Human Immunodeficiency Virus and Soil Transmitted Helminths: Measuring the Systemic Effects of Co-Infection in a Low-Resource Context

A Dissertation
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

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April, 2017

Acknowledgements

First and foremost, I am indebted to the patients who participated in this research. Without their willingness to contribute their time and of themselves, none of this research would be possible. I also thank the staff at The AIDS Support Organisation in Mbale, Uganda, in particular, my co-investigator, Dr. Miya Yunus, and Andrew Mijumbi Ojok. I also thank the staff of the Infectious Diseases Institute's Translational Laboratory, and particularly Grace Turyasingura.

I thank my primary adviser, Dr. David Boulware, for his mentorship, and commitment to independent student research. I am inspired by Dr. Boulware's incredible work ethic, appreciative of his availability and flexibility, and grateful for the multiple opportunities to learn and work that he has provided. To facilitate research, there is no task too trivial for him; and he heads a team where this spirit of teamwork is evident. I am grateful to my senior co-adviser, Dr. Claudia Muñoz-Zani, for being a sounding board through this process, providing me opportunities to teach – and through teaching learn – and for exposing me to the fascinating world of leptospirosis. The breadth and depth of her knowledge base is evident in her work, and advising.

I also thank my committee members, Dr. Richard MacLehose and Dr. Haitao Chu, for freely sharing their time, expertise, and advice. Dr. Chu never fails to provide insightful, practical, and humbling statistical feedback. I am particularly indebted to Dr. MacLehose

for going above and beyond in his advising, and asking the questions that no one else asks. His eye for quality and purpose in research is an inspiration. The dissertation would not have been possible without my committee's commitment to student training and success, and their flexibility with my process.

I am grateful to Dr. Katherine Huppler Hullsiek, who always remains focused on the process and the details, and speaks her mind. I thank her for tirelessly lending her expertise and time, as a friend, colleague, and mentor. I thank my collaborators, Dr. Rojelio Mejia (Baylor College of Medicine), and Dr. Stefanie Sowinski (University of California, San Francisco) for their technical support, input, and lab time on Aims I and III. I thank Dr. Ran Blekhman for his technical support, guidance and expertise on Aim II, as well as material support for sample sequencing. I also thank Dr. David Meya (Infectious Diseases Institute, Kampala, Uganda; Makerere University) for his mentorship during my Fogarty Scholarship, and allowing me opportunities to learn about *Cryptococcus* in Uganda. I am inspired by his devotion to improving the lives of patients, through advocacy, charity, research, and mentorship. I am also indebted to Dr. Myron Gross, who was the first person to teach me to write a paper.

The relationships that I have developed with my peers in the division have provided critical and wide-ranging support throughout this process. Their success during and after the doctoral program is a source of inspiration. I am particularly indebted to Dr. Hilary Whitham, who has fed, housed, and encouraged me, and proofread more documents than

either she or I care to remember. This process would not have been the same without the support and sacrifices from my partner, Brendan Crosby. He has endured countless weekends, evenings and vacations with me and my computer, while still being my biggest fan. I love you. And to my family, in particular Robyn, Antonio, David, Françoise and Lary, friends, colleagues, and larger community have carried and supported me in a million ways large and small, thank you. I love you.

This research was supported by two J. B. Hawley Student Research Awards from the University of Minnesota, School of Public Health, Division of Epidemiology and Community Health, the University of Minnesota Doctoral Dissertation Fellowship, the Lois and Richard King Distinguished Assistant Professorship, and by the U.S. National Institutes of Health (NIH) Research Training Grant #R25 TW009345 funded by the Fogarty International Center; the NIH Office of the Director of AIDS Research; the NIH Office of the Director of Research on Women's Health; The National Heart, Lung and Blood Institute; The National Institute of Mental Health; and the National Institute of General Medical Sciences; and awarded to the Northern Pacific Global Health Fellows Program by the Fogarty International Center.

Dedication

To Robyn and Antonio.

And, also, to Elif Yavuz, PhD.

Abstract

The impacts of helminth infection and anthelmintic therapy among HIV-infected people in co-endemic areas remains unknown. Health effects are likely species-dependent, and each species may exert countervailing effects on its host. Furthermore, there is a dearth of high-quality research conducted in the era of widely available ART. Data from two studies conducted in Mbale, Uganda were used to: 1) estimate clinical correlates of helminth infection among HIV-infected Ugandans; 2) characterize fecal microbiome composition in these participants, and correlate clinical characteristics with microbiome composition; and 3) evaluate the impact of anthelmintic therapy on markers of systemic inflammation in HIV-infected Ugandans via a randomized control trial. **Aim I** uses molecular methods to describe the prevalence and burden of 5 soil-transmitted helminth species among patients in outpatient HIV care, and quantifies the relationship between baseline helminth infection and immune status. We observed a clinically significant inverse relationship between hookworm infection and CD4+ T cells/mcL. **Aim II** analyses the fecal microbiome of HIV-infected Ugandans to identify differences in community structure across clinical characteristics, and determine if gut community structure and/or taxa are associated with change in immune status over time. Our results indicate lower bacterial community richness among participants with <100 CD4+ T cells/mcL, and identify two taxa that may be linked to CD4+ T cell recovery. **Aim III** quantifies changes in soluble CD14, C-reactive protein, and 10 pro-inflammatory cytokines in ART-initiated Ugandans randomized to either immediate or delayed albendazole therapy. Our findings indicate low helminth infection prevalence (10%) and

an increase in soluble CD14 after 1-month of follow-up among participants receiving immediate albendazole. These aims contribute to knowledge of clinical and sub-clinical correlates of helminth infection in the ART era. Results may support integration of anthelmintic therapy into adult HIV care, which is often overlooked when setting anthelmintic program priorities.

Table of Contents

List of Tables

List of Figures

1. Summary

People diagnosed with HIV are living longer lives with higher quality of life in many parts of the world, including Sub-Saharan Africa, thanks to widely available antiretroviral therapy (ART). However, as HIV-infected people live longer, they are at increasing risk for inflammation associated conditions, such as hypertension. This is due, in large part, to the state of chronic immune activation and inflammation that they experience as a function of the virus. This systemic inflammation is driven primarily from the gut, a critical area for immune function.

Helminth infections, most of which preferentially mature in the gut, are endemic in the same areas of the world where HIV incidence is high. Armed with this knowledge, and the knowledge that helminths may induce similar and countervailing immune responses in the host, we sought to elucidate relationships between gut composition or conditions, e.g. hookworm infection or fecal microbiome composition, that could impact clinical or sub-clinical markers of health in people living with HIV in low-resource areas.

This research addresses gaps in the literature with respect to our understanding of microbial translocation in the HIV- or helminth-infected person, microbiome gut composition in HIV-infected Ugandans, and the relationship between helminth infection and clinical markers of disease status in the era of widely available antiretroviral therapy (ART).

While much of this dissertation is exploratory, it does contribute to our understanding of the above-mentioned areas, and in some cases, could have direct contributions to public health programming in low-resource areas.

1.2 Specific Aims

The following research questions and corresponding aims were addressed through analyses of data collected from two cohorts, *Prevalence and Burden of Soil-transmitted Helminths among People Living with HIV in Mbale, Uganda* and *Impact of Anthelmintic Therapy on Systemic Inflammation among People Living with HIV and Invasive Helminth Infection in Uganda*, which were conducted by Bozena M. Morawski in Mbale, Uganda.

Manuscript I: What is the prevalence and burden of soil-transmitted helminths in people living with HIV in peri-urban and rural Uganda and seeking HIV care? What are clinical correlates of helminth infection in ART-initiated people?

Aim: Describe the prevalence and burden of 5 soil transmitted helminth species in patients at an HIV outpatient clinic in peri-urban Uganda. Describe the relationship between helminth infection and clinical and demographic characteristics, including the relationship between helminth infection at enrollment and immune status over time.

Manuscript II: What is the composition of the fecal microbiome in people living with HIV in Uganda? How are microbiome community structure, and differences in the relative distributions of particular taxa correlated with clinical characteristics at enrollment and over time (i.e. change in CD4+ T cell/concentrations)?

Aim: Using microbiome analysis techniques, describe the composition of the fecal microbiome of HIV-infected persons in peri-urban Uganda. Identify differences in community diversity and structure across various clinical characteristics. Determine if community structure and/or particular taxa are associated with change in immune status over time, among ART-initiated persons.

Manuscript III: How does anthelmintic therapy impact systemic inflammation markers among people who are co-infected with HIV and invasive soil-transmitted helminths?

Aim: Identify changes in soluble CD14, C-reactive protein, and 10 pro-inflammatory cytokines from baseline to 1-month of follow up via a randomized control trial among HIV-infected ART-initiated persons who are also infected with soil-

transmitted helminths.

Together, these aims offer an opportunity to investigate clinical and sub-clinical correlates of helminth infection and the impact of anthelmintic therapy among people living with HIV in the era of highly active antiretroviral therapy. Results from these studies are intended to inform programs that address the subtle but chronic effects of systemic inflammation in people living with HIV. In particular, the integration of presumptive anti-parasitic therapy into standard HIV care among adults, who are often overlooked when setting anthelmintic program priorities.

2. Introduction

In 1995, Bentwich and colleagues postulated that parasitic infection, and helminth infection specifically, was a major contributor to the scale and virulence of the African HIV epidemic, when compared to European and North American HIV epidemics.[1] They argued that helminth infections polarized the immune system towards a dominant Type 2 T helper cell response, which simultaneously induced immune activation and rendered the host less fit to respond to intracellular infections, e.g. HIV. In 2006, Bentwich and colleagues revisited their hypotheses,[2] to conclude that host immune activation remained a critical determinant of HIV pathogenesis, and that chronic host immune activation is an important aspect of helminth infections. Indeed, while evidence has shown that the natural progression of HIV is similar across geographic contexts,[3] chronic immune activation plays a central role in HIV pathogenesis.[4-7] There is also evidence linking helminth infection to chronic immune activation.[8-10] The authors conceded, however, that the decade of research attempting to elucidate the relationship between helminth infection and HIV disease progression has been inconclusive. From 1995 to 2006, one study of intestinal nematode infection found that HIV-1 RNA concentrations decreased after anthelmintic therapy,[11] while others have found no relationship.[12,13]

The call by Borkow and Bentwich[2] for larger and more definitive field studies has been answered by numerous studies between 2006 and today. As was true between 1995 and 2006, the research conducted between 2006 and today continues to paint an inconclusive picture, especially as high quality research with long-term follow-up is lacking. As recently as April 2016, the Cochran Review group concluded that presumptive anthelmintic therapy for HIV-infected adults may have small and short-term

benefits to HIV disease progression markers, viral load and CD4+ T helper cell concentrations.[14] However, the authors note that there is a low quality evidence for these recommendations, and that further studies from other populations, and with longer follow-up are required.[14] The work in this dissertation is informed by the backdrop of continued debate surrounding these helminth-HIV questions.

2.1 Research Context: Uganda

This research focuses on people living with human immunodeficiency virus (HIV) in rural and peri-urban areas of Uganda, an East African country of approximately 39 million people (2015 estimate).[15] A large proportion of Uganda's population is under the age of 15 (48.1%), while the 15-54 years old population is approximately 49.4%.[15] Life expectancy at birth (2014 est.) is 58.5 years, as compared to 74.4 years in upper middle income countries and 58.6 years in other Sub-Saharan African nations.[15] A 2013 World Bank estimate placed 19.5% of the population below the national poverty line, with a stark disparity between urban and rural populations (9.9% and 22.4%, respectively).[15,16] These numbers, while not without caveat, represent a >50% reduction in poverty since 1993. Uganda has also seen significant improvements in World Development Indicators (e.g. school enrollment, clean water source provision, life expectancy at birth) over the past decade.[16] The country compares favorably to other Sub-Saharan African countries on many health and economic indicators.[15] Overall, however, the Uganda remains economically disadvantaged in absolute terms, with apparent consequences on human development, health infrastructure, and preventive and therapeutic health care.

2.1.1 HIV Epidemiology in Uganda

With an estimated 1.5 million people living with HIV (2014 est.), Uganda is home to 4% of the global total of people living with HIV/AIDS.[17,18] In the past decade, the roll-out of large scale treatment and counseling programs has led to large relative improvements have been made in treatment and management of HIV infection. In 2014, the Ministry of Health estimated that 750,896 ART-eligible people were receiving ART, a 30% increase since the prior year alone.[17,18] The major increases in ART availability are commendable, but large numbers of ART-eligible people not receiving therapy, and new international guidelines calling for immediate therapy for all HIV-infected persons[19] may foreshadow continued national challenges for HIV care service delivery.

Beyond difficulties faced at the healthcare infrastructure level, there are numerous social and economic factors along the cascade of care that contribute to a consistently low proportion of eligible patients receiving complete and regular HIV care.[18,20] Consequently, management of HIV-associated opportunistic infections places a significant burden on the Ugandan health care system, with subsequent implications for patient morbidity, mortality, and disengagement from the care.[21,22] A 2013 study by Namutebi *et al.* found that 18% of hospital admissions among patients living with HIV were attributable to tuberculosis, 11% were related to cryptococcal meningitis, and 5% to Kaposi's sarcoma.[23] Another survey of outpatient ART patients in Kampala, Uganda, found that 11.9% had been diagnosed with tuberculosis, and 2.9% with Kaposi's sarcoma.[24] The prevalence of serum cryptococcal antigen rages from 5.7 to 9.4%

nationally (unpublished data, ClinicalTrials.gov NCT01535469);[25,26] and progression to cryptococcal meningitis carries mortality risk of up to 50%.[27,28]

2.1.2 Soil Transmitted Helminth Epidemiology

Over a billion people worldwide are infected with the five species of intestinal nematode parasites which comprise soil-transmitted helminth (STH) infections: *Ascaris lumbricoides*, *Trichuris trichiura*, hookworm species *Necator americanus* and *Ancylostoma duodenale*, and *Strongyloides stercoralis*. Estimates indicate that between 100 and 200 million people are infected with at least one of the STH species (excluding *S. stercoralis*); and a Sub-Saharan African regional loss of 1 to 18.3 million DALYs are attributed to these four parasitic infections.[29-31] These infections are largely concentrated in low-resource areas, where there is limited access to barriers against helminth infection, e.g. lack of access to clean water, lack of barriers to contact with soil.[32,33] In the Great Lakes region of Sub-Saharan Africa (Uganda, Kenya, Burundi, and neighbors), hookworm prevalence has been estimated at 50%, and *A. lumbricoides* and *T. trichiura* estimated at 7% each.[34] From 10 to >50% of the population was infected with ≥1 STH in the region.

In Uganda, recent studies have shown over 40% prevalence of *Ascaris* in adults,[35,36] although these findings are influenced by geography and occupation.[37,38] Several studies in Uganda have indicated hookworm prevalence from 26% to 45% in adults.[30,39-43] Trichuriasis has been reported in <2% to 26% of Ugandan adults, with the higher prevalence range being associated with farming occupation.[36,38] *Strongyloides stercoralis* is through to be relatively rare, due in large part to difficulties in diagnosis,

and is not included in most large-scale helminth mapping projects. However, prevalence of *S. stercoralis* has been estimated at 5% in Uganda.[44]

2.2 HIV Infection, Chronic Immune Activation, and the Gastrointestinal Immune System

HIV infection is characterized by continuous depletion of CD4+ T lymphocytes. A small portion of this CD4+ T cell depletion is caused by caspase-3-mediated apoptosis, or highly contained programmed cell death.[6] However, >95% of CD4+ T cell death is induced by caspase-1-mediated pyroptosis, triggered by non-productive HIV infection.[6] In contrast to apoptosis, pyroptosis is an intensely inflammatory cycle, wherein cells recognize that they are infected with foreign pathogenic material, and produce pro-inflammatory cytokines that lead to their destruction. At destruction, cell cytoplasm, other contents, and pro-inflammatory cytokines are released into circulation, drawing additional CD4+ T cells to the area to be infected by HIV and self-destruct, propagating a chronic pro-inflammatory state. Pyroptosis drives the state of chronic immune activation that characterizes HIV infection and disease progression.

The gut is a critical area for immune function, where a significant portion of CD4+ T lymphocytes are found, and large amount of pyroptosis occurs. These CD4+ T cell populations are destroyed early in infection and at an increased rate relative to CD4+ T cells found in peripheral blood.[7] Structural damage to the gut is another hallmark of HIV infection and progression. This structural damage in turn releases microbes into the lamina propria, the supporting loose connective tissue underneath the epithelium, which induces chronic inflammatory immune responses in the area. HIV shows a preference for infecting activated T cells, which are found in areas with existing infections and

subsequent inflammation. This local inflammation of gut tissues creates the perfect conditions for and destruction of large CD4+ cell populations and disease progression.

2.3 Soil-transmitted Helminth Infection

The gastrointestinal tract is also the preferred infection site for the five species of human soil transmitted helminths examined in this dissertation. Frequency and intensity of infection are age- and parasite species-dependent. Most high burden infections with *A. lumbricoides* and *T. trichiura* species are found in children.[45] Infections with as *A. lumbricoides* and *T. trichiura* are less prevalent in adults, given that immunity to these parasites is acquired over time, which facilitates worm destruction and expulsion, and changes in hygiene practices with increasing age reduce helminth exposure in adults. Hookworm species, however, continue to infect adults with high frequency and intensity due to an apparent lack of adaptive immunity. This lack of adaptive immunity may have important implications for host response to co-infections like HIV, and important HIV co-morbidities. The five soil transmitted helminths species have diverse life cycles, and systemic immunologic and structural interactions with the human host (**Appendix 1**).

Adult *A. lumbricoides* live in the small intestine, but their lifecycle includes obligate stages across body systems. Immature larvae cross the intestinal mucosa, and are carried to the lungs via the circulatory system. Once in the lungs, they further develop to be able to ascend to the throat, where they are swallowed and carried back to the small intestine. The average lifespan of an adult Ascaris worm is 1 to 2 years.[46]

T. trichiura eggs are ingested and hatch in the small intestine, to reside in the cecum and ascending colon upon maturation. Unlike other species, its lifecycle in

humans occurs entirely in the gut. Trichuriasis is marked by disruption of the gut mucosa in later stages of its lifecycle. As adults, these worms thread the anterior portion of their bodies into the host's intestinal mucosa, and remain stationary in that location for the duration of their life span (approximately 1 year).[47]

Both hookworm species follow similar lifecycle patterns, although the lifecycle of *N. americanus* includes an obligate stage in the lungs that is optional for the *A. duodenale* species. Third stage infective hookworm larvae, mainly in soil, penetrate the human skin and are carried, like *A. lumbricoides*, through the circulatory system to the lungs. From there, they too ascend to the throat so that they may be swallowed by the host, pass through the digestive system, to settle in the small intestine as adults. Like *T. trichiura*, hookworm species also disrupt to the gut mucosa. Hookworm, which is a well-established cause of iron-deficiency anemia in humans,[48] attaches itself to the wall of the small intestine, where they are able to cause significant host blood loss over the course of their lifetime (1 to 2 years on average, but as long as several years).[49] Hookworm infection is of particular concern in women of child-bearing age, due to increased anemia risk.[50]

S. stercoralis is the only of these five species with the capacity to be auto-infective within the human host. Strongyloides larva has two cycles, parasitic and free-living. During the free-living cycle, infective stage larvae penetrate the skin of the host and follow *Ascaris* and hookworm species in being transported to the lungs through the circulatory system, and eventually deposited to the small intestine. Female worms anchor themselves to the small intestine mucosa to for the duration of their lifespan. It is here that they generate eggs via asexual reproduction. It is during the parasitic cycle that

Strongyloides perpetuates itself in humans. The eggs develop into larvae that may re-infect the host internally (gut penetration) or externally (penetration of the perianal skin). This phase constitutes the parasitic cycle. The larva may also be passed into soil as part of the free-living cycle.[51]

These organisms induce potent immune responses in the gut, soft tissue, and circulatory system as these species develop in the human host.[52] Adults, the target population of this research, are exposed to and infected with these pathogens repeatedly, as they negotiate shared ecosystems with limited access to barriers against parasitic infection. Indeed, re-infection at 3-months post-therapy was estimated at 26% (95%CI: 16-43%) of pre-treatment prevalence for *A. lumbricoides,* 36% (95% CI: 28-47%) for *T. trichiura* , and 30% (95% CI: 26–34%) for hookworm species, and individuals with previous infection were more likely to be infected post-therapy.[53] These estimates were generated from post-mass-drug administration environments, and are likely to be higher in areas where only clinically-indicated therapy occurs.

2.3.1 Soil-transmitted Helminth Infection Immunology

As effectively reviewed by Mishra *et al*., helminth infections typically induce an anti-inflammatory, T helper type 2 (Th2) cytokine response, and a regulatory response.[52] These two responses are distinct, but complementary, and they both contribute to the overall Th2 response associated with helminth infection.[52] These responses aid the host in expelling worms more quickly and tolerating worm infestation, i.e. contribute to wound healing and dampening inflammation.[54-57] Infections with helminth worms are characterized by an increase in immune cells induced by Th2 cytokines, specifically

interleukin (IL-)4, IL-5, and IL-13.[52] These cytokines are produced by basophils, CD4+ Th2 cells, eosinophils, and innate lymphoid cells,[58-63] and are associated with an increase in non-opsonizing antibodies, i.e. antibodies that do not mark foreign extra-cellular material for destruction. Helminth infection also induces the cytokine transforming growth factor beta (TGF-β), which controls cellular proliferation and differentiation, and is important to inflammatory immune responses.[52,64]

2.3.2 The Cytokine Response to Helminth Infection

Helminth infection induces rapid changes in serum cytokines. Increases in Th2 response cytokines may be detected only hours after infection. When helminths anchor to the mucosal lining of the gut, they disrupt the intestinal epithelial cells that form the barrier between the gut lumen and intestinal tissue, including the lamina propria. The intestinal epithelial barrier is thought to be an important cytokine production site;[29] and the lamina propria maintains a diverse and dense population of immune cells. After contact with helminth organisms, IL-5, IL-13, IL-25, IL-33 and thymic stromal lymphopoietin are rapidly produced by innate lymphoid 2 cells (ILC-2). Necrotic cells release IL-33 during tissue damage, which provides some insight into the link between helminth infection and tissue damage.

