HIV AND SCHISTOSOMIASIS: STUDIES IN TANZANIA

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The printing of this thesis was supported financially by Leiden University.
The work presented in this thesis was supported by the Infectious Diseases Society of America/National Foundation for Infectious Diseases Merle A. Sande Award, the National Institutes of Health (T32 HS 000066, T32 AI 007613, UL1 RR 024996, UL1 TR000457-06, K23 AI 110238), and the Mulago Foundation.

Cover design: Jennifer Downs

Printing: Vanderperk Groep

HIV and Schistosomiasis: Studies in Tanzania

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus prof. mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 31 mei 2016
klokke 15:00 uur

door

Jennifer Downs
geboren te Verenigde Staten van Amerika
in 1978

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ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
ART	Antiretroviral therapy
CAA	Circulating anodic antigen
CCA	Circulating cathodic antigen
CCR5	C-C chemokine receptor 5
CD4	Cluster of differentiation – type 4
CD4 count	CD4+ T-cell count
CXCR4	C-X-C chemokine receptor type 4
CI	Confidence interval
DBS	Dried blood spot
epg	Eggs per gram
HIV	Human immunodeficiency virus
IQR	Inter-quartile range
ml	Milliliters
PCR	Polymerase chain reaction
SHIV	Simian-human immunodeficiency virus
STI	Sexually-transmitted infection
TAT	Turn-around time
TPPA	*Treponema pallidum* particle agglutination
UNAIDS	Joint United Nations Programme on HIV/AIDS
Viral load	Plasma concentration of HIV RNA in HIV-infected patients
WHO	World Health Organization

CHAPTER 1

GENERAL INTRODUCTION

Background

In sub-Saharan Africa, human immunodeficiency virus and acquired immune deficiency syndrome (HIV/AIDS) is the leading contributor to life-years lost (1), causing 1.1 million (73%) of the 1.5 million deaths worldwide due to HIV/AIDS in 2014 (2). In addition, the Joint United Nations Programme on HIV/AIDS (UNAIDS) reported that 70% of the 1.5 million new HIV infections worldwide occurred in sub-Saharan Africa (2). Only 37% of HIV-infected individuals in sub-Saharan Africa are receiving life-saving antiretroviral therapy (ART) (3). These numbers document the major disproportionate burden of disease inflicted by HIV in sub-Saharan Africa, as well as the substantial potential for improvement if HIV prevention, diagnosis, and management can be optimized.

In patients who have, or are at risk for, HIV infection, challenges are further exacerbated in the setting of overlapping co-infections. For example, sexually-transmitted infections (STIs) in women not only cause direct morbidity and mortality (e.g., chronic pelvic inflammatory disease leading to infertility in women and congenital infection in their babies), but they additionally increase susceptibility to HIV acquisition (4). Tuberculosis patients who are co-infected with HIV have a two-fold higher mortality rate than tuberculosis patients without HIV infection (5). Tuberculosis, STIs, and a variety of other co-infections in HIV-infected patients increase the plasma concentration of HIV RNA (viral load), which is well-recognized as a primary marker of HIV transmissibility and disease progression. A recent systematic review summarized 18 different studies, all of which demonstrated that the viral load could be reduced through appropriate treatment of tuberculosis, STIs, malaria, or helminth infections (6). Taken together, these examples clearly document the impact of multiple infections on the health of an individual, and the importance of investigating ways in which co-infections can be managed to improve morbidity and mortality.

This thesis will focus on HIV and parasitic infections caused by *Schistosoma mansoni* and *Schistosoma haematobium* in Tanzania. It will begin by investigating the overlap and interactions between these two highly prevalent infections (**Chapters 2 and 3**). It will then describe the chronic complications related to schistosomiasis of the urogenital tract that may increase a woman's susceptibility to HIV acquisition even after treatment (**Chapter 4**), and will document an innovative laboratory technique by which additional studies of the relationship between schistosome infection and HIV may be explored using dried blood spots (**Chapter 5**). After this, the focus will turn to a study of the interactions of schistosome infection and HIV in HIV-infected individuals who are taking antiretroviral medications (**Chapter 6**). Finally, the thesis will investigate the

implementation of better care to improve early infant diagnosis of HIV infection in infants born to HIV-infected mothers (**Chapter 7**). The discussion will focus on the knowledge gained through studies to this point, as well as the recommended next steps for further research to improve diagnosis, treatment, and disease prevention in individuals living in sub-Saharan Africa.

Overview of Schistosomiasis

Schistosomiasis is a parasitic infection caused by helminthic worms of the *Schistosoma* genus. The three main species of schistosomes that cause human disease are *S. haematobium*, *S. mansoni,* and *S. japonicum*. The World Health Organization (WHO) estimates that, in 2013, over 260 million individuals required treatment for schistosomiasis, and that an additional 700 million were at risk of infection, with over 90% of these individuals living in Africa where both *S. mansoni* and *S. haematobium* are endemic (7,8). Schistosomiasis is a disease of poverty acquired by contact with fresh water that has been contaminated by parasite larvae and is classified as a neglected tropical disease by the WHO (9). Schistosomiasis causes a large global burden of morbidity, with an estimated 3.31 million disability-adjusted life years in 2010. This was second only to leishmaniasis (3.32 million) among the neglected tropical diseases (10).

Schistosomiasis has a complicated life cycle that is propagated when the following requirements are met: (1) human excrement containing schistosome ova must contaminate fresh water; (2) specific intermediate snail hosts must reside in the contaminated water; and (3) humans must come into contact with contaminated water. Schistosome eggs, excreted into fresh water, hatch to release miracidium larvae that seek snail hosts, where they replicate asexually to produce multiple generations of sporocysts (11,12). Sporocysts mature into cercariae with a characteristic bifurcated tail, and are then released from snails into fresh water to seek their definitive host. Cercariae typically live for 24 hours and can penetrate unbroken skin, resulting in human infection (11,13). During skin penetration, cercariae lose their bifurcated tail and become schistosomulae, which migrate through tissue into the host blood stream, the lung, and ultimately the portal vein (11,12). Schistosomulae mature in the portal vein for 4-6 weeks into adult worms and, once mature, migrate preferentially to venules of the urinary and genital tracts (*S. haematobium*) or the gastrointestinal tract (*S. mansoni, S. japonicum*).

Adult schistosome worms living in venules form permanent reproductive male-female pairs, producing hundreds to thousands of eggs per day and living an average of 3-5 years (11,14). Schistosome miracidia within eggs secrete

proteolytic enzymes that facilitate the migration of the egg through the vessel wall and into the surrounding tissues toward the mucosal tissue and lumen of the genitourinary tract or intestine (15). Approximately one-third of eggs produced by worms ultimately arrive at the lumen of the bladder or intestine and are excreted in urine or stool (14); the remaining eggs are trapped in the tissues or are embolized to distant organs. Repeated exposure to schistosome-infected fresh water progressively increases the number of worms and eggs and the severity of the infection.

Schistosomiasis causes a plethora of immune alterations in the immune system of the host, with effects on T-helper (Th) 1, Th2, Th17, and T-regulatory (T-reg) immune responses. The initial immune response to schistosome infection is T-helper 1 (Th1)-mediated, directed against worm antigens. Once adult worms have matured and egg production begins, the immune response rapidly becomes T-helper 2 (Th-2)-mediated due to the high antigenicity of eggs as they migrate through tissue (16). This Th2-type response leads to granulomatous inflammation that surrounds eggs trapped in tissue, and, ultimately, to tissue fibrosis and chronic morbidity (17). This granulomatous response is paradoxical in its effect: though it does incite tissue fibrosis, it also protects the host tissue by sequestering toxic secretions of schistosome eggs that can otherwise result in widespread host tissue necrosis (16,18,19). Prolonged infection with granulomatous changes lead to late-stage tissue obstructive changes. Interestingly, such obstructive changes have been associated with increased tissue burdens of eggs in autopsy studies, suggesting that late-stage fibrosis may decrease transmission of the parasite from the index case (20,21).

A natural history study of morbidity caused by *S. haematobium* in an endemic region of Kenya supports this progression of tissue pathology over time (20). Investigators demonstrated that, in the initial years of infection, urinary tract lesions were inflammatory granulomata of the ureters or bladder. Over time, these lesions progressed to "established" or obstructive tissue changes such as hydronephrosis, hydroureter, and bladder fibrosis. The study investigators suggested that, because hydronephrosis predominantly affected adults and appeared to be independent of intensity of schistosome infection, the duration of parasite infection more accurately predicts risk of chronic tissue disease than the intensity of infection. Multiple studies have also demonstrated the importance of other factors including treatment, reinfection, and a complicated interplay of host genetics influencing the balance of Th1, Th2, Th17, and T-reg-type reactions to the parasite eggs in determining the degree of tissue damage and host pathology (11,17,22,23).

The major morbidity experienced in chronic schistosomiasis is attributable to the host's egg-induced granulomatous response and subsequent fibrosis in the bladder, intestine, and liver (24). Prominent clinical sequelae of various species of schistosomes depend largely on where adult worms reside. *S. haematobium* worms live in venules surrounding the urinary and genital tracts, and thus their migrating eggs typically cause bladder/ureter fibrosis leading to hydronephrosis, bladder cancer, and genital tract damage including erosions, edema, sandy patches, and neovascularization of the cervix, as well as alterations in immune cell populations in the genital mucosal tissue (25–28). Major sequelae of *S. mansoni* infection include diarrhea, intestinal inflammation, and liver fibrosis leading to esophageal varices and hematemesis. A 2003 paper estimated the annual global mortality from renal failure secondary to *S. haematobium* to be 150,000 and from hematemesis induced by *S. mansoni* to be 130,000 (29). Morbidity is also significant in those with chronic schistosome infection, who suffer from significantly higher burdens of anemia, poor nutrition, pain, and impaired academic performance and have been estimated to function at a disability level of 2-15% (30).

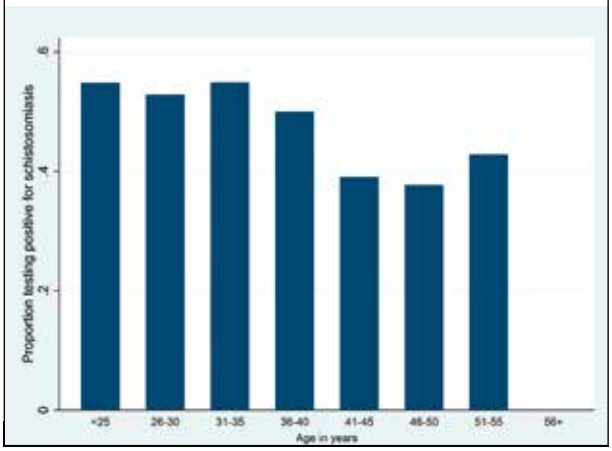
The gold standard for diagnosis, recommended by the WHO, is microscopic visualization of parasite eggs in urine or stool (8). Urine is filtered through a porous membrane filter through which schistosome eggs cannot pass, and the filter is examined directly under the microscope. Urinary infection intensity is reported as eggs per 10 milliliters (ml) of urine. Eggs in stool can be quantified using the Kato Katz technique, in which a thick fecal smear of known mass is prepared, stained, and examined for schistosome ova. Stool infection intensity is reported as eggs per gram (epg). It is well-recognized that egg excretion varies, particularly in those with light infections, and that this gold standard may be less sensitive than other diagnostic strategies including antigen testing and polymerase chain reaction (PCR) (31–33).

A highly promising diagnostic strategy is the detection of schistosome circulating antigens, which is used for much of the work done in this thesis. Circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) are produced in the gut of schistosome worms and regurgitated by the worm into the host bloodstream during active infection with either *S. mansoni* or *S. haematobium* (34). CAA becomes detectable in serum approximately 3 weeks after infection (35). It is heat-resistant and extremely stable, remaining detectable in tissue isolated from Egyptian mummies (36) and in dried blood spots (37). CAA levels fall rapidly post-treatment, with a serum half-life of 2 days, becoming undetectable within 3-6 weeks (38,39) and become positive again with reinfection (40). The CAA assay is recommended by the WHO for serologic screening programs in

which repeated parasitologic examinations of urine and stool are logistically not possible (36,41,42). CAA testing has been optimized using up-converter phosphor technology with dry reagents that are robust and straightforward for use (43,44). CCA testing has been developed into a commercially available point-of-care rapid test with high sensitivity and specificity for *S. mansoni*, though its performance for *S. haematobium* diagnosis is less reliable (44–47).

Schistosome infections occur often and early in endemic areas, beginning with babies and preschoolers and typically increasing in both prevalence and intensity throughout childhood, peaking during adolescence, then diminishing slowly as adults acquire partial but incomplete immunity (11,48–50). Our prior work has demonstrated that significant proportions of both young and middle-aged adults remain chronically infected in endemic areas (**Figure 1**). The WHO schistosomiasis treatment guidelines focus primarily on children, recommending that all children in high-risk communities should treated annually with the antihelminthic drug praziquantel, at an estimated cost of USD $0.32 per dose (51,52). Treatment is additionally extended to adults at special risk (such as those occupationally exposed to water or women using water for domestic chores) and to entire communities in endemic areas (51). In the real world, such treatment is a challenge, and the usual practice in Tanzania is to provide school-based praziquantel treatment for children annually, with no routine treatment after that. The WHO estimates that, in 2013, only 13% of those who needed treatment for schistosomiasis actually received praziquantel (8).

Figure 1. Proportion of schistosome infections by age among 477 rural Tanzanian adults living in villages highly endemic for schistosomiasis.



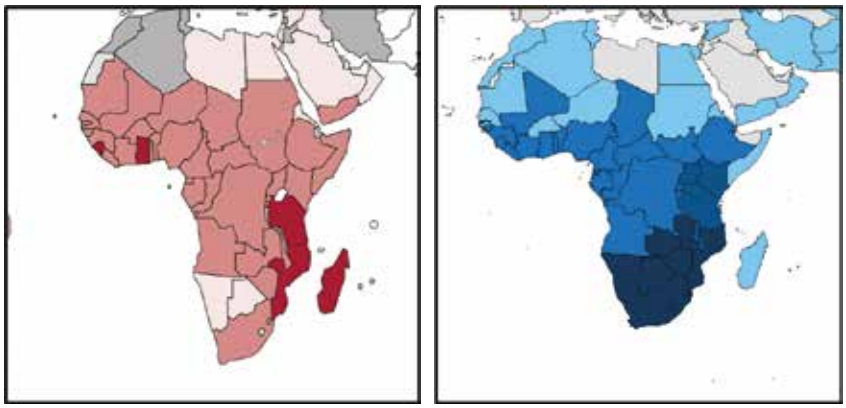
Thus it is clear that major implementation gaps remain and millions of children and adults continue to live with the chronic burden of untreated schistosomiasis. Moreover, in the absence of improved access to clean water, many individuals living in schistosome-endemic areas have no choice but to continue to use unclean water in order to sustain their livelihood. The result is a lifetime of chronic

schistosome infection and suboptimal health endured by millions of individuals living in sub-Saharan Africa.

The Confluence of Schistosomiasis and HIV Infection in sub-Saharan Africa

Approximately 78 million individuals have been infected by HIV since the epidemic began, and 35 million people are currently living with HIV worldwide (53). The HIV epidemic arose in sub-Saharan Africa and over 70% of global HIV infections (over 24 million) remain in this region (2,54). Epidemiologic mapping of HIV and schistosomiasis reveals a substantial overlap between areas in which both schistosomiasis and HIV are epidemic (**Figure 2**). In Tanzania, the prevalence of schistosomiasis in many areas is >50%, and the prevalence of HIV is 5.1% (55).

Figure 2. Schistosomiasis and HIV epidemics in Africa.



Schistosomiasis
World Health Organization, 2012

HIV Prevalence in Adults Aged 15-49
UNAIDS and Kaiser Family Foundation, 2014

Country-level epidemiologic data also suggests a relationship between schistosomiasis and HIV infection. A recent regression analysis used publicly-available data to determine the relationships between HIV infection, schistosome infection, and other epidemiologic and demographic data. Investigators reported that, after adjusting for other factors associated with HIV prevalence including male circumcision prevalence, years since the HIV epidemic began in a country, and immunization coverage, each one additional case of *S. haematobium* per 100 people was associated with a 2.9% (95% confidence interval (CI), 0.2% - 5.8%)

relative increase in the prevalence of HIV infection (56). While this data is cross-sectional and therefore not able to determine causality or to exclude potential confounders, it does lend plausibility to the existence of a relationship between the two infections.

In further support of the HIV-schistosomiasis association, the first cross-sectional study performed in individual patients was published in 2006 by Kjetland and colleagues. The study, which enrolled 527 women living in a region in Zimbabwe in which *S. haematobium* was highly endemic (with 43% of the overall study population having *S. haematobium* ova detected in urine or genital specimen), demonstrated that women who had *S. haematobium* ova found in a cervical Pap smear had a higher prevalence of HIV infection than those without ova in a Pap smear, with an adjusted odds ratio (OR) of 2.9 [95% CI, 1.1-7.5] (p=0.030) (57). Other measured factors significantly associated with HIV on multivariable analysis included Herpes Simplex Virus-Type 2 (HSV-2) infection, widowhood, infertility, and age. Investigators subsequently followed 224 women for 12 months and documented 7 new HIV seroconversions; all 7 of these occurred in women who had had signs of urogenital schistosomiasis at baseline, compared with 142/217 who had had signs of urogenital schistosomiasis at baseline but remained negative (p=0.098).

Though others had previously speculated that genital *S. haematobium* infection may predispose to HIV due to its ability to cause genital ulcer disease (25,58), the study from Zimbabwe was the first and, at the time of its publication, the only clinical study to demonstrate a clear association between the two infections. While the study design did not permit determination of causality, investigators postulated that *S. haematobium* genital infection, typically acquired during childhood prior to HIV exposure, could increase a woman's susceptibility to HIV infection. **Chapters 2 and 3** of this thesis document additional clinical studies from Tanzania that were conducted to quantitate the relationship between schistosomiasis and HIV infection in women.

Urogenital Schistosomiasis: a Risk Factor for HIV Acquisition?

Formerly termed "urinary schistosomiasis," *S. haematobium* infection was renamed "urogenital schistosomiasis" in 2009 by the WHO in recognition that up to 75% of individuals with *S. haematobium* infection have both bladder and genital tract involvement (59). *S. haematobium* worms reside in venules surrounding the urinary bladder and pelvic organs, and schistosome ova become sequestered in the not only the bladder and ureters, but also throughout both the female and male reproductive tracts (**Figure 3**). Post-mortem and surgical studies have confirmed

that, in women, the cervix is the most common site of ova sequestration, followed by vagina and Fallopian tubes (60). Eggs lodged in genital sites provoke signs and symptoms including pelvic discomfort, postcoital bleeding, itching, and vaginal discharge in women and painful ejaculation, hematospermia, and leukocytospermia in men (25,26,61–63). Findings on clinical examination in women include edema of the cervical or vaginal mucosae, erosions, ulcerations, neovascularization, and mucosal friability (26,28). These breaches in the epithelial integrity of the female genital tract, similar to those caused by trauma or STIs, have been suggested to enhance the ease of viral entry beneath the surface of the genital epithelium, thereby facilitating HIV acquisition in women with schistosomal cervical damage (25,64).

Figure 3. *Schistosoma haematobium* ova obtained in a crushed cervical biopsy (performed to exclude cervical cancer) from a 24-year-old woman in rural Tanzania.

Schistosome ova sequestered in genital tissue cause a spectrum of clinical pathology, which are best visualized using a colposcope and are used by some to make a clinical diagnosis of urogenital schistosomiasis since detection of ova or parasite DNA in genital secretions and tissues can be challenging (65,66). Classic schistosome-induced lesions of the genital mucosa have recently been photographically detailed in a "Colposcopic Atlas of Schistosomiasis" that is intended to serve as a diagnostic guidebook for clinicians and researchers (67). "Grainy sandy patches," which are rough grains located superficially within the genital mucosa that represent calcified ova beneath an atrophic epithelium, are pathognomonic for urogenital schistosomiasis (26). Another lesion, the "homogeneous yellow sandy patch," can be identified both in women with *S. haematobium* infection and in those with STIs, making this finding less specific. Sandy patches are often surrounded by abnormal blood vessels with diffuse inflammation and contact bleeding (67). The contact bleeding may provide an additional mechanism—direct access of the virus to fresh blood—by which HIV risk could be increased in the presence of *S. haematobium* infection. Importantly, sandy patches and vascular abnormalities appeared not to be completely reversible following praziquantel treatment in women (68,69). This

suggests that schistosome ova in tissue could cause longstanding and possibly permanent effects, particularly in women who are not treated until they are older than age 20 (70).

Finally, at the cellular level, schistosome ova in tissue induce a complex immune response that is characterized by infiltration of plasma cells, lymphocytes, macrophages, and eosinophils to the genital tract (71). Jourdan and colleagues quantified HIV-susceptible cells in cervicovaginal biopsies from Malawian women with and without *S. haematobium* ova and found that biopsies containing ova had higher densities of $CD4^+$ T-lymphocytes and macrophages than biopsies without ova (27). These cellular changes at the tissue level also appear to be reflected systemically in the peripheral blood of infected individuals. In Kenya, the monocytes and $CD4^+$ T-cells of car washers with *S. mansoni* infection exhibited higher densities of the chemokine receptors CCR5 and CXCR4 than the monocytes and $CD4^+$ T-cells of those who had been treated for schistosomiasis (72). Because CCR5 and CXCR4 are also HIV co-receptors, investigators postulated that the monocytes and $CD4^+$ T-cells from individuals with schistosome infection may be more susceptible to HIV infection than these same cells in uninfected or previously-treated individuals. Further, the tendency of schistosome infection to incite a Th2-type response, with an accompanying shift away from the cytotoxic Th1-type immunity that is critical to the early antiviral defense against HIV, may also promote an immune environment that is more permissive to HIV acquisition (64,73).

Taken together, the apparent interactions between HIV and schistosomiasis across a breadth of lines of investigation, and the biologic plausibility of such an association, lends credence to the hypothesis that schistosomiasis may increase susceptibility to HIV acquisition. Furthermore, the effects of schistosomiasis may not be reversible with treatment, leading to chronic enhanced HIV susceptibility. The two studies that explored response of urogenital schistosomiasis to praziquantel treatment documented clinical and parasitological findings, but did not use molecular techniques and therefore relied on tests with poorer negative predictive value to indicate whether the parasite had been eradicated or whether infection persisted. Confirming and extending these prior studies using molecular diagnostics was the goal of the longitudinal work described in **Chapter 4**.

The other clear need that emerges from this review of the relationship between HIV and schistosomiasis is the need for prospective studies that are able to document conclusively that pre-existing schistosome infection is a risk factor for incident HIV infection. Such longitudinal studies are complex and fraught with ethical issues, including the gynecological examination of pre-adolescent and adolescent

girls and/or the need to follow a group of individuals with known, untreated schistosome infection to determine whether the incidence of HIV is higher than in those without schistosome infection. One possible idea to circumvent these ethical challenges would be to use serum schistosome antigen tests in order to analyze banked serum from prior HIV-seroincidence cohorts. We have access to stored serum samples from an HIV-seroincidence cohort of 30,000 individuals in rural Tanzania who have been followed since 1994. The serum was stored in the form of dried blood spots (DBS) on Whatman 903 ProteinSaver cards. Two prior papers sought to elute CAA from two other types of filter paper and demonstrated that CAA was indeed detectable, but that available concentrations were low (37,74). Therefore, the goal of the work described in **Chapter 5** was to optimize detection of CAA in Whatman 903 cards, which are the most widely-used cards for dried blood spot collection worldwide. This would facilitate diagnosis of schistosomiasis in a variety of studies in which DBS were collected, and would pave the way for analysis of the incidence of HIV among individuals who were and were not CAA-positive prior to their HIV-seroconversion in our Tanzanian cohort.

