-Neelsen staining technique for acid fast bacteria. An extra sputum sample was collected from the adults for confirmation of diagnosis. All the gastric wash and sputum samples were refrigerated, on average 4 days, until they were sent for confirmatory microscopy and culture at the Central TB Reference Lab, Dar es Salaam (CTRL) where auramine staining was used for fluorescence microscopy and Löwenstein-Jensen media for culture. Para-nitrobenzoic acid, which inhibits MTB but not NTMs, was added in positive samples to exclude NTMs.

Table 2. Median IFN-γ U/ml in all three Quantiferon TB Gold In-Tube tubes in children and adults.

Children	Negative control	Positive control	TB antigens
	IFN-γ U/ml (IQR)	IFN-γ U/ml (IQR)	IFN-γ U/ml (IQR)
All (TB suspect)	0.15* (0.09–0.35)	2.05* (0.6–7.53)	0.19* (0.1–0.55)
Confirmed TB	0.15* (0.12–0.26)	3.8 (1.08–7.26)	0.17* (0.10–0.48)
Possible TB	0.13* (0.08–0.27)	1.69* (0.44–7.33)	0.17* (0.10–0.56)
Not TB	0.19* (0.09–0.38)	2.26 (0.80–7.61)	0.19* (0.11–0.55)
Adults			
Confirmed TB	0.36 (0.22–0.59)	2.63 (1.29–8.09)	3.28(0.99–6.58)

Comparison of median IFN-γ values in the three QFT tubes in children and adults according to diagnostic TB classificaiton. There were significantly lower levels* ($p<0.05$) in all subgroups of children compared to adults, except in the positive control where median levels in children with confirmed and not TB were not significantly different to levels in adults.
Wilcoxon's rank-sum test used to test difference between median IFN-γ in children and adults.
IQR: interquartile range.
doi:10.1371/journal.pone.0037851.t002

TB Diagnosis and Classification

The clinical TB diagnosis in children and the decision to start anti-TB treatment was made by the district TB medical doctor or the medical doctor in charge of the paediatric ward. The study team was not involved other than ensuring that the TB medical doctor was consulted on each case and providing the results of CXR, TST and HIV test. The results of the QFT test were not available to the physicians responsible for diagnosing or treating the children.

Each child was assigned to one of four predefined diagnostic classifications; "Microbiologically confirmed TB", "Highly probable TB", "Possible TB" and "Not TB". The classifications were based on microscopy and culture data, CXR results, clinical examination and follow-up data (Box S2). For data analysis children with microbiologically confirmed TB and highly probable TB were considered to have confirmed TB. Children without follow-up data were excluded, unless they had microbiologically confirmed TB. Adults with either positive culture or positive fluorescence microscopy were classified as confirmed TB.

Data Management and Statistical Analysis

Data were double-entered into a data entry database using MS-Access (Microsoft Corp, VA, USA) including error, range and consistency check programs. Statistical analyses were performed using Stata™ v 10.0 (Stata Corp, TX, USA). Epi Info™ 3.5.2 (CDC, USA) was used to generate weight-for-age z-scores to assess nutritional status in children.

Indeterminate results were excluded in the sensitivity and specificity analysis as well as in the risk factor analysis for a positive QFT result. Student's t-test was used to compare means, Wilcoxon's rank-sum test for comparing medians, using log transformation for data that was not normally distributed.

Logistic regression analysis was used for univariable and multivariable analysis of risk factors' association with positive

Table 3. Comparison of median IFN-γ responses in all TB suspect children.

		Negative control		Positive control		TB antigens	
		IFN-γ U/ml (IQR)	p-value†	IFN-γ U/ml (IQR)	p-value†	IFN-γ U/ml (IQR)	p-value†
Age	<2 yrs	0.14 (0.07–0.21)	–	1.32 (0.4–8.05)	–	0.14 (0.08–0.35)	–
	≥2 yrs	0.16 (0.1–0.44)	0.01	2.37 (1.01–7.33)	0.09	0.21 (0.12–0.64)	0.01
Sex	Male	0.17 (0.09–0.45)	–	2.18 (0.73–7.54)	–	0.21 (0.12–0.57)	–
	Female	0.14 (0.09–0.24)	0.08	1.48 (0.49–7.53)	0.27	0.15 (0.09–0.39)	0.04
HIV status	Pos	0.14(0.09–0.24)	–	1.4 (0.47–5.61)	–	0.15 (0.09–0.29)	–
	Neg	0.17 (0.09–0.52	0.09	2.49 (0.8–8.89)	0.07	0.2 (0.1–0.84)	0.01
Z-score	≤−2	0.14 (0.09–0.37)	–	1.75 (0.6–6.46)	–	0.18 (0.09–0.49)	–
	>–2	0.15 (0.09–0.45)	0.45	2.49 (0.78–9–87)	0.32	0.21 (0.11–0.79)	0.20
History contact	yes	0.17 (0.10–0.–36)		2.48 (0.99–7.39)		0.19 (0.10–0.56)	
	no	0.15 (0.09–0.37)	0.56	1.77 (0.53–8.18)	0.37	0.19 (0.10–0.54)	0.77
Living PTB⁺ *	yes	0.12 (0.1–0.25)		1.91 (0.63–4.35)		0.17 (0.09–0.55)	
	no	0.15 (0.09–0.37)	0.51	1.77 (0.53–8.18)	0.51	0.19 (0.1–0.54)	0.79
Clinical TB**	yes	0.15 (0.10–0.41)		2.28 (0.73–7.45)		0.18 (0.1–0.56)	
	no	0.15 (0.08–0.34)	0.69	1.91 (0.52–7.53)	0.56	0.19 (0.1–0.53)	0.68
Follow–up status	Healthy***	0.16(0.09–0.39)	–	2.65 (0.95–8.8)	–	0.19(0.11–0.55)	–
	Ill	0.16 (0.08–0.35)	0.59	1.08 ((0.33–2.68)	0.04	0.24 (0.1–0.58)	0.37
	Died	0.11 (0.05–0.2)	0.01	1.02 (0.19–4.39)	<0.01	0.15 (0.07–0.4)	0.18