It is from this initial exposure site that systemic Th2 responses are initiated. Once dendritic cells and other components of the innate immune response are activated, they move out of the lamina propria through lymphatic vessels to the mesenteric lymph nodes, which are located between the intestine and abdominal wall and a potentially important source of IgE and IgG1 in serum. These activated dendritic cells induce an adaptive

immune response that includes Th2 cell differentiation and B cell activation, which in turn induces IgE and IgG1 secretion. These products spread these cells throughout the body via the lymphatic vessels. It is through these processes that helminth infections of the gut cause systemic Th2 type responses, including Th2-type immune responses in cerebrospinal fluid.[65,66]

At the same time as the cytokine response, selected helminth excretory/secretory (E/S) products may simultaneously inhibit antigen presenting T cells from producing molecules that are required for Th1 cell differentiation and response. Helminth infections are classically associated with the down-regulation of the inflammatory Th1 and Th17 responses, which are active in responding to intracellular infections such as *Cryptococcus* and *Mycobacterium tuberculosis*.[67] Th1 type responses also stimulate the production of antibodies that mark pathogens for destruction, and increase Type IV (delayed type) hypersensitivity. Recent work in murine models has demonstrated that helminth infection skews the immune system composition towards Th2 regulatory responses, even after parasites have left the site of infection.[68-72] Consequently, hosts mounted weaker Th1 type responses to viral and other intracellular infections.[73] Research has also demonstrated that existing but dormant infections, e.g. latent herpes-type viruses, were re-activated upon helminth infection.[69]

2.4 Soil-transmitted helminths and HIV co-infection

Soil transmitted helminths and their effects on host response to HIV disease progression and opportunistic infections are the crux of the proposed research. Both HIV infection and helminth species are disproportionately found in Sub-Saharan Africa. Estimates of parasitic co-infection among adults living with HIV from 10 to >50%.[12,74-78]

Higher estimates are applicable to areas with reduced access to clean water and sanitation infrastructure, which is common in both urban and rural areas of Sub-Saharan Africa. Recent unpublished data from a characteristic outpatient HIV clinic in semi-rural Uganda indicated that 29.8% (95%CI: 23.7 to 36.8) of adults were infected by at least one helminth species, predominantly comprised of *N. americanus*.[42]

Bentwich *et al*. published findings that demonstrated 1) a decrease in CD4+ count and an increase in CD8+ T helper cell concentrations in chronically helminth infected persons, and 2) increased density of HIV co-receptors CCR5 and CXCR4, which play important roles in determining cell susceptibility to HIV and HIV tropism.[8,79] More recently, systemic immune activation was associated with helminth infection and HIV co-receptor expression in species-specific analyses, albeit in HIV negative patients.[9] Ascaris and *T. trichiura* infection were both linked to higher concentrations of "activated" CD4+ or CD8+ T helper cells, whereas other species, e.g. hookworm, were not associated with differences in CD4+ or CD8+ T helper cells.

Conversely, multiple papers have attempted to expand on Bentwich's theory and draw a link between clinical outcomes, e.g. CD4+ T cell densities, and helminth infection. These studies have been of mixed results, and been of varying inferential quality.[14] The effects of helminth co-infection on CD4+ T cell concentrations have overall shown relatively lower concentrations in patients with helminth infections in a mixture of observational and randomized trials.[12,13,80-83] However, these same studies did not find differences in HIV viral load between helminth infected and uninfected patients. Other research has shown that helminth infection may be beneficial in people living with

HIV due to the dampening effect that helminths have on immune system activation and inflammatory response, increasing host longevity.[12,84]

2.5 Study population

Two cohorts contributed data to this dissertation. Both cohorts were recruited at The AIDS Support Organisation (TASO) in Mable, Uganda. The first is a longitudinal cohort of HIV-infected Ugandans, seeking HIV care at TASO Mbale, which was enrolled in 2013. This cohort of adults with mixed ART experience was enrolled into the study and contributed a single stool sample and blood draw for CD4+ T cell assessment. They were then passively followed for 24 months via medical chart review. Aims I and II use data obtained from this cohort. The second cohort consists of participants in a randomized control trial of delayed versus immediate albendazole therapy. All patients in this cohort are ART-experienced, and were enrolled from June through September 2015 and followed for 3 months. Aim III draws on data collected from these participants.

3. Aim I: Evaluating the Clinical Correlates of Parasitic Infection in HIV-infected adult Ugandans.

3.1 Aim Summary

Most studies evaluating epidemiologic relationships between helminths and HIV have been conducted in the pre-ART era, and evidence of the impact of helminth infections on HIV disease progression remains conflicting. Less is known about helminth infection and clinical outcomes in HIV-infected adults receiving antiretroviral therapy (ART). We sampled HIV-infected adults for eight gastrointestinal parasites and correlated parasitic infection with demographic predictors, and clinical and immunologic outcomes. Contrasting with previous studies, we measured parasitic infection with a quantitative, highly sensitive and specific polymerase chain reaction (PCR) method.

This cohort study enrolled HIV-infected Ugandans from August-September 2013 in Mbale, Uganda and collected stool and blood samples at enrollment. Real-time PCR quantified stool: *Ascaris lumbricoides, Ancylostoma duodenale, Necator americanus, Strongyloides stercoralis, Trichuris trichiura*, *Cryptosporidium spp.*, *Entamoeba histolytica*, and *Giardia intestinalis* infection. Generalized linear models assessed relationships between parasitic infection and clinical or demographic data.

35% of participants (71/202) tested positive for ≥1 helminth, mainly *N. americanus* (55/199, 28%), and 4.5% (9/202) were infected with ≥2 stool parasites. Participants with hookworm infection had lower average $CD4^+$ cell counts (-94 cells/mcL, 95%CI: -141, -48 cells/mcL; $p<0.001$) after adjustment for sex, $CD4^+$ nadir at clinic entry, and time on ART.

The high prevalence of parasitic infection and correlation with decreased $CD4^+$ concentrations highlight the need to re-examine the effects of invasive helminth co-infection in rural, HIV-infected populations in the era of widely available ART. Elucidating the relationship between hookworm infection and immune recovery could provide opportunities for health optimization, e.g. integrated deworming, in these vulnerable populations.

3.2 Introduction

Five soil transmitted helminth species *Ascaris lumbricoides*, *Trichuris trichiura*, hookworm species *Necator americanus* and *Ancylostoma duodenale*, and *Strongyloides stercoralis* infect over a billion people worldwide.[30,32] The burden of parasitic infection is greatest in low-income areas, particularly in certain areas of sub-Saharan Africa, where human immunodeficiency virus (HIV) is also highly prevalent. Studies of African adults living with HIV have shown helminth co-infection rates that range from 10% to upwards of 45%.[12,13,45,84-87]

To date, the majority of research investigating the impact of intestinal helminth infection on HIV disease progression has occurred prior to widely available antiretroviral therapy (ART).[12,80,81,84,87-89] The effect of helminth and HIV co-infection in the presence of ART is less well characterized. Indeed, to our knowledge, only two studies to date have examined the impact of deworming on $CD4^+$ recovery in persons receiving ART.[90,91] The current literature examining the relationship between soil transmitted helminth infections and HIV in the pre-ART era presents an inconclusive picture. The large body of observational data is mixed. Two observational cohort studies found no

beneficial effect of deworming on HIV viral loads and $CD4^+$ T-cell concentrations,[12,13] while another suggested the possibility of a protective effect of helminths on decreasing HIV viral replication.[84] Of the three randomized experiments evaluating the impact of deworming on markers of HIV disease progression without ART, two found an improvement in either $CD4^+$ T-cell concentrations or HIV viral load after anthelmintic therapy.[81,87] Another larger, reflexive randomized deworming trial failed to show a statistically significant benefit of empiric deworming treatment versus standard of care in preventing HIV progression to either a $CD4^+$ count of <350 cells/mcL, first reported use of antiretroviral treatment, or death due to a non-traumatic cause (44.0 versus 49.8 events per 100 person-years; hazard ratio=0.88, 95%CI 0.74 to 1.04, P=0.10).[80] However, it is possible that there was a less extreme benefit to presumptive therapy, which they were underpowered to detect.

Interactions between soil-transmitted helminths and their human hosts are complex, and helminth infection may influence a patient's relationship with other pathogens. A recent review discusses not only the links between selected parasites and HIV susceptibility and disease progression, but also the relationship between soil transmitted helminths and the potential for increased susceptibility to malaria and tuberculosis.[67,92,93] It is also important to recognize that soil transmitted helminths, through their potent and systemic T helper cell type 2 (Th2) cytokine and regulatory responses,[52] may induce Th2 protective effects that could benefit long-term HIV survivors, e.g. protection against conditions associated with chronic inflammation.[94-97] However, this same Th2 immune response may mediate increased susceptibility for Th1-related infections.[98]

At present, integrated presumptive anthelmintic therapy in the context of HIV care is neither recommended by Ugandan National Guidelines,[99] nor is it recommended by WHO.[100] While WHO does recommend periodic so called "preventive chemotherapy" for high risk groups, including women of child bearing age and adults with occupational exposures,[101] these guidelines have generally not been integrated into any type of standard care, nor has particular emphasis been given to HIV-infection. Given the frequency and consistency with which HIV-diagnosed persons interact with their care providers, the integration of adult deworming programs into HIV care may be a logical conclusion.[102] However, given the dearth of high quality and adequately powered species-specific studies, dramatic increases in ART availability, and incomplete understanding of biological mechanisms that are impacted by helminths during HIV infection implies that research questions focused on soil transmitted helminths and HIV have not been exhausted.

Our current study evaluated the prevalence and burden of the five most common soil-transmitted helminths and three protozoal species in adults living with HIV enrolled in outpatient HIV care in peri-urban Uganda. We also evaluated the relationship between helminth infection and clinical and immunologic outcomes, and examined risk factors for helminth infection in this population.

3.3 Methods

3.3.1 Ethics Statement

Written informed consent was provided by all participants. The University of Minnesota, The AIDS Support Organisation (TASO), and the Uganda National Council of Science and Technology institutional review boards approved this protocol.

3.3.2 Participant Recruitment and Data Collection

From August through September 2013, we screened HIV-infected adults engaged in outpatient care at the TASO HIV clinic in Mbale, Uganda, during their normal clinical visit for a one-time stool sample analysis, and longitudinal follow-up via chart review. This study was powered to estimate overall parasitic infection prevalence among patients with a recent CD4+ T cell count <500 cells/mcL, a population of approximately 600. We estimated a sample size of 210, based on a true population prevalence of 30%, an alpha level of 0.05, 5% precision estimate, and a finite population size of 600.

Inclusion criteria were age ≥18 and most recent $CD4^+$ count <500 cells/mcL. We excluded persons who reported or had a record of taking albendazole or other anthelmintics in the past three months, and persons with known albendazole allergy. Pregnant women were also excluded due to potential albendazole teratogenicity.

Participant data were collected via participant interview and chart review. We collected data on age, sex, weight, village of residence, and occupation. We also collected data on date of HIV diagnosis, date of enrollment into HIV care, World Health Organization (WHO) clinical stage at clinic enrollment, $CD4^+$ at enrollment into clinical care ("nadir $CD4^+$"), 12-month history of opportunistic infections, and ART history

(regimen, duration) through review of medical records by a medical officer. Participants underwent a physical examination at study enrollment for assessment of current WHO clinical stage, weight, and presence of current opportunistic infections. Finally, we collected follow-up $CD4^+$ T helper cell concentrations, which were collected as part of TASO's routine clinical practice in the 24 months from study enrollment. Study follow-up occurred in a passive fashion, and no attempts were made beyond standard clinical practice to return patients to care if they stopped attending clinic.

3.3.3 Biological Sample Collection and Analyses

We collected blood and stool from participants during their study visit, which was also a participant's normal clinic visit. We performed a single blood draw to evaluate $CD4^+$ T cell count via the FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA) per routine TASO laboratory protocol.

Parasitic infection status was only evaluated at one time-point: study enrollment. Participants provided a single stool sample, which we froze without fixatives on site at -20^{o}C within 1-2 hours of collection. Stool specimens were transported on a weekly basis to Kampala, Uganda for long-term -80^{o}C storage during enrollment. At the Translational Research Laboratory of the Infectious Diseases Institute in Kampala, Uganda, we assessed participant stool for eight helminth/protozoa species via a modified version of a validated quantitative PCR described previously in Mejia *et al*.[103] This PCR assay was modified to increase the total volume of each reaction from 7µL to 10µL to accommodate the minimum settings on the Applied Biosystems 7900HT Fast Real-Time PCR System.

Reagent concentrations of the 10µL reaction matched those of the 7µL reaction concentrations.[103]

DNA was extracted from approximately 50mg of stool via the FastDNA™ SPIN Kit for Soil DNA Extraction (MP Biomedicals, Solon, OH) using a low reagent method developed by Mejia *et al.* for resource-limited contexts, which has been included as **Appendix 2**. An additional step was required to extract *T. trichiura* DNA, whereby the remaining insoluble pellet from one DNA extraction was re-suspended in 200µL DNA-free water, heated at 90°C for 10 minutes, and centrifuged at 14,000g for 10 minutes. We then repeated the above-described DNA extraction method to process the resulting soluble portion of the sample.

Sequences for the species-specific primers and probes and methods for the qPCR analysis are found in Mejia *et al*.[103] All control standards were tested in triplicate, and all unknown samples were tested in duplicate. A PCR cycle threshold (C_t) value >38 was considered a negative result. Each primer and probe combination has previously been demonstrated as 100% sensitive and 100% specific for its designated species. To additionally ensure that false positives were not driving our results, we conducted a post hoc experiment to bind the *N. americanus* primers and probes to the pBR322 internal control plasmid.[104] We did not observe any evidence of binding between the *N. americanus* primers or probes and the pBR322 control plasmid.

Parasite burden quantification was performed by interpolating against parasite specific sequences standards and reported as DNA fg/µl.[103,105] Briefly, egg counts were estimated from McMaster microscopy techniques of subjects infected with *N. americanus* and/or *A. duodenale* and compared directly to qPCR results. Estimated egg counts from

qPCR were calculated using $Y_{ova/g\ feces}=0.03472*X_{fg/\mu l}$ per correlation studies.[105] Similar calculations were used to estimate *Trichuris trichiura* egg counts: $Y_{ova/g\ feces}=(1.095 \times 10^{-5})*X_{fg/\mu l}$, which was derived by comparing qPCR to Kato-Katz results in infected individuals.[103]

3.3.4 Statistical Analyses

Statistical analyses focused on hookworm infection *a posteriori*, due to its unique immunologic and clinical features, and overwhelming prevalence relative to other species of helminths. Parasite infection prevalence was estimated overall, by species, and by species type (protozoa or nematoda). Infection intensity was summarized by species for helminth worms.[106,107] We used generalized linear models with a binomial distribution and log link, and a robust covariance estimator, to estimate associations between parasitic infection (overall helminth infection, hookworm infection only, and protozoa infection) and clinical and demographic characteristics, specifically, occupation (farming as primary profession versus any other), sex, age (5-year increments), weight (5-kg increments), WHO Clinical Stage (3 or 4 versus 1 or 2), and ART status (receiving or not receiving).

We also estimated the association between parasitic infection and $CD4^+$ T cells/mcL at study enrollment, and the potential effect of parasitic infection on over $CD4^+$ T cell concentrations over follow-up. Age-, sex- and weight-adjusted linear regression models estimated the mean difference in CD4+ T cells/mcL at study enrollment by parasitic infection status (any protozoa, any helminth, hookworm only). Restricted maximum likelihood linear mixed models, which included participant-specific random

intercepts, and an identity covariance matrix, evaluated change in CD4+ T cell concentrations over time across hookworm infection status among participants who were ART-initiated at baseline. These longitudinal models were adjusted for sex, age, time on ART, and weight at baseline. Additional exploratory sub-analyses of change in CD4+ T cell concentrations by hookworm infection status were performed among 1) participants who had initiated ART <1 year before enrollment, and 2) participants who had initiated ART for ≥1 year before enrollment.

We attempted to evaluate the relationship between $CD4^+$ T cell count and parasite burden (light, moderate, and heavy intensity infections per WHO classification; **Appendix 3**). However, because all infections were classified as light intensity (<2,000 eggs/gram feces), we were unable to create any clinically meaningful exposures beyond presence or absence of hookworm infection. No imputation was performed for missing data, which occurred in <2% of participants. All analyses were performed in Stata/IC 13.1 (StataCorp, College Station, Texas) and results were evaluated against an alpha level of 0.05.

3.4 Results

We consented 216 HIV-infected adults during a routine clinic visit. Of these, 14 potential participants were unable to produce a stool sample on site, and were excluded from the study. Thus, 202 participants were enrolled (**Table 1**). Women comprised 69% of participants (139/202). The participants' median age was 35 years [IQR: 30, 41]. The median overall $CD4^+$ at study enrollment was 375 cells/mcL [IQR: 243, 450], and 90% (181/202) of participants were receiving antiretroviral therapy (ART) for a median

duration of 15 (IQR: 5, 29) months. All participants were receiving primary pneumocystis jiroveci pneumonia prophylaxis with either trimethoprim/sulfamethoxazole (n=201) or dapsone (n=1).

Table 1: Baseline characteristics and demographic information by presence of stool helminth infection

Characteristic	**No helminth infection**		**Any helminth infection***		**P-value**
	N	Median [IQR] or n (%)	N	Median [IQR] or n (%)	
Age, years	140	35 [28, 40]	62	36 [30, 43]	0.16
Women	140	90 (64.3%)	62	49 (79.0%)	0.04
Weight, kg	136	53 [47, 60]	60	53 [48, 59]	0.83
CD4$^+$ nadir at clinic entry, cells/mcL	135	257 [127, 401]	59	270 [117, 432]	0.50
CD4$^+$ at study enrollment visit, cells/mcL	140	390 [280, 467]	62	319 [191, 415]	<0.001
Currently receiving ART	140	129 (92.1%)	62	52 (83.9%)	0.08
Duration of ART, months**	129	15 [5, 28]	52	15 [4, 35]	0.84
Receiving tenofovir**	129	94 (72.9%)	52	33 (63.5%)	0.21
12-month pulmonary tuberculosis history	140	2 (1.4%)	62	1 (1.6%)	0.67
Self-reported farming occupation	139	87 (62.6%)	61	44 (72.1%)	0.19

**Ascaris lumbricoides, Ancylostoma duodenale, Necator americanus, Strongyloides stercoralis, Trichuris trichiura*

**Among those participants currently receiving ART

Prevalence & Burden

Multi-parallel quantitative PCR results indicated that 35.2% (71/202) of participants were infected with at least one species of helminth or protozoa. Of these 71 participants, 10 were infected with two species. Most parasitic infections were caused by

N. americanus (27.6%, 55/199). *Giardia* had the next highest prevalence (6.1%, 12/197), followed by *Strongyloides* (4.0%, 8/202). Prevalence and infection intensity of parasitic organisms are described in **Table 2**.

Table 2: Stool parasite infection and infection intensity by species

	N	n (%)	DNA (fg/µl) Median [IQR]	Estimated eggs/g stool	WHO Classification
Overall	202	71 (35.2%)	N/A	N/A	
Helminths					
Ascaris lumbricoides	189	0 (0%)	N/A	N/A	N/A
Ancylostoma duodenale	200	1 (1%)	18.3	527	Light
Necator americanus	199	55 (27.6%)	0.025 [0.018, 0.22]	0.72 [0.53, 6.34]	Light
Strongyloides stercoralis	202	8 (4.0%)	2.1 [<0.1, 81.0]	N/A	N/A
Trichuris trichiura	201	1 (0.5%)	0.6	52,694	Heavy
Protozoa					
Cryptosporidium parvum/hominum	81	1 (1.2%)	35.9 [35.9, 35.9]	N/A	N/A
Entamoeba histolytica	201	3 (1.5%)	<0.1 [<0.1, 0.3]	N/A	N/A
Giardia intestinalis	197	12 (6.1%)	14.7 [0.3, 205.5]	N/A	N/A

Calculated egg burdens for *N. americanus* infections had a median of 0.72 eggs per gram of stool (IQR: 0.53, 6.34; maximum: 275) and 527 eggs/gram of stool for the single *Ancylostoma duodenale* infection and considered light egg burden by the World Health Organization.[108] An estimated 52,694 eggs/gram of stool was calculated for the single heavy *Trichuris trichiura* infection. *Strongyloides stercoralis* eggs generally hatch

and mostly larvae are seen in stool samples, there are no current categories for intensity of larvae in infected patients.

Factors Associated with Protozoal Infection

Results of generalized linear models analyses indicated that each 5-year increase in age was inversely related with a composite outcome of either *Giardia*, *Cryptosporidium*, or *E. histolytica* infection (Prevalence Ratio (PR) = 0.67, 95%CI: 0.49, 0.92, p=0.01); 11.5% (6/52) in participants under 30 years of age, 6.7% (6/90) in participants 31 to 40 years of age, and 1.7% (1/60) in participants greater than 40 years of age. Protozoal infection was more prevalent in farmers than other occupations, although this relationship was unstable and not statistically significant in an age- and sex-adjusted model (PR= 3.96; 95%CI: 0.89, 17.60; p=0.07). Other factors – sex, $CD4^+$ count at enrollment, ART status – were not associated with protozoa infection. (See **Table 3**.)

Factors Associated with Helminth Infection

Univariable analyses of age, sex, weight, advanced WHO stage, current receipt of ART, and occupation indicated that only sex and ART status had a relationship with a composite outcome for prevalent helminth infection, i.e. either *A. lumbricoides*, *A. duodenale*, *N. americanus*, *S. stercoralis*, or *T. trichiura*. Women were more likely to have any helminth infection (PR=1.71; 95%CI: 1.00, 2.92; p=0.05). Those who were currently receiving ART had a decreased prevalence of any helminth infection (PR=0.60; 95%CI: 0.36, 1.00; p=0.05). Multivariable models that included age, sex, occupation, and ART status yielded similar results for infection with any helminth versus no helminth

infection. Women were slightly more likely to be infected with helminths (PR=1.66; 95%CI: 0.98, 2.82; p=0.06) and people receiving ART were less likely to be infected with helminthic worms (PR=0.61; 95%CI: 0.38, 0.99; p=0.05) (**Table 3**).

Table 3: Demographic and clinical factors associated with parasitic infection

Risk Factor	**N**	**Prevalence Ratio (95% CI)**	**N**	**Prevalence Ratio (95% CI)**	**N**	**Prevalence Ratio (95% CI)**
Univariable analyses						
		Protozoa		Helminths		Hookworm spp.
Age, 5 year increments	202	0.70 (0.54, 0.91)	202	1.12 (0.97, 1.30)	198	1.19 (1.01, 1.39)
Women	202	0.73 (0.25, 2.13)	202	1.71 (1.00, 2.92)	198	1.82 (1.01, 3.28)
Weight, 5 kg increments	196	0.94 (0.78, 1.13)	196	1.00 (0.90, 1.11)	192	0.99 (0.88, 1.10)
WHO Stage 3,4	195	1.24 (0.29, 5.27)	195	1.05 (0.56, 1.94)	191	0.87 (0.42, 1.81)
Currently receiving ART	202	1.39 (0.19, 10.23)	202	0.60 (0.36, 1.00)	198	0.49 (0.30, 0.80)
Farming occupation	200	2.90 (0.66, 12.75)	200	1.36 (0.84, 2.20)	196	1.35 (0.81, 2.27)
Multivariable analyses						
		Protozoa		Helminths		Hookworm spp.
Age, 5 year increments[*]	202	0.71 (0.55, 0.92)	202	1.12 (0.97, 1.32)	198	1.20 (1.01, 1.42)
Women[**]	202	0.78 (0.27, 2.68)	202	1.71 (1.00, 2.91)	198	1.82 (1.10, 3.24)
Currently receiving ART[***]	202	1.00 (0.11, 9.41)	202	0.66 (0.41, 1.07)	198	0.57 (0.36, 0.93)
Farming occupation[***]	200	3.96 (0.89, 17.60)	200	1.28 (0.76, 2.14)	196	1.21 (0.68, 2.15)

[*] Sex-adjusted generalized linear model estimating prevalence ratios.
[**] Age-adjusted generalized linear model estimating prevalence ratios.
[***] Age- and sex-adjusted generalized linear model estimating prevalence ratios.