Schistosomiasis and HIV Disease Progression in Co-Infected Individuals

Studies of HIV-infected individuals with schistosome co-infection demonstrate that schistosomiasis may exert some effect on HIV disease progression and outcomes. In particular, meta-analyses published in 2008 and 2010 suggested, though not conclusively, that the treatment of helminthic infections (75) and of helminthic plus other co-infections (6) may decrease HIV viral load. These analyses were limited by the dearth of randomized controlled trials and other methodological problems in the existing studies on this issue.

Kallestrup and colleagues conducted the single randomized trial that explored the effect of schistosomiasis treatment on viral loads in Zimbabwe, and they reported significant viral load differences in treated and untreated groups. In this study, antiretroviral therapy (ART)-naïve HIV-infected patients with schistosome co-infection were randomized to receive single-dose praziquantel either at enrollment or after three months. Those randomized to delayed praziquantel treatment had greater increases in HIV viral loads and greater decreases in CD4 counts than those treated immediately (76). Treatment effectiveness was not reported in this population. More recently, a nonblinded, randomized trial of empiric albendazole and praziquantel in over 900 Kenyan HIV-infected patients found no effect of antihelminthic treatment on CD4 counts, viral loads, time to initiation of antiretroviral therapy (ART), or mortality (77). Of note, the prevalence of schistosomiasis in this study population was only ~5%, and the study was not

designed or powered to detect the effect of treating individual helminth species. In the wake of these conflicting findings, unanswered questions remain about the impact, or lack of impact, of schistosome co-infection on HIV disease progression. Moreover, both the Zimbabwean and the Kenyan trials enrolled only HIV-infected patients who had not yet initiated ART. To the best of our knowledge, no studies had been done to assess the impact of schistosomiasis on the response to ART, which is becoming increasingly relevant as ART coverage in sub-Saharan Africa continues to rise, recently reaching above 35% (3). Therefore, the work in **Chapter 6** was conducted to investigate, for the first time, the effect of schistosome infection among HIV-infected individuals who were receiving ART in Tanzania.

The Need for Implementation Science Research to Promote Early HIV Diagnosis in Tanzania

The 1.4 million new HIV diagnoses occurring annually in sub-Saharan Africa, together with the fact that less than 50% of people living with HIV in the region are aware of their infection status (3), demonstrate the ongoing need for major population-based interventions to prevent HIV altogether and to diagnose it expeditiously. Even when cost and logistics are not limiting factors, the journey from awareness of evidence-based best practices to implementation of these practices can be challenging. Religion, cultural values, education levels, health beliefs, and social norms all impact a person's, or a population's, willingness to adopt practices, even those that have been scientifically proven to be beneficial for health. Challenges are often exacerbated in resource-poor settings.

The growing field of implementation science strives to address these issues. Implementation science seeks to determine effective strategies by which research findings can be incorporated into healthcare policy and practice in specific settings in order to improve population health (78). The final chapter of this dissertation, inspired by the experience of spending years living and working in a country in which healthcare providers struggle to implement evidence-based guidelines, is such an implementation science study. This chapter focuses on issues related to implementing practices that, although known to be effective in early infant diagnosis of HIV, have faced implementation obstacles in Tanzania.

Chapter 7 is an implementation science project, conducted in rural Tanzania, that focused on streamlining early infant diagnosis (EID) of HIV infection in infants who were exposed perinatally to HIV. Early diagnosis is urgent because infants who become HIV-infected before, during, or shortly after birth have a mortality rate of approximately 50% before 12 months of age, with 20% dying before they

reach 3 months of age (79). In resource-poor areas without local laboratory facilities able to perform the PCR necessary to detect HIV RNA or DNA in an infant's blood, the WHO recommends collecting blood as DBS and shipping them to a reference laboratory with PCR capacity (80). Bugando Medical Centre in Mwanza, Tanzania houses the reference laboratory for 13 million people, and receives DBS for EID from 96 clinics in the region. Returning an HIV test result to an infant's mother typically takes weeks because the testing system depends on a multi-step process in which DBS are collected, transported to the laboratory, tested at the laboratory, returned to the clinic, and then communicated to the mother. This implementation science work was prompted by the observation that many mothers were never receiving their infants' test results, and those who did were receiving them extremely late. The implementation science research described in this chapter used a systems improvement methodology to make sequential interventions in this multi-step process and to monitor the effects of these interventions.

Finally, in the general discussion in **Chapter 8,** the key findings from the preceding chapters will be analyzed, and the contribution that these findings have made to the knowledge base will be summarized. We will close each section of the discussion by outlining logical next research steps to explore research questions that remain.

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CHAPTER 2

UROGENITAL SCHISTOSOMIASIS IN WOMEN OF REPRODUCTIVE AGE IN TANZANIA'S LAKE VICTORIA REGION.

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Am J Trop Med Hyg 2011; 84(3): 364-9.

Abstract

We conducted a community-based study of 457 women aged 18--50 years living in eight rural villages in northwest Tanzania. The prevalence of female urogenital schistosomiasis (FUS) was 5% overall but ranged from 0 to 11%. FUS was associated with HIV infection (OR = 4.0, 95% confidence interval: [1.2--13.5]) and younger age (OR = 5.5 [1.2--26.3] for age < 25 years and 8.2 [1.7--38.4] for age 25--29, compared to age > 35). Overall HIV prevalence was 5.9% but was 17% among women with FUS. We observed significant geographical clustering of schistosomiasis: northern villages near Lake Victoria had more *S. mansoni* infections ($p < 0.0001$), and southern villages further from the lake had more *S. haematobium* ($p = 0.002$). Our data support the postulate that FUS may be a risk factor for HIV infection, and may contribute to the extremely high rates of HIV among young women in sub-Saharan Africa.

Introduction

Female urogenital schistosomiasis (FUS) is predominantly caused by *Schistosoma haematobium* and has been estimated by the World Health Organization (WHO) to affect up to 45 million women living in sub-Saharan Africa.[1] Adult *S. haematobium* worms inhabit blood vessels surrounding the urinary bladder and female genital tract and lay eggs that migrate through tissue of proximate organs, causing chronic granulomatous inflammation most commonly in the urinary bladder, ureters, cervix, and vagina. Because the urinary and genital tracts are almost always both affected, the WHO has recently renamed this disease "urogenital schistosomiasis," with detection of *S. haematobium* in the urine or genital tract diagnostic.[1]

Chronic female genital tract inflammation caused by *S. haematobium* has been associated with vaginal itching and discharge,[2] postcoital bleeding,[3] genitopelvic discomfort,[4] marital discord,[5] and infertility.[6,7] Genital *S. haematobium* infection has been associated with HIV infection in one cross-sectional study[7] and has been postulated to be a risk factor for HIV infection.[8,9]

Tanzania's Lake Victoria region in northwestern Tanzania borders Kenya, Uganda, and Rwanda and is believed to have among the highest prevalence of *S. haematobium* in the world, with a prevalence of 50--90% reported in young schoolchildren.[10] The prevalence of *S. haematobium* infection has not been quantified in women of reproductive age, the population at risk for increased HIV infection. Therefore, we conducted a study in Tanzania's Lake Victoria region to determine the prevalence and identify risk factors for FUS among women of reproductive age.

Materials and methods

Study sites and subjects. This cross-sectional study was conducted between August 2009 and May 2010 near Lake Victoria in northwest Tanzania in partnership with a cervical cancer screening program being conducted at local health centers. Women who were receiving free cervical cancer screening, were between the age of 18 and 50 years, and who provided written consent were invited to participate in the FUS prevalence study. Women who were menstruating or who refused gynecologic examination were excluded. Pregnant and breastfeeding women were included.

Urine and gynecologic examination. A single urine sample was collected from women between 10am and 2pm and filtered and examined microscopically by a

trained parasitologist for schistosomal ova. A subset of the urine samples were read by two parasitologists for quality control.

Women who provided informed consent underwent a gynecological examination. A swab of vaginal secretions was collected for wet preparation and microscopic examination for diagnosis of Candida (using a potassium hydroxide preparation), *Trichomonas vaginalis* (using warm normal saline), and bacterial vaginosis by Amsel's criteria.[11] Next, a cervical smear was collected using a plastic spatula. Smears were stained with 0.5% Trypan Blue and were examined for schistosomal ova while fresh. Acetic acid was then applied to the face of the cervix, followed by inspection for abnormal areas after one minute. Abnormal cervical lesions were biopsied. Specimens were stained with Hematoxylin and Eosin (H&E) for histopathological examination and with Trypan Blue to examine for schistosomal ova using the "crush technique" previously described.[6,12] Biopsies were not performed on pregnant women.

Female urogenital schistosomiasis (FUS) was defined following WHO criteria as the presence of at least one schistosomal ovum seen in the urine sample, cervical smear, or cervical biopsy.[1]

Other laboratory studies. Single stool samples for *S. mansoni* ova were processed by Kato Katz technique. HIV voluntary counseling and testing was offered to all participants. For those who agreed to be tested, blood was collected and tested using a rapid test (SD Bioline). Testing was performed in the field and patients received their results immediately. Venous blood was also collected and tested for syphilis using the Rapid Plasma Reagin (RPR) test and positive tests were confirmed with the *Treponema pallidum* Particle Agglutination assay (TPPA).

Interview. Women also participated in a 20-minute structured interview about water contact, gynecologic symptoms, sexual history, and depression. The interview was administered by a nurse in Kiswahili. Women were asked to use a 4-point Likert scale to quantify how much, over the past four weeks, they had been bothered by dyspareunia, vaginal discharge, postcoital bleeding, abdominopelvic pain, infertility, menstrual abnormalities, genital itch, and incontinence. Using a 5-point Likert scale to assess sexual dysfunction, women were also asked how much they worried about pain during intercourse, made excuses to avoid intercourse, had experienced decreased frequency or quality of intercourse, and were concerned about partner infidelity. We assessed the responses to these questions as binary variables, where answers of "very much" or "somewhat" were considered positive, while answers of "rarely" or "not at all" were considered negative.

Depression was evaluated using a 9-item depression scale, the PHQ-9, that has been previously translated and validated in Kiswahili in several patient populations.[13,14] The PHQ-9 consists of nine questions, each designed to assess for one of the nine symptoms of depression delineated by the Diagnostic and Statistical Manual-IV (DSM-IV) for depression. These include anhedonia, sleeplessness or excessive sleep, hopelessness, poor or excessive appetite, difficulty concentrating, and feelings of failure. Participants receive between 0 and 3 points on each question, with 0 indicating that they experienced a given symptom "Not at all" and 3 indicating "Nearly every day". A total score of 5--9 has been classified as mild depression, 10--14 indicates moderate depression, 15--19 indicates moderately-severe depression, and above 20 indicates severe depression.[15]

Ethical considerations. The study was explained to women in a large group and subsequently one-on-one by a trained study nurse fluent in the local language. In order to participate in the study, women were asked to provide written informed consent or to place their mark on the consent form. At the local level, permission was obtained from the District Medical Officers and clinicians stationed at participating dispensaries and health centers. Ethical approval was granted by the research ethics committee at Bugando Medical Centre, by the Medical Research Coordinating Committee of the National Institute for Medical Research in Tanzania, and by the Institutional Review Board at Weill-Cornell Medical College.

Women diagnosed with urogenital or intestinal schistosomiasis, syphilis, trichomoniasis, candidiasis, or bacterial vaginosis received free treatment. Women with trichomoniasis were given medication both for themselves and their sexual partners. Women with syphilis and their sexual partners received three intramuscular injections of penicillin at the clinic. Patients diagnosed with HIV were referred to nearby district or regional hospital-based clinics for free care and treatment. Women with cervical dysplasia or cancer were referred to tertiary institutions for further management, and all of them accessed care successfully.

Statistical methods. Data were entered into a REDCap database (Vanderbilt University, Nashville, Tennessee). Continuous variables were summarized by median and interquartile range and categorical variables were summarized by frequency and percentage. Regional disparity of infections was assessed by Fisher's exact test; the association of infection status and region was evaluated using northern versus southern designation (in a 2 x 2 table) as well as individual regions (in a 2 x 8 table). Factors that were associated with the endpoint (i.e., FUS) were examined by multiple logistic regression. Backward elimination was adopted to reach the final parsimonious model that included significant factors

only – starting from a full model with all of the candidate predictors (all factors presented in Tables 1 and 3) and deleting the least significant factor one at a time until only the predictors with p-value < 0.05 remain in the model. We also confirmed that the automatic variable selection procedures (e.g., stepwise, forward, backward selection) yielded the same final model. We analyzed age in two different ways – using a continuous variable and categorized variables (with 25, 30, and 35 as cutoff points, which approximately corresponded to lower quartile, median and upper quartile, respectively).

Association between factors and the endpoint was summarized in odds ratio (OR) along with 95% confidence interval (CI) and the associated p-value. We also computed the area under the receiver-operating-characteristic curve (AUC) to ascertain the discrimination capability of the factors for FUS cases vs. non-cases—AUC of 0.5 means that the discrimination capability is no better than chance, and 1 means perfect discrimination.

SAS 9.2 (Cary, North Carolina) was used for data analyses. Two sided hypotheses/tests were assumed for computation of all confidence intervals and p-values.

Results

Patient Characteristics. Out of 550 eligible women who presented to primary care clinics and were invited to participate in the study, 457 women (83.1%) consented to participate and completed all study procedures. Characteristics of study participants are shown in Table 1. The median age was 30 years (IQR, 24--35 years). The great majority was married, had at least one child, and worked in farming and petty trade. Participants had a median number of 14 contacts with potentially-infectious water per day, and no woman reported zero contacts. One-third reported receiving treatment for schistosomiasis in the past.

Prevalence of *Schistosoma haematobium* and *mansoni* infections. The prevalence rates for *S. haematobium* and *S. mansoni* are presented in Table 2, and a map of the area with the villages where screening took place is shown in **Figure 1.** The prevalence of *S. haematobium* infections was higher among women living in southern inland villages than in northern villages along the shores of Lake Victoria. The prevalence in the south was 13/120 (10.8%) compared with 10/337 (3%) in women who lived in northern lake-side villages (p = 0.002). Of the 23 women with urogenital schistosomiasis included in the analysis, 16 had *S. haematobium* ova detected in the urine only, 6 had *S. haematobium* ova detected in a genital specimen, and one had *S. mansoni* ova visualized on a cervical smear.

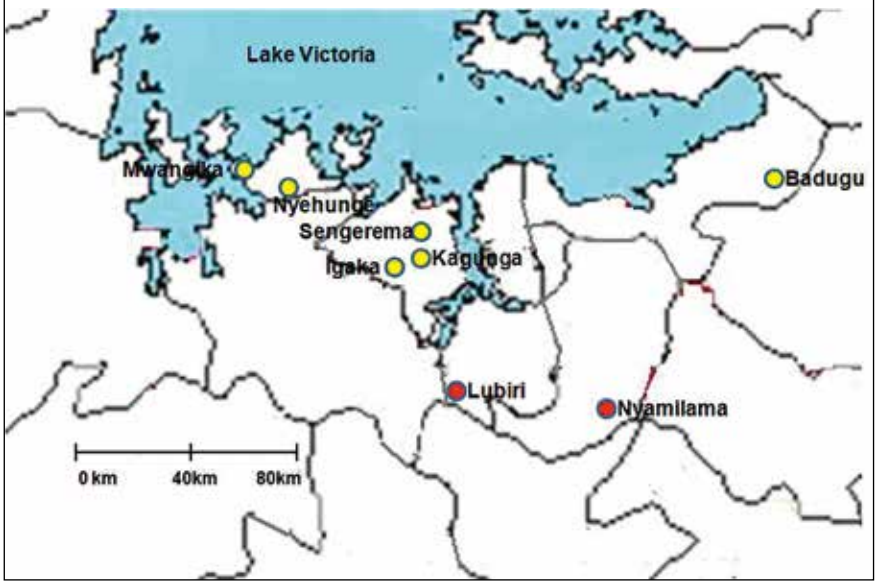
TABLE 1. Baseline characteristics of 457 women attending primary care clinics for schistosomiasis screening in Mwanza, Tanzania.

CHARACTERISTIC	VALUE
Age in years—median (interquartile range)	30 (24--35)
Marital Status—number (percent)	
Single	10 (2.2%)[a]
Living with partner	31 (6.8%)
Married	357 (78.1%)
Divorced	21 (4.6%)
Widowed	7 (1.5%)
Having at least one child—number (percent)	415 (90.8%)
People living in household—number (percent)	
0--4	163 (35.7%)
5--7	150 (32.8%)
8--10	71 (15.5%)
More than 10	56 (12.3%)
Occupation—number (percent)	
Agriculture and/or petty trade	404 (88.4%)
Homemaker	8 (1.8%)
Other	40 (8.7%)
Went to bed hungry in past month—number (percent)	27 (5.9%)
Number of water contacts per day[b]-- median (interquartile range)	14.1 (11.4--17.0)
Ever treated for schistosomiasis—number (percent)	159 (34.8%)
Received an artemesinin-containing medication for malaria in the past 3 years—number (percent)	163 (35.7%)

[a]Percentages were calculated using a denominator of the total sample size, 457. Missing data are present in some variables (with < 7%). Age was missing in 6 women and water contact data was missing in 21 women.

[b]Water contact behaviors combined the following information (swimming, bathing oneself or a child, washing clothes, hands, animals, or dishes, collecting water for use in the home, fishing, using water for crops, wading water to cross it, and cultivating rice) by summing their frequencies and dividing by 30.

Figure 1. Map of Mwanza region in Tanzania's Lake Victoria zone with village locations. Northern villages nearer to Lake Victoria, with lower rates of *S. haematobium* and higher rates of *S. mansoni,* are depicted with yellow dots. Southern villages, with higher rates of *S. haematobium* and no *S. mansoni* infection detected, are depicted with red dots.



S. mansoni infection, by contrast, was only detected among women living in northern villages near the lake. Among 337 women living in northern lake-side villages, 31 (12.2%) were infected with *S. mansoni*, and among 120 women in southern inland villages, none was infected ($p < 0.0001$).

Prevalence of other infections. Of the 457 women examined, 33 had bacterial vaginosis (7.2%), 22 had candidiasis (4.8%), and 15 had trichomoniasis (3.3%). Twenty-seven women were HIV-infected (5.9%), and 33 had reactive syphilis serology (7.2%).

Prevalence of gynecologic symptoms, sexual dysfunction, and depression. Gynecologic symptoms reported most commonly by women included abdominopelvic pain (75.5%), menorrhagia (56.0%), genital itching (54.5%), dysmenorrhea (54.3%), dyspareunia (42.9%), and foul-smelling discharge (31.1%) (Table 3). The majority of women stated that they made excuses to avoid sexual intercourse (66.1%), and 40.9% of women were worried that their partner would have sexual relations outside of the relationship. Notably, 77% of women met

TABLE 2. Prevalence of Schistosomiasis and HIV in Eight Villages in the Mwanza Region of Tanzania's Lake Zone.

VILLAGE	NUMBER ENROLLED	NUMBER WITH HIV INFECTION (%)	NUMBER WITH *SCHISTOSOMA HAEMATOBIUM* INFECTION (%)	NUMBER WITH *S. MANSONI* INFECTION (%)	REGIONAL PREVALENCE OF *S. HAEMATOBIUM*	REGIONAL PREVALENCE OF *S. MANSONI*
					Northern Villages	
Mwangika	67	5 (7.5%)	0	8 (11.9%)	10/337 (3.0%)	41/337 (12.2%)
Nyehunge	75	9 (12%)	6 (8.0%)	12 (16.0%)		
Sengerema town	71	2 (2.8%)	1 (1.4%)	12 (16.9%)		
Kagunga	30	2 (6.7%)	1 (3.3%)	1 (3.3%)		
Badugu	23	4 (17.4%)	0	0		
Igaka	71	3 (4.2%)	2 (2.8%)	8 (11.3%)		
					Southern Villages	
Lubiri	45	0	5 (11.1%)	0	13/120 (10.8%)	0/120 (0%)
Nyamilama	75	2 (2.7%)	8 (10.7%)	0		
P-value[a]			0.01	< 0.0001[b]	0.002	< 0.0001

[a]All p-values were computed by Fisher's Exact Test.
[b]For this specific p-value, Monte Carlo estimation of exact p-values instead of direct exact p-value computation was used due to time and memory problems encountered for exact computations.

criteria for depression based on the PHQ-9 scale. Of the 457 women, 259 (57%) were mildly depressed and 92 (20%) had moderate to severe depression.

Factors Associated with FUS. We examined associations between baseline characteristics, other infectious diseases, and female urogenital schistosomiasis. Younger age was significantly associated with FUS. As a continuous variable, age in years showed an odds ratio of 0.92 (95% CI 0.86--0.98), which may be interpreted as approximately an 8% decrease in odds of disease per one year increase in age. As a categorized variable, age had an odds ratio of 5.5 [95% CI 1.2--26.3] for women who were younger than 25 years old, 8.2 [95% CI 1.7--38.4] for those who were 25--29 years old, and 1.2 [95% CI 0.16--8.4] for those who were

TABLE 3. Prevalence of Gynecologic Symptoms, Sexual Dysfunction, and Depression in 457 women in Mwanza, Tanzania.

	NUMBER (PERCENT)[a]
Gynecologic Symptom:[b]	
Post-coital bleeding	24 (5.3%)
Dyspareunia	196 (42.9%)
Difficulty becoming pregnant	91 (19.9%)
Abdominal/pelvic pain	345 (75.5%)
Irregular menses	180 (39.4%)
Dysmenorrhea	248 (54.3%)
Genital itching	249 (54.5%)
Menorrhagia	256 (56.0%)
Urinary incontinence	41 (9.0%)
Foul-smelling vaginal discharge	142 (31.1%)
Sexual Dysfunction:[c]	
Fearful of pain during sexual intercourse	291 (63.7%)
Makes excuse to avoid sexual intercourse	302 (66.1%)
Decrease in quality or frequency of sexual relations	242 (53.0%)
Worry that partner will have sexual relations outside of relationship	187 (40.9%)
Score on Depression Scale:	
No depression (0--4 points)	48 (10.5%)
Mild depression (5--9 points)	259 (56.7%)
Moderate depression (10--14 points)	85 (18.6%)
Moderately severe or severe depression (≥ 15 points)	7 (1.5%)

[a]Percentages were calculated using a denominator of the total sample size, 457, in order to capture existing symptoms. Depression score was missing for 58 women, while less than 5.5% of data was missing for all other variables.
[b]Includes women that answered they were "somewhat" or "very much" bothered by symptom.
[c]Includes women that answered "I agree" or "I agree completely" that they experience the symptom.

30--34 years old, compared to women who were 35 years old or older (reference group).

HIV was also significantly associated with FUS with an odds ratio of 4.0 [95% CI 1.2--13.5]. Of the 23 women with FUS, 4 (17.4%) were HIV-infected, compared with 23 (5.3%) of the 434 women without FUS. There were no significant differences among women with and without FUS in the overall rates of other vaginal or sexually-transmitted infections including candidiasis, trichomoniasis, bacterial vaginosis, or syphilis, and no other significant differences in other variables. The final regression model with the two risk factors—age and HIV status—resulted in AUC of 0.732 (age as continuous variable) and AUC of 0.72 (age as categorical variable), which are much higher than null value of AUC = 0.5.