Comparison of median IFN-γ in subgroups of children, finding lower median levels in response to specific TB antigens in children <2 years, girls and HIV infected and lower median mitogen responses in children who subsequently died.
†Wilcoxon's rank-sum test used to test differences in median IFN-γ between subgroups of children, $p<0.05$ considered significant.
IQR: interquartile range.
*Living PTB^+ defined as living in the same household as a person with smear positive pulmonary TB.
**Clinically diagnosed with TB and put on anti-TB therapy.
***Subcategory "healthy" used for comparison both for "ill" and "died".
doi:10.1371/journal.pone.0037851.t003

and indeterminate QFT results, using 95% confidence intervals to quantify uncertainty. Risk factors that could potentially be associated with the performance of the test were identified from previous studies (age, sex, HIV status, nutritional status and contact history).

Kaplan Meier failure estimates, odds ratios and log-rank test were used to illustrate and quantify the difference in mortality according to QFT results. A p-value of <0.05 was considered statistically significant.

Results

Study Population

From April 2008 to June 2010 4713 children were screened, 347 children were eligible according to our inclusion criteria and 211 children with TB suspect signs and symptoms were included (figure 1). 107 Ziehl-Neelsen smear microscopy positive adults were screened, 90 with either positive culture and/or positive fluorescence microscopy were included. Characteristics of the study populations are shown in table 1.

Four children were culture positive, two of whom were also fluorescence microscopy positive. In adults 70 were culture and fluorescence microscopy positive, whilst an additional 4 were positive by culture only and 16 by microscopy only.

Based on the above and the criteria in box S2, 33/211 (16%) of children were classified as "confirmed TB", 85/211 (40%) "possible TB" and 93/211 (44%) "not TB". All 90 adults were classified as "confirmed TB".

TST Results

TST results were available for 197 children (6% positive) and 74 adults (85% positive). Two of four children with TST indurations measuring 5–9 mm were considered TST positive because they were HIV positive. The one adult with an induration of 5–9 mm was HIV negative.

QFT Results

QFT results were positive in 26 (12%), negative in 128 (61%) and indeterminate in 57 (27%) children. Amongst adults 73 (81%) were QFT positive, 14 (16%) negative and 3 (3%) indeterminate. One of the indeterminate results in children was due to high negative control (>8 U/ml), the remaining indeterminate results in children and adults were due to low response in the positive control.

The median levels of IFN-γ were significantly lower in children compared to adults, especially in response to the TB antigens (table 2). Even children with a positive QFT result had a lower median IFN-γ after antigen stimulation (2.08 U/ml) than adults with a positive QFT (4.09 U/ml), p = 0.03 (data not shown). The median IFN-γ in response to TB antigens was significantly lower in children under 2 years, in girls and HIV positive children (table 3). Amongst children with a positive QFT test, HIV positive children still had a significantly lower median IFN-γ response to TB antigens than HIV negative children (data not shown). Children who died before discharge or during the follow-up period, as well as those who were still ill at 6 month follow-up had lower median IFN-γ in the positive control at base-line compared to those who had regained their health.

Sensitivity of QFT and TST for Diagnosing Active TB

Amongst children, clinically diagnosed with TB and started on anti-TB treatment by the local physician, we found a sensitivity of QFT and TST of only 18% and 12% respectively. Amongst children with "confirmed TB" we found a sensitivity of QFT and TST of 19% and 6% respectively. The sensitivity of QFT and TST in children with "possible TB" was 24% and 9%, and in children with "not TB" 10% and 3% (table 4).

The specificity of QFT and TST in children was 90% and 98% respectively. The positive predictive value of QFT and TST in children for having confirmed TB was 42% and 40%, whilst the negative predictive value was 73% and 75% respectively.