Analyses of predisposing factors for infection with hookworm species *A. duodenale* or *N. americanus* alone indicated that female sex, age, and ART status were associated with hookworm infection in univariable analyses. Each 5-year increase in age was associated with increased likelihood of infection (PR=1.19; 95%CI: 1.01, 1.39; p=0.03), as was being female (PR=1.82; 95%CI: 1.01, 3.28; p=0.05). ART was associated with a decreased likelihood of hookworm infection (PR=0.49; 95%CI: 0.30, 0.80; p<0.01). These results were attenuated in multivariable models that included sex, age, ART status, and occupation, such that only receiving ART remained statistically significantly associated with likelihood of decreased hookworm infection (PR=0.53; 95%CI: 0.33, 0.86; p=0.01); 52.6% (10/19) of participants not receiving ART were infected with hookworm, and 25.7% (46/179) of participants receiving ART were infected with hookworm.

Relationship between hookworm and immune status

We assessed the relationship between hookworm infection and $CD4^+$ T helper cell concentrations at study enrollment. Participants with hookworm infection demonstrated consistently lower concentrations of $CD4^+$ cells/mcL when compared to hookworm-uninfected peers (**Table 4**). Unadjusted analyses indicated an average difference of -70 cells/mcL (95%CI: -113, -26, p=0.002) in participants with hookworm infected relative to those without detectable hookworm infection. This relationship became more pronounced when adjusting for participant age, sex, and time on ART; participants with hookworm infection had 94 fewer $CD4^+$ cells/mcL on average (95%CI -133, -55, p=<0.001) than those without hookworm. Stratified analyses on ART status (receiving or not currently

receiving ART) indicate a similar relationship among those persons receiving ART at enrollment (n=171) (mean: -102 cells/mcL; 95%CI -145, -58; p=<001). An additional stratified analysis among those persons who were ART naïve was limited by a small sample size (n=19), but did not show a statistically significant relationship between hookworm infection and $CD4^+$ T cell concentrations (mean: -43 cells/mcL; 95%CI: -118, 32; p=0.24).

Table 4: Differences in CD4+ cells/mcL between infected- and hookworm-uninfected adults living with HIV in peri-urban Uganda.

	N	Mean difference in $CD4^+$ cells/mcL (95% CI) [a]	p-value
All participants			
Protozoa			
Protozoal infection, unadjusted	202	-11 (-96, 75)	0.81
Protozoal infection, adjusted [b]	194	-23 (-118, 71)	0.63
Helminth			
Any helminth, unadjusted	202	-62 (-107, -17)	<0.01
Any helminth, adjusted [c]	194	-80 (-121, -39)	<0.001
Hookworm			
Hookworm infection, unadjusted	198	-70 (-113, -26)	0.002
Hookworm infection, adjusted [c]	190	-94 (-133, -55)	<0.001
Among ART initiated only			
Hookworm infection, adjusted [c]	171	-102 (-145, -58)	<0.001
Among ART naïve only			
Hookworm infection, adjusted [d]	19	-43 (-118, 32)	0.24

[a] Relative difference in $CD4^+$ cells/mcL in those with hookworm infection, relative to those without hookworm infection.
[b] Adjusted for nadir CD4, age, sex
[c] Adjusted for nadir CD4, sex, years on ART
[d] Adjusted for nadir CD4, sex

Among participants who had initiated ART at enrollment, results from the longitudinal analyses that participants with hookworm infection did not demonstrate a different rate of $CD4^+$ T cell immune recovery in the 24-months post-enrollment ($\beta_{hookworm\text{-}time}$=0.44; 95%CI: -0.46, 1.35; hookworm-time interaction term p-value=0.33).

Participants with hookworm did, however, have consistently lower $CD4^+$ concentrations relative to their hookworm-uninfected peers over the 24 months of follow-up (-85 cells/mcL; 95%CI -149, -21; p=0.009), based on an average of 2.3 $CD4^+$ measurements (min=1, max=5) per participant over 24 months (**Figure 1**). The mean number of measurements over time across hookworm-infected versus uninfected participants was similar (2.2 and 2.4, respectively).

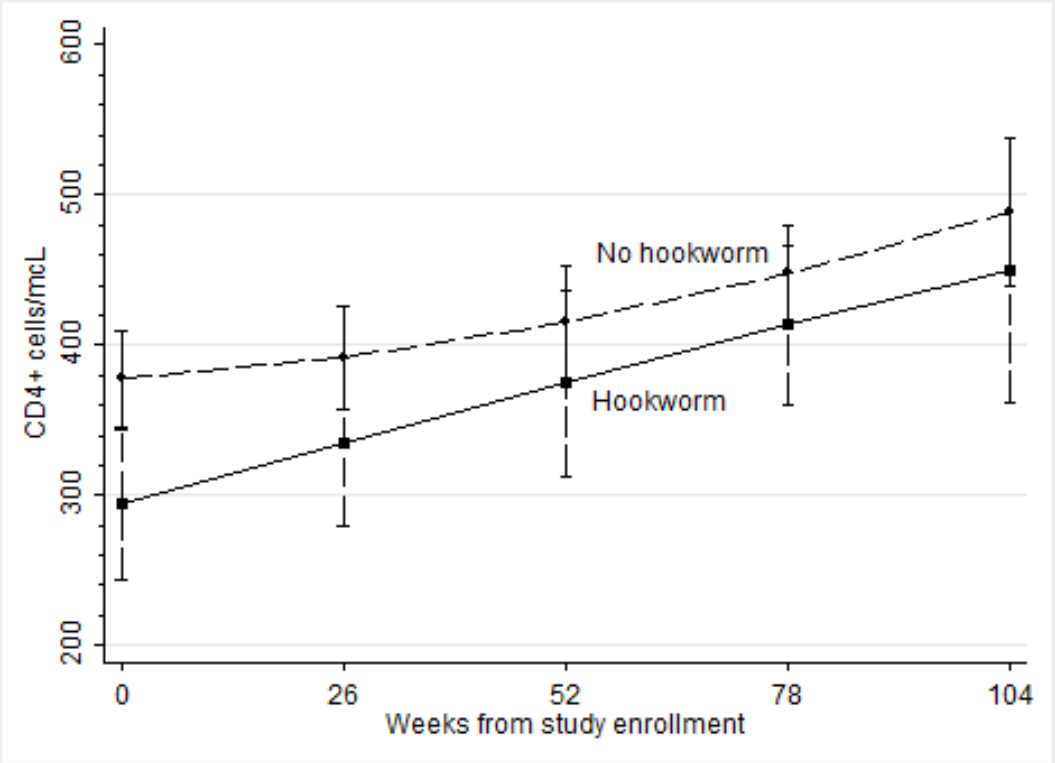


Figure 1: *Change in CD4+ T cell concentrations from enrollment to 24 months between participants infected with hookworm versus those not infected with hookworm*

At 12-months post-enrollment, when adjusting for sex, age, time on ART and weight at baseline, participants with hookworm infection had a mean 361 $CD4^+$

cells/mcL (95%CI 309, 412) versus a mean of 422 cells/mcL (95%CI 393, 451) amongst those without hookworm infection at baseline. Participants with hookworm infection at baseline had, on average, 62 fewer CD4+ cells/mcL (95%CI: -121, -3) relative to their uninfected peers. At 24-months post-enrollment, adjusting for the same co-variates, participants with hookworm averaged 438 $CD4^+$ cells/mcL (95%CI 363, 512) versus 476 cells/mcL (95%CI 435, 517) among those uninfected with hookworm at baseline. While participants with hookworm infection still had lower CD4+ cell concentrations than their uninfected peers at 24 months of follow-up, the difference in average CD4+ cells/mcL between hookworm infected versus uninfected across groups was attenuated (-39 cells/mcL; 95%CI -124, 47) and not statistically significant.

Furthermore, participants who had initiated ART ≥1 year prior to study enrollment (n=107) demonstrated a similar relationship to our overall cohort; there was no difference in rate of CD4+ cell recovery during the study period, but those with hookworm were at an immunologic deficit relative to their uninfected peers (77 fewer CD4+ cells/mcL in those with hookworm versus those without; 95%CI -154, -1). Among persons who had initiated ART less than 1 year prior to study enrollment (n=68), the effect was less pronounced and not statistically significant, with only 26 (95%CI: -121, 70) fewer CD4 cells/mcL in those with hookworm versus those without, and no difference in change over time, like other analyses.

3.5 Discussion

We demonstrate that parasitic infection, particularly with *N. americanus* hookworm species, was common in this adult, HIV-infected population in Uganda. While

these infections were generally light intensity infections, we report a clinically and statistically significant association between hookworm infection and decreased $CD4^+$ T helper cells/mcL at study enrollment. This relationship was maintained over study follow-up, where participants with hookworm infection had diminished $CD4^+$ immune status over time, relative to their peers who were not infected with hookworm. There was no difference, however, in $CD4^+$ cell recovery over 24 months among participants who were ART-initiated at baseline.

To our knowledge, only two other studies to date have examined the health impacts of helminth infection in persons receiving ART, specifically the effects of deworming.[90,91] In Uganda, Lankowski *et al*. did not find any significant beneficial effects of deworming in their overall study population. However, in a sub-group analysis of women only, they found that deworming with either albendazole or mebendazole 7 to 90 days prior to $CD4^+$ T cell measurement, for unspecified parasitic infection increased $CD4^+$ T helper cell concentrations by an average of 63 cells/mcL (95% CI: 6-120) in in the first year of ART initiation. This study was limited by the fact that a medical record of deworming was used as a proxy for helminth infection, and as such neither helminth infection prevalence nor deworming incidence were reliably captured. This may have attenuated their results towards a null finding in the overall cohort.

Ivan *et al*. found that deworming decreased HIV viral loads and increased $CD4^+$ T cell concentrations over a 12-week period in a cohort of 980 HIV-infected, ART-initiated pregnant Rwandans.[91] While the results of this study demonstrate the value in revisiting the question of deworming in the presence of increasingly available ART, it is potentially limited by a treatment cross-over effect of deworming outside of the study setting. That

said, any extra-study deworming in the control arm would likely attenuate the effects of their intervention; and one can extrapolate that the results in a completely controlled setting would have been more extreme. Additionally, this is a limited subset of the ART-receiving, HIV-infected population, and it would be important to duplicate these results in other populations of men and non-pregnant women.

Prevalence of parasitic infection

Our results are comparable with much of the available literature regarding parasite infection prevalence in adults. Other studies conducted in Uganda have found similar prevalences of hookworm infection (24 to 52%),[36,39,41,43,109] and Strongyloides (4 to 8%) [44,110,111] in adults with and without HIV. In persons with HIV in Nigeria, Senegal, and Ethiopia, *Giardia* prevalence has been observed at approximately 5%.[112-114]

Partial immunity to most parasitic infections is acquired over the life course, leading to an increased rate of parasite destruction and worm expulsion with increasing age and re-infection. Hookworm species, however, do not induce the same adaptive immunity in humans as the other soil transmitted helminths, and consequently, may continue to infect adults with high frequency and intensity.[115] In the context of frequent and repeated infection, this lack of adaptive immunity may have important implications for host response to co-infections like HIV, and important Th1-moderated HIV co-morbidities, such as tuberculosis and cryptococcal meningitis.

Relationship between hookworm infection and $CD4^+$ T cells/mcL

Our results found that participants who were infected with hookworm were at a significant $CD4^+$ T-helper cells/mcL deficit, relative to participants who were not infected with hookworm, at study enrollment, and over time. $CD4^+$ T-helper cells are

critical in mediation of the immune system's response to various pathogens, and commonly used to monitor HIV disease progression and response to ART.[116] The inverse relationship between hookworm infection and $CD4^+$ T cell concentrations was qualitatively and statistically consistent across various analyses, from unadjusted to adjusted regression, analyses restricted to persons receiving ART, and over time. We did not observe a difference in $CD4^+$ cells/mcL among persons who were ART-naïve; however, the small proportion of persons not receiving ART in this cohort (n=19) renders these analyses relatively uninformative.

Ample evidence demonstrates that soil-transmitted helminths are potent immunomodulators, and infection with soil-transmitted helminths involves many major body systems, from the gastrointestinal and circulatory systems, to soft tissues.[52] Multiple biologic mechanisms could be driving our observed relationship; and these results are likely multifactorial for any given participant. Hookworm infection in HIV-uninfected persons with celiac disease has been shown to decrease expression of interferon (IFN)-γ on intestinal T cells, and increase in $CD4^+FoxP3^+$ regulatory T cells, which could contribute to decreased differentiation to $CD4^+$ T helper cells.[117] Other research has demonstrated that hookworm antigens induce cytotoxic and pro-apoptotic activity in Jurket T Cells, contributing to an increase in $CD4^+$, $CD8^+$, and $CD19^+$ lymphocytes that were in an early and/or late stage of programmed cell death.[118] Cuellar *et al*. found that commonly excreted hookworm protein Ac-TMP-1, a Tissue Inhibitor of Metalloproteases, induced murine splenic T cells to differentiate to $CD4^+$ and $CD8^+CD25^+FoxP3^+$ regulatory T cells that expressed interleukin (IL-)10 and suppressed

naïve and activated $CD4^+$ T cells differentiation.[119] Other human studies, however, have not found similar increases in T regulatory responses to hookworm infection.[120]

Other human studies have not found differences in $CD4^+$ T cell concentrations between hookworm-infected and -uninfected groups of HIV-uninfected participants. In a quasi-experimental study by George *et al*., which measured the impact of deworming on microbial translocation (a contributor to chronic immune activation linked to decreased concentrations of $CD4^+$ T helper cells), observed that hookworm was associated with elevated levels of pro-inflammatory markers, e.g. lipopolysaccharide, soluble CD14.[121] They did not, however, observe differences in T cell subsets among naturally infected, HIV-uninfected participants at baseline.[121] The authors postulate that lack of difference in T cell subsets is mediated by a counterbalancing, anti-inflammatory effect of hookworm infection, e.g. elevated levels of IL-10, and decreased C-reactive protein, IL-17 and haptoglobin.[121]

From the standpoint of clinical endpoints, results from clinical trials conducted in ART-naïve persons remain mixed. Results from the HEAT trial, which evaluated the impact of reflexive and repeated deworming on a patient's risk for ART eligibility, i.e. a drop below 350 $CD4^+$ cells/mcL, found no difference between the reflexive deworming group (400 mg albendazole every 3 months plus 25 mg/kg praziquantel annually) versus the standard of care group (no empiric deworming).[80] The trial had 80% power to detect a hazard ratio of 0.775, which could be considered a large, albeit clinically important, difference in treatment groups. That said, actual $CD4^+$ T cell concentrations at study completion were very similar across randomization groups, supporting the idea that deworming may not dampen $CD4^+$ decline in the absence of ART. Other studies support

this conclusion.[12,13,83] However, still other studies and meta-analyses demonstrated reductions in plasma viral loads and increases in $CD4^+$ T helper cells with deworming in persons living with HIV.[81,122] The differences in these results may in part be explained by differences in methodology, and in particular the need to pool species due to limited species-specific sample sizes.

Repeated and long-term exposure to hookworm and other helminth species may cause fibrosis of the gut associated lymphatic tissues (GALT). IL-13, in particular, is increased in the presence of hookworm infection[115] and a dominant mediator of fibrotic tissue, which induces fibrosis independently and via simulation and activation of transforming growth factor (TGF)-β. In the case of chronic and repeated helminth infections, and corresponding Th2 type immune responses, IL-13 production can become pathological. Fibrosis of the GALT has been linked to the dysregulation of immune cells, including $CD4^+$ T cells, and impaired $CD4^+$ recovery.[123,124]

Chronic and repeated exposure to helminths and subsequent GALT fibrosis may have impacted the results of this and other studies. Indeed, the longitudinal analyses in this study demonstrate that hookworm-infected versus uninfected participants have a similar rate of $CD4^+$ recovery over the 24-month follow-up period, but that those infected with hookworm remained at a significant immunologic deficit relative to their uninfected peers over time. Fibrosis is not reversible with deworming or other therapy. This bears mentioning because while hookworm and other helminths are still causally implicated in the decrease in $CD4^+$ T cell concentrations, there are important implications for public health intervention design, e.g. increased deworming frequency targeting all stages of the human life course.

Limitations

It is possible that our results are spurious, either due to confounding, a misunderstanding of the directionality of the hookworm-$CD4^+$ relationship, or a type I statistical error. The primary limitations of this study arise from its observational nature. First, the temporal relationship between parasite infection and immune status remains undetermined; it is conceivable that being immunocompromised would increase the likelihood of persistent infection. Research on this topic remains mixed and parasite dependent.[78,125-127] However, most research to date suggests no difference in hookworm risk between immunocompromised and immunocompetent persons.[128-131] There is potentially one exception to this pattern. A cross-sectional study by Sanyaolu *et al*. found that 4.6% (3/65) of HIV-infected Nigerians had hookworm infection, versus 1.8% (18/1015) of HIV-uninfected peers.[132] However, this author was unable to duplicate their results based on the data provided in the paper.

Our results are based on a single stool sample, and we did not use any concentration techniques prior to DNA extraction. Diagnostic sensitivity for hookworm – and other species – when evaluating a single stool sample is lower than sensitivity when using multiple stool samples. For example, Knopp *et al*. found that a single stool sample yielded a 7.1% prevalence, while 2 samples yielded a prevalence of 15.6% via Kato-Katz.[133] These authors found that Strongyloides prevalence with 1 stool sample versus 2 samples was similar, 3.5% and 5.3%, respectively. It is unlikely that our observed hookworm prevalence would have doubled had we analyzed >1 stool sample. However, we may have misclassified parasite-infected participants as uninfected, particularly among those with a low burden.

Finally, our results may be confounded by data that would have been useful in these analyses but were not available. Hookworm, and other intestinal parasites, are considered diseases of poverty. The relationship between increased infection incidence among economically disadvantaged persons is well established.[36,134-136] Additionally, being economically disadvantaged could have impacted health outcomes in this study, e.g. $CD4^+$ T helper cells concentrations, as it has in other research.[137-139] While we collected information on place of residence, this information could ultimately only be dichotomized into participants who lived in Mbale town, where the clinic is located, versus all others, which could represent varied levels of development and corresponding hookworm exposure. Also, ART adherence data were routinely collected and reflected uniformly high adherence levels. Past research on adherence at TASO ART clinics report similarly high levels of ART adherence, with ~90% of patients reporting no missed pills in the past 30 days.[140-142] However, data from prior TASO adherence research – like adherence data for this study – are limited by the fact that they are self-reported, which consistently over-reports adherence relative to pill counts, pharmacy refill information, and/or drug concentrations in blood.[143,144]

Conclusions

Despite the limitations of this study, we feel that these results are generalizable to other adults receiving outpatient HIV therapy in low-income, peri-urban areas. The results presented herein point to a high prevalence of helminth infection in this vulnerable population, and that hookworm infection is associated with sub-optimal health outcomes, i.e. lower CD4. Therefore, further examination of these questions via a randomized trial

is warranted, especially how systematic deworming may impact the immune status of this vulnerable population in the presence of ART.

4. Aim II. Characterization of the fecal microbiome and its relationship with clinical factors among rural and peri-urban adults living with HIV in Uganda

4.1 Aim Summary

Background: Gut bacterial community composition provides insight into HIV pathogenesis, and potentially offer avenues towards low cost adjunctive HIV therapies. Western populations predominate current literature, while >70% of HIV infections occur in Africa. We examine the relationships between clinical characteristics and fecal microbiome composition in HIV care-seeking Ugandans.

Methods: 175 HIV-infected Ugandans from a cohort study examining clinical correlates of helminth infection contributed a single fecal sample to this nested analysis. CD4+ T cells/mcL were measured at baseline and over 12-months post-enrollment. Fecal samples underwent 16S bacterial rRNA sequencing. α- and β-diversity were compared against time on ART, CD4+ T cells/mcL, and parasitic infection. Parametric and non-parametric tests assessed differential relative abundances of specific taxa across ART, CD4+ T cells/mcL, and parasitic infection. The relationship between taxa and CD4+ cell recovery was assessed via linear mixed models. FDR adjustments were made for multiple comparisons.

Results: α-diversity was lower in participants with <100 CD4+ T cells/mcL (n=8) versus participants with ≥100 CD4+ T cells/mcL (n=167). We failed to observe differences community composition across clinical or demographic characteristics. Only *Anaerococcus* was inversely associated with CD4+ T cells/mcL (Spearman correlation -

0.32; p-value<0.001). *Sutterella* genus and *Alcaligenaceae* (of unknown genus) were associated with increasing CD4+ T cells/mcL over time.

Conclusions: These findings reinforce the inverse relationship between fecal microbiome community diversity and compromised immune status, and have identified a limited number of taxa associated with clinical parameters. More HIV-microbiome research should include African participants, as robust and repeated results are required to translate these findings into health-enhancing adjuvant therapies.

4.2 Background

The microbial communities of the gut are critical to the maintenance of healthy human physiology. Many human disorders – from inflammatory bowel disease[145] to diabetes[146] to cardiovascular disease[147] – have been linked to disruption of the bacterial communities found in the gut.[148,149] Human immunodeficiency virus (HIV) is no exception.[150] Evidence suggests that HIV infection causes progressive damage to the gut mucosa[151] and dysbiosis – or imbalance – of bacterial communities of the gut.[152,153] While the gut microbiota are normally protected from the host immune system by a strong mucosal barrier, HIV damages the integrity of this mucosal lining.[151] This damage potentially alters the composition of the gut flora, by exposing it to lymphocytes and other immune cells contained in the lamina propria, an intensely vascular layer of connective tissue underlying the epithelium of the gut mucous membrane.

In healthy mammals, the billions of commensal bacteria that colonize the gastrointestinal tract do so in anatomically restricted spaces of intestinal lumen, e.g. the gut epithelial surface, or within the underlying gut-associated lymphoid tissue.[154] Maintaining these anatomical niches limits inflammation and other harmful effects of community disruption and bacterial transfer. Damage to the gut mucosa allows bacteria and other organisms to traverse the gut lumen into normally protected areas of the circulatory system.[7,155] This process, referred to as microbial translocation, has been linked to chronic immune cell activation and, a perpetual state of systemic inflammation characterizing HIV infection and disease progression. Although gut flora play an important role in the development of the mucosal immune system,[152] it is unclear if

dysbiosis of the gut flora induced by HIV is a direct contributor to HIV disease progression, a byproduct of infection, or both.

Research has demonstrated that HIV-infected individuals generally – although not uniformly[156] – have less species diversity or richness in their gut flora when compared to HIV-uninfected individulas.[157,158] This within-individual gut flora species diversity or richness is broadly termed α-diversity. The fecal flora of people living with HIV has been characterized by an overgrowth of *Bacterioides uniformis*[159] and *Prevotella copri*, the latter of which has been linked to inflammatory conditions.[160] Dillon *et al*. demonstrated lower ratios of *Bacterioides spp.* to *Prevotella spp.* in persons living with HIV versus those who were HIV-uninfected.[153] Gori *et al*. observed a higher abundance of potential pathogens *Pseudomonas aeruginosa* and *Candida albicans*, and lower abundances of "healthy" *Bifidobacteria* and *lactobacilli* species in persons living with HIV relative to historical HIV-uninfected controls.[152] While α-diversity may rebound in the presence of HIV antiretroviral therapy (ART),[161] the presence and density of specific species remains fundamentally altered, potentially resulting in adverse sequelae.

