The prevalence of helminth and malaria infections and the effects of de-worming on disease progression markers in HIV-1 infected pregnant women on antiretroviral therapy in Rwanda

EMIL IVAN

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Abstract

Background: Helminth and malaria co-infections have been hypothesized to be factors driving the HIV-1 epidemic in Africa, and the fact that both cause anaemia highlights the importance of addressing the interactions between HIV/AIDS, malaria and intestinal helminthic infections in pregnancy for individuals in resource limited settings.

Aims: The aims of this thesis were to determine the prevalence and risk factors for malaria-helminthic dual infections among HIV positive pregnant women on antiretroviral therapy in Rwanda. The second aim was to determine the effect of deworming on immune markers of HIV/AIDs disease progression among HIV-infected pregnant women on antiretroviral therapy (ART), and to elucidate the benefits of deworming, specifically in targeted versus untargeted deworming.

Methods: A cross-sectional study was carried out in 328 HIV-positive pregnant women receiving ART. We determined the prevalence of helminth and malaria dual infections and the effects of ART on these infections were also examined. This cross sectional study acted as a pilot study for a deworming intervention, which took the form of a longitudinal study of targeted and untargeted deworming in which 980 HIV-infected pregnant women were randomized to 'targeted' and 'untargeted' arms with albendazole therapy. The effects of deworming on the prevalence of helminth infection and CD4 counts, viral load and haemoglobin levels were measured over time at 4 visits. Measurements were at baseline and every 3 months thereafter. The presence of *Plasmodium falciparum* was tested at each visit and anti-malarial therapy (Coartem: artemether-lumefantrine) was administered to all subjects who tested positive for *P. falciparum*. Baseline data was used to determine the risk factors for helminth infection. Helminthic infection was diagnosed using the Kato Katz method, whilst the presence of *P. falciparum* was identified from blood smears. The CD4 counts and viral load levels were also determined using standard laboratory methods.

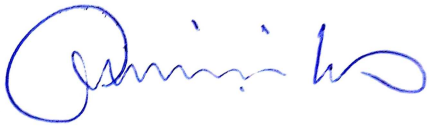
Results: Within the pilot study of 328 women residing in rural (n=166) and peri-urban (n=162) locations, 38% of those tested harboured helminths, 21% had malaria and 10% were infected with both. The most prevalent helminth species were *Ascaris lumbricoides* (20.7%), followed by *Trichiuris trichiura* (9.2%), *Ancylostoma duodenale* and *Necator Americanus* (1.2%). Helminth infections were characterized by low haemoglobin levels and low CD4

counts. Subjects treated with a d4T-3TC-NVP regimen had a reduced risk of *Trichuris trichiura* infection (OR, 0.27; 95% CIs, 0.10-0.76; $p<0.05$) and malaria-helminth dual infection (OR, 0.29; 95% CI, 0.11-0.75; $p<0.05$) compared to those receiving AZT-3TC-NVP therapy. Within the longitudinal study of deworming in 980 pregnant, HIV-infected females, analysis of the baseline data showed that education and employment reduced the risk of all types of infection whilst hand washing protected against helminth infection (0.29 [0.19-0.46]; $p<0.0005$). Logistic regression analysis, at baseline (odds ratio [95% CIs]), demonstrated that TDF-3TC-NVP (3.47 [2.21-5.45]; $p<0.0005$), D4T-3TC-NVP (2.47 [1.27-4.80]; $p<0.05$) and AZT-NVP (2.60 [1.33-5.08]; $p<0.05$) regimens each yielded higher helminth infection rates than the AZT-3TC-NVP regimen. Anti-retroviral therapy had no effect on the risk of malaria. The prevalence of *P. falciparum* infection was similar at all-time points for the targeted and non-targeted anti-helminth treatment arms, with a significant fall in helminth prevalence in both arms by visit 2. Albendazole therapy was associated with favourable changes in haemoglobin levels, CD4 counts and viral loads, in those subjects with helminth infections. Haemoglobin levels were similar in both arms at all study visits, rising significantly from visit 1 to visit 2 in both groups and peaking by visit 3. Thereafter, levels fell significantly ($p<0.0005$ for both comparisons) by visit 4.