In contrast, the diagnostic sensitivity of QFT and TST amongst adults with confirmed TB was notably higher than in children at 86% and 89% (table 4). Sensitivity of QFT was significantly lower in HIV positive adults 67% (16/24), compared to HIV negative adults 91% (57/63), p = 0.007 (data not shown). The sensitivity of TST in adults was not affected by HIV status. The specificity in adults was not calculated since the inclusion criteria was confirmed TB.

Risk Factors Association with Positive and Indeterminate QFT Results

Logistic regression analysis was used in order to identify possible causes for the low positivity rate and high indeterminate rate of QFT in children (Table 5 and 6).

Neither sex, malnutrition nor HIV infection, were associated with either positive or indeterminate QFT results in the unadjusted or adjusted analysis. Surprisingly, history of TB

Table 4. Sensitivity of Quantiferon TB Gold In-Tube test and Tuberculin Skin test in children and adults.

	QFT positive			TST positive			p-value†
Children	**n/all tested***	**(%)**	**95% CI**	**n/all tested**	**(%)**	**95% CI**	
Clinical TB diagnosis	11/60	(18.3)	8.4–28.3	8/69	(11.6)	3.9–19.3	0.28
Confirmed TB	5/27	(18.5)	3.4–33.6	2/31	(6)	−2.4–15.3	0.16
Possible TB	14/59	(23.7)	12.7–34.8	7/77	(9.1)	2.6–15.6	0.02
Not TB	7/68	(10.3)	3.0–17.6	3/89	(3.4)	−0.4–7.2	0.03
Adults							
Confirmed TB	73/87	(83.9)	76.0–91.8	63/74	(85.1)	76.8–93.4	0.85

Sensitivity of QFT and TST according to TB status in children and adults, showing low sensitivity of both T-cell based test in children, irrespective of TB classification, compared to adults with confirmed TB.
*Sensitivity analysis of QFT excludes indeterminate results.
†Difference between sensitivity in QFT and TST tested using two-sample test of proportion.
doi:10.1371/journal.pone.0037851.t004

Table 5. Risk factors association with positive QFT results in children.

QFT positive (n)						
	(N)	**n/N (%)**	**OR (95% CI)**	**p-value†**	**Adj OR[1] (95% CI)**	**p-value†**
Age	≥2 yrs	17/105 (16.2)	1	–	1	–
	<2 yrs	9/49 (18.4)	1.16 (0.48–2.84)	0.73	0.93 (0.34–2.58)	0.89
Sex	Female	11/62 (17.7)	1	–	1	–
	Male	15/92 (16.3)	0.90 (0.38–2.12)	0.82	0.95 (0.37–2.43)	0.92
HIV status	Neg	19/99 (19.2)	1	–	1	–
	Pos	6/54 (11.1)	0.53 (0.20–1.41)	0.20	0.61 (0.21–1.77)	0.37
Z-score	>−2	11/60 (18.3)	1	–	1	–
	≤−2	13/76 (11.9)	0.92 (0.38–2.23)	0.69	1.00 (0.39–2.59)	0.99
History contact	No	15/93 (16.1)	1	–	1	–
	Yes	11/60 (18.3)	1.17 (0.50–2.75)	0.72	1.37 (0.55–3.41)	0.49
Living PTB+ *	No	15/93 (16.3)	1	–	1	–
	Yes	7/27 (25.9)	1.82 (0.65–5.06)	0.25	2.83 (0.89–9.03)	0.08

Risk factor analysis using logistic regression analysis found no association between known risk factors and a positive QFT result in children.
OR: unadjusted odds ratio from univariable analysis.
Adj OR: adjusted odds ratio, adjusted for age, sex, HIV, z-score and contact TB case.
†p-value for the odds ratios.
*Compares living with a case of smear positive TB case to those reporting no contact at all.
doi:10.1371/journal.pone.0037851.t005

contact was not associated with a positive QFT result, even after adjusting for confounders in the multivariable analysis.

Age seemed to influence the test performance as children <2 years were more likely to have an indeterminate result (adjusted OR 2.39, p = 0.01).

In adults HIV infection was negatively associated with a positive QFT result (adjusted OR 0.25, p = 0.04) (table 7), whilst we found no effect of age, sex or HIV status when analysing risk factors for an indeterminate result (table 8). We were unable to determine association with BMI as none with a BMI>18.5 had an indeterminate result.

Mortality

Eleven children died during admission, whilst an additional 28 children died during the follow up period, resulting in an overall mortality of 19%. An indeterminate QFT result at baseline was associated with subsequent high childhood mortality (adjusted OR 3.87, p = 0.003, data not shown) and the cumulative mortality was significantly higher in children with an indeterminate QFT result (33%) compared to a determinate result (13%), p<0.001 (figure 2).

Discussion

The main findings of this study were a low sensitivity of both QFT and TST for diagnosing active TB in children as well as a high QFT indeterminate rate which was associated with high subsequent mortality.