Discussion

Female urogenital schistosomiasis affects young women, is associated with HIV infection, and is prevalent in inland villages of the Lake Victoria region of Tanzania. Our study builds on the findings of a previous study in Zimbabwe, which showed an association between HIV infection and *Schistosoma haematobium* detected in cervical specimens with an odds ratio of 2.9 [95% CI 1.2--3.5].[7] The authors of the earlier study postulated that genital schistosomiasis may predispose to HIV infection. Our work extends this finding by demonstrating an association between HIV infection and the newly-expanded WHO case definition of urogenital schistosomiasis. This finding, in light of the high prevalence of FUS in women of reproductive age, may have important public health implications for prevention of HIV infection.

While a causal association between FUS and HIV infection will require a prospective longitudinal study, a number of factors support the hypothesis that the risk of HIV acquisition is augmented by the presence of FUS. First, schistosomal infection is generally acquired during childhood, before the commencement of sexual activity. Second, it has been pointed out that genital schistosomiasis may increase the risk of HIV infection through its disruption of the genital tract epithelium.[8] Third, schistosomiasis can stimulate a Th-2-type immune response similar to other chronic parasitic infections. This produces changes in cytokines and an accompanying upregulation of the HIV coreceptors CC chemokine receptor 5 and CXC chemokine receptor 4 on monocytes and lymphocytes, with an overall shift in the body's immune response away from the Th-1 immunity that provides early initial control in HIV infection.[16,17] Fourth, the inflammatory reaction to schistosomal eggs in genital lesions may increase the numbers of lymphocytes and activated macrophages in the cervical tissue; these

are target cells for HIV infection. Finally, HIV-positive patients with helminth co-infections appear to have higher viral loads than those without co-infections, and thereby may experience both higher rates of HIV transmission and more rapid HIV progression.[17,18] For all of these reasons, the WHO and others have suggested that mass treatment of FUS may be an effective strategy for decreasing HIV transmission in sexually-active women in Africa.[1,9]

The association of FUS with younger age of 18 to 29 years is consistent with the natural history of schistosomiasis and has important public health ramifications. The age of peak prevalence for *Schistosoma haematobium* infection is between 8 and 15 years old, with peaks in the later end of this spectrum occurring in communities with lower prevalence of disease.[19--21] Repeated exposure to the parasite over time leads to the development of at least partial immunity in later adulthood.[19,21] Our findings demonstrate that, in communities in which the prevalence of disease is at least moderate in children [50-90% in a previous study],[10] women continue to have urogenital schistosomiasis throughout their teens and twenties. This is also the age at highest risk for HIV transmission.[22] Women between the ages of 18 and 29 are not a focus of school-based anti-schistosomal treatment campaigns in sub-Saharan Africa, but the substantial burden of FUS in this age group argues strongly for targeted FUS treatment.

Our study suggests that, while *S. mansoni* may be geographically isolated near the Lake shores, *S. haematobium* may be more widespread in inland villages throughout the region, placing more women at risk for urogenital schistosomiasis and, potentially, increased HIV transmission. A school-based survey in central Sudan reported a comparable marked variation in the prevalence and intensity of both infections, even over short distances within the same province.[23] A compilation of over 2000 studies conducted in East Africa between 1980 and 2009 revealed starkly distinct distributions for *S. mansoni* and *haematobium*, including the observations that *S. mansoni* is most prevalent along the shores of large lakes including Lake Victoria.[24] Near Lake Victoria in Tanzania, studies in schoolchildren have similarly found that the prevalence of *S. mansoni* decreased and that of *S. haematobium* increased with increasing distance from the lake.[10,25] Thus although our designation of "Northern" and "Southern" villages was not chosen a priori, our finding of significantly distinct parasite distributions in each of these regions is supported by other studies in the region.

This clustering has been attributed to differences in the ecology preferred by the intermediate snail vectors *Biomphalaria* spp. (for *S. mansoni*) and *Bulinus* spp. (for *S. haematobium*). *Bulinus* snails are capable of aestivation, and are thus able to colonize and survive in temporary water sources that dry up for months of

the year. In contrast, *Biomphalaria* snails do not aestivate and therefore require large permanent bodies of water with at least moderate aquatic vegetation for survival.[26] Because of these characteristics, transmission of *S. haematobium* is generally more widespread than *S. mansoni* but is also more seasonal, with highest transmission occurring after the rainy season when snails are not dormant.[25,26]

Another important finding from this study was the high prevalence of depression among these women in rural villages. We found that 20% of women scored 10 or above on the PHQ-9 scale, consistent with moderate to severe depression. A PHQ-9 score above 10 is 88% sensitive and 88% specific for major depression as defined by the DSM-IV criteria.[15] Other community-based studies in sub-Saharan Africa have reported a similar, though slightly lower, prevalence of major depression by DSM-IV criteria in 5--15% among women, with a higher prevalence in those living in rural settings.[27,28] Thus our findings highlight an additional prevalent healthcare need that affects rural women in sub-Saharan Africa.

This work underscores the significant burden and breadth of diseases—including parasitic, sexually-transmitted, and mental health-related—in women of reproductive age living in rural northwest Tanzania. Due to resource constraints, we relied on single urine and stool samples to estimate prevalence of intestinal and urogenital schistosomiasis. In all likelihood, had we analyzed urine and stool samples on three consecutive days, the prevalence of these infections would have been even higher than those we observed. Schistosomiasis in women of reproductive age is not currently a focus of public health screening or treatment but the infection is common and widespread among this population. It has been pointed out that control of FUS, and possibly the opportunity to curtail new HIV infections, may cost as little as 32 cents per woman.[9]

In conclusion, female urogenital schistosomiasis is a geographically-clustered infection that disproportionately affects women younger than 30 years of age and was significantly associated with HIV infection. These young women, who also have the highest risk for incident HIV infection and in whom genital lesions may be reversible if treated early, should be the focus of public-health interventions aimed to reduce further the prevalence of *Schistosoma haematobium* infection.

Acknowledgements

Acknowledgements. We wish to thank Bugando Medical Centre, Weill-Bugando University College of Health Sciences, and the National Institute for Medical Research—Mwanza Research Centre for their invaluable support in the design and conduct of this study.

Financial Support. This study was supported by the 2009 Merle A. Sande/Pfizer Fellowship Award in International Infectious Diseases, which is awarded annually by the Infectious Diseases Society of America Education & Research Foundation and by the National Foundation for Infectious Diseases. This work was also supported by T32 HS000066 (JAD) and by CTSC GRANT UL1-RR024996 (REDCap).

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CHAPTER 3

ASSOCIATION OF SCHISTOSOMIASIS AND HIV INFECTION IN TANZANIA

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Am J Trop Med Hyg 2012, 87(5): 868-73.

Abstract

Animal and human studies suggest that *Schistosoma mansoni* infection may increase risk of human immunodeficiency virus (HIV) acquisition. Therefore, we tested 345 reproductive age women in rural Tanzanian villages near Lake Victoria, where *S. mansoni* is hyperendemic, for sexually transmitted infections (STIs) and schistosomiasis by circulating anodic antigen (CAA) serum assay. Over one-half (54%) had an active schistosome infection; 6% were HIV-seropositive. By univariate analysis, only schistosome infection predicted HIV infection (odds ratio [OR] = 3.9, 95% confidence interval = [1.3–12.0], p = 0.015) and remained significant using multivariate analysis to control for age, STIs, and distance from the lake (OR = 6.2 [1.7–22.9], p = 0.006). HIV prevalence was higher among women with more intense schistosome infections (p = 0.005), and the median schistosome intensity was higher in HIV-infected than –uninfected women (400 versus 15 pg CAA/mL, p = 0.01). This finding suggests that *S. mansoni* infection may be a modifiable HIV risk factor that places millions of people worldwide at increased risk of HIV acquisition.

Introduction

Schistosomiasis is caused by a parasitic infection that affects over 200 million people worldwide, with approximately 85% of cases in Africa (1,2) Previously, in a cross-sectional study of Tanzanian women, we found that the odds of being infected with human immunodeficiency virus (HIV) were fourfold higher for subjects with *Schistosoma haematobium* infection than subjects without S. haematobium (3), and we postulated that the chronic genital inflammation caused by S. haematobium eggs pre-disposes to HIV infection. We observed a similar trend in women with S. mansoni infection, although the numbers were small, and the association did not reach statistical significance (unpublished data).

Primate models support the hypothesis that *S. mansoni* infection predisposes to HIV infection. Rhesus macaques with active *S. mansoni* infection were 17 times more susceptible to simian HIV (SHIV) acquisition after rectal inoculation than macaques without *S. mansoni* (4). Although a variety of interactions between *S. mansoni* and HIV infection in humans have been described (5–10), a direct association between active *S.mansoni* and HIV has not been documented in humans. If S. mansoni is a risk factor for HIV acquisition, this finding could have major implications for HIV prevention work in much of the world. We, therefore, performed a cross-sectional study to explore the relationship between *S. mansoni* and HIV infections in women living on the shores of Lake Victoria, including screening for other genital tract infections, which are well-known HIV risk factors, to adjust our analysis for any possible confounders.

The screening of large numbers of women in S. mansoni-endemic villages was facilitated by the use of a sensitive and specific serum test for the diagnosis of Schistosoma infection. The circulating anodic antigen (CAA) test detects a Schistosoma worm antigen in the serum, and it is 80–95% sensitive and 98–100% specific for diagnosis of schistosomiasis (11–13). CAA is a highly glycosylated excretory antigen originating from the parasite gut and released into the host blood circulation when the worm regurgitates the undigested compounds of the gut (14). Schistosome circulating antigen detection performs well in both HIV-negative and -positive individuals (9), and it is recognized by the World Health Organization as an alternative diagnostic method to parasitologic examination of multiple stool or urine samples (15,16).

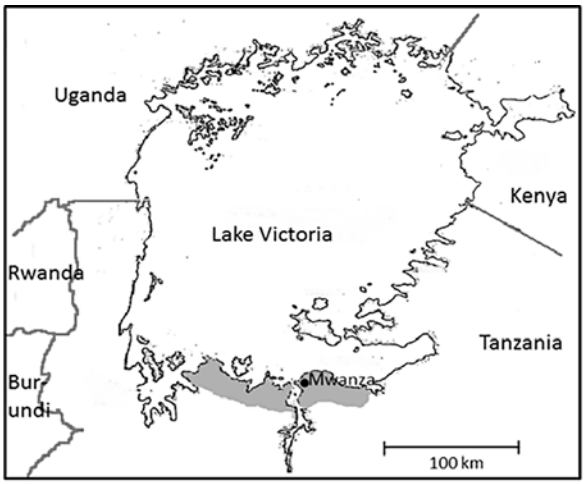
Materials and methods

Study sites and subjects. This study was conducted in seven rural villages with high rates of both *S. mansoni* and HIV, chosen for their locations at a distance

of 1–25 km from Lake Victoria in western Tanzania **(Figure 1)**. Lake Victoria is infested with the *Biomphalaria* snail, the intermediate host of *S. mansoni*, and inhabitants of the study sites have among the highest prevalence of *S. mansoni* infection in the world.

Repeat surveys conducted by our group between 1999 and 2011 have documented a high prevalence of *S. mansoni* in these villages; in contrast, the prevalence of *S. haematobium* is < 3% in adults in the same villages (3,17–20)95% confidence interval [CI] = 1.2-13.5. HIV is also prevalent in these villages; a 2004 study showed that 8% of adults greater than 15 years of age were HIV-infected (21). The major mode of HIV transmission is heterosexual intercourse, and there are more females than males infected, with a female to male ratio of HIV infection of 1.2:1.0 (21).

Figure 1. Lake Victoria, Surrounding Countries, and the Study Area.



We recruited women ages 18–50 years who were seeking routine pre-natal, post-natal, or pediatric care for themselves or their newborn children in the seven study villages. The study team visited each of the health posts for 1 week between January of 2010 and August of 2011 and invited consecutive women at the posts to participate in the study.

Study procedures and sample collection. The study included an oral questionnaire, a gynecologic exam, and phlebotomy. Gynecologic examinations included wet preparations, which were examined on-site for diagnoses of *Trichomonas vaginalis*, *Candida* species, and bacterial vaginosis by the criteria in the work by Amsel and others (22). Endocervical swabs were collected for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* testing at the laboratory of the National Institute for Medical Research (NIMR) in Mwanza, Tanzania. Venous blood was also collected, and serum was separated and stored at –20°C at the NIMR laboratory. Testing for syphilis and preparation of a portion of CAA test strips

were performed at NIMR. Additional test strip preparation and all test strip reading were performed at the Leiden University Medical Center.

Women were offered on-site voluntary HIV counseling and testing in Kiswahili by a trained nurse counselor. Rapid tests (SD Bioline; Standard Diagnostics, Inc., Kyonggi-do, South Korea) were used with confirmatory testing by a second test (Alere Determine; Inverness Medical, Princeton, NJ) as per the national testing algorithm, and patients received their results and post-test counseling on the same day.

Patients diagnosed with HIV were referred to the local HIV clinic for free care and treatment. Women with trichomoniasis, candidiasis, or bacterial vaginosis were provided treatment on the same day. Women who tested positive for gonorrhea, chlamydia, syphilis, or schistosomiasis were treated at a followup visit as soon as laboratory results were available.

Laboratory analyses. *Schistosoma.* CAA is a glycoprotein that is produced by gut epithelial cells of schistosomal worms (14) and secreted in large quantities into the host bloodstream during active infection (23). The CAA test does not distinguish *S. haematobium* from *S. mansoni* infection. The test usually becomes negative within 1 week of successful antischistosomal therapy (24). CAA testing was performed using the upconverting phosphor (UCP) technology lateral flow assay as previously described (12). Serum was treated with 4% (wt/vol) trichloroacetic acid to remove proteins and antibody complexes.

After centrifugation, the supernatant was mixed with an assay buffer containing an anti-CAA mouse monoclonal antibody conjugated to UCP reporter particles and incubated for 1 hour at 37°C. The mixture was applied to a lateral flow test strip with a capture line of the same antibody, and chromatography was permitted to continue until strips were dry. Strips were read using a modified Packard Fluorocount meter, and test line results were normalized to the control line results for each test strip. A CAA value ≥ 10 pg/mL was considered positive based on a series of negative controls (highest value plus 2 SDs). CAA values were stratified by intensity as greater or less than 3,000 pg/mL, which represents approximately 100 eggs per 1 gram of stool (25).

C. trachomatis and N. gonorrhoeae. DNA was extracted from endocervical swab specimens and tested for *C. trachomatis* and *N. gonorrhoeae* using Amplicor CT/NG specimen preparation, amplification, internal control, and detection kits (Roche Molecular Systems, Branchburg, NJ). Gonorrhea results were confirmed using 16S rRNA PCR testing.

Syphilis serology. Serum was tested for syphilis using the Rapid Plasma Reagin (RPR) test, with confirmation of positive RPR results by the *Treponema pallidum* Particle Agglutination assay (TPPA).

Ethics. The study was approved by the Institutional Review Boards at Bugando Medical Center and Weill Cornell Medical College and the National Institute for Medical Research in Tanzania. All women were informed about the study by a nurse fluent in Kiswahili and provided written informed consent before participation.

Statistical methods. Data were entered into a REDCap database (Vanderbilt University, Nashville, TN) and analyzed using Stata version 11 (Stata Corporation, College Station, TX). Continuous variables were summarized by median and interquartile range (IQR), and categorical variables were summarized by frequency and percentage. Simple logistic regression (for univariate analysis) followed by multiple logistic regression (for multivariate analysis) were used to examine factors associated with HIV. In multiple logistic regression models, comprehensive adjustment was not pursued, because it yielded failure in convergence. Associations between factors and the endpoint were summarized using odds ratios (ORs) with 95% confidence intervals (CIs) and associated P values. In regression analyses, age and distance from the lake were analyzed as continuous variables, whereas other variables were analyzed as binary variables.

We also compared the intensity of schistosome infection between the HIV-positive and -negative groups using the non-parametricWilcoxon test (because of severe non-normality of this data) and the non-parametric Jonckheere–Terpstra (JT) trend test (26). Two-sided hypotheses/tests were assumed for calculation of all CIs and P values.

Results

Patient characteristics. We invited 432 eligible women living in seven villages within 25 km of Lake Victoria to participate. Of these women, 345 (80%) women provided informed consent and completed all study procedures. The median age in this population was 30 years (IQR = 24–36) (Table 1). All but one woman reported contact with potentially contaminated water at least daily. Over three-fourths of women had not been treated for schistosomiasis in the past 5 years, and more than one-half of women had never been treated.

Prevalence of schistosomiasis, genital tract infections, and HIV. We diagnosed active schistosomiasis in 185 (54%) women in this study population (Table 2).

Table 1. Baseline Characteristics of 345 Women in *Schistosoma mansoni*-endemic Villages near Lake Victoria in Tanzania.

Characteristic	Value
Age in years	
Median	30
Interquartile range	24-36
Number of children	
Median	4
Interquartile range	2-6
Reports more than one sexual partner in past six months	12 (5%)
Reports no current sexual partner	23 (9%)
History of miscarriage	116 (39%)
Distance of village from Lake Victoria	
0-8 kilometers	123 (36%)
9-17 kilometers	125 (36%)
18-25 kilometers	97 (28%)
Daily contact with potentially-infectious water	344 (99%)
Never previously treated for schistosomiasis	178 (52%)
Never previously tested for HIV	114 (42%)

Non-missing data were included in each calculation.

Table 2. Prevalence of Infections in 345 Rural Tanzanian Women.

Infection	Prevalence—Number (Percent)
Schistosomiasis	185 (54%)
Human immunodeficiency virus-1	21 (6%)
Syphilis	26 (8%)
Gonorrhea	1 (0.3%)
Chlamydia	15 (4%)
Trichomoniasis	4 (1%)
Bacterial vaginosis	19 (6%)
Candidiasis	15 (4%)

Table 3. Associations of Potential Risk Factors with Schistosome Infection (Univariable Analysis).

Potential Risk Factor	Schistosomiasis-Positive (n=185)	Schistosomiasis-Negative (n=160)	Odds Ratio for Association with Schistosomiasis [95%CI]	p-value
Median age (IQR)	30 (25-36)	29 (23-37)	1.02 [0.99-1.05]	0.15
Received praziquantel in past 5 years	21 (19%)	16 (15%)	1.4 [0.7-2.9]	0.35
Gonorrhea and/or chlamydia cervicitis	7 (4%)	9 (6%)	0.7 [0.2-1.8]	0.42
Syphilis	17 (10%)	9 (6%)	1.8 [0.8-4.0]	0.19
Median kilometers from Lake Victoria (IQR)	10 (1-14)	12 (8-22)	0.95 [0.92-0.97]	<0.001

Non-missing data were included in each calculation.

Among those women harboring a schistosome infection, the median CAA value was 446 pg/mL (IQR = 86–2,338); 21 of 345 (6%) women were HIV-infected. The prevalence of other gynecologic infections ranged from gonorrhea in 1 (< 1%) woman to reactive syphilis serology in 26 (8%) women (Table 2).

Factors associated with schistosome infection. In both univariate and multivariate analysis, distance from Lake Victoria was the only factor that was significantly associated with schistosome infection, with an OR of 0.95 (0.92–0.97) for each increasing kilometer away from the lake ($p < 0.001$) (Table 3). The highest prevalence was 81% in a village situated < 1 km from the shore of Lake Victoria, whereas the lowest prevalence (38–42%) was observed in villages that were 12–22 km inland. Age, prior receipt of praziquantel, and genital tract infections did not significantly predict whether women were currently infected with schistosomes.

Factors associated with HIV infection. Schistosome infection was significantly associated with HIV infection, whereas other factors were not. Of 185 women with positive CAA levels, 17 (9%) women were HIV-infected, whereas of 160 women without detectable CAA, 4 (3%) women were HIV-infected (OR = 3.9 [95% CI = 1.3–12.0], p = 0.015) (Table 4). The ORs for chlamydia and syphilis

Table 4. Associations of Potential Risk Factors with HIV Infection (Univariable Analysis*).

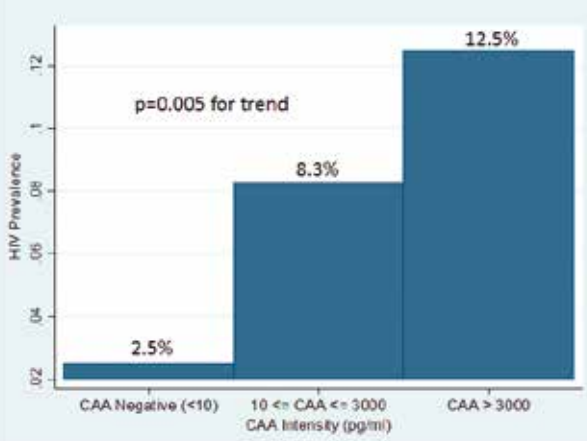
Potential Risk Factor	HIV-Positive (n=21)	HIV-Negative (n=324)	Odds Ratio for Association with HIV Infection [95%CI]	p-value for Odds Ratio
Median age (IQR)	32 (25-36)	30 (24-36)	1.00 [0.95-1.06]	0.90
Median kilometers from Lake Victoria (IQR)	12 (10-22)	10 (8-22)	1.01 [0.95-1.07]	0.71
Chlamydia infection	2 (10%)	13 (4%)	2.4 [0.5-11.4]	0.27
Gonorrhea infection	0	1 (0·3%)	---	---
Positive syphilis serology	3 (16%)	23 (7%)	2.4 [0.6-8.7]	0.19
Trichomonas infection	0	4 (1%)	---	---
Bacterial vaginosis	0	19 (6%)	---	---
Candidal infection	0	15 (5%)	---	---
Positive CAA test	17 (81%)	168 (52%)	3.9 [1.3-12.0]	0.015

*Non-missing data were included in each calculation.
For age and kilometers from the lake, the odds ratio corresponds to the odds ratio estimate for one unit increase in the exposure (i.e., per one year increase in age and one kilometer increase in distance).

were both 2.4, although neither was statistically significant. The OR for each increasing 1 year of age was 1.00 (0.95–1.06; p = 0.90). The OR for each increasing 1 km away from Lake Victoria, which correlates with living closer to major roads, was 1.01 (0.95–1.07; p = 0.71). In multivariate analysis, schistosome infection was the single best predictor of HIV infection. Other variables were not significant and did not affect the relationship between *Schistosoma* and HIV infection. Specifically, when multiple regression models were used to control for age, sexually transmitted infections (STIs), and distance from the lake, the association between *Schistosoma* and HIV remained statistically significant with an OR of 6.2 (1.7–22.9; p = 0.006).

The median intensity of schistosome infection was significantly higher among HIV-infected than -uninfected women (400 versus 15 pg/mL, p = 0.01). When women were stratified by CAA intensity, the prevalence of HIV infection significantly increased among groups (p = 0.005 by JT test) **(Figure 2).**

Figure 2. Prevalence of HIV Infection by Intensity of Schistosome Infection.



Factors associated with other STIs. Cervicitis caused by gonorrhea and/or chlamydia was significantly associated with younger age (OR = 0.87 [0.79–0.96] for each increasing year of age, p = 0.006). No other significant associations were observed.

Discussion

In women living in *S. mansoni*-endemic areas near Lake Victoria, more than one-half were schistosome-infected, and *Schistosoma* infection was strongly associated with HIV. This finding suggests that *S. mansoni* infection and the chronic inflammation from the gut caused by S. mansoni eggs may be a risk factor for HIV acquisition. Schistosomiasis may be placing millions of women throughout sub-Saharan Africa at increased risk of becoming HIV-infected, and mass therapy of women of reproductive age for schistosomiasis may be an effective HIV prevention strategy.