Conclusions: The prevalence of helminth infection in HIV infected pregnant women on antiretroviral therapy is common in rural and peri-urban settings in Rwanda. This study clearly shows that, albendazole treatment is associated with an increase in CD4 counts, a fall in viral loads and an increase in haemoglobin levels. The effects of albendazole are mediated by the eradication of helminth infection. The study also shows that treatment with albendazole using a targeted or non-targeted regimen is equally effective. The mechanism by which certain ART regimens reduce the risk of helminth infection warrants further study.

Declaration

I, Emil Ivan, hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by neither another person nor materials which to large extent has been accepted for a ward of any other degree or diploma of the University or other higher learning institutions where due acknowledgment has been made in the text

Signed: 

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Dedication

This work is dedicated to my children:

Elvis, Elizabeth and I. Natasha

Published papers

1. Ivan E, Crowther NJ, Rucogoza AT, Osuwat LO, Munyazesa E, Mutimura E, Njunwa KJ, Zambezi KJ, Grobusch MP: Malaria and helminthic co-infections among HIV-positive pregnant women: Prevalence and effects of antiretroviral therapy. Acta Trop; 2012 Dec; 124(3): 179-84 **(Appendix 5**)
2. Ivan E, Crowther NJ, Mutimura E, and Osuwat LO, Janssen S, Grobusch MP: Helminthic Infections Rates and Malaria in HIV-Infected Pregnant Women on Anti-Retroviral Therapy in Rwanda. PLoS Negl Trop Dis. 2013 Aug 15; 7(8): e2380 (**Appendix 6)**
3. Ivan E, Crowther NJ, Mutimura E, Rucogoza A, Janssen S, Njunwa KK, and Grobusch MP. Effect of deworming on markers of disease progression in HIV-1 infected pregnant Rwandan women on ART: a longitudinal cohort study in Rwanda: Journal of Infectious diseases 2015; 60: 135-142 (**Appendix7)**

Contributions to the published papers by each author

Ivan Emil: Design of the study, data collection, analysis and writing of manuscript.

Nigel J Crowther: Study design, supervision, data analysis and review of manuscript.

Eugene Mutimura, Aniceth Rucogoza and Kato Njunwa: Data collection, monitoring, analysis and drafting of manuscript and Janssen Saskia: Review of manuscript.

Martin P. Grobusch: Study design, study supervision: Data analysis and final review of manuscript.

AWARDS, CONFERENCE PRESENTATIONS, AND ABSTRACTS

I obtained WHO-TDR LTG-2009 studentship award- Leadership training grant on NTDs for DECs, Switzerland – Geneva, February 2009 to attend University of the Witwatersrand. Grant consisting of tuition fees and stipend for three years: ***Index n^{o}, HQA80325***

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Ethical committee presentations

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Oral presentations in conference proceedings

4th ISNTD (International Conference for Neglected Tropical Diseases) at Imperial College, London, UK, from **12th-15thFebruary, 2013**: Oral presentation of this research article was named among the best top 20 of the Pub Med Articles that featured on co-infections of NTDs-HIV in Pregnancy from January-December 2012-2014 selections.

ORAL PRESENTATION TITLE: NTDs-HIV/AIDS Co-infections and Malaria among HIV infected pregnant women in Rwanda: Prevalence and effects of ART.