We found a surprisingly low sensitivity of QFT in children with confirmed TB (19%) compared to adults with confirmed TB (84%). The sensitivity of TST was similarly poor in children (6%) compared to adults (85%). Using other criteria for

Table 6. Risk factors association with indeterminate QFT results in children.

QFT indeterminate (n)						
	(N)	**n/N (%)**	**OR (95% CI)**	**p-value†**	**Adj OR (95% CI)**	**p-value†**
Age	≥2 yrs	29/134 (21.6)	1	–	1	–
	<2 yrs	28/77 (36.4)	2.07 (1.11–3.85)	0.02	2.39 (1.22–4.68)	0.01
Sex	Female	25/87 (28.7)	1	–	1	–
	Male	32/124 (25.8)	0.86 (0.47–1.59)	0.64	0.79 (0.40–1.54)	0.49
HIV status	Neg	32/131 (24.4)	1	–	1	–
	Pos	24/78 (30.8)	1.38 (0.74–2.57)	0.32	1.28 (0.63–2.63)	0.50
Z-score	>−2	19/79 (24.1)	1	–	1	–
	≤−2	33/109 (30.3)	1.37 (0.71–2.65)	0.35	1.09 (0.53–2.21)	0.82

Risk factor analysis using logistic regression analysis found increased odds of an indeterminate result in children <2 years.
OR: unadjusted odds ratio from univariable analysis.
Adj OR: adjusted odds ratio, adjusted for age, sex, HIV and z-score.
†p-value for the odds ratios.
doi:10.1371/journal.pone.0037851.t006

Table 7. Risk factors association with positive QFT result in adults.

QFT positive (n)						
	(N)	n/N (%)	OR (95% CI)	p-value†	Adj. OR (95% CI)	p-value†
Age years*	–		1.0 (0.96–1.04)	0.93	0.10 (0.95–1.04)	0.89
Sex	Female	11/16 (68.8)	1		1	
	Male	62/71 (87.3)	3.13 (0.88–11.12)	0.08	2.07 (0.49–8.81)	0.33
HIV status	Neg	57/63 (90.5)	1		1	
	Pos	16/24 (66.7)	0.21 (0.06–0.70)	0.01	0.25 (0.07–0.92)	0.04
BMI	≥18.5	28/33 (84.9)	1		1	
	<18.5	45/54 (83.3)	0.89 (0.27–2.94)	0.85	0.81 (0.23–2.84)	0.74

Risk factor analysis using logistic regression analysis found lower odds of a positive QFT result in HIV infected adults.
OR: odds ratio from univariable analysis.
Adj. OR: adjusted odds ratio adjusted for age, sex, HIV infection and BMI.
*Odds ratio calculated as per year increase in age.
†p-value for the odds ratios.
doi:10.1371/journal.pone.0037851.t007

diagnosing TB, such as the clinical TB diagnosis or by including children with possible TB in the group of TB cases, did not alter the overall picture of an extremely low sensitivity. In addition the positive and negative predictive values of QFT in children were low.

We found a high specificity (≥ 90%) for both QFT and TST, either suggesting that false positive results due to latent TB or BCG vaccination did not influence the performance of the tests or reflecting the overall poor performance of the test, with low proportions of positive results in this population.

The few existing previous paediatric studies have reported QFT sensitivity for active TB varying from 50%–100% [12]. To our knowledge only one other study has evaluated QFT in a high burden setting and they found only 8 children with confirmed TB and a sensitivity of 63% [13]. Studies evaluating T-Spot®-TB, another commercially available IGRA (Oxford Immunotec, Abingdon, UK), in children in a high endemic setting are equally few, one study in South Africa study with 10 culture confirmed cases of TB, found a sensitivity of 50% [14].

We found only 12 children with a positive TST, which is surprising given that 72 (35%) reported contact to person with TB and 92.9% were BCG vaccinated. The sensitivity of TST (6%) was even lower than QFT.

Infants are known to have poor production of cytokines, such as IFN-γ, even beyond infancy [15] and there is evidence that an effective antigen specific response to the MTB infection is delayed and less effective in infants compared to adults [16] which could explain our comparatively low positivity rate. Not only young age [13] but also HIV infection [17] and malnutrition, [18] were expected to affect test sensitivity, due to immature immune response or suppression of T-cell response. We found no clear association in our risk factor analysis between these factors and the QFT positivity rate, possibly due to small sample size, however we did find reduced IFN-γ responses in children <2 years, HIV infected and children who were still ill or had died by 6 month follow-up. Children in low-income settings often present with severe TB after a long period of illness, and consequent suboptimal immune response [19,20]. These findings suggest that the performance of QFT as well as TST, in these children with chronic and severe illness, were affected by immature or impaired immunity and progressive exhaustion of T-cells ability to react adequately to antigen response [21].

Table 8. Risk factors association with indeterminate QFT result in adults.