Few studies to date have examined the fecal microbiome composition of adults living with HIV from Sub-Saharan Africa. This is despite the fact that >70% of HIV infections are clustered in the region,[17] and that high levels of regional variation in gut flora have been demonstrated across different geographic and cultural contexts.[149,162,163] To our knowledge, only two studies have characterized the fecal microbiome of adults living with HIV in Sub-Saharan Africa.[164,165] These studies had slightly contradictory results. The first demonstrated that the fecal microbiome composition of participants became less phylogenetically diverse with decreasing CD4+ T cell counts, and that

specific potentially pathogenic bacteria became more abundant with decreasing CD4+ T cell counts. Another study indicated that HIV therapy, and not the infection itself, was associated with a shift towards a predominance of pathogenic bacteria. Parasitic infection is common in low-income areas like Sub-Saharan Africa, where HIV is also common. Parasite and HIV co-infection prevalence estimates range by context, but average approximately 30% in this context.[42,80,166] Parasitic colonization of the gut is also associated with gut flora composition shifts, although these relationships are inconsistent and parasite-dependent.[167-171]

The present analysis characterizes the fecal microbiome of 175 adult Ugandans living with HIV who are enrolled in care at an outpatient HIV clinic in peri-urban Uganda. Over 90% of these participants were receiving ART at the time of enrollment. To date, this is the largest cohort of HIV-infected Africans who have undergone 16S sequencing, a common molecular method used to identify bacterial taxa, of their fecal microbiome. We hypothesize that either composition of the fecal microbial community and/or specific taxa will be associated with duration of ART, CD4+ T cell concentrations, and infection with intestinal parasites. Furthermore, we examine the relationship between gut community composition and CD4+ T cell recovery over time, a proxy for overall immune system recovery and health. These analyses – and longitudinal analyses in particular – are exploratory in nature, and should be further examined in future cohorts.

4.3 Methods

4.3.1 Ethics statement

All participants provided written informed consent. The University of Minnesota, The AIDS Support Organisation (TASO), and the Uganda National Council of Science and Technology institutional review boards approved this protocol.

4.3.2 Study participants and study design

We recruited adult Ugandans receiving outpatient care at TASO's HIV clinic in Mbale, Uganda, into a study evaluating the prevalence and burden of eight gastrointestinal parasites and assessing clinical correlates of parasitic infection. Conduct of this study has been described previously.[42] In brief, the TASO Mbale clinic provides care for the rural and peri-urban population surrounding Mbale, located in southeastern Uganda. A blood draw was performed at enrollment for same-day CD4+ T cell concentrations; and participants were passively followed for 12 months for CD4+ T cell/mcL values over time.

Each fecal sample was self-collected by participants at study enrollment during their standard HIV care clinical visit in a polypropylene collection container (Globe Scientific, Paramus, New Jersey). The sample was maintained at room temperature for ≤4 hours, prior to freezing at -20°C. Upon being thawed, the sample was split to perform molecular parasitological analysis per previously described methods.[42,103] The remaining portion was re-frozen until thawed a second time for ribosomal ribonucleic acid (rRNA) extraction, the result of which was used for sequencing analyses. Each sample underwent two freeze-thaw cycles prior to extraction of genetic material and sequencing.

Bacterial 16S rRNA extraction, sequence preparation, and operational taxonomic unit selection

Genetic material was extracted from stool samples using the MP BIOMEDICALS FastDNA™ SPIN Kit for Soil (Santa Ana, CA, USA) per the manufacturer's protocol. The Illumina MiSeq platform (Illumina, San Diego, CA) sequenced the V5-V6 region of the bacterial 16S rRNA gene at the University of Minnesota Genomics Center. The 16S region is a highly-conserved region, and its stability over time makes it ideal for taxonomic identification. Forward and backward sequence reads from each sample were trimmed to their maximum value of 280 base pairs using the Cutadapt program.[172] Adapter sequences were removed from forward reads* and backward reads†, and sequences were trimmed using a quality threshold of 20. To apply this quality threshold, the Cutadapt program subtracts the quality value threshold from the quality assigned to each base read. These new values are summed, sequentially, from each end, inwards, and the process stops at the point where the adjusted base quality value is greater than 0. Forward and backward sequences were aligned using the resync.pl script in the "riss_util" module developed by the Research Informatics Support Systems at the University of Minnesota. QIIME v1.8.0 was used to join forward and backward sequences via the "join_paired_ends.py" script (≥50 paired bases).

To identify operational taxonomic units from these sequences, closed-reference selection was employed in QIIME v1.8.0 using the USEARCH method,[173,174] whereby sequences from unknown samples were compared against the Greengenes reference database v13_8 at a 97% similarity cutoff, which is sufficiently specific to accurately

*CTGTCTCTTATACACATCTCCGAGCCCACGAGACNNNNNNNNATCTCGTATGCCGTCTTCTGCTTG
†CTGTCTCTTATACACATCTGACGCTGCCGACGANNNNNNNNGTGTAGATCTCGGTGGTCGCCGTATCATT

group sequences originating from the same genera.[‡] A closed-reference operational taxonomic unit selection method minimizes spurious classification, e.g. chimeras attributable to assay amplification error, by using pre-existing and validated taxonomic information. Any sequences that are not matched to the reference database are discarded from downstream analyses; this sequence "loss" is typically 1 to 10% when classifying bacteria from well-characterized environments such as the human gut. Operational taxonomic units are used to construct downstream metrics based on actual taxonomic classification, e.g. phylogenetic diversity. Samples with a very small number of reads (<5,000) were excluded from further analyses.

As a data quality measure, rarefaction analyses were performed to verify that each sample was sequenced to a sufficient degree to accurately capture the diversity of each participant's fecal bacterial community. These analyses entail visual assessment of alpha diversity, i.e. the number/richness and/or distribution of taxa for a single bacterial community,[175] plotted against an increasing number of randomly sampled without replacement sequences. In rarefaction analyses, samples that have been sequenced to a sufficient depth will exhibit an alpha diversity plateau as the sampling proportion increases. We performed rarefaction analyses using three different alpha diversity metrics: i) species counts, ii) phylogenetic diversity, and iii) the Chao1 estimator. Alpha diversity metrics used in this paper are described in **Table 5**, along with brief explanations of other microbiome analysis terminology. Rarefaction plots are shown, by CD4+ T cell categories, in **Figure 2**.

[‡] 99% similarity was a commonly used similarity cutoff for species-level operational taxonomic unit determination. However, species in the same genus are indistinguishable up to 42% of the time. [Jovel, *et al*. 2016; Pei, *et al*. 2010] Thus, we have favored genera-level classification for this paper, like most peer-reviewed literature.

Table 5: *Glossary of microbiome analyses terms*

Term – description
Species counts – alpha diversity metric calculated by the total number of unique operational taxonomic units.
Phylogenetic diversity – alpha diversity metric based on total taxonomic branch length per sample.
Chao1 estimator – alpha diversity metric that estimates the actual number of species in a community, given the observed sample, while accounting for the relative distribution of species within each sample.[176]
Shannon-Weaver diversity – alpha diversity metric that accounts for number and distribution of species
Simpson diversity – bacterial community diversity metric, with a 0 - 1 distribution scale.[177]
Principal coordinates analysis (PCoA) – Multivariate, distance-based technique used to visualize the relationships between communities. Each individual's sample, or bacterial community, is represented by a point in two- or three-dimensional plot, and similar communities exhibit clustering in a PCoA plot. "Community" for purposes of these visualizations may be constructed by comparing different categories of clinical or demographic characteristics, e.g. ART-initiated vs. ART-naïve.[178]

From the results of this rarefaction analysis and the smallest number of common sequences per sample, an additional rarefaction step was performed to harmonize the number of S16 sequencing reads per sample. Samples were rarefied, i.e. randomly sampled without replacement, at a depth of 17,000 sequences. This rarefied operational taxonomic unit table was subsequently normalized by taxonomic family and genus, whereby taxa counts were collapsed within participant, and a relative abundance metric

was created (number of taxa-specific counts divided by the total number of counts per sample).

Exposure Classification

For purposes of these analyses, clinical indicators were *a priori* categorized as outlined in **Table 6**. CD4+ cells/mcL were also categorized *a posteriori* and along clinically relevant lines as <100 cells/mcL, 100 to <350 cells/mcL, and ≥350 cells/mcL.

Table 6: *Description of clinical and demographic variables included in baseline and longitudinal analyses*

Clinical or Demographic Characteristic	Categorization of variable for analysis
Parasitic infection	• Any parasitic infection vs. no infection • Hookworm infections vs. no hookworm infection • *G. intestinalis* vs. no *G. intestinalis* • Protozoal infections vs. no infections • Helminth infections vs. no infections
Time on ART	• ART-naïve • >0 to 3 months ART • >3 months to 6 months • >6 to 12 months ART • >12 to 24 months ART • >24 months ART
CD4+ cells/mcL at study enrollment	• <100 cells/mcL • 100 to <200 cells/mcL • 200 to <350 cells/mcL • 350 to <500 cells/mcL • ≥500 cells/mcL

Cross-Sectional Diversity Analyses

We evaluated the relationship between α-diversity metrics (number of observed species, phylogenetic diversity, Chao1, Shannon-Weaver diversity) and clinical

characteristics (CD4+ T cells/mcL, time on ART at enrollment, parasitic infection) via univariable and age-adjusted analyses-of-variance (ANOVA). Continuous diversity metrics were the dependent variables in these analyses. CD4+ T cells were analyzed as continuous and categorical variables. For categorical CD4+ analyses, when ANOVA indicated a significant overall relationship between the alpha diversity and categorical CD4+ concentrations, Tukey-Kramer pairwise comparisons further explored the relationship. Kruskal-Wallis non-parametric tests evaluated the correlation between Simpson diversity, which is not-normally distributed, and clinical characteristics. These relationships were evaluated against a 5% Type I error rate, with no FDR adjustment.

We also visually examined community composition across different levels of clinical characteristics, e.g. <100 CD4+ T cells/mcL versus ≥100 CD4+ T cells/mcL, via beta diversity measures, which function as an assessment of similarity between populations.[§] β-diversity was calculated via weighted and unweighted "unique fraction", or unifrac, methods.[179-181] As summarized by Lozupone *et al*. "unifrac measures the phylogenetic distance between sets of taxa in a phylogenetic tree as the fraction of the branch length of the tree that leads to descendants from either one environment or the other, but not both."[180] For example, two samples with completely unique phylogenetic trees would have a unifrac β-diversity score of one. Conversely, completely identical specimens would have a unifrac β-diversity score of zero. Unweighted unifrac only accounts for the presence or absence of bacterial species, and will give more weight to

[§] Morgan *et al*. (doi: 10.1371/journal.pcbi.1002808) offer a helpful contrast of alpha and beta diversity: "Additionally, when comparing multiple populations, beta diversity measures including absolute or relative overlap describe how many taxa are shared between them. An alpha diversity measure thus acts like a summary statistic of a single population, while a beta diversity measure acts like a similarity score between populations, allowing analysis by sample clustering or, again, by dimensionality reductions such as PCA."

less common species, relative to weighted unifrac. Weighted unifrac accounts for the contribution of each bacterial species to the sample composition by its abundance.

The unifrac measure was calculated for pairwise sample combinations, and used to create distance matrices for the entire cohort. These matrices were visually assessed for within-clinical characteristic similarity, i.e. clustering, via principal coordinates analysis (PCoA) across parasitic infection; time on ART; and CD4+ cells/mcL at study enrollment categories. PCoA is a multivariate ordination technique that generates uncorrelated axes, which describe sources of variability in the data via eigenvalues and % variation explained. Participant-level data are plotted against these axes, and the relationships between characteristics are visually assessed.[178] Permutational multivariate analysis of variance (PERMANOVA) was also used to quantify differences in weighted unifrac distance matrices by clinical characteristic categories.[164,168,181] Pseudo-F-test statistic p-values from 1,000 PERMANOVA permutations were evaluated against unadjusted Type I error rate of 5%. The % variation explained from weighted unifrac beta-diversity matrices were 31.2% (PC1), 10.1% (PC2), and 7.8% (PC3). Unweighted unifrac beta-diversity matrices were excluded from PERMANOVA analyses, due to their low eigenvector values and % variation explained (PC1 = 8.9%; PC2 = 4.3%; PC3 = 3.4%).

Cross-sectional Analyses

We qualitatively examined the abundance of particular taxa that are characteristically associated with an HIV-associated loss in microbial diversity, namely the phyla *Proteobacteria*, *Fusobacteria*, *Bacteroidetes*, and *Firmicutes*.[150] Kruskal-Wallis equality-of-populations rank tests correlated individual genera with clinical parameters (e.g. parasitic infection, time on ART, CD4+ cells/mcL at study enrollment, as described

above). Taxa that were identified in <5% of participants were not considered in analyses. P-values for each outcome were adjusted for multiple comparisons via the Benjamini–Hochberg technique, or FDR.[182]

Longitudinal Analyses

Longitudinal analyses explored the relationship between alpha diversity and specific genera and 12-month CD4+ T cell recovery among participants with more than 1 CD4+ measurement (n=84). Restricted maximum likelihood estimation linear mixed models using unstructured covariance matrices, included participant-specific random intercepts and random effects for time, and an interaction term for time from enrollment and the genus-level operational taxonomic unit or diversity metric. These models adjusted for potential confounders of time on ART at enrollment and age. Statistical analyses were conducted in Stata/SE 14.2 (StataCorp LP, College Station, TX, USA), and tests were conducted using an overall alpha level of 0.05. P-values for each outcome were adjusted for multiple comparisons via the Benjamini–Hochberg technique as described above.[182]

4.4 Results

We enrolled 202 study participants of whom 181 (90%) had specimens available for sequencing. Of 181 samples, 6 had <5,000 reads and were excluded from analyses. Thus, data from 175 participants were included in analyses. These samples had a median of 46,515 sequences (min. 17,058, max. 149,393). Among these 175 participants, 121 (69%) were women. The median age was 35 (interquartile range (IQR): 30-41) years. At time of study entry, the median CD4 count was 379 (IQR: 252, 454) cells/mcL, with 38

(22%) having <200 CD4+ T cells/mcL, and 8 (5%) having <100 CD4+ T cells/mcL. Overall, 156 (89.1%) were receiving ART, and for a median duration of 15 (IQR: 5, 29) months. Trimethoprim-sulfamethoxazole (cotrimoxazole) primary prophylaxis was used by 174 (99.4%) participants, with the remaining participant receiving dapsone prophylaxis. Overall, 63 (36.0%) participants had stool parasites identified by PCR, with hookworm species *Necator americanus* being the most common (50/175, 28.6%), followed by *Giardia intestinalis* (12/175, 6.9%).

Fecal microbiome composition among these subjects demonstrated a predominance of *Bacteroidetes* and *Firmicutes*, which collectively accounted for a median relative abundance of 86.6%. The median relative abundance of *Bacteroidetes* in this cohort was 47.9% (IQR: 36.3, 59.2), the median for *Firmicutes* was 38.7% (IQR: 28.2%, 48.3%), and 0.8% (IQR: 0.2%, 2.5%) for *Proteobacteria*. In this population, 86.9% of participants exhibited no *Fusobacteria*, and the maximum relative abundance was 1.1%.

Diversity analyses

Of the clinical factors examined for their association with alpha diversity, only CD4 T+ cell count was associated with differences in within participant community richness (observed species and phylogenetic tree, Chao1 alpha diversity metrics) (**Table 7; Figure 2**). No differences in Shannon and Simpson diversity were observed across CD4+ cell categories. The differences in alpha diversity were largely driven by differences between participants with <100 CD4+ T cells/mcL (n=8), who had markedly lower alpha diversity scores by the observed species, phylogenetic tree, and Chao1 metrics than participants with ≥100 CD4+ cells/mcL (**Table 7**). We observed no

association between weighted or unweighted beta diversity and participant characteristics, e.g. CD4+ T cell concentrations, time on ART, or parasitic infection status, as assessed visually or via PERMANOVA. PERMANOVA pseudo-F-test statistics and p-values are shown in **Table 8**. Weighted unifrac beta diversity PCoA plots are shown in **Figure 3**.

Table 7: *Fecal Microbiome α-diversity stratified by CD4+ T cells/mcL*

	<100 CD4 cells/mcL (n=8)	**100 - 199 CD4 cells/mcL (n=30)**	**200 - 349 CD4 cells/mcL (n=34)**	**350 - 499 CD4/mcL (n=80)**	**≥500 CD4 cells/mcL (n=23)**	**p-value**
Observed species, mean (SEM)[a]	349.3 (29.3)	475.5 (24.4)	498.8 (20.6)	473.9 (14.7)	498.9 (26.4)	**0.03**[*]
Whole tree Phylogenetic Diversity, mean (SEM)[b]	25.3 (1.8)	32.6 (1.2)	34.4 (1.1)	32.8 (0.8)	34.1 (1.4)	**0.02**[*]
Chao1, mean (SEM)[c]	571.5 (45.1)	765.6 (38.4)	798.9 (31.2)	755.3 (23.2)	800.5 (39.8)	**0.03**[*]
Shannon, mean (SEM)	4.56 (0.2)	5.2 (0.1)	5.3 (0.1)	5.0 (0.1)	5.0 (0.2)	0.21[*]
Simpson, median (25^{th}% – 75^{th}%)	0.89 (0.85-0.93)	0.92 (0.88-0.95)	0.93 (0.89-0.95)	0.92 (0.87-0.95)	0.90 (0.83-0.95)	0.34[**]

[a] Tukey-Kramer pairwise comparisons critical value: <100 CD4 cells/mcL vs. 100-349 CD4 cells/mcL= 4.1; <100 CD4 cells/mcL vs. >349 CD4 cells/mcL=4.0; 100-349 cells/mcL vs >349 cells/mcL= 0.5897 versus a critical value (0.05, 3, 170) = 3.34.

[b] Tukey-Kramer pairwise comparisons critical value: <100 CD4 cells/mcL vs. 100-349 CD4 cells/mcL= 4.5; <100 CD4 cells/mcL vs. >349 CD4 cells/mcL=4.3; 100-349 cells/mcL vs >349 cells/mcL= 0.61 versus a critical value (0.05, 3, 170) = 3.34.

[c] Tukey-Kramer pairwise comparisons critical value: <100 CD4 cells/mcL vs. 100-349 CD4 cells/mcL= 4.1; <100 CD4 cells/mcL vs. >349 CD4 cells/mcL=3.8; 100-349 cells/mcL vs >349 cells/mcL= 0.81 versus a critical value (0.05, 3, 170) = 3.34.

[*]ANOVA F-test p-value, adjusted for time on ART and age

[**]Kruskal-Wallis equality-of-populations rank test

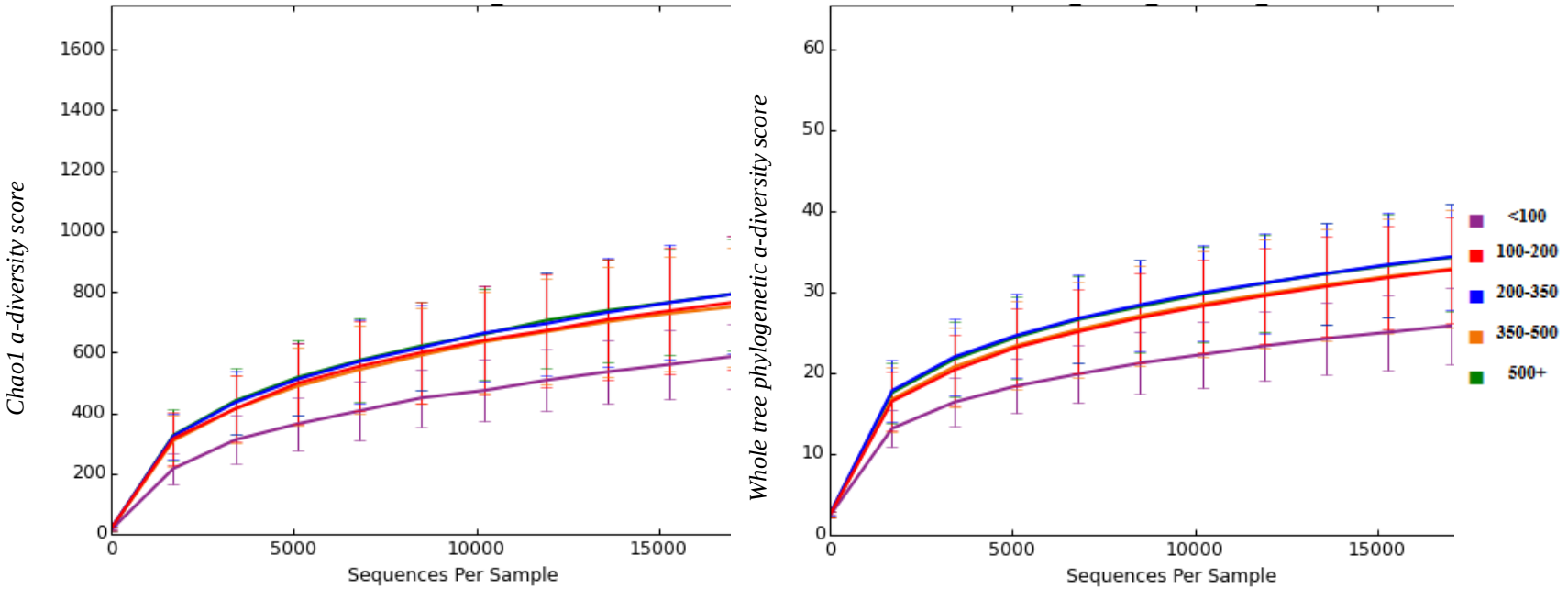


Figure 2: *Rarefaction Plots of A) Chao1 alpha diversity (left); B) Whole Tree Phylogenetic Diversity (right) by CD4+ T cells/mcL category.*

Participants with <100 CD4+ T cells/mcL (n=8) demonstrated relative reductions in alpha diversity as measured by the Chao1 and phylogenetic tree metrics, i.e. they demonstrated reductions in the richness and diversity of bacterial species relative to participants with ≥100 CD4+ T cells/mcL (n=167).