Abstracts presented at conferences as posters

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List of acronyms and abbreviations

ITEM:	MEANING
ART	: Antiretroviral therapy
AZT-3TC-NVP	: AZT (Zidovudine) +3TC (Lamivudine) +Nevirapine (NVP)
BCA	: Benefit cost analysis
BCR	: Benefit cost ratio
BMI	: Body mass index
cART	: Specific antiretroviral therapy
CBA	: Cost-benefit analysis
CD4	: Cluster of Differentiation- 4
CDC	: Centre for Disease Control
CEA	: Cost effectiveness analysis
CRF	: Case report form
D4T-3TC-NVP	: When AZT is replaced with d4T (Stavudine)
DALYs	: Disability-adjusted life years
DEC	: Disease endemic countries
DHS	: Demographic health system
DVL	: Detectable viral loads
Epg	: Eggs per gram in faeces
GCLP	: Good clinical laboratory practice
GoR	: Government of Rwanda
HAART	: Highly active antiretroviral therapy
Hb	: Haemoglobin
HIV	: Human immune deficiency virus syndrome
HRP-2	: Histidine-rich protein-2
ID	: Identification number
ITN	: Insecticide treated bed net
KHI	: Kigali Health Institute
LBW	: Low birth weight
LF	: lymphatic filariasis
LTG	: Leadership training grant
MDA	: Mass drug administration
MoH	: Ministry of Health

NRL	: National Reference Laboratory
NTDs	: Neglected tropical diseases
OR	: Odds ratio
PAPFAR	: Presidential fund for AIDs Relief
PMTCT	: Prevention of mother-to -child transmission
PZQ	: Praziquantel
R&D	: Research and development
RBC	: Rwanda Biomedical Centre
REB	: Rwanda Education Board
SFAR	: Student Funding Agency of Rwanda
STH	: Soil-transmitted helminths
TB	: Tuberculosis
TDF-3TC-NVP	: Abacavir (ABC) + Tenofovir (TDF) + Lopinavir (LOPV/r)
TDR	: Tropical disease research
TRAC-Rwanda	: Treatment and AIDS Research – Rwanda
UDVL	: Undetectable viral loads

Definitions of technical terms used

TERM	: DEFINITION
Deworming	: Giving of an anthelminthic drug to subject who may be infested with helminths.
Disease progression markers	: Markers associated with disease susceptibility able to advance the disease progression pathways.
HIV type 1	: Human immune deficiency virus type-1
Targeted deworming	: The giving of an anthelmintic drug (albendazole) as Treatment only to those tested positive for helminthic infection.
Non targeted deworming	: The giving of anthelmintic drug to subjects in the study Whether without testing or not they are affected by helminths
ART-regimens	: Highly active antiretroviral drugs medically proven to treat and reduce the status of HIV/AIDS among infected humans.
Pregnancy trimester	: Pregnancy being the fertilization and development of one or more offspring in a mother's uterus, for reference purposes the average nine months of human gestation is arbitrarily divided into three trimester periods of three months each.
Differential effect of treatment	: The effects of different treatment regimens, derived from the statistics referred to in this thesis.
Haemoglobin trajectories	: Red blood cell or haemoglobin levels as they change over time among subjects in the study. As an example, a lower trajectory is defined as <11g/dl of mean Hb.
CD4 Trajectories	: Changes in the mean cluster of differentiation-4 (T cells or T lymphocytes) for subjects in the study.
EPG count	: Eggs per gram of faecal matter, counted to represent the worm burden of an individual.

Detectable viral load RNA-copies	: RNA copies of viral loads that were at detectable levels or would diminish to undetectable levels in AIDS patients.
Undetectable viral load RNA-copies	: HIV-1 infected individuals among AIDs patients found without detectable viral load copies

Preface

This thesis arises from the author's experience and close observations regarding the paucity of data on malaria, helminths among neglected tropical diseases (NTDs) and HIV co-infections in Rwanda. This was during work co-ordinating the teaching of laboratory students and supervising projects in community outreach modules for rural health centre clinical placements for laboratory students at the Kigali Health Institute.