Animal models support the hypothesis that *S. mansoni* infection increases host susceptibility to HIV infection. Studies in macaques showed that the rectal inoculum of SHIV required for SHIV acquisition was 17 times lower in macaques with than without *S. mansoni* infection (4). In contrast, prior *S. mansoni* infection did not significantly change the required infectious dose when SHIV was inoculated intravenously (4,27). Researchers postulated that *S. mansoni* infection increased the number of activated CD4+ T cells in the gut-associated lymphoid tissue (GALT) and thereby increased the optimal targets for SHIV infection. Mouse models of schistosomiasis show that S. mansoni eggs induce a Th17 CD4+ T-cell response in the gut mucosa (28,29). A growing body of literature suggests that HIV preferentially infects Th17 CD4+ T cells in gut and genital tissue mucosa and that increased numbers of these cells may increase susceptibility to HIV infection (30,31). Additional human studies are needed to determine if *S. mansoni* eggs induce a Th17 cell immune response in the GALT and if these Th17 cells express HIV susceptibility factors, such as CC Chemokine Receptor 5 (CCR5) and integrin α4β7.

Our work is also supported by recent findings from the Rakai region of Uganda, where *S. mansoni* is endemic (32). As noted perceptively in the work by Secor (33), HIV-infected individuals more often had antibodies to schistosome antigens than HIV-uninfected individuals. Unlike in our study, in which CAA positivity indicates live worm infestation, the detection of antibodies to schistosome antigens does not prove active schistosomiasis. Coupled with our findings, this population-based study lends additional clinical support to our finding that *S. mansoni* infection and not only *S. haematobium* infection (as previously reported) is associated with HIV.

In addition, *S. mansoni*-infected individuals displayed higher densities of the HIV chemokine receptors CCR5 and CXCR4 on their CD4+ T cells and monocytes than individuals with schistosomiasis that had been previously treated (34). *S. mansoni* infection was also shown to increase the HIV RNA viral load in HIV-positive patients with untreated *S. mansoni* infection compared with patients with *S. mansoni* infections that had been treated (35). Several earlier non-randomized studies did not find an effect of praziquantel treatment on viral load (5,6,36,37), but this result has been postulated to be caused by transient increases in the schistosomiasis-conducive Th2 environment immediately after treatment (5,38). S. mansoni-infected individuals are also reported to excrete fewer ova than individuals without HIV (7,10). Our results support these findings that show complex interactions between *Schistosoma* and HIV infections as well as the growing consensus that schistosome infection, including *S. mansoni*, may be a risk factor for HIV acquisition (1,33).

Our work suggests that, among rural African women in whom the prevalence of genital tract infections is low, schistosome infection may be a major contributor of risk for HIV acquisition. Over one-half of our population was infected with schistosomes, leading to an estimated population-attributable fraction for HIV acquisition caused by schistosome infection of 69% (36–81%) using previously described methods (39). In contrast, the population-attributable fractions for genital tract infections in our population were 7% and below. The direction of our findings supports the well-described association between HIV and STIs, but the inability of our study to show statistical significance may be caused by the low prevalence of STIs in this rural population. We postulate that, in our population and other rural populations with few traditional HIV risk factors, such as multiple sexual partners and high rates of genital tract infections, schistosome infection may play a role as a key driver of HIV transmission. Of note, this study addresses the risk only in women aged 18–50 years who were seen at rural health clinics, and it does not address whether men or younger adolescent girls are also at increased risk.

The CAA test is a valuable diagnostic test for schistosome infections. The antigen becomes detectable in serum approximately 5 weeks after infection (40). CAA levels fall rapidly posttreatment, with a serum half-life of 2 days (24), and they rise with reinfection (41). The test is, therefore, highly time-specific for active schistosome infections. CAA assays have been recommended for serologic screening programs, in which repeated parasitologic examinations of urine and stool are logistically complicated (42). The CAA test has recently been developed into a very sensitive, robust lateral flow strip test, making test performance in rural settings increasingly feasible (12). The test is unable to distinguish between species of schistosomal infections. Therefore, projects relying on CAA testing either will only allow species-specific conclusions when performed in regions with sharp geographic demarcation of schistosome species, like in our study, or will not be able to differentiate between schistosome species.

Schistosomiasis is usually acquired in childhood, infecting 50–90% of children living in endemic areas by early adolescence (2). Thus, acquisition of schistosome worms typically predates sexual activity and concomitant exposure to HIV. The World Health Organization recommends that children in endemic areas receive targeted, periodic school-based praziquantel treatment (1). Women do not often receive mass treatment. In our population, more than one-half of women had never received antischistosomal treatment, despite living in a hyperendemic area and coming into daily contact with unclean water. A policy of routine periodic praziquantel administration for women seeking reproductive healthcare services (including family planning, cervical cancer screening, and pre-natal/post-natal care) would be a safe (1), efficient, and inexpensive way to control schistosomiasis and, moreover, potentially to prevent HIV acquisition in this vulnerable group.

This cross-sectional study shows a strong association but does not prove a causal role of schistosomiasis in HIV acquisition. An interventional trial would be necessary to show causality. However, we feel that the best explanation of our findings is that pre-existing schistosome infection modifies mucosal immunity and predisposes to HIV infection. The highest incidence of schistosome infection typically occurs in childhood between the ages of 5 and 15 years, and in individuals with ongoing exposure, it produces a chronic infection over decades. More than 80% of our study participants denied receiving praziquantel in the past 5 years, and more than one half reported never being treated. Therefore, it seems most likely that the large proportion of our study participants had chronic, untreated schistosome infection that pre-dated their exposure to HIV. Although it is conversely possible that HIV infection increases susceptibility to schistosomiasis (43), the former explanation for the association

most aptly combines both natural history and biological plausibility data. Also, it should be noted that the use of ORs in this study could possibly overestimate the relationship between HIV and schistosome infection compared with relative risk because of the fact that schistosome infection was common in our population (44).

In conclusion, more than one-half of rural Tanzanian women seen at health posts in *S. mansoni*-endemic areas of the Lake Victoria region had evidence of active schistosome infection, and the prevalence of HIV among these women was markedly higher than among those women without schistosome infection. Active *S. mansoni* infection may be a modifiable HIV risk factor that is contributing to high rates of new HIV infections among millions of women living in sub-Saharan Africa.

Acknowledgments: We greatly appreciate the assistance of Dr. Jaco J. Verweij in transporting materials for circulating anodic antigen testing to the National Institute for Medical Research laboratory and instructing laboratory technicians in Mwanza in the performance of the test. We also thank Bugando Medical Centre, the National Institute for Medical Research, Mwanza Research Centre, the Mwanza Interventional Trials Unit, Leiden University Medical Center, and Sengerema District Hospital for their support. Most of all, the authors thank the Tanzanian women for their willing participation in this study.

Financial support: This work was supported by the 2009 Merle A. Sande/Pfizer Fellowship Award in International Infectious Diseases, which is awarded annually by the Infectious Diseases Society of America Education & Research Foundation and the National Foundation for Infectious Diseases. This work was also supported by Agency for Healthcare Research and Quality Grant T32HS000066, National Institute for Allergy and Infectious Diseases Grant T32AI007613, and Clinical and Translational Science Center at Weill Cornell Medical College Grant UL1-RR024996 (to J.A.D.). Various improvements of the upconverting phosphor-LF assay for circulating anodic antigen detection, involving the implementation of dry reagents (allowing worldwide shipping), increased sensitivity, and handheld upconverting phosphor readers, were developed, in part, with financial support from US National Institutes of Health Grant UO1DE017855 and the University of Georgia Research Foundation, Inc. (Schistosomiasis Consortium for Operational Research and Evaluation [SCORE] project).

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CHAPTER 4

DETECTABLE UROGENITAL SCHISTOSOME DNA AND CERVICAL ABNORMALITIES SIX MONTHS AFTER SINGLE-DOSE PRAZIQUANTEL IN WOMEN WITH *SCHISTOSOMA HAEMATOBIUM* INFECTION

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Trop Med Int Health 2013 Sep;18(9):1090-6.

Abstract

We explored the response to single-dose praziquantel therapy in a cohort of 33 women with *Schistosoma haematobium* infection in rural Mwanza, Tanzania. Women with *S. haematobium* infection confirmed both by eggs in urine and by polymerase-chain reaction (PCR) received single-dose praziquantel and treatment for concomitant sexually-transmitted infections. Macroscopic cervical abnormalities were also quantified. After six months, microscopically-detectable egg excretion was eliminated but 8 of 33 women (24%) were persistently positive for *S. haematobium* by PCR and 11 (33%) had cervical abnormalities potentially attributable to schistosomiasis. This suggests that praziquantel treatment more frequently than every six months may be necessary for complete elimination of the parasite and prevention of genital tissue pathology. This aggressive therapy may in turn play a key role decreasing HIV susceptibility in millions of people living in regions in which *S. haematobium* is endemic.

Introduction

Schistosoma haematobium is a parasitic worm infection acquired through contact with infested fresh water that affects an estimated 112 million people living in sub-Saharan Africa (1). Adult *S. haematobium* worms live for years in host pelvic venous plexi and lay hundreds of eggs per day (2). The schistosome miracidium within the egg secretes proteolytic enzymes through the egg's shell that facilitate the migration of the egg through the vessel wall and into the surrounding tissues toward the mucosal tissue and lumen of the genitourinary tract (2). Some eggs reach the lumen and are excreted in urine or genital secretions (3), but a significant fraction become trapped in the tissues of urogenital organs, where they cause acute and chronic inflammation. Histopathological and autopsy studies of women with *S. haematobium* infection demonstrate eggs throughout the upper and lower genital tract (4–6). *S. haematobium* infection in women has been associated with HIV infection in cross-sectional studies (7–9) and is postulated to increase HIV susceptibility both by damaging the mucosal integrity of the female genital tract and by causing local mucosal and systemic immune responses (8,10–14).

The World Health Organization (WHO) recommends empiric anti-schistosome treatment with praziquantel for at-risk individuals in sub-Saharan Africa to reduce disease morbidity and with the possibility of preventing HIV (15,16). In highest-prevalence areas (>50%), annual single-dose praziquantel is recommended for both children and adults (17). Self-report of even one anti-schistosome treatment before the age of 20 years in girls has been associated with significantly lower rates of genital manifestations of schistosome infection such as sandy patches and contact bleeding (18).

Two longitudinal studies have suggested that single-dose praziquantel significantly reduces urinary egg excretion but does not completely cure *S. haematobium* infection, particularly its gynecological manifestations. The smaller study reported regression of genital sandy patches in 2/4 women re-examined nine weeks after treatment but persistence in the other two (19). In the larger study of 338 women seen after 12 months, praziquantel treatment was not associated with improvements in gynecological abnormalities or contact bleeding (20). In order to explore and extend these findings, we added molecular diagnostics to investigate response to single-dose praziquantel in a cohort of women with *S. haematobium* infection in rural Tanzania.

Methods

Study design. We conducted a six-month cohort study in which women with *Schistosoma haematobium* infection were recruited from the *S. haematobium*-endemic villages of Lubiri and Nyamilama, treated with praziquantel, and retested after six months. This was a proof-of-concept study to explore the utility of PCR for monitoring treatment response in urogenital schistosomiasis. We enrolled women only for this preliminary study because our work was incorporated into an ongoing women's health program administered by the Tanzanian Ministry of Health.

As previously-described, women aged 18-50 had been screened for cervical cancer using visual inspection with acetic acid (the standard of care in Tanzania) in partnership with a governmental outreach program (7). We enrolled women who screened negative for cervical cancer and who had *S. haematobium* infection confirmed by visualization of eggs in urine plus were PCR-positive for schistosome DNA in urine. We additionally examined cervical smears for eggs and tested cervicovaginal lavages for schistosome DNA. A nurse collected socio-demographic data using a structured questionnaire in Kiswahili, the local language.

Women received free observed treatment with single-dose praziquantel (40mg/kg) at enrollment. We included pregnant and lactating women as praziquantel is recommended for these groups by the WHO (17). Women and their sexual partners also received free treatment for the sexually-transmitted infections (STIs) for which testing can be routinely done in Tanzania, including syphilis, trichomoniasis, chlamydia, gonorrhea, candidiasis, and bacterial vaginosis if indicated. Women participated in an educational seminar about schistosomiasis, STIs, and possible associations with HIV infection on the day of study enrollment. Study participants were re-evaluated for *S. haematobium* and gynecological infections at the six-month follow-up visit and again received indicated treatment without cost.

Field investigations. Gynecological examinations were performed at enrollment and six months to document any macroscopic cervical abnormalities including sandy patches, irregular blood vessels, contact bleeding, condylomata, and abnormal discharge. Prior to any sample collection, the cervix was visually inspected using a flashlight and magnifying glass. Macroscopic abnormalities were identified by the physician and recorded and drawn to scale on a standardized data collection form. Abnormal discharge, defined as discharge of any color other than white or white discharge that was thick and/or copious, was also noted and described on the form. The physician was blinded to the prior examination findings at the time of follow-up data collection.

The first sample collected was a wet preparation for candidiasis, trichomoniasis, and bacterial vaginosis, followed by an endocervical swab for chlamydia and gonorrhea as previously described (7,21). Next, cervical smears were taken from abnormal-appearing regions if present and otherwise from the transformation zone. Lastly, cervicovaginal lavage samples were prepared using four milliliters of normal saline to wash the face of the cervix three times.

Single urine samples at enrollment and follow-up were collected between 10am and 2pm, filtered, and examined microscopically for schistosome ova by a trained microscopist. Serum was tested by Rapid Plasma Reagin (RPR), with *Treponema pallidum* particle agglutination (TPPA) testing performed on positive samples for diagnosis of syphilis.

Real-Time PCR. DNA isolation and PCR was performed as described previously (12). Briefly, DNA was isolated from well-mixed pre- and post-treatment urine and cervicovaginal lavages using QIAamp Tissue Kit spin columns (QIAgen, Hilden, Germany). Quantitative real-time PCR testing was performed using *Schistosoma* genus-specific primers and detection probe. PhocineHerpes Virus 1 (PhHV-1) was used as an internal control in each sample along with PhHV-1-specific primers and detection probe to detect any inhibition. Amplification, detection, and analysis were performed with the CFX96 real-time detection system (Bio-Rad laboratories). The PCR output from this system consists of a cycle-threshold (Ct) value, representing the amplification cycle in which the level of fluorescent signal exceeds the background fluorescence, and reflecting the parasite-specific DNA load in the sample tested. Negative and positive control samples are included in each amplification run.

Statistics. Continuous variables were summarized by median and interquartile range (IQR) and categorical variables were summarized by frequency and percentage. Proportions were compared using Fisher's Exact test and continuous variables by the Wilcoxon rank-sum test. Pre-post treatment values were compared with the Wilcoxon matched-pairs signed-rank test. Two-sided hypotheses/tests were assumed for all confidence intervals and p-values. Data were analyzed using Stata Version 11 (College Station, Texas).

Ethics. Written informed consent was obtained by a trained study nurse fluent in the local language who read the consent form to all study participants. Women who decided to participate signed their names or made their mark/thumbprint (if illiterate) on the consent form. Ethical permission was granted by Bugando Medical Centre, the Tanzanian National Institute for Medical Research, and Weill Cornell Medical College.

Results

Patient characteristics. Between November 2009 and May 2010, we enrolled 39 women with *S. haematobium* infection detected by both microscopy and PCR. Of these, 33 (85%) were re-examined a median of 188 (IQR, 183-188) days after treatment and included in the analysis. There were no significant differences between age or number of urinary ova between women who were lost to follow-up (n=6) and those who were not.

Socio-demographic information is detailed in Table 1. Patients were young, with a median age of 22 (20-28) years, and 6 patients were aged 18-19. The large majority (90%) were married. Sexual risk factors were assessed indirectly. Nearly one-third of women had children with at least two different men, and more than half of women stated that their husbands had children with another woman during the marriage. Ten women (30%) reported going to bed hungry in the past month

Table 1. Demographic Characteristics of 33 Adult Women (>18 years) Treated for Urogenital Schistosomaisis and Followed Up After Six Months.

CHARACTERISTIC	VALUE
Age in years—median (interquartile range)	22 (20—28)
Married—number (percent)	28 (90%)
Number of children	
0	3 (9%)
1--2	16 (49%)
3--4	9 (27%)
≥ 5	5 (15%)
Number of fathers of a woman's children	
1	23 (70%)
2	8 (24%)
3	2 (6%)
Husband had children outside of marriage during the marriage—number (percent)	18 (55%)
Went to bed hungry in past month—number (percent)	10 (30%)
Reported no prior treatment for schistosomiasis—number (percent)	8 (24%)

Non-missing data were included for each calculation.

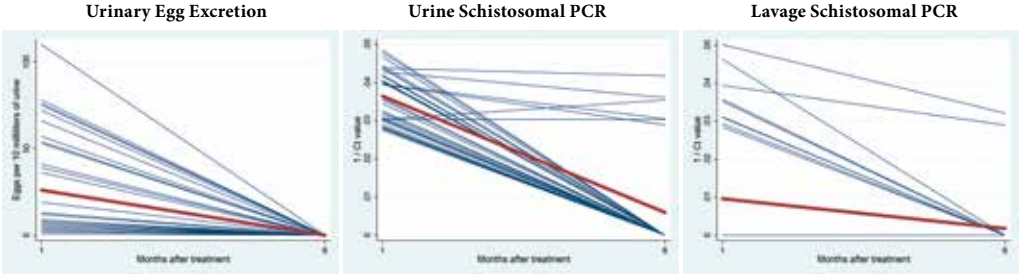
because their household did not have enough food, a measure of food insecurity (22,23).

Microscopy and PCR pre- and post-treatment. At enrollment, all women had urinary ova with a median of 8 (4-53) per 10 milliliters. Three women additionally had viable *S. haematobium* ova in cervical smears. All 33 women had positive urine PCR results. Urine Ct values ranged from 20.6-36.3 (median 27.8). Cervicovaginal lavage PCRs were positive in 9 women (27%), ranging from 19.9-35.1 (median 28.3). Two of the three women with eggs in cervical smears had PCR-positive lavages.

Six months after treatment, ova were no longer detected in any urine or cervical sample. Eight women (24%) still had detectable schistosome DNA by PCR with Ct values <35 (six urines and two lavages, **Figure 1**). In women with persistently positive PCR findings, the DNA quantity remained approximately unchanged. Urine PCRs became zero in the two women with persistently-positive lavages.

Gynecological abnormalities pre- and post-treatment. STIs diagnosed included: four chlamydia (12%) and one each (3%) of syphilis, gonorrhea, trichomoniasis, and multiple cervical condylomata. At enrollment, 9/33 women (27%) were negative for STIs but had at least one macroscopic gynecological abnormality

Figure 1. Pre- and Post-Treatment Urine Egg, Urine Schistosome PCR, and Cervico-vaginal Lavage Schistosome PCR.



Results for each patient are shown at the time of praziquantel treatment (Month 0) and follow-up examination six months later (Month 6). PCR, Real-Time Polymerase Chain Reaction to detect schistosome DNA. Bold red line indicates the mean value of all women tested (n=33).

that, in the absence of STIs or malignancy, could be consistent with *Schistosoma* involvement of the genital tract (Table 2). These included sandy patches, contact bleeding, convoluted blood vessels, and abnormal discharge.

After treatment, the same macroscopic abnormalities persisted in 7 of these 9 women, and four others had new abnormalities that could be consistent with cervical *Schistosoma* infection in the absence of STIs and malignancy. Thus we observed, in total, 11 women (33%) with gynecological abnormalities that were possibly consistent with schistosomiasis six months after praziquantel treatment. Four of these 11 (36%) were persistently PCR-positive (3 urines and one lavage), compared with 4 (18%) of the 22 who had no gynecological abnormalities (p=0.25). Ages were not significantly different between groups of women who remained positive by PCR or who had persistent gynecological abnormalities and those who did not.

In total, 10 women had evidence of *S. haematobium* involvement of the genital tract by eggs or PCR at the time of enrollment, and 6 months after treatment, 2 (20%) remained PCR-positive in cervicovaginal lavages. Schistosome DNA additionally was detectable in urine of 6 other women at 6 months. Of the 25 women who were negative for both schistosome eggs and DNA at 6 months, 7 (28%) had gynecological abnormalities in the absence of STIs or cervical cancer, suggestive of *S. haematobium*-induced pathology.

Discussion

In this cohort of 33 young adult women with *Schistosoma haematobium* infection who received single-dose praziquantel therapy, *Schistosoma* DNA was detectable 6 months later in the genital tract of 2/10 women with initial genital tract involvement and in urine of 6 additional women. Moreover, nearly one-third had ongoing cervical pathology potentially attributable to schistosome infection. Our findings suggest that, after single-dose praziquantel, which is the WHO's current recommendation for schistosomiasis (24), parasite remnants and genital tract tissue pathology persist in a substantial minority of adult women. Other groups have advocated higher and/or repeated praziquantel doses (25–27). Our work supports this concept and raises serious concern given recent findings that schistosomiasis may be a risk factor for HIV acquisition (7,8,21).

Schistosoma real-time PCR has been evaluated as an indicator of genital *S. haematobium* infection (28) but not as a marker of effectiveness of praziquantel treatment. Our work documents that *Schistosoma* DNA was detectable six months after treatment in 8/33 women whose negative egg counts appeared to indicate

Table 2. Findings in 33 Adult Women (>18 years) Treated for *Schistosoma haematobium* Infection and Re-tested after Six Months.

Measurement	Pre-Treatment	Six Months Post-Treatment	p-value
Urine PCR (Ct value)			
Positive urine PCR—number (percent)	33 (100%)	6 (18%)	<0.001
Median	27.8	0	<0.001
Interquartile range (IQR)	23.8-32.9	0-0	
Range	20.6-36.3	0-34.6	
Urinary ova (per 10 ml)			
Positive urinary ova—number (percent)	33 (100%)	0	<0.001
Median	8	0	<0.001
IQR	4-53	0	
Range	1-110	0	
Lavage PCR (Ct value)			
Positive lavage PCR—number (percent)	9 (27%)	2 (6%)	0.044
Median	0	0	0.069
IQR	0-19.9	0-0	
Range	0-35.08	0-34.4	
Positive cervical smear ova—number (percent)	3 (9%)	0	n.s.
Convoluted blood vessels—number (percent)	1 (3%)	2 (6%)	n.s.
Sandy patches—number (percent)	7 (21%)	9 (27%)	n.s.
Contact bleeding—number (percent)	1 (3%)	1 (3%)	n.s.
Abnormal discharge—number (percent)	2 (6%)	1 (3%)	n.s.
Any gynecological abnormality potentially consistent with genital *S. haematobium* infection—number (percent)	9 (27%)	11 (33%)	n.s.

n.s. = not significant

resolved infection. Previous studies in Zimbabwe and Malawi have similarly reported unresolved macroscopic gynecological pathology (sandy patches, blood vessel abnormalities, and/or contact bleeding) in women treated for genital *S. haematobium* infection and any STIs and re-examined up to a year later (19,20). Our work now substantiates these clinical reports by showing that, after praziquantel treatment, a sizeable percentage of women have not only ongoing cervical pathology but also detectable parasite DNA in urine and cervicovaginal lavage fluid.

The persistence of gynecological lesions potentially caused by *S. haematobium* suggests that *S. haematobium*-induced pathology may be irreversible. Liver fibrosis due to *S. mansoni* infection is often permanent even after parasite death (29). Similarly, gynecological lesions are believed to begin as inflammatory tissue reactions to migrating ova (30,31) which, over time, become fibrotic "sandy patches" (18,30) that may not be curable. In support of this hypothesis, women from an *S. haematobium*-endemic region who reported receiving praziquantel before age 20 were less likely to have gynecological contact bleeding and sandy patches than women treated after age 20 (18). Thus regulartreatment beginning in childhood appears optimal to prevent potentially-irreversible genital lesions that may predispose to HIV infection (11,16,18). Establishment of the most effective treatments for genital *S. haematobium* infection remains an urgent research priority.