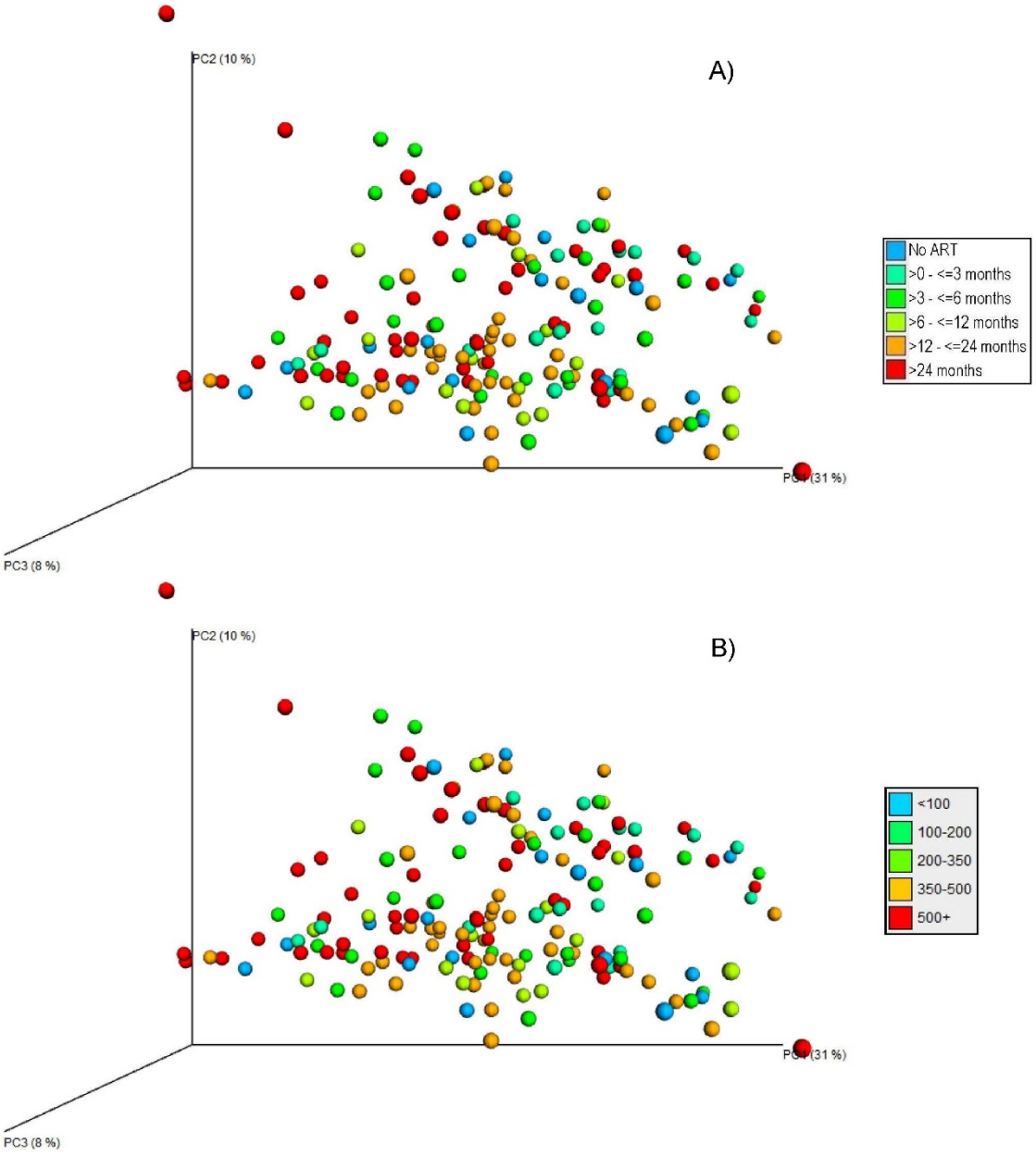


Figure 3: *Weighted unifrac beta diversity plots of A) time on ART and B) CD4+ cells/mcL among 175 HIV-infected Ugandans.*

No clustering of samples, i.e. no distinct bacterial communities, as classified by beta diversity, is observed by time on ART or clinically CD4+ T cells/mcL.

Table 8: *Pseudo-F-test statistics and p-values for PERMANOVA analyses of fecal microbiome diversity by clinical characteristics.*

Clinical Characteristic	Pseudo-F-test statistic	p-value
<100 CD4+ cells/mcL vs. ≥100 CD4+ cells/mcL	0.4914	0.822
CD4+ T cell concentrations **<100 cells/mcL** **100 to <200 cells/mcL** **200 to <350 cells/mcL** **350 to <500 cells/mcL** **≥500 cells/mcL**	1.1949	0.228
Time on ART **ART-naïve** **>0 to 3 months ART** **>3 months to 6 months** **>6 to 12 months ART** **>12 to 24 months ART** **>24 months ART**	1.4119	0.087
ART-initiated vs. ART-naïve	0.3459	0.954
Protozoa or helminth infection vs. no protozoa or helminth infection	0.9381	0.429
Hookworm infection vs. no hookworm infection	1.0669	0.361
Protozoa infection vs. no protozoa infection	0.7714	0.531

Baseline microbiome composition analyses

Cross-sectional analyses of the relative abundance of individual genera across clinical characteristics of CD4+ T cells, time on ART, and parasitic infection yielded no statistically significant correlations after adjustment for multiple comparisons, with one exception. We did observe an inverse relationship between the *Anaerococcus* genus and CD4+ T cells/mcL (Spearman correlation -0.3207; p-value=0.0000152 (**Figure 4**).

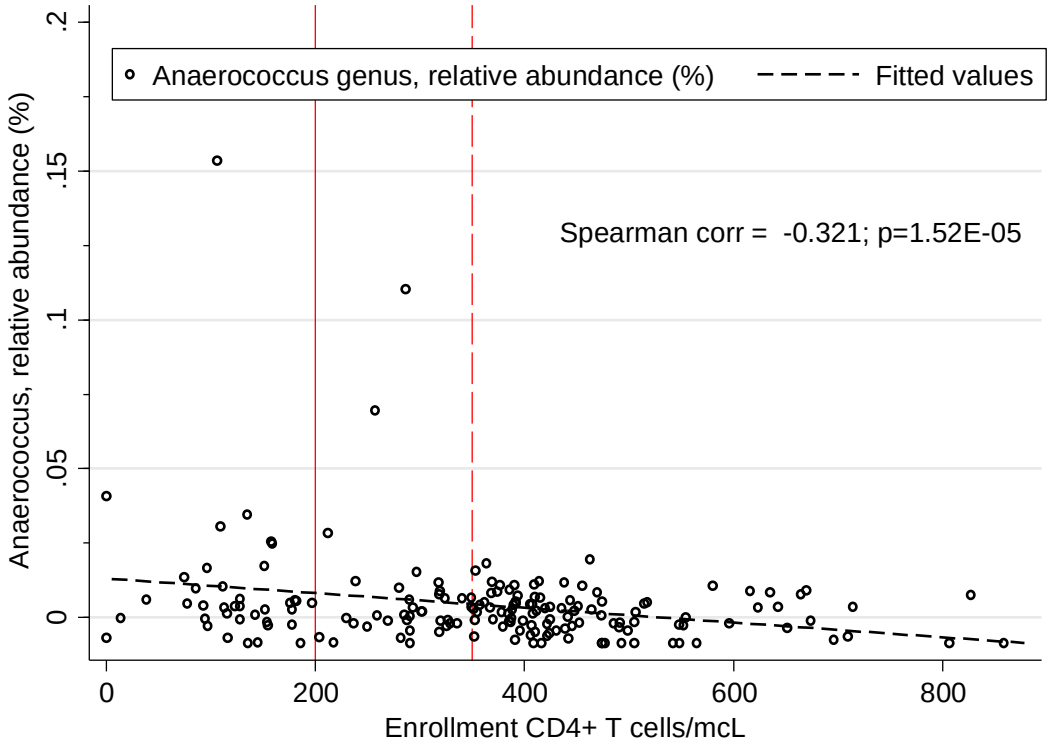


Figure 4: *Distribution of CD4+ T Cells relative to* Anaerococcus *genus relative abundance at study enrollment.*

Results of analyses of other common genera that had p-values <0.01 are presented in **Table 9**.

Table 9: *Selected results (p<0.01) from genera-level operational taxonomic unit baseline analyses.*

Genera	Direction*	p-value	B-H p-value†
Hookworm infection			
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae	H+↓	0.002488	0.000179
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Faecalibacterium	H+↓	0.0082	0.000357
p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Bulleidia	H+↑	0.009417	0.000536
Continuous CD4+ T cells/mcL			
p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Anaerococcus	CD4↑ → ↓	**1.52E-05**	**0.000179**
p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae]	CD4↑ → ↓	0.00142	0.000357
p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Finegoldia	CD4↑ → ↓	0.003105	0.000536
p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Porphyromonas	CD4↑ → ↓	0.006863	0.000714
p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g__Paraprevotella	CD4↑ → ↑	0.008509	0.000893
Helminth infection			
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae	H+↓	0.005596	0.000179
p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Bulleidia	H+↑	0.009058	0.000357
Protozoa infection			
p__Actinobacteria; unspecified family, genus	P+↓	0.006154	0.000179
Giardia intestinalis infection			
p__Actinobacteria; unspecified family, genus	P+↓	0.006388	0.000179

*Direction of bacterial relative abundance in the presence of either hookworm (H), protozoa (P) or CD4+ T cells/mcL.
†Benjamini-Hochberg adjusted comparison P-values.[182]

Longitudinal analysis – baseline composition and CD4+ T cell/mcL

All participants had a baseline CD4+ cell concentration, and 84 (48%) had one follow-up CD4+ T cell measurement, 11 (6%) had 2 follow-up CD4 measurements, and one participant had three follow-up CD4+ T cell measurements in the 12-months post enrollment. Data on the reason for CD4+ T cell assessments in the 12-months post-follow up are unknown, although standard clinical practice at the time dictated a CD4+ T cell assessment every 6 months. Results of age-, weight-, and time on ART-adjusted hierarchical models indicated that two operational taxonomic units, both *Proteobacteria*, were correlated with relative increases in CD4+ T cell concentrations over time in age, weight and time on ART-adjusted models: *Sutterella* ($\beta_{\text{relative abundance*time}}$: 38.4 cells/mcL 95%CI: 19.4, 57.5; p-value<0.001) and an unclassified genus of the *Alcaligenaceae* family ($\beta_{\text{relative abundance*time}}$: 36.5 cells/mcL, 95%CI: 17.9, 55.2; p-value<0.001). Beta coefficients from models with statistically significant interactions between genera and time were used to generate visual representations of the change in CD4+ T cells over time by percentage relative distribution (0%, 0.5%, 1%) of the two genera listed above, using mean cohort values for age (34.5 years), weight (75.9 kgs), and time on ART (1.6 years) (**Figure 5**).

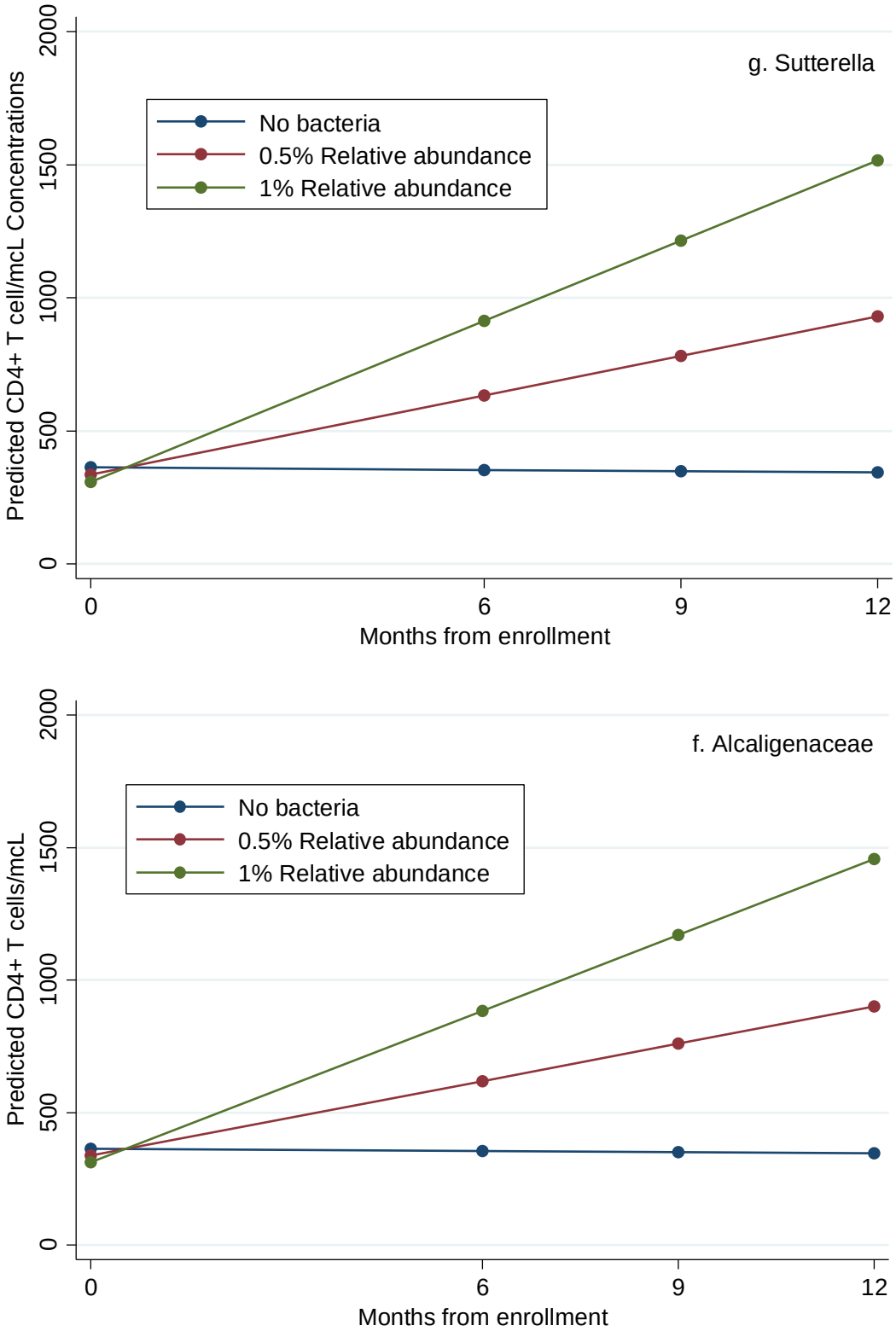


Figure 5: CD4+ T cell predictions from linear mixed model estimates at varying levels relative abundadnce of genus *Sutterella* and f. *Alcaligenaceae*, unspecified genus.

4.5 Discussion

Alpha diversity in this cohort of Ugandan adults with HIV was similar across most clinically significant levels of CD4+ T cell/concentrations, apart from persons living with AIDS and <100 CD4+ T cells/mcL. Severely immunocompromised participants demonstrated decreased overall bacterial community diversity, relative to their peers, even after adjusting for participant age. We found an inverse correlation between the relative CD4+ T cell concentrations and *Anaerococcus*, but no other baseline clinical factors were associated with differential distribution of taxa after correction for multiple comparisons. We also that found two taxa – *Sutterella* and an unspecified genus of the *Alcaligenaceae* family – were positively associated with increases in CD4+ T cell trajectory over the 12-months post-study enrollment.

Monaco *et al*. compared the fecal microbiome of 40 HIV-negative participants, 42 HIV-infected and ART-naïve participants, and 40 HIV-infected participants who had been receiving ART for a median duration of 7 years (interquartile range (IQR): 6.4 to 7.5).[164] They, too, demonstrated that the fecal microbiome composition of participants became less phylogenetically diverse with decreasing CD4+ T cell counts, and that specific bacteria, including inflammation-associated *Enterobacteriaceae*, became more abundant with decreasing CD4+ T cell counts. Second, Nowak *et al*. examined the rectal microbiota of 41 HIV-infected and untreated, 34 HIV-infected and receiving ART, and 55 HIV-uninfected men who have sex with men in Nigeria.[165] Their findings indicated that HIV infection itself did not alter the rectal microbiome, relative to their uninfected peers, but that treatment for HIV was associated with a shift towards a predominance of pathogenic bacteria.

A study evaluating the gut microbiome of healthy Ugandan controls found similarly high mean relative abundance of *Bacteroidetes* (47.4%; Standard Deviation=3.8%) and Firmicutes (41.0%; Standard Deviation=2.8%), similar to the present cohort.[183] We also confirm what has been observed in other cohorts, both African[164,165] and Western,[150,184] with respect to alpha diversity, which demonstrate lower within-community diversity and richness as immune function decreases – albeit only at the extremely low end of the immunologic spectrum here and in Monaco *et al*. However, as demonstrated in Nowak *et al*. these effects may be related to ART and cotrimoxazole therapy.[165] An increase in the potentially pathogenic *Anaerococcus* was correlated with HIV therapy in Nowak *et al*. Additionally, the presence or increases of *Anaerococcus* in semen has been linked to low sperm quality,[185] non-alcoholic fatty liver disease,[186] and it has been found in the vaginal fluid of HIV-infected women with bacterial vaginosis, whereas not in their HIV-uninfected peers.[187]

In Cameroon, *Entamoeba* protozoal infections were predictive of having a microbiome profile associated with autoimmune disorders.[168] Conversely, a cohort from Côte d'Ivoire demonstrated that *Entamoeba* or *Blastocystis hominis* colonization was associated with a higher ratio of *Faecalibacterium prausnitzii* to *Escherichia coli*, suggestive of community balance.[169] Another protozoal infection, giardiasis, was associated with gut flora dysbiosis in this cohort.[169] Helminth infection has been postulated to promote reductions in inflammation through their interaction with the gut microbiota.[170] This assertion is supported by at least two studies.[167,171] Lee *et al*. observed greater species diversity and higher relative abundance of *Paraprevotellaceae* operational taxonomic units amongst Malaysians infected with helminth species, in particular

Trichuris infection.[171] Cantacessi *et al*.'s study of experimental hookworm infection found that hookworms induced small increases in alpha diversity, but neither differences in overall community structure nor differences in the distribution of relative abundance of individual taxa were observed.[167] Our results diverge slightly from past studies, which could be attributable to multiple factors – including the general limitations of the study discussed below. It could also be due to the light infections that we observed in this cohort.

Sutterella – and generally an overabundance thereof – has been associated with a variety of disorders, from inflammatory[188,189] to Down's syndrome[190] to autism spectrum disorders,[191] yet *Sutterella* has not been previously linked to immune status. A recent paper, however, has identified *Sutterella* as a potential commensal and immunomodulator that does not significantly disrupt the epithelial homeostasis critical to inflammation control.[192] This provides a more intuitive biological basis for the relationship between increasing relative abundance of this taxa and immune recovery observed here. Nevertheless, this observation must be confirmed in future cohorts.

Additionally, the *Alcaligenaceae* family has been identified as a commensal bacterial family of the gut associated lymphatic tissues, a critical area for HIV infection.[154] As part of normal biological mechanisms to promote epithelial homeostasis, this taxon is normally contained to the underlying gut-associated lymphoid tissues, and the presence of *Alcaligenaceae* elsewhere may be pro-inflammatory in nature.[193] Thus, the association observed here with increasing CD4 over time may be indicative of a particularly robust *Alcaligenaceae* community in the gut associated lymphatic tissues,

despite displacement related to damage to gut associated lymphatic tissues. This relationship should also be investigated in future cohorts.

This study diverges from the one prior study evaluating the gut microbiome of Ugandans living with HIV, which has a similar purpose as the present study, in that Monaco *et al*. performed their analysis at the species level. This may have led to a higher number of individual species-specific relationships being elucidated as statistically significant,[164] despite the potential for misclassification at the species level.[194,195] For example, *Enterobacteria*-associated operational taxonomic units were associated with <200 CD4+ T cells/mcL versus those participants not infected with HIV, as well as those not receiving ART.

Both Nowak *et al*. and Monaco *et al*. note that cotrimoxazole primary prophylaxis may contribute to the decreased diversity in microbial communities observed in persons who have initiated HIV therapy, similarly driving the results seen herein with respect to similar levels of within-in participant diversity and abundance across many levels of CD4+ T cell concentrations. Given our knowledge of the importance of the gut microbiome's impact on human health and inflammation, the relationship between long-term cotrimoxazole use, its impact on the bacterial composition of the gut deserves new scrutiny in the era of widely available HIV chemotherapy.

While this is a relatively large cohort, the limitations of this study include the lack of a direct comparison group of HIV-uninfected persons from the same region and background. Given the variation in dietary and regional impacts on the microbiome,[196,197] it would be valuable to compare relative abundances in Uganda across HIV-infected and uninfected people. However, in comparing to Monaco *et al*. it is at least possible to view

some similarities in some alpha diversity metrics (~700 Chao1 score and ~22 in phylogenetic diversity at 5,000 sequence depth for HIV-negative and CD4>200 participants), although the different mechanisms by which these values were calculated may render this comparison less informative. It would also have been valuable to correlate soluble CD14 and other pro-inflammatory markers to support our findings, as well as additional stool samples over time within this population to support longitudinal findings. While most the CD4+ T cell concentrations in this cohort are with the healthy range, additional data related to reasons for CD4 blood would have been valuable, to help account for potential confounding by indication. Furthermore, and perhaps most importantly, we were unable to ascertain nutritional status (or dietary patterns) in these patients. It is possible that the results that we see here – and particularly the relationship between *Anaerococcus*[198] and CD4+ cells/mcL is confounded by nutritional status, which is driving CD4 depletion and bacterial community composition. It is also possible that while unique species are driving clinical and biological processes, the decision to analyze at the genera level may have diluted the effect of some of these distinct species. However, normally genera-level analyses are preferred due to the assumption that grouping taxonomically and therefore functionally similar bacteria will increase power as well as the effect, if any. It is also possible that the multiple freeze-thaw cycles contributed to overgrowth of one species versus others, so that the sample failed to accurately represent the gut environment, essentially leading to misclassification of either exposure or outcome.

The data presented herein represent the largest cohort of HIV-infected adults from Sub-Saharan Africa to participate in research on the gut microbiome, its composition, and

the relationship between that composition and clinical correlates. Genetic regional differences notwithstanding, these participants are largely generalizable to other HIV-infected persons seeking care across Uganda. These data also provide some exploratory pathways for future investigation of how the gut microbiome composition may impact CD4+ T cell immune recovery while receiving ART, which is novel for this population. Furthermore, while patterns have emerged internationally in terms of gut profile and inflammation, given the links between gut microbiome composition, microbial translocation, and subsequent inflammation in people living with HIV,[7,152,159,184,199] further research with an eye towards interventions and specific to Sub-Saharan African populations is required.

5. Aim III. Impact of anthelmintic therapy for invasive helminth infection on microbial translocation, inflammation, and immune response among Ugandans living with HIV: a randomized proof of concept study

5.1 Aim Summary

Background: Microbial translocation is considered a major driver of chronic immune activation, which is responsible for HIV disease progression. Invasive parasitic gut nematodes also induce microbial translocation. We evaluated the impact of albendazole anthelmintic therapy on serum markers of microbial translocation and inflammation among concurrently helminth- and HIV-infected Ugandans.

Methods: Participants were randomized to immediate or delayed 1200mg albendazole therapy, and followed for 1 month. Baseline stool analysis determined parasitic infection prevalence. Baseline and follow-up blood draws evaluated soluble CD14 (sCD14), C-reactive protein (CRP), and 10 pro-inflammatory cytokines. Parametric and non-parametric tests examined the change in biomarker concentrations over time and across randomization arms.

Results: We randomized 224 HIV-infected, antiretroviral therapy (ART)-experienced adults in Mbale, Uganda. 24 (10.7%) participants were infected with either *Necator americanus* or *Strongyloides stercoralis*, 12 in the immediate albendazole arm, and 12 in the delayed albendazole arm. We observed increased concentrations of CRP, interleukin (IL)-4, IL-6, IL-10, and tumor necrosis factor (TNF)-α among persons with current helminth infection relative to uninfected participants at baseline. Participants in the

immediate therapy arm had higher sCD14 concentrations at follow-up relative to participants in the delayed arm. We did not observe effects of anthelmintic therapy on any other biomarker concentrations among helminth-infected participants.

Conclusion: Increases in sCD14 post-anthelmintic therapy in this cohort require further investigation in larger cohorts and for longer follow-up durations. However, incorporating anthelmintic therapy into regular adult HIV care may provide subtle health benefits in this potentially vulnerable population.