The aims of this study were: to determine the prevalence and risk factors for helminth-malaria co-infections in pregnant HIV-positive females receiving ART; to investigate whether treatment of helminth infections in HIV infected pregnant women have any beneficial effect on HIV disease progression markers, such as viral load and CD4 cell counts; to determine the effects of de-worming on haemoglobin levels and body mass index; to determine the prevalence of malaria infections in the absence and presence of a helminth infection; to determine the efficacy of two de-worming intervention methods i.e. targeted and untargeted.

This thesis is presented in six chapters. Chapter one outlines the general introduction and perspectives of the HIV and NTDs co-infections and ends with literature review, Chapter two describes the materials and methods used to conduct the study, whereas chapters three, four and five describes the results of the study presented in form of published scientific papers to various types of peer-reviewed journals. Chapter six gives the conclusions and implications of the main results, limitations of the study, recommendations and further studies, and then offers concluding remarks about the findings with regard to the study hypotheses and objectives of the thesis. Chapters end with combined list of references. All published manuscripts are attached as pdf appendices and presented at the end of the thesis

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1. Chapter 1: Literature Review

1.1. Introduction

This chapter includes a review of the literature that is relevant to the study. It will cover the treatment of soil-transmitted helminths in HIV-infected patients on antiretroviral therapy (ART) and the global burden and epidemiology of helminths. Helminthic species, diagnosis and treatment with albendazole have also been considered. The role of co-infections in the immunity deterioration of HIV-infected individuals and the effects on HIV disease progression are all considered. A review of this literature identifies a research gap on which this study has been based. This is covered at the end of this chapter with a brief statement on the research question covered by my study, and a description of the hypothesis tested, and the aims and objectives of the study.

1.1.1. Helminth infection in HIV/AIDs patients on ARTs

Helminth infections are well-known to affect human immune system and rarely infect the human host in isolation, particularly in the settings of tropical countries, where co-infections with HIV virus are extremely common [1–3]. Both helminths and HIV-1 pandemic mainly affects people living in resource-constrained settings [2–5]. Infections due to soil-transmitted helminths (STHs) are said to have inflicted more than 1 billion individuals from the poorest socio-economic levels of the developing countries [2,6–9]. Previous research studies have suggested that helminths co-infection with HIV-1 may result in a more rapid progression of HIV-1 disease [2,7,10]. Helminthic infections make the infected host more susceptible to HIV infection and enhance disease progression due to the chronic immune activation. Co-infections of helminthic parasites with HIV may ultimately lead to a more rapid destruction of the host immune system, and potentially lead to progression of HIV-1 infection to AIDS [11,12]. A study concluded that chronic helminth infection may suppress immune responses directed against HIV-1 and that concurrent immune activation may directly lead to more rapid loss of CD4 cells in HIV-1 infected individuals [13,14,15]. It is important to determine if prevalent helminthic infections affect the progression of HIV-1 in co-infected individuals living in disease- endemic countries among populations at high risk, such as pregnant women taking highly active antiretroviral

therapy (HAART). There is limited data on effects of anthelminthic utilization along with antiretroviral drugs among pregnant women in resource-limited countries, where co-infection of helminths and malaria is prevalent. The effects of de-worming on the prevalence of helminth infection and the level of malaria and HIV co-infection has been poorly investigated and no studies on these outcomes have been udetaken in subjects receiving antiretroviral therapy (ARTs). The study aim was to test the hypothesis that treatment for helminths and malaria co-infections positively affects HIV/AIDS immune progression markers among HIV-infected pregnant women on ART, using targeted and untargeted deworming strategies in Rwandan antenatal care settings.

Importantly, in Rwanda, the rollout of antiretroviral therapies is on a large scale, particularly for HIV-infected pregnant women. However, information regarding helminthic co-infection with HIV in rural and peri-urban settings is currently very limited particularly in the context of the recent Rwandan Health Ministry policy on early initiation of ART for pregnant women irrespective of CD4 cell count levels [16]. We hypothesise that, HIV co-infection with helminths may be common among pregnant women in these settings. To date, the public health relevance of anthelminthic use in helminth infected pregnant women on ART in Rwanda have not been extensively studied, and data covering the effects of the anthelminthic drug albendazole for pregnant women on antiretroviral drugs could be very useful for public health program managers.