A limitation of this study was our inability to determine whether *Schistosoma* DNA detected at six months was due to re-infection, sample contamination by semen of infected sexual partners or by stool, partial treatment failure, post-treatment maturation of juvenile worms which are less susceptible to praziquantel (32), or lingering ova and parasite products in tissue and blood (33). We believe that the latter three explanations are the most likely sources of DNA in urine samples, which were clean-catch and showed no ova. Cervicovaginal lavages are inherently not sterile and could more easily have been contaminated. Second, neither testing for Herpes simplex virus and Human papillomavirus infections, nor exclusion of premalignant lesions, was possible. Lastly, the sensitivities of cervical smears (34), PCR of cervicovaginal lavages (particularly in women older than age 25) (28), and single rather than double urine samples are all suboptimal for detection of *S. haematobium* infection. Lower sensitivity would bias our study towards finding less residual infection in patients with confirmed *S. haematobium* at the study outset. Thus our detection of persistent schistosome DNA in 8/33 women may be low. Either way, our findings suggest that treatment may need to be repeated more frequently than the WHO recommends (annually) and more frequently than even the six-month duration of this study in order to maximize parasite elimination and minimize tissue pathology.

In conclusion, our results suggest that single-dose praziquantel does not completely eliminate *S. haematobium* DNA in a substantial portion of women. Moreover, one dose of praziquantel appears not to reverse cervical pathology. Our data supports using praziquantel early and often in girls and women living in endemic areas. Given that *S. haematobium* may be a risk factor for HIV acquisition, this low-cost ($0.32) (11) intervention may have the potential not only to lessen schistosome-related morbidity, but also to prevent new HIV infections among millions of at-risk women in Africa.

Acknowledgements

We thank Eric A.T. Brienan and Pystje Hoekstra for performing the PCR assays and the Tanzanian women for their enthusiastic participation in this study.

This work was supported by the 2009 Merle A. Sande/Pfizer Fellowship Award in International Infectious Diseases, which is awarded annually by the Infectious Diseases Society of America Education & Research Foundation and by the National Foundation for Infectious Diseases. This work was also supported by grants T32 HS000066 from the Agency for Healthcare Research and Quality (AHRQ), T32 AI007613 from the National Institute for Allergy and Infectious Diseases, and CTSC grant UL1-RR024996 (J.A.D.).

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CHAPTER 5

CORRELATION OF SERUM AND DRIED BLOOD SPOT RESULTS FOR QUANTITATION OF SCHISTOSOMA CIRCULATING ANODIC ANTIGEN: A PROOF OF PRINCIPLE

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Acta Trop 2015 Jul 3; 150:59-63.
doi: 10.1016/j.actatropica.2015.06.026

Abstract

Circulating Anodic Antigen (CAA) testing is a powerful, increasingly-used tool for diagnosis of active schistosome infection. We sought to determine the feasibility and reliability of measuring CAA in blood spots collected on Whatman 903 Protein Saver cards, which are the predominant filter papers used worldwide for dried blood spot (DBS) research and clinical care.

CAA was eluted from blood spots collected from 19 individuals onto Whatman 903 cards in Mwanza, Tanzania, and the assay was optimized to achieve CAA ratios comparable to those obtained from the spots' corresponding serum samples. The optimized assay was then used to determine the correlation of serum samples (n=16) with DBS from cards that had been stored for 8 years at ambient temperature. Using a DBS volume equivalent to approximately four times the quantity of serum, CAA testing in DBS had a sensitivity of 76% and a specificity of 79% compared to CAA testing in serum. CAA testing was reliable in samples eluted from Whatman 903 cards that had been stored for 8 years at ambient temperature. The overall kappa coefficient was 0.53 (standard error 0.17, $p<0.001$).

We conclude that CAA can be reliably and accurately measured in DBS collected onto the filter paper that is most commonly used for clinical care and research, and that can be stored from prolonged periods of time. This finding opens new avenues for future work among more than 700 million individuals living in areas worldwide in which schistosomes are endemic.

1. Introduction

The schistosome Circulating Anodic Antigen (CAA) assay is a test of high importance for both the estimated 260 million individuals worldwide with schistosome infection, as well as the 700 million who live in endemic areas and are at risk of schistosome acquisition (1,2). CAA is a glycoprotein produced in the gut of schistosome worms (3) that is secreted into the host bloodstream during active schistosome infection. CAA correlates closely with infection status, making the test useful not only for diagnosis but also for monitoring treatment response (4–7).

Compared to the traditional diagnosis of schistosomiasis by microscopic examination for eggs in multiple urine and stool samples, schistosome antigen testing offers advantages of single sample collection, elimination of labor-intensive work with excrement, and enhanced sensitivity with the potential to detect as little as one worm pair (8). Antigen testing has also been recommended for serologic screening programs in which repeated examinations of urine and stool are logistically not possible (9) and for diagnosis of young children, from whom obtaining urine and stool samples is difficult (10). Given that the CAA assay has recently been developed into a dry-reagent lateral flow assay with a portable reader that can be easily transported to, and operated in, resource-limited settings (11), its utilization will likely continue to increase.

From a laboratory standpoint, the CAA assay is an appealing test. The antigen's unique carbohydrate structure has no known biological equivalent (11,12), and recent modifications make the assay highly sensitive (13). CAA is heat-resistant and extremely stable, remaining detectable in tissue isolated from Egyptian mummies (14). While this would suggest that CAA might be easily measured in dried blood spot (DBS) samples, two early studies that explored this issue in several types of filter paper have shown that CAA was detectable but that available concentrations were low (15,16). Of note, these studies did not evaluate Whatman 903 Protein Saver cards, which cost approximately USD $1.50 each and are the most commonly-used filter papers for HIV testing and monitoring worldwide, including early infant HIV diagnosis and HIV drug resistance genotyping (17,18). Whatman 903 cards have additionally been validated for detection of malaria gametocyte RNA by qRT-PCR (19,20). Given that numerous projects currently collect DBS from regions in which schistosomiasis, HIV, and malaria are co-endemic and that the ability to test these DBS for schistosomiasis would be useful for future patient care and research, we sought to determine the feasibility and reliability of measuring CAA in DBS on Whatman 903 cards, as compared to serum, from patients in Tanzania where all three infections are co-endemic.

2. Materials and Methods

2.1 Study site. Samples for this study were collected in the Kisesa ward in northwest Tanzania, located approximately 20 kilometers east of Mwanza city. We have previously demonstrated that the prevalence of schistosomiasis by CAA in serum is ~50% among adult women in this region near Lake Victoria (21), with a similar prevalence in adult men (unpublished data). Urine and stool microscopy demonstrated that the predominant species in the region is *S. mansoni*, with approximately 25% of community-based participants in prior studies having *S. mansoni* ova visualized in stool and approximately 2-3% having *S. haematobium* ova visualized in urine.

2.2 Sample collection for assay optimization. In April 2012, we invited women of childbearing age who were seeking care for themselves or their children at the Kisesa Health Centre to participate in this study. Four milliliters of blood were collected by venipuncture from the antecubital fossa and five spots of blood (each ~13 millimeters in diameter) were collected by fingerstick lancet onto Whatman 903 Protein Saver cards (GE Healthcare Life Sciences, Piscataway, NJ, USA). Cards were dried away from direct sunlight, placed into individual zip bags 24 hours after collection, and stored at room temperature until processing. Venous blood was centrifuged upon return to the National Institute for Medical Research Laboratory in Mwanza City approximately 20 kilometers away, and serum was stored at -20C. All women were given empiric praziquantel (40mg/kg) in accordance with World Health Organization guidelines (22).

2.2 Dried blood spot sample preparation. To elute dried samples from cards, we cut sections from the DBS and placed them into eppendorfs containing 100μL of phosphate-buffered saline. The sections were incubated overnight at 4C, then placed on a shaker for 1 hour at 37C, after which 100μL of 4% (w/v) tricholoroacetic acid (TCA) was added and the mixture vortexed and centrifuged. We first eluted a 24mm^2 DBS into a total volume of 200μL. In order to increase the sensitivity, we subsequently eluted DBS with a total area of 144mm^2 into final concentration of 2% TCA. The supernatant from these elutions was concentrated to a final volume of 20-30μL using a 10kDa concentration device (Amicon Ultra-0.5ml Centrifugal Filters, Millipore Corp).

2.3 CAA test strip preparation and testing. Serum samples (20μL) were mixed with an equal volume of 4% TCA, vortexed, and centrifuged. Twenty microliters of this supernatant, 20μL of the supernatant of the eluted samples from the small circles, or all of the concentrated eluate supernatants were subsequently mixed with assay buffer containing UCP reporter particles labelled with anti-CAA

McAb, incubated, and applied to CAA-specific lateral flow test strips as previously described (8,11,23). Strips from serum samples were prepared at the local laboratory in Mwanza, Tanzania and then shipped with DBS to Leiden University Medical Centre for subsequent testing. Elutions, CAA strip preparation for eluted samples, and CAA strip scanning using a modified Packard Fluorocount meter were performed at Leiden University Medical Centre. The test line signal was normalized to the control line signal for each individual sample. Standards of known concentrations, run with each assay, were used to construct a standard curve and to determine the cut-off point above which samples were considered positive, which corresponded with 30pg/ml.

2.4 Additional testing in banked dried blood spots. Following optimization of the assay, we obtained additional paired serum and DBS samples from the same Kisesa region that had been collected onto the same Whatman 903 cards from adults aged 15-49 years in 2005. The cards had been stored with desiccant packs in separate zip bags at room temperature. CAA strips from serum samples were prepared in Mwanza; DBS elution and subsequent processing were performed in the Netherlands. Elutions were performed using 216mm^2 of dried sample and 600 µl PBS as described above, with the subsequent addition of 138 µl of 12% TCA (resulting in a final 2% (w/v)). The total volume of supernatant was concentrated to 20-30 µl.

2.5 Statistical analysis. Results were entered into Microsoft Excel and analyzed using Stata/IC Version 13 (College Station, TX, USA). Correlation between log-transformed CAA concentrations was determined using Pearson's correlation coefficient. Agreement between positive and negative test results was assessed using Cohen's kappa coefficient.

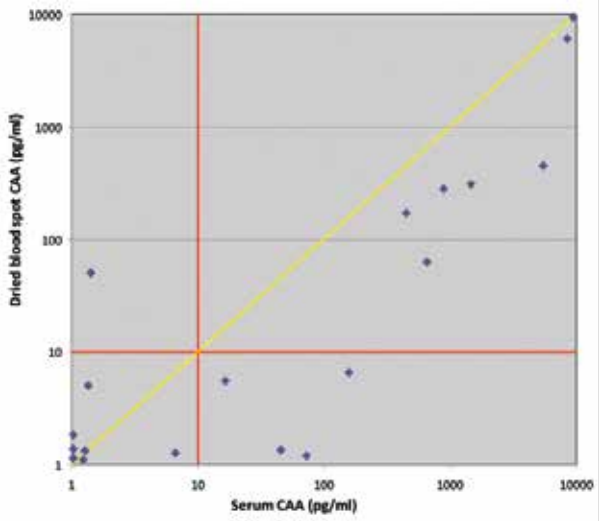
2.6 Ethics. This project was approved by the joint Bugando Medical Centre/ CUHAS Research and Publications Committee, the National Institute for Medical Research in Tanzania, and Weill Cornell Medical College. Study participants provided informed consent for their participation.

3. Results

3.1 Available samples. Paired serum and DBS were available from a total of 35 individuals. Nineteen participants provided fresh blood and DBS in April 2012, which were used for test optimization between April and July 2012. The other 16 patients had both serum and DBScollected and banked in 2005; these samples were tested in March 2013. If serum results from 2005 diverged strongly from DBS results both samples were retested in August 2013.

3.2 Optimization of dried blood spot testing. By serum testing, 11 of the 19 samples (58%) collected for optimization were positive for CAA, with concentrations greater than 10 pg CAA/ml. Testing of DBS was initially performed using small 24mm^2 DBS. This yielded positive results only in four of the samples that had been most strongly CAA-positive on serum testing (CAA >200pg/ml). When the DBS area was subsequently increased to 144mm^2 (corresponding to approximately 30μL of dried serum), 8 of 11 spots were positive (**Figure 1**). Among the 8 serum-negative samples, one was positive for CAA on DBS (50pg/ml, versus 1pg/ml in serum). The remaining seven were negative for CAA both in serum and DBS.

Figure 1. Serum CAA versus optimized dried blood spot values (n=19).

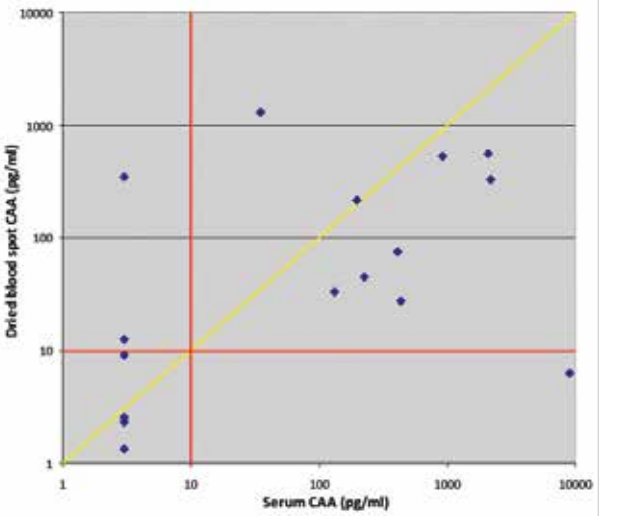


CAA values from serum and from 144mm^2 DBS samples were plotted against each other. Red solid vertical and horizontal lines indicate the cut-off value of 10pg/ml, above which a patient is considered "CAA-positive." The diagonal line indicates perfect 1:1 correlation. The majority of samples' plotted values fall below the diagonal line, indicating higher concentrations obtained from serum than from DBS.

3.3 Application of optimized assay to banked dried blood spots. By serum testing, 10/16 patients (63%) who had serum collected eight years previously were CAA-positive. Their corresponding DBS, which had been stored at room temperature for eight years, were positive in 9 of these 10 patients. **Figure 2** demonstrates the linear correlation between serum and DBS CAA concentrations, with a Pearson's correlation coefficient of 0.70 ($p<0.001$). The three samples that appear most divergent were re-tested blindly and again had similarly divergent values. As seen in the samples used for optimization, CAA concentrations measured in serum appear to be slightly higher than those obtained from the DBS.

CAA values from eight-year-old serum and from DBS samples were plotted against each other. Red solid vertical and horizontal lines indicate the cut-off value of 10pg/ml, above which a patient is considered "CAA-positive." The diagonal line indicates perfect 1:1 correlation. The majority of samples' plotted values fall below the diagonal line, indicating higher concentrations obtained from serum than from blood spots. DBS testing correctly identified as CAA-positive 9 of 10 samples that were CAA-positive in serum.

Figure 2. Serum CAA versus Dried Blood Spot Samples after Eight Years of Storage.



3.4 Overall performance measures. Considering all 35 samples tested with serum as the "gold standard," the overall sensitivity and specificity of DBS testing were 76% (16/21) and 79% (11/14), respectively. The overall kappa coefficient was 0.53 (standard error=0.17, $p<0.001$).

4. Discussion

Our work demonstrates that schistosome CAA can be reliably and accurately quantitated in DBS stored on Whatman 903 Protein Saver cards, yielding close correlation with serum values. Because Whatman 903 cards are the most commonly-used filter paper for DBS collection and preservation in clinical care and research, this finding has important implications for future work among more than 700 million individuals living in areas in which schistosomes are endemic. First, we have validated a simple means by which DBS that have been banked for cohort studies can be tested to determine whether pre-existing schistosomiasis was a risk factor for later patient events, such as subsequent HIV acquisition or development of cancer. In addition, our work demonstrates an opportunity to enhance both patient care and clinical research by coupling schistosome testing with HIV- or malaria-related testing in the same DBS. The ability to test for CAA in DBS represents a streamlined way in which schistosome testing can be

enhanced, either on its own or in concert with other projects, thereby increasing attention to this neglected parasitic infection.

In contrast to a prior report that very little CAA could be detected in DBS stored at room temperature for longer than three months (15), we successfully eluted and quantified CAA in Protein Saver cards that had been stored at room temperature for approximately eight years. Our finding that the CAA sensitivity remained high in eight-year-old spots suggests minimal degradation of CAA during long-term storage at ambient temperatures on these optimized storage papers. While long-term Protein Saver card storage at ambient temperatures has been demonstrated to be suboptimal for subsequent study of nucleic acids (24) and antibodies (25), our findings support the conclusion that this is not an issue for this schistosome-derived glycoprotein that is stable enough to be detected in Egyptian mummies (14).

Our results support and further the findings previously reported for CAA testing in DBS on other paper types—that CAA is indeed detectable in DBS but that it is incompletely eluted. In order to achieve CAA ratios comparable to those obtained from 20 μL of serum, we had to use an area of blood on the Protein Saver card that corresponded to approximately 30 μL of serum. An earlier study similarly reported recovering less than one-third of a known quantity of CAA from several other common filter papers (15). In contrast, 80-100% of the original CAA quantity was obtained from blood dried onto an inert polypropylene fiber web matrix (15). Investigators postulated that poor CAA recovery from filter papers compared to the matrix was related to the papers' higher fiber densities, smaller pore sizes, and the interaction of CAA with reactive hydroxyl and carboxylic acid groups on the hydrophilic surface of the cellulose. Whatman 903 Protein Saver cards are similarly made from nearly pure cellulose, with a comparable pore size and a larger fiber density than papers previously tested (26). Our data shows that these challenges, while not trivial, become surmountable by increasing the area of blood spot used. Our prolonged overnight elution, as compared to 30 minutes in the prior study (15), may also be an important factor in maximizing recovery.

Our study had to deal with some practical limitations. Mainly, due to small sample size and the finite quantity of sample available from DBS, we were not able to test multiple iterations of our elution protocol in order to optimize the performance of CAA testing in DBS. It is likely that the protocol can be further optimized to increase sensitivity (e.g. extended (24-hour) elution time, larger elution-volume to card-surface ratio, elevated temperature and shaking, and use of detergents were not explored to the fullest). We also did not have microscopy data from stool or urine, and therefore designed this study to determine the correlation between

CAA testing in DBS and in serum rather than to compare CAA testing in DBS with parasitological data.

In conclusion, our work demonstrates the feasibility and reliability of CAA testing in DBS collected onto Whatman 903 cards. Such DBS collection is a cost-effective, convenient way to obtain, transport, and store blood samples. Many of the remote resource-poor settings in which the simplicity of DBS collection is most needed are also those plagued by schistosomiasis. Our work opens new avenues for testing these at-risk populations, with the flexibility to test both recent samples for clinical care and older samples, regardless of storage temperature, to explore research questions. Increased implementation of CAA testing has the potential to benefit 700 million people living in schistosome-endemic areas through exploration of new hypotheses related to interactions between schistosomiasis, co-infections, and non-communicable diseases in these vulnerable individuals.

Acknowledgements
The authors wish to thank the staff at Kisesa Health Centre for assisting with this project and the study participants for their enthusiastic participation. We are also grateful to our colleagues at the National Institute for Medical Research – Mwanza Research Centre, and particularly to its Director John Changalucha, for their support. This work was supported in part by the National Institutes of Health (K23 AI110238) and by the University of Georgia Research Foundation, Inc. (Schistosomiasis Consortium for Operational Research and Evaluation [SCORE] project).

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CHAPTER 6

SCHISTOSOMIASIS AND IMPAIRED RESPONSE TO ANTIRETROVIRAL THERAPY AMONG HIV-INFECTED PATIENTS IN TANZANIA

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J Acquir Immune Defic Syndr. 2013 April 15; 62(5): e153–e156.

Schistosomiasis affects over 230 million people worldwide, 90% of whom live in sub-Saharan Africa (1). Data suggests helminthic infections like schistosomiasis may hasten HIV progression in co-infected patients (2,3). Helminths induce chronic immune activation, shifting from a T-helper cell type 1 (Th1) to type 2 (Th2) immune response. Th2- lymphocytes down-regulate cytotoxic effects of CD8+ T-lymphocytes, leading to an altered cytokine profile with increased viral replication (4–7). Studies have shown that treating ascariasis or filariasis improves CD4+ T-cell counts (CD4 counts) and viral loads in HIV-infected patients (8,9).

Little research has explored the specific interaction between schistosomiasis and HIV, but one study yielded concerning results. Antiretroviral therapy (ART)-naive HIV-infected patients with schistosome co-infection who were randomized to delayed anti-schistosome treatment with praziquantel after three months had larger increases in HIV RNA levels and greater declines in CD4 counts than patients treated immediately (10). Despite this possible interaction, schistosomiasis screening is not currently recommended for HIV-infected patients in many endemic countries, including Tanzania. Also, no study has yet assessed impact of schistosomiasis on ART response.

We hypothesized that schistosome infection may adversely affect HIV-infected patients' responses to ART. We conducted a retrospective cohort study to explore this issue at Bugando Medical Centre (BMC) near Lake Victoria in Tanzania, where schistosomiasis is hyper-endemic.

Methods

Study participants

This study was conducted from August-December 2011 at BMC's HIV clinic. HIV-infected adults who had taken ART for 6–15 months were enrolled serially. Patients who had received praziquantel since beginning ART, or who were currently receiving antituberculous therapy and therefore had another concomitant infection that could cause CD4 decrease (11), were excluded.

Data collection

Demographic information was collected by structured questionnaire. Baseline data (at ART initiation) was obtained from the patient database, including CD4 count, weight, and height.

At the time of enrollment and sample collection, we measured CD4 count by FACSCount system (BD Biosciences, San Jose, CA), height, and weight. We used

the World Health Organization (WHO) definition of immunological failure as either CD4 count falling below baseline or CD4 count persistently <100 cells/ mm^3 (11).

Patients provided single mid-day stool and urine samples. Kato-Katz stool smears were prepared using 41.7mg templates (Vestergaard Frandsen, Switzerland). Five slides were prepared from different sites of each stool sample, which has a reported sensitivity comparable to examination of specimens from different days (12). A trained parasitologist quantified *S. mansoni* eggs/gram.

Urine was examined microscopically and tested for circulating cathodic antigen (CCA) (Rapid Medical Diagnostics, South Africa). CCA, an antigen secreted into the bloodstream by adult schistosomes during active infection, is detectable in urine by rapid reagent test strip (13,14). Schistosome infection was defined as ova in stool or urine and/or a positive CCA test, a strategy shown to increase diagnostic sensitivity without compromising specificity for low-intensity *S. mansoni* infections typical of adult populations (15).

Data analysis

Logistic regression models (adjusting baseline CD4 count, which we call 'bivariate analysis,' and multivariate analysis adjusting for additional factors) were used to examine factors associated with immunological failure. In all models, we adjusted baseline CD4 count for more valid analyses, noting that the outcome, immunological failure, is defined as a function of baseline CD4 count. We used backward elimination, deleting variables with the largest p-value one by one, to reach a final parsimonious model including all factors with $p<0.05$.

We used analysis of covariance (ANCOVA) to compare CD4 count increases between groups with and without schistosomiasis while adjusting for baseline CD4 count (16). Two-sided 95% confidence intervals and p-values were used throughout. Data were analyzed using Stata IC/10.1 (College Station, Texas).

Ethics

Ethical approval was obtained from BMC and Weill Cornell. Patients diagnosed with schistosomiasis immediately received praziquantel (40mg/kg).

Results

Patient characteristics

Of 364 eligible HIV-infected outpatients coming to clinic during the study period, one had received praziquantel and 10 were being treated for tuberculosis.

Table 1. Baseline factors associated with immunological failure in HIV-infected adults after at least six months of antiretroviral therapy.