5.2 Background

Continuous CD4+ T cell depletion and subsequent impairment of immune function is the primary causal mechanism behind HIV-related morbidity and mortality.[200] While treatment with antiretroviral therapy (ART) significantly improves quality of life and life expectancy, CD4+ T cell concentrations never fully recover from the impacts of HIV infection. This progressive CD4+ T cell depletion, while the product of complex and multifactorial biological processes, is predominantly thought to be caused by programmed cell death, in the forms of pyroptosis[6] and apoptosis, which is exacerbated by immune activation.[201]

Heightened and chronic immune activation is a hallmark of the chronic stage of HIV infection, predictive of HIV progression and negative health outcomes,[202,203] and can often persist in HIV-infected persons despite viral suppression.[4,201] Microbial translocation, the process by which microbes normally sequestered in the gut lumen, traverse the gut epithelial barrier and enter host circulation via the immune cell-rich lamina propria,[7] is considered a major driver of chronic immune activation. Early in HIV infection, even prior to seroconversion, high levels of viral replication and CD4+ T cell depletion in gut-associated lymphatic tissues are correlated with dysregulation of epithelial barrier maintenance gene expression related to epithelial barrier maintenance, and increased transcription of immune activation-, inflammation-, and apoptosis-associated genes.[204] These processes induce damage to the gut epithelial barrier, and in turn allows for sustained high levels of microbial translocation.[5,205] Microbial translocation markers, lipopolysaccharide (LPS)[199] and soluble CD14 (sCD14),[206] have been independently linked to accelerated HIV disease progression and increased

mortality, respectively. LPS is a Gram-negative bacteria cell wall component; and sCD14 is a marker of actual monocyte activation in response to LPS, and correlated with LPS concentrations.[7]

Invasive soil transmitted helminths, i.e. those that burrow into the gut mucosa, such as hookworm species, *Strongyloides stercoralis*, and *Trichuris trichiura*, also damage the gut epithelial layer and contribute to microbial translocation.[121,207-209] To date, only two studies in humans have investigated the relationship between these invasive soil-transmitted helminths, microbial translocation, and pro-inflammatory responses.[121,207] George *et al*. found that hookworm infection was associated with increases in concentrations of microbial translocation markers LPS and sCD14, and increases in the anti-inflammatory cytokine interleukin (IL)-10.[121] A study investigating the change in microbial translocation biomarkers, acute-phase proteins, and inflammatory biomarkers after treatment for *S. stercoralis* observed decreases in all of these markers post-therapy, suggesting that infection was associated with a pro-inflammatory immune response.[207]

However, even while helminths cause damage to the gut mucosa and are associated with increases in microbial translocation marker concentrations, these highly potent immunomodulators[210,211] have been shown to simultaneously downregulate the expression of pro-inflammatory cytokines, which could off-set any deleterious effects of increases in LPS and sCD14.[96,115,212] Indeed, the same study by George *et al*. also found no difference in tumor necrosis factor (TNF)-α or type-I T helper cell (Th_1) response cytokines interferon (IFN)-γ or IL-12 concentrations between hookworm-infected and hookworm-uninfected persons.[121] Furthermore, concentrations of the pro-inflammatory

cytokine IL-17 were decreased in hookworm-infected relative to their hookworm-uninfected peers.[121] Should helminth infection increase concentrations of microbial translocation biomarkers, the extent to which anti-inflammatory immune responses mitigate the adverse immune activating effects of helminth infection among people living with HIV is unknown.

To date, two human studies have addressed the mutual effects of parasitic infection and microbial translocation, with varied results.[121,207] Furthermore, the relationship between helminths and microbial translocation has not been examined in humans living with HIV, who demonstrate differential responses to immune challenges as a hallmark of their disease. Finally, this relationship has not been evaluated in a setting where intestinal helminth and HIV co-infection is highly prevalent, such as Sub-Saharan Africa.[42,213]

This randomized trial examines the short-term impact of one-time anthelmintic treatment on microbial translocation and inflammatory processes in the HIV- and helminth-infected human host. Our primary hypothesis is that helminth-infected participants receiving immediate anthelmintic therapy will have decreased circulating concentrations of microbial translocation marker, soluble CD14 (sCD14) and non-specific pro-inflammatory biomarker, C-reactive protein (CRP), relative to participants in the delayed therapy arm at 1-month of follow-up. We also hypothesize that concentrations of pro-inflammatory cytokines interferon (IFN)-γ, interleukin (IL)-10, IL-12, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8, tumor necrosis factor (TNF)-α will be higher among helminth-infected participants randomized to immediate therapy at 1-month follow-up, relative to helminth-infected participants randomized to the delayed. This

second hypothesis is premised on the idea that helminths exert a dampening effect on inflammatory processes, and that helminth infection has some protective – and therefore beneficial – properties against inflammation. Enrolled participants were randomized to either immediate (same day as enrollment) or delayed (at the three-month visit) receipt of albendazole therapy at a 1:1 ratio.

5.3 Methods

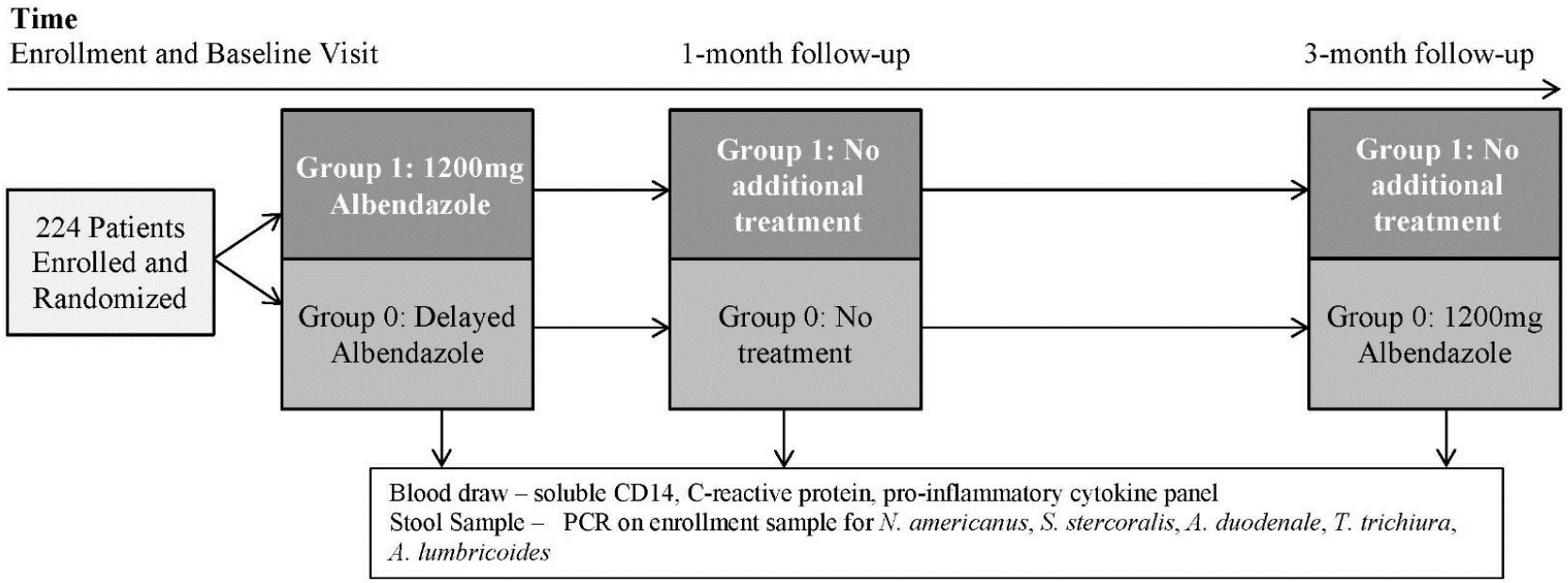
5.3.1. Ethics Statement

Written informed consent was provided by all participants. The AIDS Support Organisation (TASO), University of Minnesota, and the Uganda National Council of Science and Technology institutional review boards approved this protocol. All participants received albendazole at least once during the trial. No clinically indicated albendazole or other deworming medication was withheld from participants.

5.3.2 Study participants and study design

From June 2015 through September 2015, we screened outpatients at The AIDS Support Organisation (TASO)'s HIV care clinic in Mbale, Uganda for study participation. Demographic data and biological samples (stool and blood) were collected at the patient's regular outpatient ART clinic, at study enrollment, 4-weeks, and 12-weeks post-enrollment. However, only baseline and 4-week samples were considered in these analyses. Parasitic infection status was unknown at randomization. Albendazole was administered as a 1200mg cumulative dose over 3 days, with the first dose (400mg) received in clinic as directly observed therapy. No placebo was administered during the trial. The study design is illustrated in **Figure 6**.

Figure 6: *Diagram of enrollment and visit flow for a randomized control trial of immediate versus delayed albendazole among HIV- and helminth-infected Ugandans*



Study inclusion criteria were age ≥18 years, known HIV-infection, as determined by Ministry of Health HIV testing algorithm, and receipt of ≥3 months of antiretroviral therapy (ART). Participants were excluded if they had received albendazole or similar anthelmintic treatment in the past 4 weeks, demonstrated allergies or other contraindications to albendazole, were experiencing any unresolved opportunistic infections including tuberculosis; were pregnant; or were unable to provide informed consent.

5.3.3 Clinical Evaluation

Participants were evaluated at enrollment for World Health Organization HIV clinical stage, which includes opportunistic infection screening, by a study clinical officer. This classification is detailed in **Appendix 4**. They were screened for new infections at each study visit thereafter. The clinical officer reviewed the participant chart for HIV diagnosis date, trimethoprim-sulfamethoxazole (cotrimoxazole) or dapsone primary prophylaxis initiation date, ART initiation date and current regimen, and any history of tuberculosis. Body mass index (BMI; kg/m^2) and ART adherence (pill counts) were evaluated by the clinical officer at each visit. During follow-up visits, participants were asked if they had received anthelmintic chemotherapy in the interim since the last visit, to gauge any deviations from the randomization arm interventions. Finally, symptoms of possible gastrointestinal parasite/helminth infections were assessed at each visit, including: diarrhea, bloating, stomach pain, weakness, lethargy, and blood in stool.

5.3.4 Parasitologic evaluation

A single self-collected stool sample was collected in the clinic at baseline and each follow-up visit in a polypropylene collection container (Globe Scientific, Paramus, New Jersey) and stored in this same container for a maximum of 2 hours at room temperature before freezing at -20 °C. Samples were moved to -80 °C storage for up to 3 months before assessing helminth infection via molecular methods. Low-reaction volume multi-parallel real-time polymerase chain-reaction (PCR) was used to determine presence or absence of infection with soil transmitted helminth species *Necator americanus* and *Ancylostoma duodenale* (hookworm species), *Ascaris lumbricoides*, *Trichuris trichiura*, and *Strongyloides stercoralis* per previously described methods.[42,103] Species-specific primer and FAM-labeled minor groove binder probe sequences are listed in **Appendix 5**. All unknown samples were tested in duplicate, and samples with cycle threshold (C_t) values >38 were considered negative. Helminth infection at baseline was determined after randomization.

5.3.5 Evaluation of microbial translocation and pro-inflammatory biomarkers

We measured microbial translocation marker sCD14 concentrations in serum samples via ELISA assay (Human sCD14 Quantikine ELISA kit, R&D Systems, Minneapolis, MN) per the manufacturer's instructions at a dilution factor of 1000. The minimum detectable dose of human sCD14 is 0.000125 µg/mL. C-reactive protein (CRP) concentrations were measured via V-PLEX assay (V-PLEX Human CRP Kit, K151STD-1, Meso Scale Diagnostics, Rockville, MD) per the manufacturer's instruction. Serum concentrations of 10 pro-inflammatory human cytokines – interferon (IFN)-γ, interleukin (IL)-10, IL-12, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8, tumor necrosis factor (TNF)-α – were

also measured via V-PLEX assay (V-PLEX Human Proinflammatory Panel, K15049D-1, Meso Scale Diagnostics, Rockville, MD), per the manufacturer's instructions. Serum biomarkers concentrations were evaluated at enrollment and 1-month follow-up. Evaluation of biomarkers at 1-month follow-up was chosen because changes in biomarker values if any were likely able to be observed post-therapy,[214-217] and re-infection risk is minimized in this uncontrolled and endemic setting.[53] Biomarker concentrations at additional time points may be explored at a later date.

5.3.6 Statistical Analyses

Given that our primary hypothesis was to measure the impact of anthelmintic therapy on inflammation and microbial translocation among participants with known parasitic infection, participants who tested negative for all five species in question were excluded from the primary analyses. From the enrollment stool assessment, 24 participants were infected with ≥1 helminth species (12 in the immediate and 12 in the delayed arm). Two of these participants (delayed arm) were lost-to-follow-up before the 1-month visit. To evaluate change over time across arms, t-tests of the difference in baseline and 1-month follow-up biomarker concentrations were performed, with unequal variances as needed (IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-13).[218]

Given the unexpectedly low observed helminth prevalence (~10% instead of the expected 30%), we also analyzed these data via non-parametric tests. Specifically, we evaluated non-transformed biomarker concentrations across randomization arms at 1-month follow-up via Monte Carlo permutation tests.[219] The z statistic from the observed data was compared against permutation distribution of z statistics from the randomly

permuted data (n=10,000), and p-values (and 95%CI) were calculated from the number of time that that observed z statistic was as or more extreme than the permuted z statistic. These analyses were performed in Stata/SE 14.2 (StataCorp, College Station, TX, USA) using the "permute" command.

Due to a possible imbalance in duration of ART across arms, we elected to perform *a posteriori* CD4+ T cell-adjusted linear regression on the differences in biomarker concentrations over time, with a robust covariance estimator. CD4+ was selected instead of ART duration because CD4+ is – in part – an effect of ART duration, and CD4+ T cell concentrations account for potential confounders that could be related to time on ART, such as time from HIV diagnosis to therapy initiation, and overall immune status.

As a reference, we performed the same t-tests of difference on the entire available cohort of 74 baseline positive and negative participants. Finally, we assessed the change in gastrointestinal symptom prevalence between enrollment and 1-month follow-up among those infected with helminths across randomization arm. All statistical analyses were conducted in Stata 14.2 (StataCorp, College Station, TX, USA) against an alpha level of 0.05.

5.4 Results

From June 2015 through September 2015, 231 people were screened for enrollment in the study. Five people were excluded due to insufficient duration on ART (n=1) or incarceration (n=4). Two declined participation. We enrolled 224, and

randomized 112 to the immediate albendazole intervention and 112 to delayed albendazole therapy (CONSORT diagram featured in **Figure 7**).[220]

Necator americanus and *Strongyloides stercoralis* were the only species, of the 5 assayed, found in this cohort (**Table 10**). Of the 25 (11.2%) enrolled participants who were found to be infected with ≥1 soil transmitted helminth species, 12 (48%) were randomized to immediate anthelmintic chemotherapy, and 12 (48%) were randomized to delayed anthelmintic chemotherapy. One person with helminth infection who was randomized to the delayed arm was not included in the analyses due to insufficient serum sample volume. Of the 199 participants who were not infected with any soil transmitted helminth species, 54 were randomly selected and included in secondary analyses. Of these 27 (50%) had been randomized at enrollment to immediate anthelmintic chemotherapy, and 27 (50%) to delayed anthelmintic chemotherapy.

Participant demographics are described in **Table 10**, and illustrate similarities across randomization groups among persons infected with helminths, apart from a possible imbalance in duration of ART. The immediate albendazole arm had a median duration of 37 months of ART [IQR: 13, 64], whereas the delayed albendazole arm had a median duration of 15 months [IQR: 13, 32]. The median ART duration of participants in this cohort was 26 months [IQR: 14, 44]). Despite medium- to long-term ART use and reported high adherence, these participants represent the spectrum of immunocompetence. Median CD4+ T cells in this cohort were 409 cells/mcL (IQR: 331, 570; min. 45; max. 1092), with no differences across helminth infection status. This cohort was 70.5% (55/78) female, with a median age of 42 years (IQR: 37, 51). The median participant body mass index (BMI) was 22.1 kg/m^2 [IQR: 19.8, 24.8]. Among

participants with helminth infection, non-specific gastrointestinal symptoms were reported in 33.3% (8/24) of participants upon enrollment physical exam; bloating was reported most frequently (25.0%; 6/24), followed by diarrhea (12.5%; 3/24), and stomach pain (8.3%; 2/24).

Figure 7: *CONSORT Diagram of randomized control trial evaluating the impact of immediate versus delayed 1200mg albendazole in people living with HIV in Mbale, Uganda*

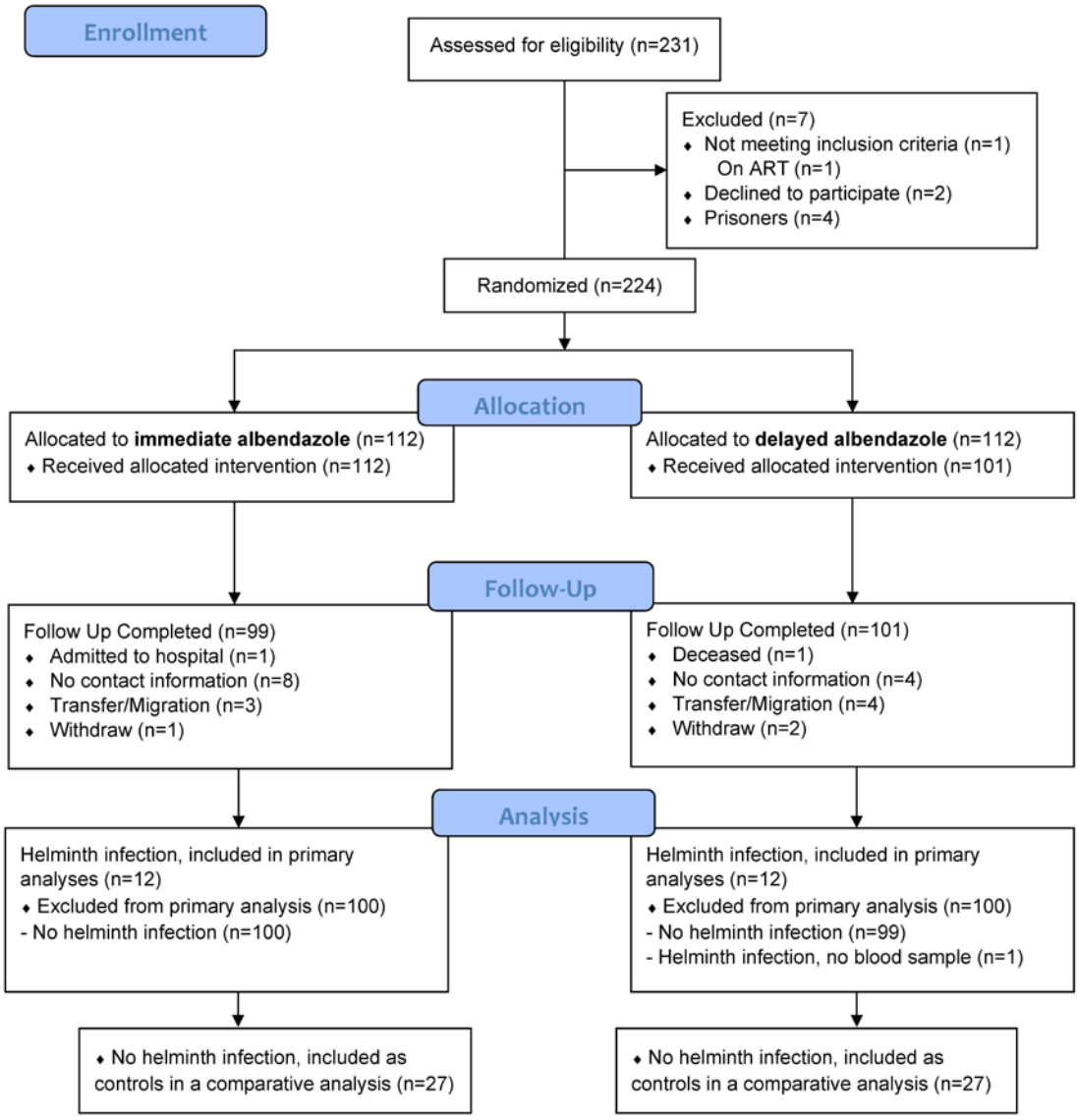


Table 10: *Demographic Characteristics of Participants with Helminth and without Helminth Infection, by Randomization Arm*

	Immediate Anthelmintic Therapy		**Delayed Anthelmintic Therapy**	
	Helminth infection (n=12)	**No helminth infection (n=27)**	**Helminth infection (n=12)**	**No helminth infection (n=27)**
	Median [IQR] or n (%)	Median [IQR] or n (%)	Median [IQR] or n (%)	Median [IQR] or n (%)
Demographic Characteristics				
Age, years	42 [36, 51]	43 [37, 52]	46 [36, 58]	42 [37, 47]
Women, %	8 (67%)	20 (74%)	9 (75%)	18 (66.7%)
Clinical Characteristics				
BMI (kg/cm^2)	22.0 [20.2, 23.5]	21.4 [19.5, 25.1]	21.2 [19.0, 23.9]	23.1 [20.1, 26]
WHO Stage III/IV, %	1 (8.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
CD4+ T cells/mcL	457 [296, 563]	470 [334, 668]	447 [341, 719]	332 [186, 545]
Months from HIV diagnosis	75 [32, 105]	29 [22, 104]	58 [31, 145]	89 [24, 128]
Months on ART	37 [13, 64]	25 [14, 44]	15 [13, 32]	28 [15, 51]
ART adherence, %	95 [95, 95]	95 [95, 95]	95 [95, 95]	95 [95, 95]
Receiving cotrimoxazole	12 (100%)	27 (100%)	12 (100%)	27 (100%)
Current Gastrointestinal Symptoms				
≥1 symptom	4 (33%)	10 (37.0%)	4 (33%)	8 (29.6%)
Diarrhea	2 (16.7%)	1 (3.7%)	1 (8.3%)	1 (3.7%)
Bloating	3 (25%)	9 (33.3%)	3 (25%)	4 (14.8%)
Stomach pain	1 (8.3%)	7 (25.9%)	1 (8.3%)	6 (22.2%)
Weakness/Lethargy	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Blood in stool	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Parasitic Infection[±]				
A. lumbricoides	0 (0%)	0 (0%)	0 (0%)	0 (0%)
A. duodenale	0 (0%)	0 (0%)	0 (0%)	0 (0%)
N. americanus	6 (50.0%)	0 (0%)	9 (81.8%)	0 (0%)
S. stercoralis	6 (50%)	0 (0%)	5 (41.7%)	0 (0%)
T. trichiura	0 (0%)	0 (0%)	0 (0%)	0 (0%)

[±] Overall prevalence rate was 11.2% (25/224). Two participants were infected with both hookworm and *S. stercoralis* species. One additional person listed as positive in the overall cohort, but not included in analyses due to lack of blood sample.

Results among helminth infected persons

Among participants infected with helminths, sCD14 concentrations at baseline were median 1.44 µg/ml [IQR: 1.05, 2.31] overall. sCD14 was 1.31 µg/ml [IQR: 0.91, 2.18] in helminth-infected participants randomized to immediate albendazole, and 1.76 µg/ml [IQR: 1.16, 2.56] among helminth-infected participants randomized to delayed albendazole (**Table 11**). CRP was elevated (≥5 mg/L)[221] in 58% (14/24) of this cohort. Among helminth-infected participants, median CRP concentrations were higher in the delayed albendazole arm (median 15.7 mg/L; IQR: 3.5, 111.6) than the immediate albendazole arm (median 11.3 mg/L; IQR: 3.5, 32.5). Cytokine concentrations were mostly equivalent across randomization arms (**Table 11**) among participants infected with helminths, although IL-2, IL-6, and IL-8 were slightly elevated in the immediate albendazole arm.