1.1.2. The burden and epidemiology of neglected tropical diseases

The burden of disease estimates are widely used for priority setting in public health [17–19] Infections due to soil transmitted helminths and other neglected tropical diseases affects over a billion people globally [19–21]. However, the global funding priorities underestimated the resources for management and control of neglected tropical diseases and this affects the health programs targeting the bottom poor billions affected with high morbidity and mortality rates [22]. In many parts of African countries, the public health issues with these ailments have less attention from public health managers [19]. Earlier studies have proposed that the only way forward is the integration of control efforts, with efficacious programs such as HIV and malaria combined to provide a package of diagnostic tools and treatment with drugs against the common, neglected tropical diseases underscored in the world health organisation control agenda [23,24].

However, despite the several efforts invested in research, no conclusive evidence linked NTDs epidemiological studies with HIV pandemic in areas where helminths and malaria co-infection is prevalent to elucidate effects of deworming among pregnant women on ARTs in a targeted and untargeted deworming strategy.

1.1.3. Global perspectives on soil-transmitted helminths

Soil-transmitted helminths (STHs) are a group of parasitic metazoan worms, affecting billions of people in most poorest populations [25,26]. Soil transmitted helminthic infections are among the most common in developing countries, but precise estimates of the populations at risk of infection, morbidity and mortality are difficult to determine and careful evaluation of the global distribution and disease burden of helminths is essential to determine the cost-effectiveness of control and to ensure that control programmes are focused appropriately [23]. In turn, understanding the disease burden depends on a summary measure of health as well as reliable data on risks of infection, morbidity and mortality [20,23,27]. The cosmopolitan distribution and, the public health importance of the common roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichiura*), and hookworms (*Necator Americanus* or *Ancylostoma duodenale*) are described globally [28,29]. They are considered together because it is common for a single individual, especially a child living in a less developed country, to be chronically infected with all three worms [30]. This is possible in developing countries where individuals are estimated to be earning less than US$2 per day [31].

Moreover, helminthic infection has been estimated to account for between 5 and 39 million disability-adjusted life years, largely attributable to anaemia, stunting, and reduced cognitive development in children and this causes debilitating effects in adults and women in their reproductive age [17,18,32–34].The current control strategies have focused on preventive chemotherapy through mass drug administration (MDA), with albendazole as a single dose of 400mg [23]. Preventive chemotherapy can greatly reduce morbidity from helminths infection, but re-infection typically occurs more rapidly after treatment in settings with poor sanitation facilities which calls for frequent treatment strategies [35]. In areas with high burden, prevalence is commonly pooled with worm burden which is the intensity of infection, that is frequently determined by the number of eggs per gram (EPGs) of faeces for intestinal helminths and other NTDs [35].

To summarise the global burden of helminthiasis, Table 1.1 below shows the common NTDs affecting mostly populations in developing countries. This sheds more insights on the global burden of 13 major NTDs (adapted from reference 36). This data demonstrates that populations at high risk include regions of sub-Saharan Africa and cuts across the Americas, South East Asia and regions of the Pacific.

Table 1.1. The global burden of NTDs [35]