Factors	Immunological failure (n=25)	No Immunological failure (n=326)	Bivariable Analysis Odds Ratio	p-value	Multivariable Analysis Odds Ratio	p-value
Male gender	7 (28%)	96 (29%)	1.1 [0.4-2.7]	0.91		
Age in years	36 (32-49)	36 (31-43)	1.03 [0.99-1.08]	0.14		
Level of education*			0.4 [0.2-1.05]	0.062	0.3 [0.1-0.9]	0.036
No formal education	7 (28%)	24 (7%)				
Primary education	17 (68%)	270 (83%)				
Secondary education	0	18 (6%)				
College/higher education	1 (4%)	14 (4%)				
Occupation			---	---		
Business/professional	3 (12%)	34 (10%)				
Petty trading	13 (52%)	202 (62%)	0.7 [0.2-2.8]	0.52		
Unemployed	7 (28%)	68 (21%)	1.0 [0.2-4.2]	0.97		
Farming	1 (4%)	18 (5%)	0.5 [0.05-5.3]	0.45		
Fishing	1 (4%)	4 (1%)	2.3 [0.2-28.7]	0.36		
CD4 count (cells/μL) at time of ART initiation**	258 (189-298)	168 (73-239)	1.006 to 1.007	0.002 to 0.004	1.005 [1.001-1.009]	0.010
BMI (kg/m^2) at time of ART initiation	22.6 (20.8-24.4)	21.5 (19.5-23.8)	1.1 [0.98-1.2]	0.13	1.12 [1.01-1.24]	0.035
WHO clinical stage at time of ART initiation	3 (2-3)	2 (2-3)	0.9 [0.5-1.5]	0.72		
Days since ART initiation	336 (225-380)	333 (265-373)	1.0 [0.99-1.01]	0.91		
Schistosome infection	15 (60%)	82 (25%)	3.9 [1.6-9.1]	0.002	4.6 [1.9-11.2]	0.0009

Of the remaining 353 patients, 351 provided written informed consent, urine, and stool and were enrolled. Of these, 248 (71%) were females. The median age was 36 (interquartile range 31–43) years. Over 90% had primary school education or less, and >80% were unemployed or petty traders. Baseline CD4 count at ART initiation was 173 (76–249) cells/μL and baseline body mass index (BMI) was 21.6 (19.7–23.9) kg/m2. Baseline WHO clinical stages were: Stage 1--54 patients (15%), Stage 2—130 (37%), Stage 3—127 (36%), Stage 4—40 (11%). Patients had taken ART for a median of 338 (265–376) days.

Prevalence of schistosomiasis

Schistosomiasis was diagnosed in 97 patients (27.6%). All 97 were CCA-positive, and 46/97 had ova in urine or stool. All 46 of these had *Schistosoma mansoni*, and one had concurrent *S. haematobium*. All 46 patients with schistosome ova were CCA-positive, and 41/46 had low-intensity infections of <100 eggs/gram of stool.

Outcomes after ART

The median CD4 count change was +190 (104–303) cells/μL. Median BMI increase was +1.4 kg/m2. Twenty-five patients (7%) met ≥1 WHO criterion for immunological failure: 22 had CD4 counts below baseline and 7 had CD4 counts persistently <100 cells/μL.

Factors Associated with Immunological Failure

Table 1 shows univariate and multivariate analyses. In the multivariate model, education and baseline BMI were moderately associated with immunological failure (p=0.04), while schistosome infection was strongly associated (Odds ratio 4.6 [95% confidence interval: 1.9–11.2], p=0.0009).

Secondary analysis by ANCOVA, with change in CD4 count as a continuous outcome, showed that CD4 count increase on ART was significantly associated with schistosome infection, baseline CD4 count, and age in the multivariate,

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Binary variables are reported with number and percent, and continuous variables are reported with median and interquartile range. For continuous variables/ factors, odds ratios correspond to that for a one unit increase in variable (e.g., age 50 to 51).

*In regression models, education was modeled as a continuous variable.

**In all regression models, baseline CD4 was always adjusted as the outcome is defined as a function of baseline CD4. Thus, in bivariable analyses, results for baseline CD4 are summarized as a range rather than a single number.

parsimonious model. These effects were most strongly driven by schistosome infection status, with an estimated difference of 65.5 cells/μL in CD4 count change between those with and without schistosomiasis (p=0.0004). Unadjusted and adjusted mean CD4 count changes were +163 versus +226 cells/μL and +161 versus +227 cells/μL, respectively.

Discussion

Among these adult HIV-infected outpatients living in a schistosome-endemic area, nearly one-third had active schistosome infection. Odds of developing immunological failure were four times greater in patients with schistosome co-infection. To our knowledge this is the first study assessing the association between schistosomiasis and ART treatment failure. Our findings have major implications for ART management in millions of HIV-infected outpatients living in schistosome-endemic areas who are managed based on immunological and clinical criteria because viral load measurements are not routinely available.

Schistosome-infected patients also had significantly lower CD4 count increases on ART than schistosome-uninfected patients. More frequent immunological failure and smaller CD4 count gains in schistosome-infected patients could both be explained by chronic helminth-induced Th2-type immune activation, which may permit increased viral replication (4). Our work extends findings of a study in which Zimbabwean HIV- and schistosome-co-infected patients randomized to delayed praziquantel had larger HIV RNA increases and CD4 count declines than patients treated immediately (10). Other studies have suggested similar effects from other helminth infections, but not unanimously (8,9,17). Notably, other studies have not explored helminth infections' effects on patients receiving ART.

Our finding that schistosomiasis is both common and associated with immunological failure supports implementation of schistosomiasis screening at ART initiation. Unfortunately, stool and urine testing alone, particularly when done as the thin preparation of stool and unfiltered urine typical of many African clinical laboratories, has low sensitivity for detecting schistosome ova. Antigen tests including urine CCA used in this study may provide rapid, more sensitive screening for schistosomiasis, particularly since HIV-infected individuals may excrete fewer eggs (18,19). Further operational research is needed to determine costs, benefits, and optimal screening strategies.

In a previous study that compared baseline characteristics of schistosome-infected and uninfected HIV-positive patients, schistosome-infected patients had higher

baseline CD4 counts and CD4:CD8 ratios but comparable viral loads (6). Patients in that study were ARTnaive, as were ours during baseline investigations. Neither study observed other baseline differences between patients with and without schistosomiasis that might explain the higher CD4 counts. Schistosomiasis may cause distinct immunological alterations in peripheral blood CD4+ T-lymphocyte subsets, and these alterations may impair patients' responses to ART. Additional studies are needed to better-characterize peripheral blood CD4+ T-lymphocyte subsets in HIV and schistosomiasis co-infection.

The retrospective nature of our study is an ethically-necessary limitation since our hypothesis could not be studied prospectively. Based on the Zimbabwean study showing worse virological and immunological outcomes in HIV-infected patients with untreated schistosomiasis, it would not have been ethical to leave schistosomiasis untreated in patients initiating ART. Another limitation was our inability to test viral loads. Without virological data, we cannot determine whether schistosomiasis was associated with immunological failure alone or with concomitant virological failure. We plan further studies using viral load testing to explore this question.

In conclusion, nearly one-third of our Tanzanian HIV-infected outpatients had schistosome infection. Schistosome infection was significantly associated with immunological failure and poorer CD4 count gain following ART use. Untreated schistosomiasis may be a major cause of immunological failure among HIV-infected patients in schistosome-endemic areas. Further studies are needed to investigate whether this represents an immunological phenomenon or true virological failure, and whether screening and treatment for schistosomiasis among HIV-infected patients will improve response to ART. This is an urgent finding with major implications for ART management in resource-limited settings, where choices of antiretroviral medications are limited and success of patients on first-line ART must be maximized.

Acknowledgments

We thank Bugando Medical Centre for their support. In addition, we thank the National Institute for Medical Research—Mwanza Research Centre, and particularly Mr. Oswald Kaswamila, for their hard work. This project was supported in part by a grant from the United States National Institutes of Health Fogarty International Center (TW 00018) and a scholarship program at Weill Cornell Medical College supported by Pfizer Inc. The sponsors were not involved in study design or the preparation of the manuscript.

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CHAPTER 7

SHORTENING TURNAROUND TIMES FOR NEWBORN HIV TESTING IN RURAL TANZANIA: A REPORT FROM THE FIELD

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PLoS Med 2015; 12(11): e1001897.

Introduction

Newborns infected with HIV before, during, or shortly after delivery have high mortality with 50% dying before one year of age, and 20% of these early deaths occurring between the first and third months of life (1). Early diagnosis and treatment reduces mortality by up to 76% (2), but neonatal diagnosis is difficult. Antibody-based rapid HIV tests frequently yield false positive results because of transplacental transfer of maternal antibodies that can persist in the child's circulation for up to 18 months (3). For this reason, polymerase chain reaction (PCR)-based viral nucleic acid tests are recommended instead (4). At present, viral DNA or RNA can only be identified in a laboratory with the technical capacity to perform PCR.

The demonstration that HIV RNA and DNA can be detected in dried blood spots (DBS) revolutionized newborn HIV testing in resource-poor areas in which laboratory facilities are limited (5). Unlike serum samples that must either be tested within hours of collection or frozen for transport, DBS can be stored in warm, humid climates and later transported to reference laboratories for testing while still yielding accurate results (6). These findings led the World Health Organization to endorse DBS testing as the single screening tool for all infants born to mothers with HIV infection or unknown HIV status (4). Given that DBS are additionally now being used for viral load monitoring, drug resistance genotyping and even neonatal screening for hereditary diseases (7–9), ensuring the feasibility and reliability of this system is crucial.

In northwestern Tanzania, where we work, Bugando Medical Center (BMC) serves as the reference laboratory for early infant diagnosis (EID) of HIV-exposed children. The laboratory's EID program was first piloted in 2006 (10). BMC's laboratory serves a population of 13 million people, receiving DBS from 96 clinics in the seven regions surrounding the medical centre.

The lead investigator for this project (S.M.) is a pediatric nurse providing primary care to HIV-infected children at a clinic in Magu, Tanzania, located ~70 kilometers east of the Bugando laboratory. She grew frustrated with the protracted turn-around time (TAT) for results of DBS collected from HIV-exposed infants attending the clinic. DBS collected during infants' six-week immunization visits were still pending at children's subsequent ten-week visits. Sometimes mothers never received their infants' results. These delays in diagnosis led to preventable deaths, particularly given the ~30% mortality of perinatally-infected infants during the first six months of life (1) and the advanced stage of HIV disease that affects approximately half of HIV-infected infants who do not start ART before

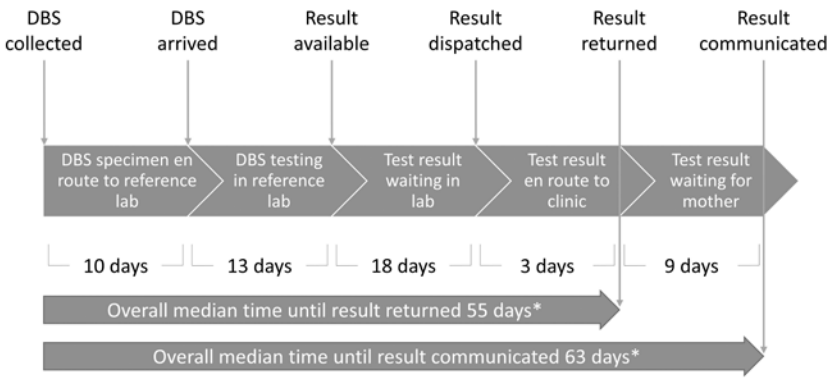
12 weeks of age (11). She therefore designed a systems improvement project to identify and address the root causes of these delays.

Systems Improvement Project Methodology

In the project's first phase, each step in the EID DBS process was carefully mapped. Five steps in the process between collection of a DBS and reporting of the result to a caregiver were identified. Each time a DBS was collected, infant demographics, date of collection, and date of shipment to Bugando were recorded consecutively in a log book in Magu. When the DBS result was returned, nurses in Magu used the lab report to fill in the dates for the remaining steps in the process.
The study's lead investigator collected this data from the handwritten log book and entered it into Microsoft Excel. Minor errors in dates with an obvious alternative were corrected (e.g. correct month and day but wrong year, or aberrant month in an otherwise consecutive series of dates). Ambiguous dates without an obvious alternative were recorded as missing data. The duration of each step was calculated based on the available (non-missing) data.

Two significant clinical process endpoints were identified: 1) TAT between DBS collection and return of test result to the Magu clinic (when the result is available to the clinician and the local staff) and 2) TAT between DBS collection and communication of test result to the infant's mother. **Fig 1** demonstrates the 5 steps in this process, and lists the median number of days between each step during a baseline assessment in 2011.

Figure 1. Flow diagram of steps required for rural dried blood spot HIV viral load testing and median times to completion at baseline.



In the second phase, the team implemented sequential interventions to decrease the time of individual steps. After each intervention, the team measured resultant duration of each step as well as the cumulative TAT between DBS collection and the two designated endpoints. Consecutive interventions were designed and implemented based on observations and experience from prior interventions using a systems improvement method based on the Plan-Do-Study-Act model (12).

Data analysis was performed using Stata Version 13 (College Station, Texas). Due to non-normal distribution of the data, medians and interquartile ranges (IQRs) were calculated. Overall differences between groups were determined using the Kruskal-Wallis test. If the p-value was less than 0.10, we subsequently performed between-group comparisons using the Wilcoxon rank-sum test.

Results

Between July 2011 to October 2013, 383 HIV-exposed infants were seen at Magu Health Centre and had DBS collected and sent to the Bugando reference laboratory for testing. Sixteen infants were entered into the Magu log book but had no further dates recorded and were excluded from further analysis. Infants were a median of 61.5 (IQR 39-133.5) days old at DBS collection. Baseline data were collected from July 2011 to January 2012 for 85 patients for whom DBS were sent, and these data are presented in **Fig 1**. The median overall baseline TAT from DBS collection to result availability at Magu was 55 (IQR 35–68.5) days, and TAT from DBS collection to result communication to mothers was 63 (IQR 35-88) days. Of note, due to the skewed distribution of the data, the total median turn-around times differ from the sum of the median values presented for each step in **Fig 1**.

We first attempted to decrease time between arrival of DBS at BMC and completion of testing (**Step 2**, **Fig 1**). The laboratory tests 44 samples per day in a single run on the COBAS Ampli-Prep/Taqman System (Basel, Switzerland) and receives 150-200 samples per day from health centers. Samples testing positive on the first run are repeated the following day. Due to staffing levels, the laboratory was unable to complete a second run per day on the machine. Our study team therefore turned its focus to other steps. Indeed, from 2011 to 2013 the median number of days that samples spent in the BMC laboratory actually increased slightly (**Table 1**).

We next attempted to decrease time between completion of testing at BMC and return of results to Magu (**Steps 3 and 4, Fig 1**). Beginning in February 2012, the study team provided a laboratory staff member with phone vouchers to call a nurse at Magu weekly and verbally relay results from the prior week. Verbal results

Table 1. Median (interquartile range) number of days required for sequential steps for DBS testing over consecutive intervention periods.

	DBS specimen en route to reference lab	DBS testing in reference lab	Test result waiting in lab	Test result en route to clinic	Test result at clinic waiting for mother	TAT collection to Magu	TAT collection to mother
Baseline ***July 2011—January 2012***	10 (4-17.5)	13 (11-17)	18 (10-34)	3 (3-7)	9 (3-31)	55 (35-68.5)	63 (35-88)
Period 1 ***February 2012 – May 2012*** **Phone voucher**	9 (5-18)	34 (24-40)	8 (5-13)	1 (0.5-1)	6.5 (1-8)	48 (43-65)	69 (60-82)
p-value for Wilcoxon rank sum test comparing Period 1 with Baseline	0.813	**<0.001**	**<0.001**	**<0.001**	**0.042**	0.867	0.199
Period 2 ***June 2012 – October 2013*** **Transport + home nurse**	6 (3-9)	21 (11-30)	14 (5-17)	0 (0-0.5)	6 (2-14)	38 (29-51)	58 (42-78)
p-value for Wilcoxon rank sum test comparing Period 2 with Baseline	**0.001**	**0.001**	**<0.001**	**<0.001**	0.072	**<0.001**	0.708
p-value for Wilcoxon rank sum test comparing Period 2 with Period 1	<0.001	**<0.001**	**0.014**	**<0.001**	0.561	**<0.001**	**0.012**
p-values for Kruskal-Wallis test comparing all 3 time periods	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.088	**<0.001**	0.057

were recorded in the Magu log book and acted upon, and they were subsequently confirmed when the written results arrived at Magu and were entered formally into the log book. This led to significant decreases in the time that the test result was ready in the lab waiting for transport (18 (IQR 10-34) days to 8 (IQR 5-13) days, p<0.001) and to the amount of time taken for the test result to be transmitted from the lab to Magu (3 (IQR 3-7) days to 1 (IQR 0.5-1) day, p<0.001). The overall TAT from DBS collection to return of result to Magu decreased, though not yet significantly, from the baseline value of 55 (IQR 38-68.5) days to 48 (IQR 43-65) days among patients who had blood spots collected from February to May 2012 (p=0.87). The lack of significant decrease in cumulative TAT is likely attributable to the significant increase in time required for test performance in the reference laboratory (13 to 34 days, p<0.001). The team noted that, despite this improvement, 18/51 results (35.3%) that returned to Magu were not communicated to mothers and room for improvement remained.

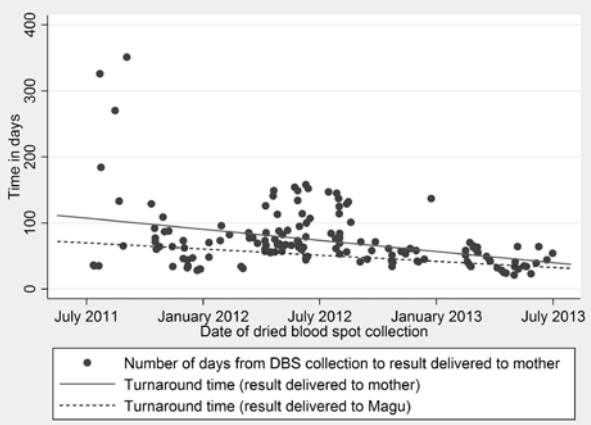
After June 2012, the team focused on two separate steps: time between collection of DBS and arrival at BMC (**Step 1, Fig 1)** and time between result arrival at Magu and result communication to mothers (**Step 5, Fig 1**). It was discovered that nurses at Magu were not sending DBS to Bugando until they had collected at least 3-5 samples. Instructions to send DBS as soon as possible after collection were reinforced, and nurses began to ask drivers of hospital vehicles between Magu and Bugando to carry blood spots to the laboratory whenever possible (usually 1-2 times weekly) rather than waiting to send samples in batches in a dedicated car from the Bugando HIV clinic. Median time between a DBS collection and arrival at Bugando decreased from 10 (IQR 4-17.5) days at baseline to 6 (IQR 3-9) days after June 2012 (p=0.001). This led to an overall improvement in median TAT from 48 (IQR 43-65) days during the previous intervention period to 38 (IQR 29-51) days (p<0.001).

After June 2012, the study team also partnered with an ongoing home visit nurse program in Magu in which clinic nurses spend one afternoon per week visiting homes in the community. The study team ensured that visiting nurses focused preferentially on visiting HIV-infected mothers to remind them to return to clinic to obtain their infants' results. Additionally, nurses at the Magu clinic now leave a blank space on infants' vaccination cards and fill in the DBS result when mothers obtain it. Taken together, these interventions led to consistent communication of results to mothers in 90.5% (95/105) of blood spot results that arrived at Magu after June 2012, compared with 47.2% (34/72) at baseline (p<0.001).

Since the project began, the overall TAT from DBS collection to return of results to Magu has decreased from a median of 55 (IQR 35-68.5) to 38 (IQR

29-51) days (p<0.001, **Fig 2**). Dates of DBS collection and timepoints continue to be recorded in the log book by the Magu nursing staff, and data from 2014 demonstrates that these levels have been sustained, with ongoing oversight from a nurse who visits Magu once monthly from Bugando but no further cost investment. The ongoing costs are weekly vouchers for phone calls (USD $1 per week); other interventions have built on and/or redirected existing systems at negligible incremental cost. For example, the visiting nurse program is not a new program but instead reprioritizes the goals of an existing visiting nurse program. Since the start of this project, 18 HIV-infected infants' mothers have been notified of their results, and these babies have been started on lifesaving antiretroviral therapy.

Figure 2. Turnaround times from dried blood spot collection until result available at health center (dashed line) or result given to mother (solid line).



Project Assessment and Discussion

Our work has demonstrated that significant improvements can be made in the implementation of neonatal HIV testing in a rural setting with dedicated attention and application of a simple, locally-driven system-improvement methodology. During this two-year project, the study team's intervention decreased DBS TAT from 55 to 38 days, and improved result communication rates to mothers from 47% to 91%. These interventions required minimal incremental cost in the study setting and have been sustained. Our study team has now presented our experience to other nurses providing primary pediatric care in rural clinics in our region to equip them to devise their own locally-relevant solutions to address implementation problems at their respective health centres.

A major strength of our work is that it models the critical role for quality improvement interventions within HIV programming, and particularly how such interventions can and often should be driven by local health centre staff. Health workers caring for patients in rural settings have first-hand knowledge of system breakdowns and, often, can propose innovative, low-cost, sustainable ideas for improvement if given the opportunity to do so. In our case, our principal investigator initially sought to

change procedures in the reference laboratory (largely beyond her control). When that proved impossible, she subsequently devised a series of simple solutions that, taken together, shortened the TAT by ~17 days, bringing major health benefits to her patients and empowerment to local staff. Such quality improvement work, in which locally-devised innovations promote "buy-in" and rejuvenation for overburdened staff, was recognized as a key factor in multiple successful quality improvement projects in South Africa (13).

To the best of our knowledge, no studies have been published on small scale, low-cost systems improvement projects to EID such as ours. Three countries have published marked improvements in their EID TAT after large scale technological or logistical investment. Rwanda made multiple changes to their system, including integrating EID into the immunization program, instituting a national pickup system to collect samples twice weekly from facilities, and implementing an automatic SMS system that sends results back to the mobile phone of the provider who ordered the test and the laboratory technician at the facility where the DBS originated. This package of interventions decreased median TAT from DBS collection to return of results to the clinic from 144 to 20 days (14). Uganda invested in a national pickup system, providing a motorbike and driver for each of their collection hubs and a national SMS printer system that sends results back to the hubs automatically from the reference laboratory. Uganda's TAT from DBS collection to return of results decreased from 49 to 14 days (15). Zambia piloted a similar automatic SMS system, and TAT from DBS collection to result communication to the mother dropped from 67 to 35 days (16).

In contrast to these large projects, our work, at a fraction of the cost, demonstrates the feasibility of local interventions in achieving TATs comparable to those reported through expensive, country-wide systems. It is our hope that other local clinics will use our work as a model to devise low-cost action plans relevant to their own contexts. In our case, simple changes such as writing reminders to obtain results on children's health cards and providing a nurse with dedicated time to seek out caregivers who have not obtained their children's results were effective interventions that required investment of relatively little money and time. Of note, Magu benefits from existing infrastructure like the visiting nurse program and a close transportation connection with BMC that might not be present in other settings. We outline key barriers and opportunities for change in Table 2. We recognize that this project's focus on neonatal DBS testing represents only one aspect of highly complex prevention of mother to child transmission of HIV (PMTCT) programs. But improving this aspect of PMTCT programs needs to be a nonnegotiable, urgent goal.

Table 2. Barriers to Expeditious Dried Blood Spot Testing and Potential Solutions.