QFT indeterminate (n)						
(N)		n/N (%)	OR (95% CI)	p-value†	Adj. OR (95% CI)	p-value†
Age years*		–	1.06 (0.99–1.15)	0.10	1.10 (0.99–1.22)	0.07
Sex	Female	2/18 (11.1)	1	–	1	–
	Male	1/72 (1.4)	0.11 (0.01–1.32)	0.08	0.02 (0.00–1.28)	0.07
HIV status	Neg	2/65 (3.1)	1	–	1	–
	Pos	1/25 ((4)	1.31 (0.11–15.15)	0.83	0.47 (0.01–17.02)	0.68
BMI **	≥18.5	0/33	–	–	–	–
	<18.5	3/57 (5.3)	–	–	–	–

Risk factor analysis using logistic regression analysis found no association between suspected risk factors and an indeterminate QFT result in adults.
OR: odds ratio from univariable analysis.
Adj. OR: adjusted odds ratio adjusted for age, sex, HIV infection and BMI.
*Odds ratio calculated as per year increase in age.
**Logistic regression analysis not possible for association between BMI and indeterminate result, since there are no indeterminate results in those with BMI ≥18.5.
†p-value for the odds ratios.
doi:10.1371/journal.pone.0037851.t008

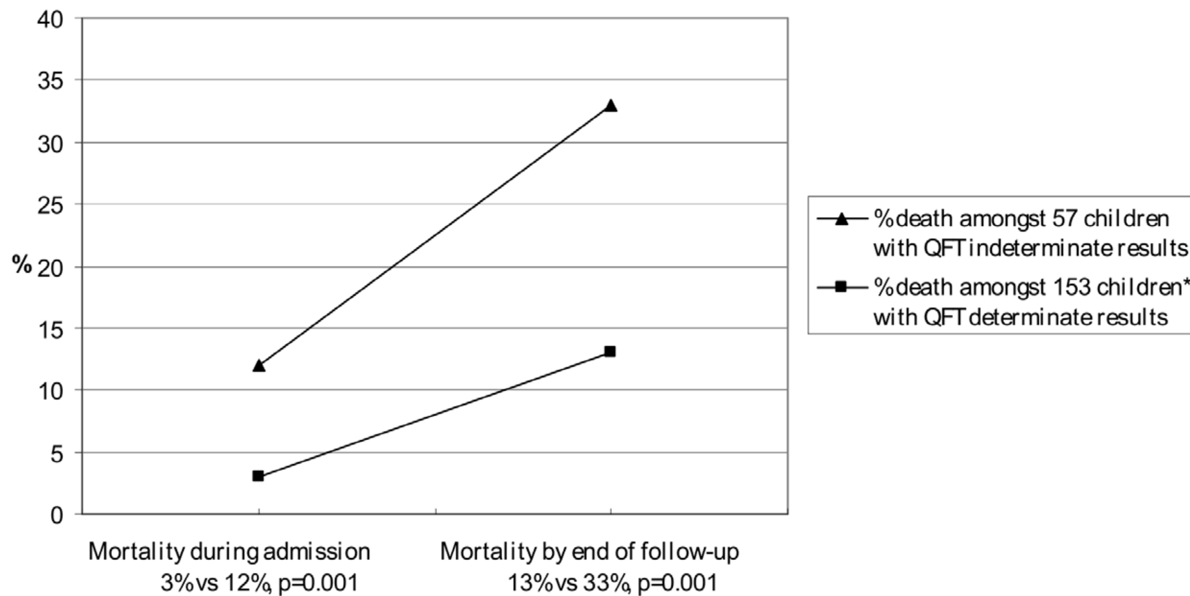


Figure 2. Childhood mortality according to Quantiferon TB Gold In-Tube result. Childhood mortality recorded during admission and at 6 month follow-up, according to QFT results. Children who did not attend scheduled follow-up at 6 months were traced and follow-up visits were conducted in their homes within 7–12 months of inclusion. Both the mortality during admission and the overall mortality in children with an indeterminate QFT result was significantly higher than in children with a determinate QFT result, $p<0.001$. * One of 154 children with a determinate QFT result did not have follow-up data.
doi:10.1371/journal.pone.0037851.g002

Due to the lower levels of IFN-γ in response to specific antigens seen in HIV infected and young children it has been suggested that a lower cut-off value for a positive QFT for children may be relevant [22]. However applying a cut-off of 0.26 U/ml to our data only increased the number of positives by two, both of whom were classified "not TB". Thus our study does not indicate added value of a lower cut-off in terms of sensitivity of QFT for active TB.

The poor sensitivity of, not only QFT but also TST, could also be explained by misclassification and over-diagnosis of TB however we included microbiological confirmation, good clinical response to treatment, in addition to either typical CXR or clinical features, which is in accordance with the recommendations of the recent expert consensus report on evaluating TB diagnostics in children [23].

Like other studies including TB suspect children we found only a small proportion of culture confirmed cases (2%). In the four culture confirmed cases of TB there was only one QFT and one TST positive result (in the same child), indicating that, rather than a classification problem, the performance of T-cell based assays in this particular group of children is suboptimal. Two of the remaining children with culture confirmed TB, were both HIV positive and severely malnourished, and it reasonable to conclude that they were not able to mount an adequate immune response.