Table 11: *Baseline Biomarker Values Across Immediate vs. Delayed Treatment Group among Helminth-infected Participants*

Biomarker	**Delayed Therapy (n=12)**	**Immediate Therapy (n=12)**
	Median [IQR]	Median [IQR]
sCD14, µg/ml	1.76 [1.16, 2.56]	1.31 [0.91, 2.18]
CRP, mg/L	15.7 [3.5, 111.6]	11.3 [3.5, 32.5]
IFN-γ, pg/mL	4.1 [2.5, 8.4]	8.3 [3.0, 22.5]
IL-1β, pg/mL	0.1 [0.1, 0.2]	0.1 [0.1, 0.3]
IL-2, pg/mL	2.6 [1.5, 8.3]	4.1 [1.7, 6.7]
IL-4, pg/mL	<0.1 [<0.1, 0.1]	<0.1 [<0.1, 0.1]
IL-6, pg/mL	2.6 [1.5, 8.3]	4.1 [1.7, 6.7]
IL-8, pg/mL	98.5 [60.6, 252.1]	144.6 [64.5, 358.3]
IL-10, pg/mL	0.6 [0.4, 1.7]	0.6 [0.3, 0.8]
IL-12, pg/mL	0.3 [0.1, 0.6]	0.2 [0.1, 0.5]
IL-13, pg/mL	0.9 [0.2, 2.0]	1.1 [0.7, 1.6]
TNF-α, pg/mL	5.4 [4.1, 12.4]	6.0 [5.0, 9.3]

All analyses were conducted as intention to treat. No participants reported taking anthelmintic medication in the period between enrollment and 1-month follow-up. Two participants in the delayed albendazole arm were lost to follow-up in the first month, and were unable to be included in tests of differences in means at one month. Serum biomarker concentrations by arm among helminth infected persons at follow-up are shown in **Table 12**. Results from t-tests of the difference in serum biomarker concentrations from baseline to 1-month follow-up across randomization arms are shown in **Table 13**. Follow-up showed higher concentrations of sCD14 at 1-month follow-up in immediate therapy participants versus delayed (mean difference in sCD14 increase 1.40 µg/ml; 95% CI: -0.17, 2.98; p-value=0.08; non-parametric permutation p-value=0.05; 95%CI: 0.05, 0.06; **Table 13**; **Figure 8**). CD4+ T cell-adjusted linear regressions indicated slightly pronounced effects of therapy relative to the t-test of the difference. CD4-adjusted linear regression indicated that the difference in sCD14 change over time across arms was 1.51 µg/mL (95%CI: 3.00, 0.02) higher in participants randomized to immediate therapy, versus those randomized to delayed therapy. Additionally, the change across arms for TNF-α became more extreme, with participants receiving therapy at baseline having 11.7 pg/mL (95%CI: -0.85, 24.3) greater change in TNF-α concentrations over time than those randomized to delayed therapy, despite this relationship not being statistically significant. No other relationships were observed.

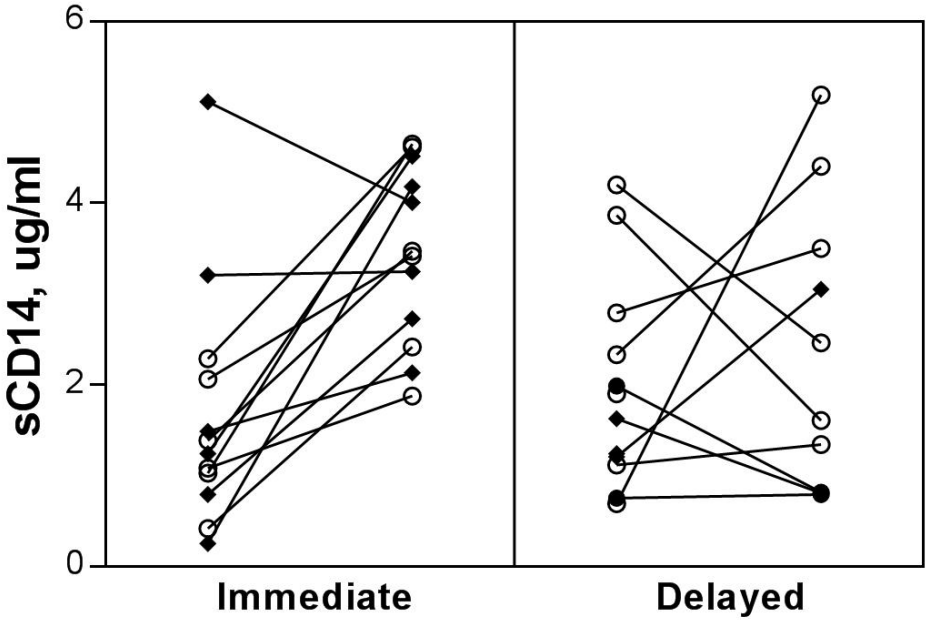
Table 12: *Inflammation-related biomarkers by early versus delayed albendazole administration helminth infected participants, at 1-month follow-up*

	Biomarker Concentration at 1-month follow-up	
Biomarker	Delayed Therapy Arm at 1-month (n=10)	Immediate Therapy Arm at 1-month (n=12)
	Median [IQR]	Median [IQR]
sCD14, µg/ml	2.04 [0.82, 3.50]	3.45 [2.57, 4.35]
CRP, mg/L	14.1 [2.2, 96.5]	6.34 [2.26, 40.6]
IFN-γ, pg/mL	3.27 [2.35, 5.11]	7.46 [2.96, 11.71]
IL-1β, pg/mL	0.93 [0.73, 1.14]	0.85 [0.25, 1.72]
IL-2, pg/mL	0.22 [0.19, 0.48]	0.53 [0.34, 0.71]
IL-4, pg/mL	0.03 [<0.01, 0.03]	0.03 [0.03, 0.04]
IL-6, pg/mL	11.3 [9.4, 15.8]	10.4 [5.5, 20.0]
IL-8, pg/mL	459.1 [353.1, 1046.7]	448.1 [193.1, 846.5]
IL-10, pg/mL	0.57 [0.43, 3.10]	0.76 [0.57, 1.26]
IL-12, pg/mL	0.45 [0.30, 0.61]	0.58 [0.19, 0.61]
IL-13, pg/mL	2.06 [1.30, 2.55]	1.87 [1.30, 2.52]
TNF-α, pg/mL	1.81 [1.00, 2.04]	13.33 [10.34, 31.25]

Table 13: *Change in biomarker concentration from enrollment to 1-month follow-up across arms, among participants infected with ≥1 helminth species*

	Mean Change		**Difference in Change by Arm**	
Biomarker	Delayed Therapy Arm (n=10)	Immediate Therapy Arm (n=12)	Paired t-test results	Wilcoxon rank-sum permutation
	Mean (95% CI)	Mean (95% CI)	Mean (95%CI)	p-value (95%CI)
sCD14, µg/ml	0.34 (-1.12, 1.80)	1.74 (0.80, 2.69)	1.40 (-0.17, 2.98)	0.06 (0.05, 0.06)
CRP, mg/L	-8.9 (-63.0, 45.3)	7.1 (-35.1, 49.3)	15.9 (-47.1, 79.1)	0.76 (0.75, 0.77)
IFN-γ, pg/mL	-59.2 (-181.7, 63.3)	14.0 (-32.5, 60.4)	73.1 (-40.3, 186.6)	0.48 (0.47, 0.49)
IL-1β, pg/mL	0.87 (0.20, 1.54)	0.74 (-0.53, 2.01)	-0.13 (-1.57, 1.31)	1.00 (0.99, 1.00)
IL-2, pg/mL	0.33 (-0.03, 0.70)	0.67 (-0.61, 1.95)	0.34 (-1.02, 1.71)	0.81 (0.80, 0.82)
IL-4, pg/mL	-0.03 (-0.06, <-0.01)	-0.02 (-0.04, 0.01)	0.01 (-0.02, 0.05)	0.34 (0.33, 0.35)
IL-6, pg/mL	8.3 (0.6, 16.0)	20.0 (-17.2, 57.2)	11.7 (-27.6, 51.0)	0.81 (0.80, 0.82)
IL-8, pg/mL	491.3 (115.8, 866.8)	90.11 (-651.3, 831.5)	-401 (-1236, 433)	0.45 (0.44, 0.46)
IL-10, pg/mL	2.2 (-1.5, 5.8)	0.1 (-0.5, 0.8)	-2.1 (-5.2, 1.1)	0.52 (0.51, 0.53)
IL-12, pg/mL	0.06 (-0.21, 0.32)	0.18 (-0.04, 0.41)	0.12 (-0.20, 0.45)	0.58 (0.57, 0.59)
IL-13, pg/mL	1.62 (-0.16, 3.40)	0.55 (-0.09, 1.19)	-1.06 (-2.70, 0.57)	0.34 (0.33, 0.35)
TNF-α, pg/mL	3.5 (-3.6, 10.6)	13.5 (3.0, 24.1)	10.0 (-2.5, 22.5)	0.25 (0.24, 0.26)

Figure 8: *Change in soluble CD14 µg/ml by immediate versus delayed albendazole randomization arms among participants with helminth infection at baseline.*



● = *N. americanus* and *S. stercoralis* co-infection; ○ = *N. americanus* only;
♦ = *S. stercoralis* only

Species-specific analyses indicated that the increase in sCD14 across randomization arms was higher among participants who were infected with *Strongyloides* only (n=8; mean sCD14 increase 19.5 µg/ml, 95%CI: -16.7, 60.3) versus those infected with hookworm species (n=12; mean sCD14 increase 6.1 µg/ml, 95%CI: -5.5, 17.8), although these values had wide confidence intervals and were not significantly different. No other biomarker concentrations were different across randomization arms.

Finally, among participants with helminth infection, there was a 28.8% (95% CI: 8.0%, 49.6%) absolute decrease in self-reported gastrointestinal symptoms between enrollment and follow-up. Among participants in the delayed albendazole arm, there was a 23% (95%CI: -9.2, 55.8%) absolute decrease in gastrointestinal symptoms; and among

participants in the immediate albendazole arm, there was a 33.3% (95%: 6.7%, 60.0%) absolute decrease in gastrointestinal symptom prevalence.

Results among helminth infected and helminth uninfected persons

We also examined the change in biomarker concentration over time in the entire available cohort (n=78; n helminth=24; n no helminth=54) by arm. Two additional participants were lost to follow-up in the helminth-uninfected group, resulting in 37 participants in the immediate albendazole are, and 37 in the delayed albendazole arm. We found no difference in change over time across randomization arms (**Table 14**) when examining the entire cohort with available data.

Table 14: *Change in biomarker concentration from enrollment to 1-month follow-up across arms, among helminth-infected and helminth-uninfected participants*

	Mean Change		**Difference in Change by Arm**
Biomarker	Delayed Therapy Arm (n=37)	Immediate Therapy Arm (n=37)	Paired t-test results
	Mean (95% CI)	Mean (95% CI)	Mean (95%CI)
sCD14, µg/ml	0.33 (-0.27, 0.92)	0.87 (0.23, 1.50)	0.54 (-0.32, 1.40)
CRP, mg/L	3.2 (-16.3, 22.6)	-6.7 (-23.7, 10.3)	-9.8 (-35.2, 15.6)
IFN-γ, pg/mL	-9.8 (-41.3, 21.7)	5.5 (-8.2, 19.3)	15.3 (-18.5, 49.2)
IL-1β, pg/mL	1.06 (0.65, 1.48)	0.90 (0.35, 1.45)	-0.17 (-0.85, 0.51)
IL-2, pg/mL	0.29 (0.08, 0.49)	0.43 (0.04, 0.82)	0.14 (-0.29, 0.58)
IL-4, pg/mL	-0.01 (-0.02, <-0.01)	-0.01 (-0.02, <-0.01)	<0.01 (-0.01, 0.01)
IL-6, pg/mL	17.7 (8.7, 26.7)	16.4 (3.2, 29.6)	-1.3 (-17.0, 14.4)
IL-8, pg/mL	634 (355, 913)	363 (62, 664)	-271 (-674, 133)
IL-10, pg/mL	4.19 (-0.25, 8.63)	1.69 (-0.50, 3.87)	-2.50 (-7.37, 2.36)
IL-12, pg/mL	0.20 (0.08, 0.33)	0.24 (0.12, 0.35)	0.03 (-0.13, 0.20)
IL-13, pg/mL	1.46 (0.81, 2.11)	1.03 (0.60, 1.46)	-0.43 (-1.20, 0.34)
TNF-α, pg/mL	6.7 (2.8, 10.7)	12.7 (6.1, 19.3)	6.0 (-1.6, 13.5)

Helminth infected versus helminth uninfected persons

As a reference, we also examined the difference in biomarker concentrations at baseline between participants with and without helminth infection. **Table 15** shows cytokine concentrations by helminth infection status, indicating relatively higher concentrations in CRP, IL-4, IL-6, IL-10, and TNF-α among persons with current helminth infection relative to uninfected participants at baseline.

Table 15: *Baseline biomarker values across participants with helminth infection versus no helminth infection*

Helminth uninfected persons

As a final reference point, we also summarized the change in biomarker concentrations over time for participants who did not receive albendazole and who were

not found to be infected with hookworm at baseline. These results (**Table 16**) indicate variability in biomarker concentrations over time in the absence of an intervention.

Table 16: *Acute-phase proteins, pro-inflammatory cytokines between baseline and follow-up among helminth-uninfected participants randomized to delayed albendazole therapy.*

5.5 Discussion

This randomized trial attempted to demonstrate that invasive intestinal helminths were associated with increases in microbial translocation and cytokine response in persons co-infected with HIV, and that albendazole intervention would reduce circulating markers of microbial translocation and impact of pro-inflammatory cytokine concentrations. Contrary to our expectation, we observed a marked increase in sCD14 concentrations among helminth-infected participants who were randomized to immediate albendazole, relative to their peers in the delayed albendazole arm (1.50 μg/ml higher mean concentration; 95%CI: -0.17, 2.98; p-value=0.06). Change over time in sCD14 concentrations was also significant in this cohort. We observed elevated concentrations of CRP, IL-4, IL-6, IL-10, and TNF-α at enrollment among helminth infected participants versus their uninfected peers. However, apart from sCD14, we failed to observe any statistically significant effects of albendazole therapy on serum biomarker concentrations across randomization arms among hookworm- and *Strongyloides*-infected participants.

Our results align with some studies, which show increases in sCD14 and TNF-α, along with other pro-inflammatory cytokines after therapy for strongyloidiasis.[212] Unlike George *et al*., Anuradha *et al*. found that therapy removed the down-regulation of inflammatory processes. This is consistent with the idea that helminths hypo-modulate the immune system. In this sense, our results (increasing sCD14 and TNF-α) post therapy are logical results of removing an inflammation suppressing organism.

To our knowledge, this is the first study to directly evaluate the effect of albendazole therapy on markers of microbial translocation and systemic inflammation using a control arm. It is also the first study to evaluate the relationship between

anthelmintic therapy and these biomarkers – particularly sCD14 – in people living with HIV in a low-resource context, which is a critical population to evaluate given that the global burden of HIV and helminths are predominantly in Sub-Saharan Africa.[29,222] The two prior studies evaluating the relationship between helminth infection and microbial translocation focused on a particular pathogen each – hookworm species and *Strongyloides* – and provided treatment for all infected participants.[121,207] While both studies included an exhaustive analysis of biomarkers, neither study included any participant controls, i.e. helminth infected participants that did not receive therapy, to guard against conflating a causal relationship of therapy with other factors that influence these biomarkers to change over time. Indeed, in the current randomized trial, we observed changes in multiple cytokine concentrations (IL-1β, IL-13, IL-6, IL-8, IL-2, TNF-α, IL-12, IL-5) between enrollment and follow-up among participants who were not identified as having helminth infection and randomized to delayed albendazole.

Rajamanickam *et al*. evaluated microbial translocation markers, acute phase proteins, inflammatory markers, and proinflammatory cytokines in participants that were infected with *S. stercoralis*.[207] They collected data from 58 individuals (30 with asymptomatic strongyloidiasis, 28 uninfected healthy adult controls from the same area), and followed those infected with *S. stercoralis* for 6 months. The authors made a compelling case for the relationship between elevated levels of acute-phase proteins and inflammation markers and strongyloidiasis via exhaustive biomarker assessments. This study was, however, limited by lack of a comparison group, the absence of infection burden information to evaluate a potential dose-response, and the absence of correction for multiple comparisons.

George *et al*. conducted a similar study focusing on hookworm infection, which included 46 hookworm-infected adults and 45 healthy controls from the same communities. Infected participants were followed for three months post-anthelmintic therapy.[121] Their findings indicated that while hookworm infection is indeed characterized by increased concentrations of microbial translocation markers, hookworm did not appear to induce acute-phase proteins, e.g. CRP, or pro-inflammatory cytokine IL-17,[223] contrary to the study focused on strongyloidiasis. The authors noted that hookworm infection was associated with increased concentrations of IL-10, a potent anti-inflammatory cytokine that mediates the host's immune response to pathogens while promoting tissue homeostasis and preventing damage.[224] George *et al*. also found that hookworm infection was associated with diminished concentrations of CD8+ T cells, CD4+ and CD8+ effector memory T cells, and diminished proportions of plasmacytoid and myeloid dendritic cells, which are associated with pro-inflammatory responses to infection.[225] Taken together, these finding may have particular relevance to HIV-infected persons, who could benefit from systemic anti-inflammatory effects.

These anti-inflammatory effects may become clinically important when viewed through the lens of host response to other HIV-associated illnesses, e.g. tuberculosis. A second paper from George *et al*. investigated the impact of strongyloidiasis on tuberculosis severity in HIV-uninfected persons, and demonstrated that participants with active tuberculosis who are also infected with *S. stercoralis* have decreased concentrations of systemic immune activation markers (sCD14 and others) and acute phase proteins (CRP and others), as well as other markers of pro-inflammatory immune response. The lack of follow-up and intervention limits our ability to draw causal

inference from these data, which the authors themselves acknowledge. However, they postulate that strongyloidiasis decreases pro-fibrotic factor production and other inflammatory factors, and thus may decrease tuberculosis severity via decreased lung pathology.[10] Boef and colleagues also investigated the relationship between current helminth infections and immune responsiveness in a rural Ghanaian population, as measured by pro-inflammatory, regulatory, and Type 2 cytokine responses.[226] They found no relationship between *N. americanus* and cytokine concentrations, and particularly of note, no relationship between helminth infection and IL-10. While these results were surprising, the authors postulated that this lack of response could be attributed to their inability to assess infection duration.

Clinically relevant changes in sCD14 are not well established. However, this study had 70% power to detect a difference of approximately 0.3 ng/mL in sCD14 concentrations across intervention arms,[227] and the sCD14 concentrations in this cohort reflect those of other similar Ugandan cohorts.[164] Prior research had demonstrated helminth prevalence of 30% in this population.[42] Thus, 240 participants were to be randomized to ensure participation from at least 72 participants with helminth infection. The observed helminth prevalence during the trial, however, was one third of what had previously been observed just two years prior;[42] and we were only able to enroll 24 participants with helminth infection (12 in each arm). Thus, the major limitation of this study was the small number of helminth-infected persons who comprised our primary study population.

Current first line therapy for strongyloidiasis is a 200 μg/kg orally for 1-2 days, and alternative therapy for strongyloidiasis is 400 mg of albendazole, twice per day for 7

days. Participants in this cohort receive 400 mg albendazole for 3 days, which is standard therapy for trichuriasis and enhanced therapy for hookworm. This regiment was chosen for this study was based on the very high prevalence of hookworm infection that was anticipated in this cohort,[42] and similar to the recommended preventive chemotherapy regimen (400 mg albendazole).[101] The cure rate for strongyloidiasis for this 1200mg albendazole regimen has been documented at 38%,[228] while for hookworm cure rates have been documented at 92%.[229] The lack of complete cure rates is another limitation of this study, especially in light of the small sample size. It is also possible that partial action against both parasites, in particular *Strongyloides*, disrupted parasite gut homeostasis, which encouraged adult migration in the gut, causing additional perforation of the mucosa, and leading to the observed increases in sCD14.

Other limitations of this study include the non-blinded nature of the intervention and lack of "cure rate" information at follow-up. We asked participants to not take any anthelmintic chemotherapy outside of the study for its duration, and no one reported taking ex-study anthelmintic chemotherapy. However, a placebo, which was not possible in the context of this study, would have provided an additional safe guard against treatment cross-over and dilution of effects by arm. Future analyses of follow-up stool samples for parasitic infection could also illuminate if participants had sought therapy outside of the study, as *N. americanus* and *S. stercoralis* infections persist in the host gut for at least several years and we would expect to see an infection unless otherwise treated.[115,230] If participants in the delayed albendazole arm did seek therapy outside of the study, it would have further attenuated the difference in effect, if any. Conversely,

assessing parasitic infection at follow-up would also allow us to identify which participants did not entirely clear their infection.

This study would have been strengthened by additional evaluation for other sub-clinical infections, such as *Schistosoma* spp. and filarial worms, which could have similar effects on serum biomarkers as helminths.[231] However, prior studies evaluating gastrointestinal schistosomiasis in this population indicated 0% prevalence (unpublished data), and lymphatic filariasis is more common in the northern region of Uganda, as opposed to the eastern region where this study took place.[232] Future analyses will be conducted in the entire cohort, instead of focusing on only those 24 participants who were found to be infected with helminths. First, it is possible that some participants who were infected with soil transmitted helminths of interest here were not identified as such, and excluded from the primary analysis. Additionally, albendazole has activity against a wide range of parasites, beyond those invasive species investigated here, e.g. *Giardia*. In expanding the analysis to the entire cohort, we may include participants with undetected infections, and register some effects of anthelmintic therapy on a broader range of species. Finally, given that the current literature evaluating this topic provides differing immune responses by species, it would have been potentially informative to analyze the effects of deworming by species. However, the small number of species-specific infections (*S. stercoralis* n=11; *N. americanus* n=16) makes it difficult to reliably assess these relationships.

Despite these limitations, this trial represents patients from a typical HIV clinic in Uganda. It also benefits from the use of molecular diagnostics, which has increased sensitivity and specificity for helminths relative to traditional methods.[233] Our results

likely reflect an inability to answer our primary question about the relationship between these invasive parasites and systemic inflammation in immunologically compromised Ugandans. There may be subtle effects on chronic immune system activation that we were unable to detect here. Future analyses focusing on the longitudinal change in serum biomarkers at different time-points would help assess if the increase in sCD14 observed here is transient, and sCD14 concentrations will approximate the results of George *et al.* and Rajamanickam *et al.* over time,[121,207] or if they would stay elevated as in Anuradha et al.[212]

The patterns of elevated post-therapy sCD14 identified here were also observed in a single study of primate colitis.[234] From their 5 primate subjects, one subject demonstrated increased sCD14 *post T. trichiura* therapy, a similar pathogen to *Strongyloides*. This observation lead authors Broadhurst *et al*. to postulate that subjects with inherently "leaky" mucosal barriers – also true for people living with HIV – may express higher concentrations of sCD14 after anthelmintic therapy due to partial gut mucosa restoration and increased bacterial attachment. However, this conclusion is difficult to contextualize given the sample size of one.