Delayed transport from collection site to central laboratory	
Problems	**Solutions**
1) Busy health center staff responsible for coordination of transport	1) Shift burden of collection of samples to be transported to a driver rather than health center staff
2) Health center staff trying to decrease costs by shipping more samples at once	2) Standardize and routinize specimen pick-up at regular, frequent intervals
	3) Emphasize to health center staff the larger cost of later seeking out patients whose results returned late
Delayed transmission of results to health centre	
Problems	**Solutions**
1) No person in laboratory responsible for result transmission	1) Place log or register in laboratory requiring documentation of result transmission
2) Laboratory's emphasis is maximizing number of test results rather than sending results	2) Implement measurement of time to result transmission in laboratory as a quality-control standard
	3) Subsidize phone/SMS communication or institute automated SMS transmission method
Difficulty providing results to mothers/caregivers	
Problems	**Solutions**
1) Caregivers do not understand urgency of obtaining results	1) Improve counseling provided to caregivers at time of dried blood spot collection
2) Many caregivers return routinely for vaccinations but not at other times	2) Ensure that home visit/outreach nurse reminds caregivers to return for results
3) Some caregivers grow frustrated with returning to clinic only to learn that results have still not arrived	3) Insert space for result on child's health card so caregiver notices that it is incomplete

Despite these successes, our work also demonstrates the need for an ongoing discussion about the feasibility of relying on such a complex multi-step process for provision of laboratory testing in remote settings. Our local neonatal testing algorithm is a five-step process that works smoothly only when laboratory supply and functionality, transport, communication, and patient follow-up systems are simultaneously operational. A breakdown at any step creates an impasse and delays HIV diagnosis for hundreds of children in northwest Tanzania. In the face of these challenges, the growing optimization of point-of-care nucleic acid testing for infant HIV diagnosis offers hope that EID will eventually become a simple, streamlined service that provides same-day results, allowing earlier initiation of ART for infected infants and earlier cessation of ART for uninfected ones (17,18). In conclusion, our work highlights the major importance of working, both on local and national scales, to improve the implementation of HIV programs with proven effectiveness. With the impending expansion of DBS testing for HIV viral load monitoring and genetic disease screening, our successes and challenges highlight the importance of a realistic assessment of this system's limitations. Yet we hope that our experience also engenders hope that simple, practical interventions can have real impact on the health and well-being of patients in resource-poor settings. We continue to strive towards additional improvements in TAT, with particular focus on increasing the percentage and frequency of mothers returning for results by providing community education on the importance of EID. We also are now evaluating the percentages of infants who are ultimately started on lifesaving ART, which is the ultimate goal of efforts to expedite EID. We share our experience in hopes that we, other local clinicians, and national leaders will resolve to work together to streamline and/or de-centralize this system in order to make essential diagnostic testing more accessible.

Acknowledgements

We thank the clinical staff at Magu District Hospital and the laboratory team at Bugando Medical Centre for their enthusiastic support of this project. This project was generously supported by a leadership training grant from the Mulago Foundation, as well as by the National Institute of Allergy and Infectious Diseases (K23 AI110238) and the Fogarty International Center (TW009337). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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CHAPTER 8

SUMMARIZING DISCUSSION

The work presented in this thesis was conducted in Mwanza, Tanzania, and was inspired by the tremendous burden of disease in the Tanzanian population. Neglected tropical diseases such as schistosomiasis, which may be seen once or twice in a lifetime by a physician practicing in a high-income country, are weekly if not daily diagnoses in health centers and hospitals, and disproportionately affect younger patients of lower socioeconomic status. HIV, though a worldwide epidemic, has also particularly afflicted sub-Saharan Africa. Over 1 in 20 Tanzanian adults is HIV-infected, and nearly 60% of these are young to middle-aged women (1). Moreover, like many other illnesses in Tanzania, HIV is often not diagnosed until patients have developed advanced disease, at which point it may be too late.

The goal of the thesis has been to conduct studies that, ultimately, will lessen the disproportionate burden of infectious diseases in sub-Saharan Africa, whether by preventing them altogether, by diagnosing them earlier, or by working to alleviate complications attributable to co-infections. The work has focused primarily on schistosomiasis and HIV in Tanzania, building on prior work in the field from Zimbabwe, Kenya, and several other countries in sub-Saharan Africa.

The discussion will be divided into three sections: Schistosomiasis and HIV Risk; Managing Schistosomiasis-HIV Co-Infection; and an Implementation Science Study to Improve Early Infant HIV Diagnosis. Each of these topics will be first discussed with reference to the contribution made by the findings presented in the preceding chapters, and then will turn towards an examination of the needed next research steps.

I. Schistosomiasis and HIV Risk

Schistosoma haematobium Infection as a Risk Factor for HIV Acquisition

S. haematobium infection was postulated to be a risk factor for incident HIV infection at least as early as 1994, long before Kjetland and colleagues published their clinical findings from Zimbabwe (2). A causal relationship between *S. haematobium* infection, usually acquired in childhood well before sexual debut, and HIV infection is biologically plausible for a number of reasons, as outlined in the introduction. Kjetland and colleagues documented for the first time a clinical association between *S. haematobium* infection and HIV in Zimbabwean women (3). Because of the biologic plausibility and multiple other lines of evidence suggesting that schistosomiasis could be a risk factor for HIV acquisition, the Zimbabwe findings were compelling. However, that work was conducted at a single site that may have had uniquely high rates of both *S. haematobium* (39%) and HIV (25%) infections. In addition, due to the study's cross-sectional design,

it was not able to demonstrate that *S. haematobium* infection preceded HIV infection, but merely that the two were significantly associated with one another.

Our work presented in **Chapter 2** provides strong new, independent evidence in support of the HIV-*S. haematobium* association. We enrolled 457 women living in eight rural villages in Tanzania; the prevalence of *S. haematobium* infection varied by village from 0 to 11% and the overall prevalence of HIV infection was 5%. In this moderately-endemic setting, *S. haematobium* infection was again shown to be strongly associated with HIV, with an OR of 4.0 [IQR, 1.2-13.5]. Our OR was comparable to the OR of 2.9 reported by Kjetland and colleagues, despite the markedly higher prevalence of both *S. haematobium* infection (40-50%) and HIV infection (28%) that were observed in Zimbabwe (3). Thus our study provided important corroboration of the Zimbabwe study, because there now exist two separate studies, conducted in different countries with varying intensities of *S. haematobium* and HIV infections, both demonstrating the disturbing finding that women with *S. haematobium* infection have a 3-4 -fold higher odds of being HIV-infected than women without *S. haematobium* infection.

Worry about the potential HIV risk caused by *S. haematobium* infection is further increased by results reported in our longitudinal study in **Chapter 4**. We documented that praziquantel treatment of urogenital schistosomiasis resolved neither the gynecologic abnormalities associated with *S. haematobium* infection, nor the presence of the parasite as demonstrated by detectable schistosome DNA, 6 months after treatment. Our work extended others' findings that the clinical signs of schistosomiasis did not resolve up to 12 months after treatment (4,5) by documenting that, additionally, schistosome DNA could be identified in both urine and cervical lavage. Women in our study had a median age of 22 [IQR, 20-28], and one-fourth reported never being previously treated for schistosomiasis. Many of them may therefore have resembled the older Zimbabwean women, described by Kjetland and colleagues, in whom praziquantel treatment after age 20 was not effective in reversing cervical pathology (6). If *S. haematobium* infection is indeed a risk factor for HIV acquisition, and if the effects that lead to increased HIV susceptibility are incompletely reversed when treated according to the current standard of care, this is an area for urgent future research.

Moreover, if *S. haematobium* is a risk factor for HIV acquisition and it is at least partially irreversible, then this must prompt a redoubling of efforts to eradicate the disease. Vaccines against *S. mansoni* are currently in Phase I clinical trials in the United States and Brazil, while a Phase III clinical trial for a vaccine for *S. haematobium* is expected to be completed shortly in Senegal and Nigeria (7). The vaccine development process for schistosomiasis and other neglected

tropical diseases has been fraught with challenges including the complexity of the eukaryotic parasite genome, the difficulty measuring effectiveness in infections that cause more disability than death, and the dearth of fully-translatable animal models (7). In the interim until vaccines become available, early and regular provision of anti-schistosome medications to prevent disease morbidity must be urgently prioritized, particularly since only approximately 15% of people who needed treatment actually received it in 2013 (6,8,9). As the current gold-standard medication praziquantel is made more widely available to populations in need, its administration must be accompanied by robust studies to determine optimum dose and frequency of administration, as well as to quantify clinical effectiveness. This is particularly important given the findings of a recent Cochrane review that treating soil-transmitted helminth infections among children did not improve mean nutritional status, cognition, hemoglobin level, or survival (10). The ultimate aim, listed as one of the 2015-2030 United Nations Sustainable Development Goals is to eliminate schistosomiasis and other neglected tropical diseases altogether (11).

Other investigators have also explored additional implications of the association between *S. haematobium* and HIV infection. Men with *S. haematobium* infection have been shown to have bloody ejaculate containing higher levels of lymphocytes and inflammatory cytokines than men without schistosomiasis, which may facilitate HIV transmission in men with both *S. haematobium* and HIV infections (12). For women living in endemic settings, *S. haematobium* infection may render them doubly susceptible to HIV acquisition, due to both their own increased susceptibility and to the increased HIV transmissibility of their *S. haematobium*-infected male partners. It has been recently suggested that the failure to account for *S. haematobium* as a potential HIV risk factor may have been a major confounding factor in 8 randomized controlled trials of treatment of STIs to prevent HIV acquisition in sub-Saharan Africa (13). In fact, in several of the studies, "control" treatment included albendazole or provision of improved sanitation—both of which would effectively decrease *S. haematobium* infections and bias the study towards a null result. If *S. haematobium* infection does indeed increase the odds of HIV acquisition by 3-4-fold, as in these two clinical studies, then the effect size of *S. haematobium* infection is comparable to the increased odds of HIV acquisition attributable to STIs (14) and *S. haematobium* infection could certainly have obscured the effects of STI treatment in these randomized trials.

Schistosoma mansoni Infection as a Risk Factor for HIV Acquisition

The findings described in **Chapter 3** were surprising. Our hypothesis had been that *S. haematobium* increased HIV susceptibility predominantly by causing

lesions in the genital mucosa. However, we observed that women with *S. mansoni* infection also had an approximately four-fold increased odds of HIV infection, and that increasing intensity of *S. mansoni* infection, as measured by CAA level, was strongly and significantly correlated with the prevalence of HIV infection. One other study, performed in fishing villages in Uganda, also suggested that *S. mansoni* could be associated with HIV acquisition. In that study, HIV-infected individuals living in an area endemic for *S. mansoni* were found to more frequently have antibodies to schistosome antigens than did HIV-uninfected individuals (15). As with our study, the Uganda study was cross-sectional and unable to demonstrate which infection preceded the other. The Uganda study also relied on an antibody test that can be positive both during and after infection, in contrast to the CAA assay that we used, which is positive during active infection and falls rapidly after treatment (16,17).

In contrast to our findings, two recent clinical studies have reported no association between *S. mansoni* and HIV infection, but they have been limited by methodological issues. Mazigo and colleagues screened 1,785 adults in Tanzanian fishing villages for HIV and *S. mansoni* infections, and found no association between these infections (adjusted OR 1.01 [IQR 0.84-1.21]) (18). This study relied on a single Kato-Katz smear and therefore may have missed lighter infections, which is notable given several reports that HIV-infected individuals may excrete fewer schistosome eggs (19,20). Impaired egg excretion by HIV-infected individuals would result in misclassification of some HIV-infected patients as being negative for *S. mansoni* infection, thereby biasing the study towards a negative finding. Sanya and colleagues in Uganda tested 1,412 adults using both a single Kato-Katz smear and a rapid Circulating Cathodic Antigen (CCA) point-of-care test (21). They found no significant relationship between *S. mansoni* and HIV infection by stool microscopy (OR 1.04 [IQR 0.74-1.47], p=0.81). CCA testing was performed in only 650 individuals, yielding an OR for association with HIV infection that trended towards significance (OR 1.53 [0.78-3.00], p=0.19). Importantly, this sub-analysis only had the power to detect a difference of more than 10% in the prevalence of *S. mansoni* infection using CCA as the diagnostic tool, so the authors' conclusion that their study demonstrates no association between *S. mansoni* and HIV infection raises questions.

Strong animal data from rhesus macaques clearly demonstrates that macaques with *S. mansoni* infection are more susceptible to simian HIV (SHIV) infection than uninfected macaques. Macaques have been previously demonstrated to be a robust model for HIV infection in humans, with macaques exhibiting parallel differential transmission risks across various mucosae and similarly slow progression of SHIV after infection (22,23). Macaques were inoculated rectally with increasing doses

of SHIV until they ultimately developed systemic SHIV infection. Macaques chronically infected with *S. mansoni* (n=8) developed SHIV at a median inoculum that was 17 times lower than macaques without *S. mansoni* (n=9) ($p<0.001$) (24). Median peak SHIV viral load was also >1 log copies/ml higher in *S. mansoni*-infected macaques than the macaques without *S. mansoni* ($p=0.004$). Of note, no significant difference in inoculum size or viral load was found when the experiment was repeated using intravenous inoculation instead of rectal mucosal inoculation (23). These findings directly implicate changes in the rectal mucosa, rather than in the systemic circulation, as the major contributors to increased HIV susceptibility in macaques with *S. mansoni* infection.

Our findings in women with *S. mansoni,* together with these elegant macaque studies, have led us to reformulate our hypotheses about the mechanisms of HIV susceptibility in the setting of schistosome infection. It seems very likely that the epithelial breaches incited by *S. haematobium* ova migrating through the cervix are an important contributor to HIV risk. In addition, because *S. mansoni* eggs do not typically damage the genital mucosa in humans, we further postulate that schistosome infection triggers a generalized mucosal immune response, which may involve both rectal and genital mucosa. We hypothesize that this mucosal inflammation could lead to recruitment of HIV-susceptible cells to the mucosal surface and could thereby foster an immune environment in the mucosal tissue, at the initial site of HIV exposure, which is permissive to HIV infection.

Next Steps: Schistosomaisis and HIV Risk

Therefore, a variety of evidence suggests that both *S. haematobium* and *S. mansoni* infections increase the odds of HIV acquisition. Mathematical modeling predicts that routine praziquantel administration to adults living in schistosome-endemic areas would be highly cost-effective at $295 per HIV infection averted (25,26). However, until a robust prospective study quantitates the importance of schistosomiasis in HIV acquisition and definitively documents the causal relationship between schistosomiasis and HIV infection, schistosomiasis treatment will not be a public health priority for HIV prevention.

Given the ethical complexity of conducting such a study prospectively, our work in **Chapter 5** lays important groundwork that will make a retrospective longitudinal study possible. In Tanzania, serum samples have been stored as DBS from a cohort of 30,000 adults in rural villages who have been followed for over 20 years for HIV-seroconversion. In the proof-of-concept study described in Chapter 5, we documented that CAA can be reliably, accurately quantified in DBS as compared to serum, even in DBS that had been stored for up to 8 years. Our optimization of a technique to elute and quantify CAA from Whatman 903

paper, the most commonly-used DBS paper worldwide, opens new possibilities for other research studies on interactions between schistosomiasis and a variety of other communicable and noncommunicable diseases. We are currently using the technique that we have described to quantify CAA in banked DBS collected from adults prior to their HIV-seroconversion, and to compare this to the quantity of CAA in DBS from adults who did not HIV-seroconvert. We are additionally determining HIV-1 RNA viral load set-points as copies per milliliter of blood in new HIV-seroconverters who had and did not have schistosome infection at the time of HIV-seroconversion. This will provide human prospective data on the relationships between HIV susceptibility, schistosomiasis, and early HIV virologic control, with the potential to impact health policy and HIV prevention strategies throughout sub-Saharan Africa.

To investigate further the mucosal immunity hypothesis, studies are needed to identify and quantify differences in frequency, function, and types of immune cells in the cervical mucosal tissue of women with and without *S. haematobium* infection. This can ultimately be expanded to men and to individuals with and without *S. mansoni*. In addition, it will be important to determine whether, as reported with interleukin-17 production in mouse models, abnormal cytokine levels in tissue are reflected in peripheral blood (27).

II. Managing Schistosomiasis-HIV Co-infection

Association between Schistosome Infection and Immunological Failure in HIV-Infected Patients Receiving Antiretroviral Therapy

Growing evidence suggests that schistosomiasis may be not only a risk factor for HIV acquisition, but that it also may play an important role in HIV disease progression in individuals with HIV-schistosome co-infection. The single randomized trial in HIV-schistosome co-infected patients documented that patients treated with praziquantel had lower viral load increases than those who received delayed praziquantel treatment (28). A large randomized trial that treated helminth infections more generally and did not observe improvements in viral load was conducted in a setting with a low prevalence of schistosome infections (29). Neither of these trials enrolled HIV-infected patients on ART, which is a growing proportion of the HIV-infected population in sub-Saharan Africa. We hypothesized that schistosome infection could affect clinical outcomes and/or response to ART in HIV-infected patients and therefore performed the study in **Chapter 6** to explore this question.

This project was conducted among 351 HIV-infected adult outpatients who had been taking ART for at least six months and resided in an area in which

S. mansoni is highly endemic. We reported that 28% of patients had concurrent schistosome co-infection, as documented by a positive CCA rapid test. This finding alone suggests the importance of screening and treating HIV-infected patients in our setting for schistosome infection in order to prevent schistosome-associated morbidity and mortality. More troubling, HIV-infected patients with schistosome co-infection had a four-fold higher odds of having immunological failure than HIV-infected patients without schistosome co-infection. Patients with schistosome co-infection also had significantly lower CD4 count increases than those without schistosome co-infection after controlling for level of education, baseline CD4 count, and body mass index. Limitations of this study include our inability to study patients prospectively to determine the impact of untreated schistosome infection on immunological response, lack of information about other potential confounders including STIs, and the unavailability of HIV-1 RNA viral load level quantification at Bugando Medical Centre.

A study of CD4 counts in South African girls and young women demonstrated no significant differences in CD4 counts of girls with versus those without *S. haematobium* infection, as defined either by ova in urine or by gynecological abnormalities (30). In contrast to our study, in which all HIV-infected patients had been taking ART for at least 6 months, only approximately 15% of the HIV-infected South African women were on ART. Another key distinction is different species of parasites (*S. haematobium* is endemic in South Africa and stool was not tested, while the vast majority in our study had *S. mansoni*). We also used both microbiological and antigen testing for diagnosis, which may have higher sensitivity for diagnosis of schistosome infection particularly in HIV-infected individuals who have been suggested to excrete fewer eggs (19). Therefore it is possible that the effects on CD4 counts are limited to *S. mansoni* infection, which has been associated with increased density of HIV-co-receptors on the surfaces of peripheral blood CD4 cells (31). Another possibility is that the plethora of systemic immune alterations caused by chronic schistosomiasis, including increased T-regulatory cells, increased Th2 immune response, and impaired innate Th1 immunity, impair the body's ability to control viremia and in this way may precipitate true virological failure (32–34).

Gaining a clearer understanding of these phenomena is urgent. Clinicians caring for most HIV-infected patients in sub-Saharan Africa still do not have access to gold-standard viral load monitoring for patients on ART, and are therefore dependent on CD4 counts to determine whether patients' antiretroviral therapy is succeeding or failing, in accordance with the WHO's clinical and immunological criteria for treatment failure (35). If schistosomiasis affects CD4 count measurements, whether or not it actually impairs patients' response to

antiretroviral therapy, then this will strongly impact treatment decisions for HIV-schistosomiasis co-infected patients in sub-Saharan Africa.

Next Steps: Managing Schistosomiasis-HIV Co-infection

A major limitation of the work presented in Chapter 6 was our inability to measure viral loads. We were therefore unable to determine whether the immunological failure that was associated with schistosome co-infection was reflective of true virological failure in patients on ART. It is certainly possible that schistosome infection could increase the risk of virological failure, perhaps through its induction of a Th2-type immune environment that is permissive to viral replication (32,36). Conversely, it is also possible that schistosome co-infection in HIV-infected patients could cause an immunological phenomenon that is independent of viral load suppression. Elliott and colleagues reported a difference in CD4:CD8 T-cell ratios between HIV-infected patients with versus without schistosome co-infection who were not on ART (37), and others have described a mechanism by which schistosome infection may induce the mild neutropenia observed clinically in patients (38). It is therefore possible that schistosome infection affects blood lymphocytes, and particularly that it could impair the ability of HIV-infected patients to mount high CD4 counts, even though they are successfully virologically suppressed. Impaired CD4 count recovery, even in patients with virological suppression, has been associated with increased AIDS-related clinical outcomes, AIDS-related mortality, and non-AIDS related mortality (39).

Therefore, a clear next step is to assess whether schistosomiasis is associated with virological failure and/or impaired CD4 count recovery in patients on ART. This could be examined using CAA testing in a cohort of patients for whom serum or dried blood spots were stored at the time of ART initiation. Because the majority of patients in sub-Saharan Africa are still managed based on CD4 counts, it is essential to ascertain whether immunological failure in the setting of schistosome co-infection represents true treatment failure, legitimizing a switch from first- to second-line ART. If it does not represent true treatment failure, millions of patients with HIV-schistosomiasis co-infection are at risk of unnecessarily being started on costlier, more toxic second-line ART.

Other studies in this field should further characterize the interactions between HIV and CD4 counts in patients with schistosome co-infection, particularly with regard to the rate of decrease following HIV acquisition and to the functionality of CD4 cells in preventing opportunistic infections. Moreover, regardless of whether schistosomiasis is associated with only immunological or both immunological and virological failure, future studies should assess whether patients with HIV-

schistosomaisis co-infection have poorer outcomes.

III. Implementation Science: Improving Efficiency of Early Infant Diagnosis of HIV by Dried Blood Spot Testing

Finally, our work in **Chapter 7** provides an example of a low-budget implementation science project that, with sequential interventions, led to sustained improvement in early diagnosis for HIV-exposed infants. The local challenges that inspired this study typify the indispensable nature of quality improvement work to make scientific advancements in HIV care accessible in resource-limited settings. Importantly, the genesis of the project came from a local health centre nurse, who had experienced first-hand the frustrations of an ineffective system for HIV diagnosis. She proposed innovative, inexpensive ideas that decreased the turn-around time from dried blood spot collection to result availability at the rural clinic from 55 to 38 days. While there remains room for additional improvement (38 days is still far from optimal), this project has invigorated local staff to devise new solutions that will both continue to shorten the turn-around time for DBS and will solve other local healthcare delivery problems. Moreover, this work can serve as a model for others seeking to address programmatic challenges in resource-poor settings.

Next Steps: Implementation Science Studies for Further Systems Improvement and to Maintain Impact

Implementation science is an essential aspect of providing HIV care and treatment in sub-Saharan Africa. As the WHO advises a shift toward viral load monitoring for management of HIV-infected patients on ART (40), the availability of viral load testing will continue to increase. In addition, point-of-care viral load tests, which could provide same-day results in HIV-exposed newborns, are also on the horizon (41,42). As these technologies and others like them are scaled up in sub-Saharan Africa, it will be vital that the scale-up is implemented in ways that ensure reliability, quality, and sustainability. Operational projects to assess the effectiveness and longevity of new programs must be integrated into the normal workflow of overburdened clinics in ways that empower, rather than add to the work burden of, local staff (43).

Implementation science needs also to be a cornerstone of public health prevention measures. As public awareness grows regarding the relationship between schistosomiasis and HIV infection, it will be essential to ensure that individuals with schistosomiasis do not become stigmatized (44), whether due to public perceptions of their poverty or uncleanliness or to incorrectly-perceived sexual promiscuity. Uptake of routine anti-schistosome treatment, vaccination, or other

novel interventions to treat schistosomiasis and decrease HIV risk will likely not be maximized if public perceptions are not managed appropriately.