Disease [references]	Global cases	Population at risk	Regions with highest prevalence
Ascariasis [37,38]	807 million	4.2 billion	East Asia and Pacific Islands, India, Sub Saharan Africa, South Asia, China and Caribbean
Trichuriasis [39]	604 million	3.2 billion	Sub Saharan Africa, East Asia –Pacific, Latin America, India and South Asia
Hook worm infection [40],[41]	576 millions	3.2 billion	Sub Saharan Africa, East Asia, India, South Asia and Latin America)
Schistosomiasis [20,42]	207 million	779 million	Sub Saharan Africa, Latin America and Caribbean
Lymphatic Filariasis [43]	120 million	1.3 billion	India, South Asia, East Asia and pacific islands, sub Saharan Africa
Trachoma [44]	84 million	590 million	Sub Saharan Africa, Middle East, North Africa, South Asia, East Asia –Pacific
Onchocerciasis [45]	37 million	90 million	Sub Saharan Africa and Latin America
Leishmaniosis[46]	12 million	350 million	Latin America
Chagas' disease [47]	8-9 million	25 million	Latina America and Caribbean,
Leprosy[34]	0.4 million	NA[d*]	India, Sub Saharan Africa and Latin America
Bruli Ulcer[48]	NA	NA[d*]	Sub Saharan Africa
Human African Trypanasomiasis [34]	0.3	60million	Sub Saharan Africa
Dracunculiasis [49]	0.01 million	NA[d*]	Sub Saharan Africa

*donates data NOT available

1.1.4. Effects of helminths and malaria on HIV disease progression

Sub-Saharan Africa is highly burdened with various infectious diseases such as malaria, and those caused by the human immunodeficiency virus (HIV)-1 and intestinal helminths [50-52]. The prevalence of helminthic infections in most developing countries is very high, and a quarter of the world's population is infected [53,54]. Dual helminthic and HIV-1 co-infections are extremely common in Africa [55]. Furthermore, literature indicates that helminthic infections play a role in the pathogenesis of HIV-1 infection in Africa, due to their profound effects on the host immune system, which makes those infected with helminths more susceptible to HIV-1 infection and more vulnerable to the disease's effects [56].

Helminthic infections also increase mother-to-child transmission of HIV [57]. Chronic immune activation with a dominant T-helper 2 profile is seen as hallmark of chronic helminths infection [1,13,14].These immune changes are usually characterised by several alterations in the normal immune response, particularly those of the cellular immune response, which accounts for the possible effects of chronic helminth infection on the host's ability to handle HIV and other infections such as malaria. In a meta-analysis that analysed all the studies that have investigated the effects of treatment of helminthic co-infections on HIV-1 disease progression, five studies were identified, of which one was a randomised controlled trial [58]. As a result of the meta-analysis, the authors concluded that, despite the void in conclusive evidence demonstrating the efficacy of anthelminthic treatment in slowing HIV-1 disease progression, there is a negative correlation between the treatment of helminth infection and HIV plasma viral load [59].The fact that only five studies, out of over 6,000 reviewed studies addressing this issue, were finally included in the meta-analysis clearly illustrates the paucity of high-quality data in this area. Furthermore, even within this small group of studies, significant differences in methodology and evaluation are evident. Thus, the review rightly concludes that there is an urgent need for larger and wider studies of this question, and that they should be double-blinded and controlled [2,58,60]. Since the publication of this review, there has been one additional report of a study performed in Tanzania, which showed that the eradication of filarial infection significantly decreased plasma HIV viral load [61].

An increasing body of evidence indicates that co-infection with neglected tropical diseases (NTDs) also adversely affects the natural history and progression of malaria and HIV/AIDS [62].

The immunological interplay between malaria and helminths or malaria and HIV has been reported in several studies which highlight the severity of the resulting clinical malaria [51,52,63-65]. A study from Malawi shows that women infected with hookworms were at a higher risk of having malaria than uninfected women [66] and studies from Thailand show an increased susceptibility to malaria in patients with soil-transmitted helminth infections [75]. In addition to inducing increased susceptibility to malaria, helminth infections, especially hookworms and schistosome infections, exacerbate the anaemia caused by malaria. Studies from Kenya show that anaemia resulting from helminths and malaria is profound [67]. Both helminths and malaria causes anaemia and anaemia in pregnancy contributes to maternal deaths and may also contribute to adverse birth outcomes, including intrauterine growth retardation and prematurity, and to prenatal morbidity and mortality [67–70]. In pregnancy, anaemia is also a leading contributor to maternal morbidity and mortality, and is associated with shock, risk of cardiac failure, decreased ability to work, and adverse prenatal outcomes [67]. In coastal Kenya, malaria was identified as the most important cause of anaemia in primigravidae, whereas hookworm infection was an important cause of anaemia in multigravidae [71]. By some estimates, NTDs are second only to HIV/AIDS as a cause of disease burden, resulting in a loss of approximately 57 million disability-adjusted life years (DALYs) annually [72].