Our results highlight the difficulties in diagnosing TB in children and we cannot conclude whether QFT suffers from extremely low sensitivity in young children with active TB in a high burden country or whether TB was over-diagnosed in these very sick children. However in light of the review by Mandalakas et al [12] which found that sensitivity of IGRAs and TST was lower in children <5 years, HIV positive children and in low-middle income countries, our finding of low sensitivity is not unlikely.

In adults we found a sensitivity of 84% which compares to the pooled sensitivity of 80% in a recent review of IGRA studies [8] and in line with another Tanzanian study [17] and the review by Metcalfe [24], we also found a lower sensitivity amongst HIV positive adults.

We found a high indeterminate rate in children (27%) compared to adults (3%) ($p<0.01$) with higher rates amongst children <2 years and in children who subsequently died after inclusion. Surprisingly our data did not support a significant association with HIV infection or malnutrition possibly due to low numbers.

Indeterminate rates in other paediatric QFT studies range from 0–35% [13,25], high rates correlated with young age [26], immune suppression [25] and malnutrition [27]. Thomas et al [27] found, in a study in Bangladesh that indeterminate results were associated not only with malnutrition but also with helminth infection, suggesting that children in a generally poor nutritional state with added infections are less likely to have a determinate QFT result. Hook worm and schistosomiasis are known to be highly prevalent amongst school children in the study area [28] and we can reasonably assume that many of the study participants were co-infected, which may have influenced the performance of QFT [29].

In our study 58% of children were defined as being malnourished. Malnutrition leads to an impaired cellular immunity which could affect the performance of the IGRA tests [18,30] however several studies conducted in populations with significant malnutrition [13,31], including the current study, did not find malnutrition to be associated with reduced positivity rates or increased indeterminate results. It is possible that weight-for-age z-score is not the optimal measure for assessing the effect of malnutrition on IGRA responses. A study of Peruvian adults found that malnutrition as measured by corrected arm muscle area, but not BMI or body fat, was associated with decreased TST positivity [32].

No previous paediatric studies have shown that an indeterminate result is a risk factor for death. To our knowledge only one

study in TB-suspect HIV positive adults in Uganda has shown that mortality was higher in those with an indeterminate T-SPOT.TB result [33]. It is reasonable to hypothesise that the children who died after inclusion were severely ill and therefore unable to mount an appropriate immune response. Thus, we suggest that the care for children with indeterminate results should be intensified with extensive diagnosis and treatment.

We found a low indeterminate rate of 3% amongst adults. Even amongst the HIV positive adults, the indeterminate rate was only 4% which contrasts to other studies, including HIV infected participants, where indeterminate rates reach as high as 21% [34]. HIV infection is known to be associated with lower positivity rates [35] and our data support these findings.

The samples of both children and adults were taken and processed concurrently, at the same hospital using the same staff, procedures and equipment. As such, it is most unlikely that the poor performance in children was due to technical errors.

Our study suggests that T-cell based assays, such as QFT and TST, have poor performance in children with immature or impaired immune systems and that indeterminate results are a predictor for death. With a sensitivity of 19% amongst children with confirmed TB and indeterminate rate of 27%, QFT is not the answer for providing clinicians with a reliable tool for diagnosing childhood TB in a high burden setting. This conclusion is in line with recent reports that underline that IGRA cannot be used as a test to exclude active TB in children [36], that use of IGRAs is not recommended in children under 5 years [7] and according to WHO should not be used in low and middle income countries at all [37]. Whether low sensitivity is due to poor antigen response, misclassification or over-diagnosis remains a major challenge for the evaluation of any new diagnostic test for TB in children however the evidence of high mortality in this vulnerable group serves to underline the continued importance of research in this field.

Supporting Information

Box S1 Inclusion criteria.
(DOC)

Box S2 Diagnostic classifications. Classifications in children in line with previous paediatric studies (Liebeschuetz 2004, Marais 2006, Bamford 2010) and in accordance with a recent expert consensus on TB classifications for the use in childhood TB research (Graham 2012).
(DOC)

Acknowledgments

The authors would like to thank Teule Hospital patients and caretakers, the staff and administration for facilitating the research, lab staff at Teule Hospital, CTRL and NIMR-Mbeya Medical Research Programme, with a special thank you to Ben Amos for invaluable help with laboratory issues, The Regional and District TB coordinators, Dr Kijazi and Dr Fadhili for assistance and supportive supervision throughout the study and the Joint Malaria Program for administrative support.

Author Contributions

Conceived and designed the experiments: MVR PR IB GK SGM. Performed the experiments: MVR GK IK TNN. Analyzed the data: MVR TNN GK IK SGM PR IB. Contributed reagents/materials/analysis tools: MVR PR IK MH GK SGM. Wrote the paper: MVR SGM IB PR. Final approval of the manuscript: MVR GK TNN IK MH SGM IB PR.