Evaluating parasitic clearance, and re-infection rates over time will also help to contextualize our results. However, incorporating regular chemotherapy into care for this vulnerable population may be justified given the health effects of helminth infection, e.g. anemia due to blood loss, protein loss, potential strongyloidiasis hyper-infection, and HIV-clinical considerations, e.g. misleading laboratory results,[235] possible effects on CD4+ T cell concentrations.[90,91]

6. Contributions and Conclusions

This research examined several aspects of the gut environment among people living with HIV in a low-resource context, with a focus on clinical immune markers and correlates (fecal microbiome composition and systemic inflammation). The contributions of this dissertation to the literature are discussed below.

The first aim of this dissertation contributed to outstanding questions surrounding HIV and helminth coinfection.[14] It did so by applying a molecular diagnostic tool to helminth (and protozoa) detection in a longitudinal cohort of rural and peri-urban adult Ugandans. This allowed us to identify a high prevalence of hookworm infection in an otherwise apparently healthy but HIV-infected, and therefore immunologically vulnerable, population. Application of this molecular diagnostic allowed us to detect and quantify low burden infections, likely lower than would have been detected with microscopy. We were also able construct a longitudinal cohort of HIV-infected adults who had initiated antiretroviral therapy, in contrast to most research on HIV and helminths, which has centered on HIV progression prevention when ART was not widely available. This aim was approached through the lens of health optimization for a vulnerable group.

The second aim of this dissertation focused on the fecal microbiome, which has been linked to inflammatory conditions and disease progression. It contributed to our knowledge of the fecal microbiome composition among populations where HIV is the most prevalent. It is only the second of its kind in Uganda, and provides a novel addition of longitudinal CD4+ T monitoring. Longitudinal exploratory analyses were able to identify two taxa that were positively correlated with increasing CD4+ T cell recovery.

There is some documented biological basis for the relationship between CD4+ recovery and increased concentrations of these taxa, *Sutterella* genus and *Alcaligenaceae* (of unknown genus), which may be worth exploring in future cohorts. Probiotic or other low-cost adjuvant therapies that could harness the communities of the gut to reduce inflammation in people living with HIV are perhaps far from available in African – or other – contexts. However, this research contributes to characterizing the gut environment among people living with HIV where the global burden is highest, and low-cost adjuvant therapies could be impactful.

Finally, the third aim of this dissertation has contributed to the literature by addressing a topic that is frequently discussed in studies of anthelmintic therapy, microbial translocation, and systemic inflammation: the importance of these relationships for people living with HIV. This is the first study, to our knowledge, that evaluates the effect of anthelmintic therapy on markers of systemic inflammation and microbial translocation in HIV-infected people from an area where HIV and helminths are endemic. While the final number of helminth-infected participants was relatively small, our results indicate that treating helminth-infected persons may result in increases inflammation (as witnessed by increases in sCD14 and TNF-α, the latter of which was not statistically significant at an 0.05 level). These findings disagree with similar research on this topic (impact of therapy on sCD14 in HIV-uninfected persons), but do agree with other research on the topic of helminths, therapy and deworming. The findings from this study merit further investigation, particularly with respect to the change in sCD14 over a longer time period, and investigation in larger HIV- and helminth-infected cohorts. This aim also gives us pause about recommending medical interventions that could have undesired

long-term effects, i.e. removing an immunomodulating sub-clinical infection that decreases inflammation, in favor of removing other, albeit potentially serious, negative health effects.

In summary, this research has added to our knowledge base surrounding hookworm infection prevalence in rural and peri-urban HIV-infected Ugandans in the era of widely available ART, and the relationship between hookworm infection and potential impacts on CD4+ recovery. It has reinforced some of the findings from one study that examined the fecal microbiome from HIV-infected Ugandans, and identified two taxa that were linked to immunologic recovery in people living with HIV, which could be explored in future cohorts. Finally, it is the first study to evaluate anthelmintic therapy on serum markers of microbial translocation among HIV-infected people living in co-endemic areas, the findings from which stand in contrast to prior work. These results from this dissertation will hopefully help focus future research, and more importantly help inform interventions and programs when warranted, among these and similar populations.

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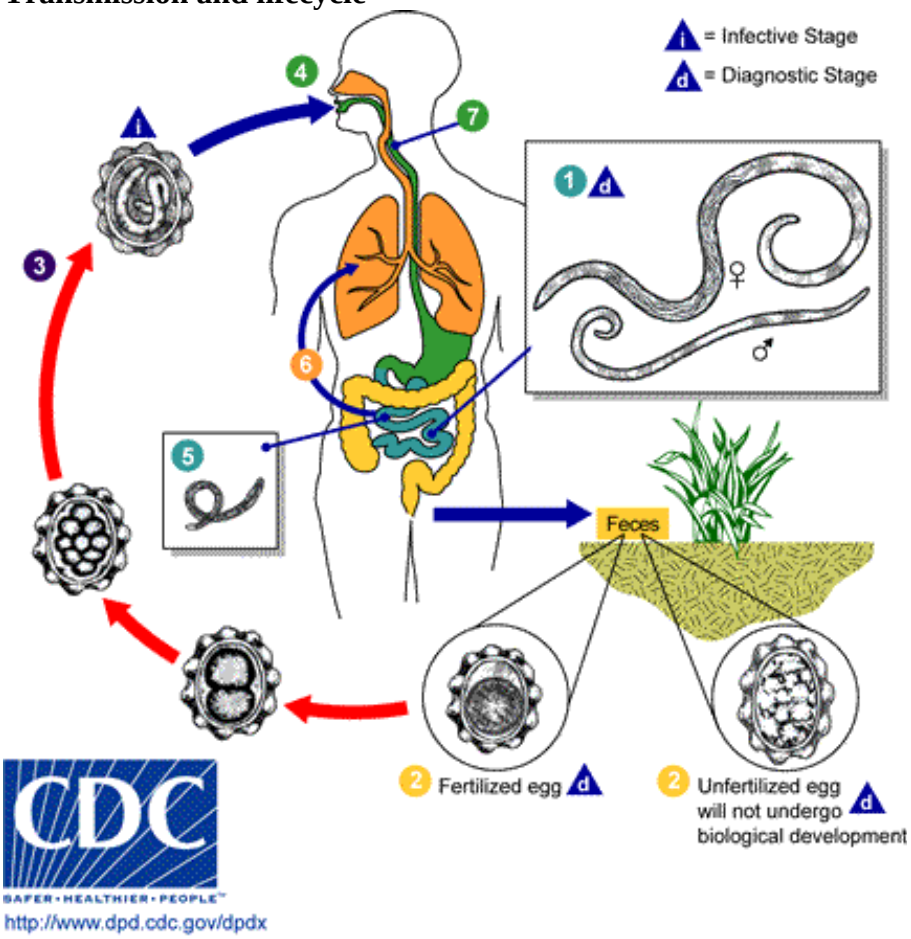
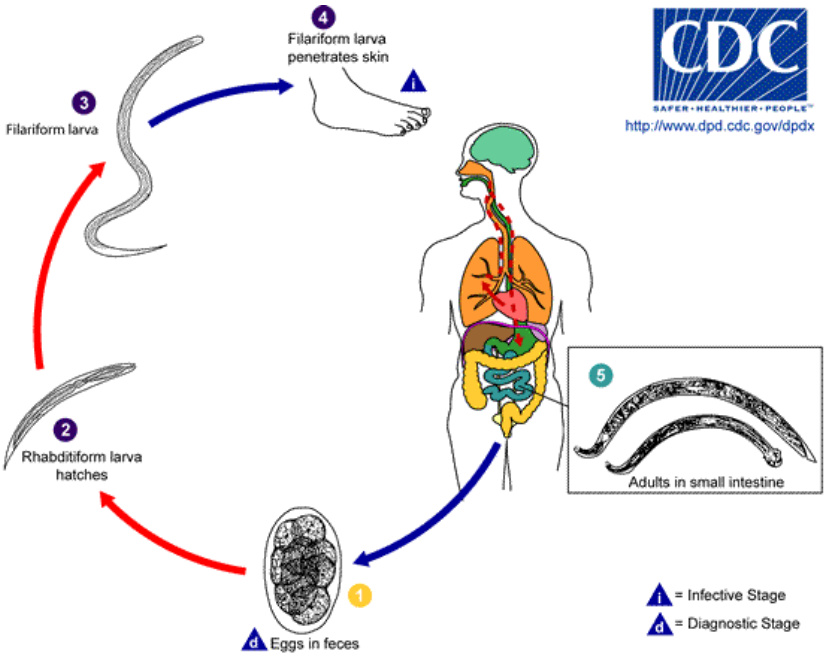
8. Appendices

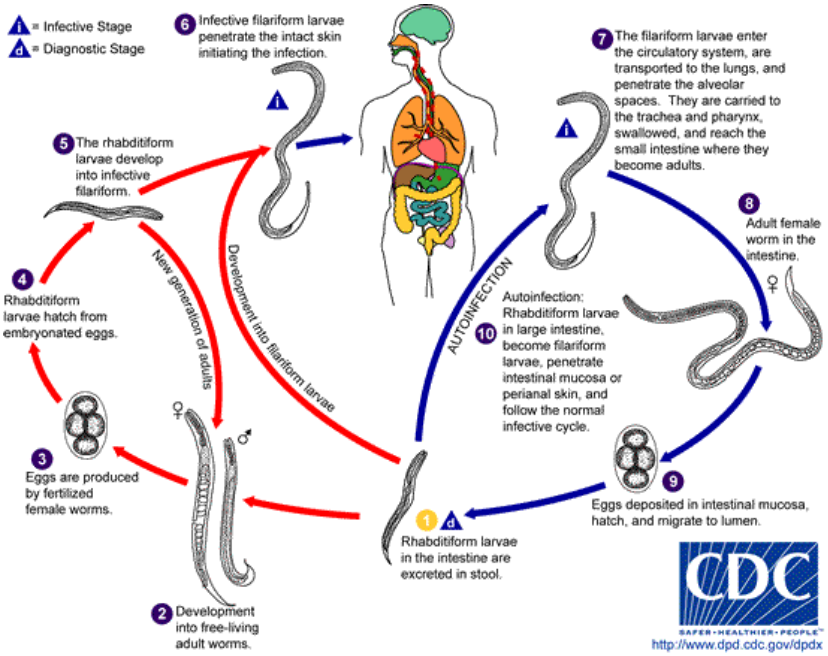
Appendix 1: Soil-transmitted helminth transmission lifecycles

Infection	Transmission and lifecycle
Ascariasis[46]	

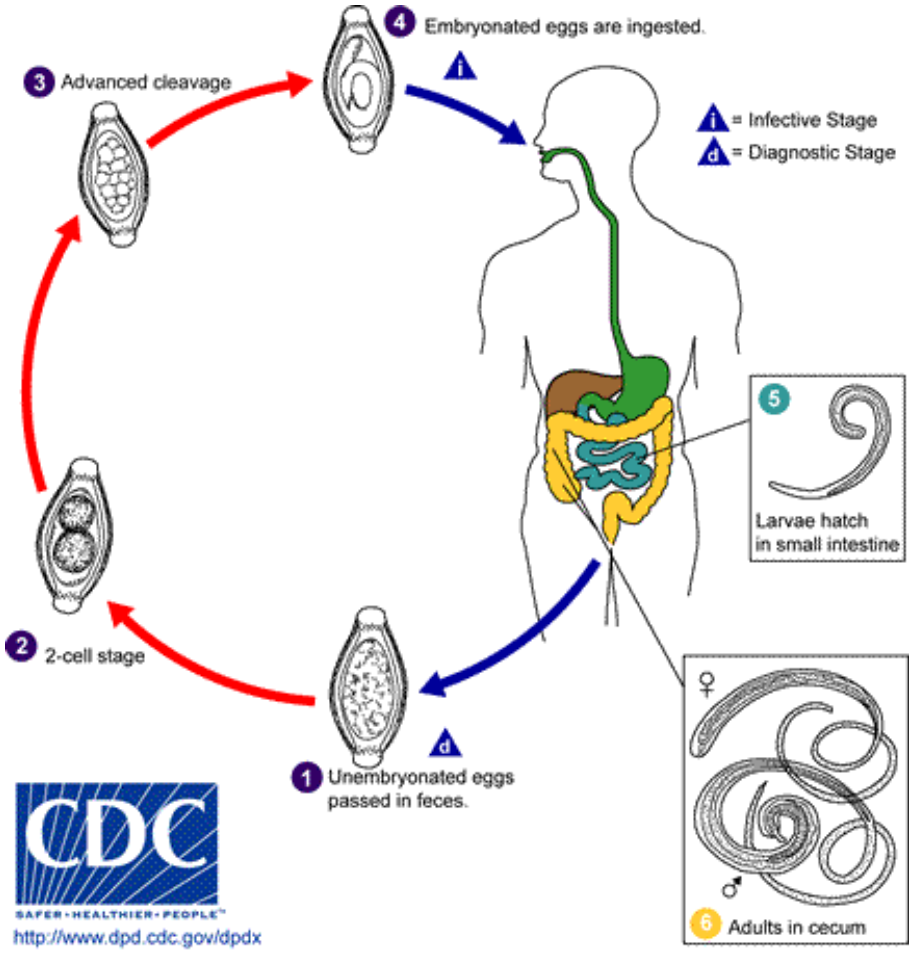
Hookworm[49]



Strongyloidiasis[51]



Trichuriasis[47]



Appendix 2: Low-reagent DNA Extraction Protocol
Using the MP Biomedicals FastDNA™ SPIN Kit for Soil DNA Extraction

1. Add 978 µl Sodium Phosphate buffer to Lysing Matrix E tube.
    a. Use same tip to load all samples
2. Add 122 µl MT Buffer.
    a. Use same tip to load all samples
3. Add 50 mg of stool to each tube.
    a. Use new toothpick or new pipet tip to load
4. Homogenize
    a. FastPrep Instrument 40 sec on speed setting 6.0
    b. Mini Beadbeater 2 min
    c. Disruptor Genie 5 min 3000 rpm
5. Centrifuge 14,000 g for 10 min
6. Add 250 µl PPS to new 2 ml tube
    a. Use same tip
7. Pipet supernatant from (Step 5) into 2 ml tube (Step 6) and invert 10 times by hand
8. Centrifuge 14,000 g for 10 min.
9. Add 2µl Internal control (PBr322, 104), E. coli plasmid
    a. Do not MIX
10. In 2 new tubes, add 500 µl binding matrix
    a. Re-suspend binding matrix vigorously and pipet from bottom of bottle
    b. Use same tip to load binding matrix
    c. Mix binding matrix every 10 tubes
11. Add supernatant from step 9 approximately 600 µl to each tube
    a. Volumes may vary, divide the supernatant equally
    b. Use same tip per sample, but change between numbers
12. Invert for 2 min (Can store at 4 C for 1 hour at this step.)
13. Let sit for 5 min at room temperature.
14. Remove almost all supernatant from each tube.
    a. Remove at angle, careful not to disrupt matrix
    b. Remove enough supernatant leaving approximately 1 mm of volume behind
    c. Use same tip per sample, but change between numbers
15. Remove entire binding matrix
    a. Use same tip from Step 14
16. Add to spin filter with tube
17. Centrifuge 14,000 g for 2 minutes.
    a. Depending on consistency of sample, more spins maybe needed to elute fluids
18. Empty catch tube and discard fluid
19. Add 500 µl of PREPARED SEWS-M to matrix and mix with gentle pipetting.
    a. Careful not to pierce membrane at bottom of filter tube
20. Centrifuge 14,000 g for 2 min
21. Empty catch tube and discard fluid

22. Centrifuge 14,000 g for 2 min to help dry the matrix.
23. Replace the tube with final catch tube.
24. Air dry the filter for 5 min at room temp
    a. Leave lid open
25. Add 100 µl of DES and mix matrix with gentle STIRRING
26. Centrifuge 14,000 g for 2 min
27. Store sample at 4 C or -20 C for long term storage

Trichuris extraction
Trichuris extraction can be done at same time as above extraction. *From Step 5 above:*

6. Add 200 µl H20 to used Lysing Matrix E tube
7. Shake vigorously for 1 min
8. Heat at 90 C for 10 min
9. Shake vigorously for 1 min
10. Centrifuge 14,000 g for 10 min
11. Using the same tip, add 50 µl PPS to new 2 ml tube
    a. Can also use same tube from (Step 6 above) for the same patient sample
12. Pipet supernatant from (Step 5) into 2 ml tube (Step 6) and invert 10 times by hand.
13. Centrifuge 14,000 g for 10 min
14. Add 2 µl Internal control (PBr322, 104)
    a. Do not MIX
15. In 1 new tube, add 500 µl Binding matrix
    a. Re-suspend binding matrix vigorously and pipet from bottom of bottle
    b. Use same tip to load binding matrix
    c. Mix binding matrix every 10 tubes
16. Add supernatant from step 9
17. Invert for 2 min (Can store at 4 C for 1 hour at this step.)
18. Let sit for 5 min at room temperature
19. Remove supernatant and discard.
    a. Remove at angle, careful not to disrupt matrix
    b. Remove enough supernatant leaving approximately I mm of volume behind
    c. Use same tip per sample, but change between numbers
20. Remove entire binding matrix
21. Add to spin filter with tube
22. Centrifuge 14,000 g for 2 min
    a. Depending on consistency of sample, more spins maybe needed to elute fluids
23. Empty catch tube and discard fluid
24. Add 500 µl of PREPARED SEWS-M to matrix and mix with gentle pipetting
    a. Careful not to pierce membrane at bottom of filter tube
25. Centrifuge 14,000 g for 2 min
26. Empty catch tube and discard fluid

27. Centrifuge 14,000 g for 2 min to help dry the matrix
28. Replace tube with final catch tube
29. Air dry the filter for 5 min at room temp with lid open
30. Add 50 µl of DES and mix matrix with gentle STIRRING
31. Centrifuge 14,000 g for 2 min
32. Store sample at 4 C or -20 C for long term storage

Appendix 3: Parasite Infection Intensity Metrics

Parasite egg and larval counts per gram feces used to describe infection intensity

Causative pathogen	**Infection Intensity**		
	Light	Moderate	Heavy
	Egg count per gram feces (EPG)[108]		
A. lumbricoides	1-4,999	5000-49,999	≥50,000
Hookworm spp.	1-1,999	2,000-3,999	≥4,000
T. trichiura	1-999	1,000-9,999	≥10,000
	Larva per gram feces (LPG)[106]		
S. stercoralis	≤1	2-9	≥10

Appendix 4: WHO staging for HIV infection and disease in adults and adolescents, including screened opportunistic infections, from Uganda Ministry of Health Guidelines

Clinical Stage I:

1. Asymptomatic
2. Persistent generalized lymphadenopathy

And/or Performance Scale 1: Asymptomatic, normal activity

Clinical Stage II:

1. Moderate weight loss (<10% of presumed or measured body weight)
2. Minor mucocutaneous manifestations (seborrhoeic dermatitis, prurigo, fungal nail infections, recurrent oral ulcerations, angular stomatitis)
3. Herpes zoster within the last five years
4. Recurrent upper respiratory tract infections, e.g., bacterial sinusitis, tonsillitis, otitis media and pharyngitis

And/or Performance Scale 2: Symptomatic but normal activity

Clinical Stage III:

1. Severe weight loss (>10% of presumed or measured body weight)
2. Unexplained chronic diarrhea for more than one month
3. Unexplained prolonged fever, intermittent or constant, for >1 month
4. Oral candidiasis
5. Oral hairy leukoplakia
6. Pulmonary tuberculosis (current)
7. Severe bacterial infections such as pneumonia, pyomyositis, empyema, bacteremia or meningitis
8. Acute necrotizing ulcerative stomatitis, gingivitis or periodontitis
9. Unexplained anemia (<8gm/dl), neutropenia ($<0.5\times 10^9$ per liter), or chronic thrombocytopenia ($<50\times 10^9$ per liter)

And/or Performance Scale 3: Bed-ridden for less than 50% of the day during the last month

Clinical Stage IV:

1. HIV wasting syndrome – weight loss >10%, and either unexplained chronic diarrhea for more than one month or chronic weakness or unexplained prolonged fever for more than one month
2. Pneumocystis pneumonia (PCP)
3. Recurrent severe bacterial pneumonia
4. *Toxoplasmosis* of the brain
5. Cryptosporidiosis with diarrhea for >1 month
6. Chronic isosporiasis
7. Extrapulmonary cryptococcosis including meningitis
8. Cytomegalovirus infection (retinitis or infection of other organs)
9. Herpes simplex virus (HSV) infection, mucocutaneous for >1 month, or visceral at any site
10. Progressive multifocal leukoencephalopathy (PML)
11. Any disseminated endemic mycosis such as histoplasmosis, coccidioidomycosis
12. Candidiasis of the oesophagus, trachea, bronchi or lungs
13. Atypical mycobacteriosis, disseminated
14. Recurrent non-typhoid salmonella septicemia
15. Extrapulmonary tuberculosis
16. Lymphoma
17. Invasive cancer of the cervix
18. Kaposi's sarcoma
19. HIV encephalopathy
20. Atypical disseminated leishmaniasis
21. Symptomatic HIV-associated nephropathy or symptomatic HIV-associated cardiomyopathy

And/or Performance Scale 4: Bed-ridden for more than 50% of the day during the last month

Appendix 5: Sequence information for eight parasites tested by using a high-throughput quantitative multi-parallel real-time polymerase chain reaction, from Mejia *et al.*

Parasite Species[103]	Forward primer sequence Reverse primer sequence Probe sequence (FAM)	Target Region	GenBank Accession N$^{o.}$
Ascaris lumbricoides	TGCACATAAGTACTATTTGCGCGTAT CCGCCGACTGCTATTACATCA GAGCCACATAGTAAATT	ITS-1	AB571301.1
Cryptosporidium parvum/hominis	AACTTCACGTGTGTTTGCCAAT CCAATCACAGAATCATCAGAATCG CATATGAAGTTATAGGGATACCAG	DNA J-like protein	XM_625506.1
Ancylostoma duodenale	GAATGACAGCAAACTCGTTGTTG ATACTAGCCACTGCCGAAACGT ATCGTTTACCGACTTTAG	ITS-2	EU344797.1
Necator americanus	CTGTTTGTCGAACGGTACTTGC ATAACAGCGTGCACATGTTGC CTGTACTACGCATTGTATAC	ITS-2	AJ001599.1
Strongyloides stercoralis	GAATTCCAAGTAAACGTAAGTCATTAGC TGCCTCTGGATATTGCTCAGTTC ACACACCGGCCGTCGCTGC	18S rRNA	AF279916.2
Giardia intestinalis	CATGCATGCCCGCTCA AGCGGTGTCCGGCTAGC AGGACAACGGTTGCAC	16S rRNA	AJ293299.1
Entamoeba histolytica	GTTTGTATTAGTACAAAATGGCCAATTC TCGTGGCATCCTAACTCACTTAGA CAATGAATTGAGAAATGACA	18S rRNA	X75434.1
Trichuris trichiura	TCCGAACGGCGGATCA CTCGAGTGTCACGTCGTCCTT TTGGCTCGTAGGTCGTT	ITS-1	FM991956.1

ITS = internal transcribed spacer; rRNA = ribosomal RNA