Therefore, the presence of helminths may affect the risk of malaria and severity of the disease; and the occurrence of malaria infection may in turn increase the risk of helminths infection and related morbidities [72-74]. As a result, the disease due to one of these infections could be exacerbated due to co-existence of the other, resulting in synergistic effects on the infected host [75-77]. It is well known that HIV infection has extremely detrimental effects on the immune system [77,78], resulting in an increased incidence of opportunistic infections among co-infected individuals [1,80]. The hypothesis is that, if considered together, helminths among other NTDs would represent the fourth most important communicable disease in Africa. Recent data suggest that control of NTDs could actually become a powerful tool for combating HIV/AIDs, tuberculosis and malaria [20,62,72]. Therefore, achieving success in the global fight against HIV/AIDs, tuberculosis and malaria may well require a concurrent attack on these NTDs.

1.1.5. HIV Co-infection with NTDS in Rwanda

The most important risk factor for HIV-1 acquisition in Rwanda is heterosexual mode of transmission [81] and most of the HIV-infected women are in their reproductive years [81,82]. Co-infection of NTDs with HIV is reported to be common in countries within the east African region, and it is widely documented that helminthic and HIV infection have an overlapping geographical distribution [3,53,72,81]. Data for helminthic prevalence and co-infection with HIV-1 in Rwanda is nonetheless very scarce. Health survey data available indicate that HIV prevalence is 3 % [83] Countrywide, there are 207 sites providing public access to highly active anti-retroviral therapy (HAART) and there are 130,000 adults currently on HAART, of whom 32,031 are females. Among 212 501 pregnant women previously HIV tested in antenatal care 6059 (3.8%) were HIV positive [83].

Rwandan data available on the prevalence of helminthic infection is for school-going children, indicating a prevalence of 64.5% in eight surveyed districts [84]. However, there are no data available on the prevalence of helminthic and helminthic-HIV co-infection in pregnant women on ART in Rwanda. The World Health Organization recommends that anthelminthic treatment be given to pregnant women after the first trimester (13 weeks) in areas where the prevalence of hookworm or other soil-transmitted helminths exceeds 20-30% [7,85]. However, the use of antenatal anthelminthic treatments remains uncommon in most developing countries, and research into its effects has been limited for common neglected tropical diseases.

1.1.6. Diagnosis, treatment, and management of NTDS

Accurate methods of diagnosis and optimal strategies to sample the population are essential for the reliable mapping and surveillance of infectious diseases [86]. The current standard for detection of soil-transmitted helminths (STH) entails use of the Kato-Katz diagnostic method [86,87].The reliable mapping, surveillance and evaluation of infectious diseases rely upon two key factors: accurate methods of diagnosis and optimal strategies to sample the population. For the soil-transmitted helminths (STH: *Ascaris lumbricoides*, *Trichuris trichiura* and hookworm), the commonly used diagnostic technique is the Kato-Katz method [87]. This technique allows for the quantification of intensity of infection on the basis of faecal egg counts[88]. In most endemic

countries reliable diagnostic tools are not easily available for disease monitoring and surveillance yet very critical components for treatment-related complications [81].