References

1. Chintu C, Mudenda V, Lucas S, Nunn A, Lishimpi K, et al. (2002) Lung diseases at necropsy in African children dying from respiratory illnesses: a descriptive necropsy study. Lancet 360: 985–990. S0140-6736(02)11082-8 [pii];10.1016/S0140-6736(02)11082-8 [doi].
2. McNally LM, Jeena PM, Gajee K, Thula SA, Sturm AW, et al. (2007) Effect of age, polymicrobial disease, and maternal HIV status on treatment response and cause of severe pneumonia in South African children: a prospective descriptive study. Lancet 369: 1440–1451. S0140-6736(07)60670-9 [pii];10.1016/S0140-6736(07)60670-9 [doi].
3. Marais BJ, Gie RP, Schaaf HS, Beyers N, Donald PR, et al. (2006) Childhood pulmonary tuberculosis: old wisdom and new challenges. Am J Respir Crit Care Med 173: 1078–1090. 200511-1809SO [pii];10.1164/rccm.200511-1809SO [doi].
4. WHO/HTM/TB/2006/371 (2006) Guidance for national tubeculosis programmes on the management of tuberculosis in children.
5. Van RP (2002) The use of the paediatric tuberculosis score chart in an HIV-endemic area. Trop Med Int Health 7: 435–441. 882 [pii].
6. Du TG, Swingler G, Iloni K (2002) Observer variation in detecting lymphadenopathy on chest radiography. Int J Tuberc Lung Dis 6: 814–817.
7. Mazurek M, Jereb J, Vernon A, LoBue P, Goldberg S, et al. (2010) Updated guidelines for using Interferon Gamma Release Assays to detect Mycobacterium tuberculosis infection - United States, 2010. MMWR Recomm Rep 59: 1–25. rr5905a1 [pii].
8. Sester M, Sotgiu G, Lange C, Giehl C, Girardi E, et al. (2011) Interferon-{gamma} release assays for the diagnosis of active tuberculosis: a systematic review and meta-analysis. Eur Respir J 37: 100–111. 09031936.00114810 [pii];10.1183/09031936.00114810 [doi].
9. Lewinsohn DA, Lobato MN, Jereb JA (2010) Interferon-gamma release assays: new diagnostic tests for Mycobacterium tuberculosis infection, and their use in children. Curr Opin Pediatr 22: 71–76. 10.1097/MOP.0b013e3283350301 [doi].
10. Muheza District Council (2010) Muheza District Council Report 2009.
11. Mbago F (2010) Annual TB and Leprosy Report. Muheza District 2009.
12. Mandalakas AM, Detjen AK, Hesseling AC, Benedetti A, Menzies D (2011) Interferon-gamma release assays and childhood tuberculosis: systematic review and meta-analysis. Int J Tuberc Lung Dis. 0631 [pii];10.5588/ijtld.10.0631 [doi].
13. Dogra S, Narang P, Mendiratta DK, Chaturvedi P, Reingold AL, et al. (2007) Comparison of a whole blood interferon-gamma assay with tuberculin skin testing for the detection of tuberculosis infection in hospitalized children in rural India. J Infect 54: 267–276. S0163-4453(06)00147-2 [pii];10.1016/j.jinf.2006.04.007 [doi].
14. Nicol MP, Davies MA, Wood K, Hatherill M, Workman L, et al. (2009) Comparison of T-SPOT.TB assay and tuberculin skin test for the evaluation of young children at high risk for tuberculosis in a community setting. Pediatrics 123: 38–43. 123/1/38 [pii];10.1542/peds.2008-0611 [doi].
15. Jaspan HB, Lawn SD, Safrit JT, Bekker LG (2006) The maturing immune system: implications for development and testing HIV-1 vaccines for children and adolescents. AIDS 20: 483–494. 10.1097/01.aids.0000210602.40267.60 [doi];00002030-200602280-00001 [pii].
16. Newton SM, Brent AJ, Anderson S, Whittaker E, Kampmann B (2008) Paediatric tuberculosis. Lancet Infect Dis 8: 498–510. S1473-3099(08)70182-8 [pii];10.1016/S1473-3099(08)70182-8 [doi].
17. Aabye MG, Ravn P, PrayGod G, Jeremiah K, Mugomela A, et al. (2009) The impact of HIV infection and CD4 cell count on the performance of an interferon gamma release assay in patients with pulmonary tuberculosis. PLoS One 4: e4220. 10.1371/journal.pone.0004220 [doi].
18. Pai M, Lewinsohn DM (2005) Interferon-gamma assays for tuberculosis: is anergy the Achilles' heel? Am J Respir Crit Care Med 172: 519–521. 172/5/519 [pii];10.1164/rccm.2506003 [doi].
19. Bjerrum S, Rose MV, Bygbjerg IC, Mfinanga SG, Tersboel BP, et al. (2012) Primary health care staff's perceptions of childhood tuberculosis: a qualitative study from Tanzania. BMC Health Serv Res 12: 6. 1472-6963-12-6 [pii];10.1186/1472-6963-12-6 [doi].
20. Marais BJ, Schaaf HS (2010) Childhood tuberculosis: an emerging and previously neglected problem. Infect Dis Clin North Am 24: 727–749. S0891-5520(10)00031-0 [pii];10.1016/j.idc.2010.04.004 [doi].
21. Yi JS, Cox MA, Zajac AJ (2010) T-cell exhaustion: characteristics, causes and conversion. Immunology 129: 474–481. IMM3255 [pii];10.1111/j.1365-2567.2010.03255.x [doi].
22. Lighter J, Rigaud M, Eduardo R, Peng CH, Pollack H (2009) Latent tuberculosis diagnosis in children by using the QuantiFERON-TB Gold In-Tube test. Pediatrics 123: 30–37. 123/1/30 [pii];10.1542/peds.2007-3618 [doi].
23. Graham SM, Ahmed T, Amanullah F, Browning R, Cardenas V, et al. (2012) Evaluation of Tuberculosis Diagnostics in Children: 1. Proposed Clinical Case Definitions for Classification of Intrathoracic Tuberculosis Disease. Consensus From an Expert Panel. J Infect Dis. jis008 [pii];10.1093/infdis/jis008 [doi].
24. Metcalfe JZ, Everett CK, Steingart KR, Cattamanchi A, Huang L, et al. (2011) Interferon-gamma release assays for active pulmonary tuberculosis diagnosis in