Concluding Remarks

This thesis began by describing the persistent burden of infectious diseases in sub-Saharan Africa at a time when many wealthier parts of the world, having gained relative control of many infectious diseases, are now focusing on non-communicable diseases. It also touched on the morbidity and mortality caused by schistosomiasis, which, in and of itself, merits dedicated efforts at treatment. The bulk of the work presented in this thesis, displaying additional overlap between HIV infection and schistosomiasis, only further strengthens the imperative to treat patients suffering from this neglected tropical disease. Operational studies on other topics related to HIV prevention and diagnosis can serve as models for implementation science work that will improve upon the recent estimates that only 13% of those needing schistosomiasis treatment were treated in 2013 (9). Not only would treatment optimization decrease suffering from schistosomiasis itself, but its impact may be far broader if indeed schistosomiasis is a risk factor for HIV infection.

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ADDENDUM:

ENGLISH SUMMARY
SAMENVATTING
ACKNOWLEDGEMENTS
CURRICULUM VITAE
LIST OF PUBLICATIONS

English Summary

HIV infections have claimed more than 39 million lives since the HIV epidemic began more than 35 years ago. The HIV epidemic began in sub-Saharan Africa and continues disproportionately to affect this region. Though sub-Saharan Africa accounts for only ~12% of the world's population, it is home to over two-thirds of global HIV infections, incident new infections, and 2/3 of HIV-attributable deaths.

It is well-known that individuals with infections such as gonorrhea, syphilis, and herpes simplex virus have a higher risk of becoming HIV-infected than individuals without these infections. In addition, people with HIV who have concurrent infections—including the sexually-transmitted infections mentioned above and tuberculosis—have higher concentrations of HIV-1 RNA in their blood. This increased HIV viral load causes these individuals with co-infections to transmit HIV more readily to their sexual partners, and also it speeds their progression from HIV to symptomatic AIDS and death in the absence of treatment. In addition, a growing body of evidence suggests that some tropical parasitic infections may also have these effects on HIV viral load and on an individual's susceptibility to HIV. Thus it is plausible that co-infections such as these may have played a major role in the spread of the HIV epidemic in sub-Saharan Africa.

Schistosomiasis is a helminthic worm infection that affects 260 million people worldwide, 90% of whom live in sub-Saharan Africa. In Tanzania, where the research in this thesis was conducted, two species of schistosomes are highly endemic (*Schistosoma haematobium* and *S. mansoni),* with more than 50% of adults infected with one or both schistosome species in many regions. In and of itself, schistosomiasis causes significant morbidity and mortality, with an estimated 200,000 deaths annually and 3.31 million disability-adjusted life-years. The possibility that it additionally impacts HIV transmission and disease progression render treatment and control of this neglected tropical disease even more urgent.

This thesis focuses on HIV prevention and disease management in sub-Saharan Africa. It will first describe population-based epidemiological work in Tanzania associating HIV with *S. haematobium* and with *S. mansoni.* Subsequent chapters focus on treatment of *S. haematobium* infection in women, where it causes genital tract disease, and on the effects of schistosome infection on immunological response to treatment in people living with HIV infection. The final chapters focus on implementation science work with high potential to improve HIV prevention and early diagnosis in Tanzania.

In **Chapter 1**, the background and context of the work presented in this thesis is presented. The literature to date is reviewed, and objectives of the thesis are outlined.

In **Chapter 2**, we conducted a community-based study of 457 women from 8 different villages in northwest Tanzania. Women were screened for *S. haematobium* and *S. mansoni* as well as for trichomoniasis, syphilis, and HIV infection. We observed a significant association between HIV and *S. haematobium* infection, with women with *S. haematobium* having a four-fold greater odds of being HIV-infected than women without *S. haematobium*. This finding independently confirmed results from a single other study done in Zimbabwe, which reported a similar odds ratio for HIV infection among women with *S. haematobium*. Taken together, these two studies argue strongly for the urgency of additional work to determine prospectively whether *S. haematobium* is a risk factor for HIV acquisition, as well as whether efforts to control *S. haematobium* infection will decrease the incidence of HIV in endemic communities.

Chapter 3 describes a similar community-based study of 345 women living in communities in which *S. mansoni* is endemic. Women received HIV voluntary counseling and testing and had schistosome Circulating Anodic Antigen (CAA) quantitated in their serum. We found that over 50% of women had schistosome infections. In this population, the odds of HIV infection among those with *S. mansoni* infection was 3.9. Moreover, the prevalence of HIV was highest (12.5%) among women with the highest quantities of CAA in serum, as compared to the HIV prevalence in women with light to moderate schistosome infection (8.3%) or the HIV prevalence among those with no detectable schistosome antigen (2.5%). These findings were novel, as previous studies had focused on women with *S. haematobium*, and led us to generate new hypotheses about the mechanisms of HIV susceptibility in women with schistosomiasis.

In **Chapter 4**, we explored the problem that women with urogenital schistosomiasis, caused by *S. haematobium*, had been reported to have persistent clinical disease even after treatment with the anti-schistosome medication praziquantel. We treated women with praziquantel and performed periodic follow-ups over the subsequent 6 months. This work expanded on prior studies by using polymerase-chain reaction (PCR) and serum schistosome antigen measurements to quantify schistosome infections with increased sensitivity. We documented that, even six months after praziquantel treatment, women with urogenital schistosomiasis had persistent gynecologic abnormalities and detectable schistosome DNA in urine and genital specimens. This highlights the urgency of preventing gynecologic damage and controlling schistosomiasis early, since its effects may not be entirely reversible.

Chapter 5 represents the laying of important groundwork that will facilitate new studies of schistosomiasis. We demonstrated that schistosome CAA can be eluted from, and quantitated in, dried blood spots, which are used widely in resource-poor settings for both research and clinical care. CAA values obtained from the dried blood spots were strongly correlated with serum CAA levels, and the correlations remained strong even among dried blood spots that had been stored for 8 years. We used the Whatman ProteinSaver 903 cards for this work, which are the most widely-used dried blood spot collection cards worldwide.

We then turned to interactions between HIV and schistosomiasis in **Chapter 6**. We enrolled 351 HIV-infected outpatients who had been taking antiretroviral therapy for at least six months. We documented that the ~30% of HIV-infected individuals with schistosome co-infection had a four-fold greater odds of immunological treatment failure than did HIV-infected individuals without schistosomiasis. Because many HIV-infected patients in sub-Saharan African are switched from first- to second-line antiretroviral treatment if they develop immunological failure, these findings are clinically relevant and highlight the critical need for additional studies in this area.

The final chapter of this thesis maintains the focus on improving HIV management in Tanzania, albeit via different research techniques. In **Chapter 7**, we present an implementation science study conducted to optimize the efficiency of dried blood spot testing for early infant diagnosis of HIV in a rural clinic in Tanzania. Using a series of low-cost, locally-driven interventions implemented sequentially, we were able to decrease the turn-around time between collection of the dried blood spot from the infant and the provision of the HIV test result to the patient's caretaker from 55 to 38 days, and to ensure that over 90% of infants' caretakers received results. This project is not only locally valuable, but is also more broadly applicable because it serves as a model for implementation of inexpensive, innovative interventions led by local healthcare staff.

The work presented in this thesis was conducted by a physician-scientist who both provides patient care and conducts research in Mwanza, Tanzania. Many of the findings have direct clinical implications for patient management and HIV prevention, as well as sparking essential follow-up studies to further improve health in Tanzania and beyond.

Samenvatting

Sinds de ontdekking van het humaan immunodeficiëntievirus hiv, ruim 35 jaar geleden, zijn meer dan 39 miljoen mensen overleden aan de gevolgen van een hiv-infectie. De hiv-epidemie heeft haar oorsprong in sub-Sahara Afrika, en nog steeds wordt deze regio het hardst getroffen door de gevolgen hiervan. Hoewel slechts 12% van de wereldbevolking zich in Afrika bevindt, woont daar tweederde van het aantal hiv-geïnfecteerde mensen. Daarbij is Afrika het werelddeel waar zich tweederde van alle nieuwe hiv- infecties ter wereld voordoen, evenals tweederde van het totaal aantal sterfgevallen ten gevolge van de ziekte aids.

Het is algemeen bekend dat mensen die lijden aan seksueel overdraagbare aandoeningen (soa's), zoals gonorroe, syfilis, en herpes simplex-virus, een groter risico lopen om met hiv geïnfecteerd te raken in vergelijking tot degenen die vrij zijn van soa's. Daarbij zijn de concentraties van het virus in het bloed veel hoger bij mensen die een hiv-infectie hebben in combinatie met een andere aandoening, zoals één van bovengenoemde soa's of tuberculose. Deze toename in concentratie van virusdeeltjes zorgt ervoor dat juist deze mensen met een dubbele infectie het virus gemakkelijker kunnen overdragen naar hun partner. Daarbij versnelt deze hogere concentratie de overgang van het hiv-dragerschap naar de symptomatische ziekte aids, en leidt het tot een snellere dood indien niet tijdig een behandeling wordt ingezet. Onderzoek van de laatste jaren laat steeds overtuigender zien dat sommige tropische parasitaire infecties ditzelfde effect kunnen bewerkstelligen. Met andere woorden: het lijkt er op dat co-infecties van hiv en parasieten een belangrijke rol spelen in de verspreiding van hiv en aids in sub-Sahara Afrika.

Schistosomiasis is een worminfectie waaraan naar schatting 260 miljoen mensen lijden, van wie 90% in sub-Sahara Afrika woont. In Tanzania, het land waar het onderzoek werd gedaan dat in dit proefschrift wordt beschreven, komen twee soorten *Schistosoma* endemisch voor: *Schistosoma haematobium* en *S. mansoni*. In sommige regio's blijkt meer dan 50% van de volwassenen een infectie te hebben met ten minste één van deze twee soorten *Schistosoma*. Op zich veroorzaakt schistosomiasis al substantiële ziekte en sterfte, met naar schatting 200.000 doden per jaar en een jaarlijks verlies van 3.31 miljoen DALYs (disability-adjusted life-years). De mogelijkheid dat schistosomiasis bovendien de overdracht van hiv en de progressie van aids bevordert, maakt de noodzaak van de juiste behandeling en bestrijding van deze verwaarloosde tropische worminfecties zelfs nog urgenter.

De focus van dit proefschrift ligt op het voorkomen van de overdracht van hiv en het beheersen van ziekte ten gevolge van deze infectie in sub-Sahara Afrika. Als eerste zal een beschrijving worden gegeven van het uitgevoerde epidemiologische bevolkingsonderzoek in Tanzania, waarbij het verband tussen infecties met hiv en infecties met *S. haematobium* en *S. mansoni* in kaart werd gebracht. De daarop volgende hoofdstukken richten zich op de behandeling van infecties met *S. haematobium* bij vrouwen, teneinde verdere genitale afwijkingen te voorkomen, alsmede de immunologische reacties op de behandeling bij hiv-positieve vrouwen gunstig te beïnvloeden. De afsluitende hoofdstukken zijn gewijd aan de mogelijke implementatie in Tanzania van de wetenschappelijke bevindingen met als doel hiv-infecties te voorkomen of in elk geval tijdig te diagnosticeren.

In **Hoofdstuk 1** worden achtergrond en context belicht van het onderzoek dat wordt weergegeven in dit proefschrift. Tevens wordt een overzicht gegeven van de bestaande publicaties en worden de doelstellingen van het onderzoek gepresenteerd.

In **Hoofdstuk 2** wordt een bevolkingsonderzoek besproken onder 457 vrouwen woonachtig in acht verschillende dorpen in het noordwesten van Tanzania. De vrouwen werden zowel onderzocht op infecties met *S. haematobium* en *S. mansoni* infecties, als op trichomoniasis, syfilis, en hiv. We constateerden een duidelijk verband tussen *S. haematobium* en hiv-infecties, waarbij vrouwen met een *S. haematobium*-infectie een viermaal hogere kans bleken te hebben om met hiv te zijn geïnfecteerd, in vergelijking tot vrouwen zonder schistosomiasis. Deze bevindingen bevestigen een eerdere studie in Zimbabwe waarbij vergelijkbare Odds Ratio's werden gevonden voor hiv-positieve vrouwen met een *S. haematobium*- infectie. Samen leveren deze twee onafhankelijke studies voldoende argumenten om prospectief vervolgonderzoek te initiëren naar de onderliggende mechanismen, zodat bepaald kan worden of een infectie met *S. haematobium* daadwerkelijk een risicofactor vormt voor het oplopen van een hiv-infectie, en tevens om aan te tonen dat bestrijding van *S. haematobium* zal leiden tot een afname van de incidentie van hiv-infecties binnen de gemeenschappen waar *S. haematobium* endemisch is.

Hoofdstuk 3 beschrijft een vergelijkbaar bevolkingsonderzoek onder 345 vrouwen in een gebied waar *S. mansoni* endemisch is. Vrouwen konden zich vrijwillig opgeven om zich op een hiv-infectie te laten testen, inclusief de bijbehorende begeleiding, en de status van hun *Schistosoma*-infectie te laten bepalen door middel van het testen op concentraties in het serum van het Circulerend Anodaal Antigen (CAA). Een *Schistosoma*-infectie werd zo bij meer dan 50% van de onderzochte vrouwen aangetoond, waarbij de kans op het hebben van een

hiv-infectie bij de *S. mansoni* positieve vrouwen 3.9 keer groter bleek. Daarbij was de prevalentie van hiv het hoogst (12.5%) bij vrouwen die ook de hoogste concentratie van CAA in het serum vertoonden, terwijl de prevalentie van hiv 8.3% was bij de vrouwen met een lage CAA- concentratie en 2.5% bij vrouwen bij wie dit *Schistosoma* antigeen niet kon worden aangetoond. Deze bevindingen waren nieuw aangezien voorafgaande studies zich voornamelijk waren gericht op infecties met *S. haematobium*. Dit gaf aanleiding tot nieuwe hypotheses over het mechanisme waardoor schistosomiasis kan leiden tot het toenemen van het risico op een infectie met hiv.

In **Hoofdstuk 4** onderzoeken we waarom vrouwen met urogenitale schistosomiasis, welke wordt veroorzaakt door *S. haematobium*-infectie, ziekteverschijnselen behouden zelfs na het toedienen van de juiste behandeling met praziquantel, het middel tegen schistosomiasis. Hiertoe behandelden we vrouwen met praziquantel en gedurende de daaropvolgende zes maanden herhaalden we regelmatig de bepalingen. Dit onderzoek ging verder dan eerder uitgevoerde studies door het toepassen van nieuwe diagnostische methoden om de *Schistosoma*-infectie op zeer gevoelige wijze kwantitatief aan te tonen, te weten de polymerase kettingreactie (PCR) en de bepaling in het serum van het *Schistosoma* antigeen CAA. Hiermee toonden we aan dat vrouwen met urogenitale schistosomiasis aanhoudend gynaecologische afwijkingen vertoonden, zelfs tot zes maanden na de behandeling met praziquantel, en dat er tevens nog Schistosoma DNA aantoonbaar bleek in de urine en de afgenomen genitaalmonsters. Deze bevindingen tonen het belang aan van vroegtijdige diagnostiek en behandeling van schistosomiasis, aangezien de gynaecologische schade in een later stadium onomkeerbaar kan zijn.

Hoofdstuk 5 legt de basis voor een nieuwe aanpak van onderzoek naar schistosomiasis. Hier laten we zien dat het *Schistosoma* antigeen CAA kan worden geëxtraheerd en gekwantificeerd vanuit kaartjes met ingedroogde bloeddruppels. Dit is een methode van verzamelen van bloedmonsters die vaak wordt toegepast in armere streken, zowel voor wetenschappelijke doeleinden als voor klinisch onderzoek. De hoeveelheid CAA, bepaald vanuit gedroogde bloedmonsters op de kaartjes, bleek significant te correleren met de hoeveelheid CAA die rechtstreeks was bepaald in het serum. Deze correlatie bleek ook niet te zijn beïnvloed als de kaartjes met de ingedroogde bloeddruppels tot acht jaar werden bewaard. Voor dit onderzoek werden *Whatman ProteinSaver 903* kaartjes gebruikt, het type dat wereldwijd het meest wordt gebruikt voor het verzamelen van bloeddruppels.

In **Hoofdstuk 6** borduren we voort op de interactie tussen hiv-infecties en schistosomiasis. Hierbij werden 351 hiv-positieve poliklinische patiënten gerekruteerd die de in de voorafgaande zes maanden antivirale behandeling

hadden ondergaan. We toonden aan dat de 30% van de hiv-geïnfecteerde patiënten die tevens een infectie met *Schistosoma* hadden, een vier keer zo grote kans hadden op immunologisch falen van de hiv-behandeling in vergelijking met diegenen die geen gelijktijdige *Schistosoma*-infectie vertoonden. Deze bevindingen zijn klinisch relevant omdat vele hiv-positieve patiënten in sub-Sahara Afrika van eerste-lijn behandeling overstappen naar een tweede-lijn behandeling zodra er sprake is van een immunologisch falen. Tevens benadrukt dit hoofdstuk het belang van verder onderzoek naar dit onderwerp.

Tenslotte worden in **Hoofdstuk 7** de resultaten gepresenteerd van een toegepast onderzoek naar het verder optimaliseren van de wijze van verzamelen en testen van de kaartjes met de gedroogde bloeddruppels bij pasgeborenen om daarmee het onderzoek naar de hiv-status in een rurale kliniek in Tanzania te verbeteren. Door middel van het toepassen van diverse opeenvolgende goedkope en lokaal aangestuurde interventies werd het mogelijk om de doorlooptijd tussen het verzamelen en het testen van de 'bloedkaartjes' terug te brengen van 55 dagen naar 38 dagen, waarbij er nadrukkelijk op werd gelet dat 90% van de verzorgers van deze kinderen ook daadwerkelijk de juiste testuitslagen ontvingen. Dit onderzoek was niet alleen van belang voor de lokale gemeenschap, maar is tevens breder toepasbaar omdat het tegemoet komt aan een algemeen model voor goedkope en vernieuwende interventies, voornamelijk aangestuurd door lokale gezondheidswerkers.

Het werk dat in dit proefschrift wordt gepresenteerd, werd uitgevoerd door een klinisch onderzoeker die zich in Mwanza, Tanzania, zowel bezighield met de patiëntenzorg als wetenschappelijk onderzoek verrichtte. Veel van de bevindingen zijn rechtstreeks klinisch toepasbaar, zowel in de patiëntenzorg als bij de preventie van hiv-infecties, maar de resultaten dienen eveneens ter stimulans voor vervolgstudies met als doel de gezondheidssituatie in Tanzania verder te verbeteren.

Acknowledgements

With deep thanks I acknowledge my collaborators, colleagues, role models, study team members, and partners. This thesis would not have been possible without the support, mentorship, and encouragement of so many people. You have been indispensable to my work!

First, I thank the study participants in Tanzania for your enthusiastic participation in our projects, your trust, and your honest answers to probing questions and examinations. I am inspired by you and grateful for your patience with me as I learn more of your culture and as we work together to learn better how to treat and prevent disease in Tanzania and sub-Saharan Africa.

I greatly appreciate my Tanzanian colleagues and study team members. I offer many thanks to the hardworking nurses (Jane Mlingi, Ndalloh Paul, Inobena Tosiri) and parasitologists (John Igogote, Donald Miyaye, Petro Mnyeshi, Ruth Magawa) who have spent long days for the past seven years working by my side in rural villages. I thank my physician colleagues at Bugando Medical Centre (Samuel Kalluvya, Adolfine Hokororo, Lucheri Efraim) and my visionary HIV clinic nurse colleague Sabina Manumbu for their hard work, bright ideas, and strong partnership. I also thank the diligent laboratory team at the National Institute for Medical Research (NIMR) (Julius Mngara, Donald Miyaye, Eric Lyimo, Crispin Mukerebe, Pius Ikigo, Peter Lutonja) for your reliable and careful work on multiple projects. I thank my scientific colleagues at NIMR (Godfrey Kaatano, Safari Kinung'hi, Jim Todd, Mark Urassa) for your wisdom and scientific advice. I am particularly grateful to the Director General of NIMR, John Changalucha, who has provided patient and wise mentorship and guidance since 2009.

I deeply thank my colleagues at Leiden University Medical Centre, who have been strong partners and friends since 2010. I think back fondly to the beginning of our partnership, when we met briefly at a meeting in Copenhagen. Shortly thereafter, you welcomed and taught me so much during a month in Leiden in 2011. Thank you to Maria Yazdanbakhsh for serving as an excellent and supportive Promotor and Lisette van Lieshout for serving as my dedicated Co-Promotor and close colleague. I am also grateful to other colleagues in the Departments of Parasitology (Govert van Dam, Eric Brienen, Jaco Verweij, Dieuwke Kornelis, Pytsje Hoekstra) and Molecular Cell Biology (Paul Corstjens, Claudia de Dood, Elisa Tjon Kon Fat) for all that you have taught me and for our ongoing collaborations. Special thanks to Pytsje Hoekstra and Claudia de Dood for also serving as my paranymphs. It is a pleasure to have you all as friends and colleagues.

I am immensely grateful to my colleagues and mentors at Weill Cornell Medical College. I would not be doing the work I am doing today without the strong support and mentorship I have received from Warren Johnson and Daniel Fitzgerald in the Center for Global Health. I am also grateful to Mary Charlson, Augustine Choi, and Trip Gulick for their support. I thank my fellow junior faculty colleagues and close friends Kate Dupnik, Luke Smart, and Rob Peck for your ongoing scientific partnership in many projects.

Finally, I deeply thank my family. Thanks to my parents and wonderful sister for always encouraging me, and for visiting us in Mwanza! Thanks to my in-laws for your love and acceptance of your daughter-in-law, even when that means your son and grandchildren are far away. To my sweethearts Emily Christine (age 5), Luke William (age 3), and Elijah Clement (age 1)—you have lived half of your lives in Tanzania and patiently shared your mom with so many colleagues and patients in Tanzania. I adore each of you. To my darling husband—you have brought endless joy and love into my life for more than 20 years now, and have shaped me into the person I am today. Thank you.

Curriculum Vitae

Jennifer Alzos Downs grew up in Wilmington, Delaware, United States of America and obtained her undergraduate degree from the University of North Carolina at Chapel Hill (Bachelor's of Science in Biochemistry). From North Carolina, she moved to New York City where she obtained her Doctor of Medicine at Weill Cornell Medical College in 2004. During an elective rotation in rural Central America during medical school, she decided that she would pursue a career in global health.

Dr. Downs completed her internship and residency in Internal Medicine at Columbia University College of Physicians and Surgeons in 2007 and her Infectious Diseases fellowship at Weill Cornell Medical College in 2010. She made her first trip to Tanzania in 2007 when she worked as a resident and teacher on the wards at Bugando Medical Centre in Mwanza for six weeks. Following the completion of her clinical infectious diseases training, she returned to Mwanza where she established a home and gained fluency in the local language (Kiswahili).

She is currently Assistant Professor of Medicine and Assistant Professor of Microbiology and Immunology in the Center for Global Health at Weill Cornell Medical College in New York. She is also Lecturer in the Department of Medicine at Weill-Bugando School of Medicine in Mwanza. She devotes her time to research, teaching, and clinical care in Mwanza. She additionally serves as a teacher, mentor, and dissertation supervisor for Master's of Medicine post-doctoral Tanzanian students in Mwanza. She has collaborated with colleagues at Leiden University Medical Center since 2010 and began her PhD at LUMC in 2014, and looks forward to continuing her work on schistosomiasis and HIV in Tanzania for many years to come.

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