adults in low- and middle-income countries: systematic review and meta-analysis. J Infect Dis 204 Suppl 4: S1120–S1129. jir410 [pii];10.1093/infdis/jir410 [doi].
25. Haustein T, Ridout DA, Hartley JC, Thaker U, Shingadia D, et al. (2009) The likelihood of an indeterminate test result from a whole-blood interferon-gamma release assay for the diagnosis of Mycobacterium tuberculosis infection in children correlates with age and immune status. Pediatr Infect Dis J 28: 669–673. 10.1097/INF.0b013e3181a16394 [doi];00006454–200908000–00001 [pii].
26. Bergamini BM, Losi M, Vaienti F, D'Amico R, Meccugni B, et al. (2009) Performance of commercial blood tests for the diagnosis of latent tuberculosis infection in children and adolescents. Pediatrics 123: e419–e424. 123/3/e419 [pii];10.1542/peds.2008–1722 [doi].
27. Thomas TA, Mondal D, Noor Z, Liu L, Alam M, et al. (2010) Malnutrition and helminth infection affect performance of an interferon gamma-release assay. Pediatrics 126: e1522–e1529. peds.2010–0885 [pii];10.1542/peds.2010–0885 [doi].
28. Tatala SR, Kihamia CM, Kyungu LH, Svanberg U (2008) Risk factors for anaemia in schoolchildren in Tanga Region, Tanzania. Tanzan J Health Res 10: 189–202.
29. Hamm DM, Agossou A, Gantin RG, Kocherscheidt L, Banla M, et al. (2009) Coinfections with Schistosoma haematobium, Necator americanus, and Entamoeba histolytica/Entamoeba dispar in children: chemokine and cytokine responses and changes after antiparasite treatment. J Infect Dis 199: 1583–1591. 10.1086/598950 [doi].
30. Cegielski JP, McMurray DN (2004) The relationship between malnutrition and tuberculosis: evidence from studies in humans and experimental animals. Int J Tuberc Lung Dis 8: 286–298.
31. Liebeschuetz S, Bamber S, Ewer K, Deeks J, Pathan AA (2004) Diagnosis of tuberculosis in South African children with a T-cell-based assay: a prospective cohort study. Lancet 364: 2196–2203. S0140673604175922 [pii];10.1016/S0140–6736(04)17592–2 [doi].
32. Pelly TF, Santillan CF, Gilman RH, Cabrera LZ, Garcia E, et al. (2005) Tuberculosis skin testing, anergy and protein malnutrition in Peru. Int J Tuberc Lung Dis 9: 977–984.
33. Cattamanchi A, Ssewenyana I, Davis JL, Huang L, Worodria W, et al. (2010) Role of interferon-gamma release assays in the diagnosis of pulmonary tuberculosis in patients with advanced HIV infection. BMC Infect Dis 10: 75. 1471–2334–10–75 [pii];10.1186/1471–2334–10–75 [doi].
34. Hoffmann M, Ravn P (2010) The use of Interferon-gamma release assays in HIV positive individuals. European Infectious Disease, 2010; 4(1): 23–29.
35. Raby E, Moyo M, Devendra A, Banda J, De HP, et al. (2008) The effects of HIV on the sensitivity of a whole blood IFN-gamma release assay in Zambian adults with active tuberculosis. PLoS One 3: e2489. 10.1371/journal.pone.0002489 [doi].
36. European Centre for Disease Prevention and Control (2011) Use of interferon-gamma release assays in support of TB diagnosis.
37. WHO (2011) Use of tuberculosis interferon-gamma release assays (IGRA) in low- and middle-income countries.