Soil-transmitted helminths (STHs), primarily Ascaris, Trichuris and hookworm, inflict a substantial morbidity burden on poor populations living in tropical and subtropical regions of Africa constituting a big share of the bottom poor population of the world [89]. Chronic STH infections can cause intestinal blood loss and nutrient loss and malabsorption, which can result in or exacerbate iron deficiency, anaemia and other nutritional deficiencies [89,90]. More than 1 billion people are infected with at least one STH, and at least 44 million pregnant women are infected with hookworm alone [89]. Pregnant women are especially vulnerable to the harmful consequences of these parasitic infections due to increased nutritional demands during pregnancy [89,90]. It important to control STHs using available mass drug admiration where it exists and to keep track records of diseases transmission dynamics for monitoring transmission and identify possibilities of interruption [32]. Diagnosis and treatment are partly important in identifying disease resurgence [28].However, diagnostic tools in low-resource setting lack sufficient laboratory infrastructure, equipment and trained health practitioners [91].This remains a big challenge while trying to design control interventions for neglected tropical diseases and other comorbidities. Resource limited countries and more at risk of getting infected with dozens of nematode worms but have limited control of disease surveillance laboratory capacity [92]. Therefore, multiple health interventions approach has the potential to minimise costs and expand intervention coverage. Thus, the integration of mass drug administration should be strategically encouraged in resource-limited settings for the delivery of preventive chemotherapy to control soil-transmitted helminthiasis and other NTDs in Sub-Sahara African health facilities [32].Therefore, policy implementers should embark on integrated NTD control to have a big impact on diseases affecting the majority bottom poor [72].

1.2. Methods of Diagnosis of NTDS

Methods available for detection of human helminths include wet mount or stained smear preparations, but the number of parasitic forms isolated by these two methods is often too low to be observed microscopically [88]. In most cases, the use of concentration techniques is preferred

as this increases the chances of detecting parasitic organisms, thus increasing the sensitivity of the microscopic techniques. The two most commonly used stool concentration techniques are Kato Katz sedimentation and flotation techniques [45]. Sedimentation techniques by Kato Katz are performed in general diagnostic laboratories because they are easier to perform and with few technical errors [93].

Unlike S. heamatobium infection, which is diagnosed by detecting eggs in urine [94], other soil dwelling helminthic infections are detected mainly from stool samples using the traditional 'wet mount method' for detection of helminths eggs. Other diagnostic methods can be used like concentration techniques using Kato Katz methods for qualitative detection of worm burden for schistosoma spps eggs and hookworm eggs [93,95,96]. It is also possible to detect eggs in stool using formal ether concentration techniques for the detection of eggs of *Ascaris lumblicoides*, *trichuris trichiura* and hookworm [97].

1.2.1. Kato Katz techniques for estimating worm burdens for STHs

The Kato-Katz technique is a laboratory method for preparing human stool samples prior to searching for parasitic eggs [93]. Published methods vary in details however; one involves staining a sieved faecal sample and examining it under a microscope. The eggs of any soil-transmitted helminths can be detected by experienced technician [98]. The total number of stained eggs are counted and used to calculate the number of eggs per gram (epg), also known as the worm burden, following known standard methods [96,99].

1.2.2. Formal ether concentration for identification of STHs

In developing countries, the diagnosis of parasitic infections in humans is challenging and requires the recognition of parasite stages based on size, morphology, colour, and movement of parasitic organisms by trained and experienced individuals [100].The size and morphology are the major diagnostic microscopic features for their identification [100,101]. A formal ether concentration technique is one concentration procedure that should be performed as a routine part of a complete examination for parasites [28]. Such stool concentration allows the detection

of parasites present in small numbers. Otherwise, the diagnostic features of helminthic eggs or their larva may be missed by using direct wet mount methods [102].

1.2.3. Concentration techniques over traditional methods

During concentration techniques, parasitic elements are concentrated through sedimentation to enhance the recovery of eggs in faecal specimens [102]. The concentration of eggs is possible because the specific gravity of helminthic eggs is greater than that of water [103]. In principle, formalin acts both as a fixative and preservative of helminthic eggs. During this process, faecal fragments are extracted into the ether phase so that the parasitic forms can be separated by centrifugation s [28]. The advantage of using this method over wet mount preparations is that of high sensitivity and specificity which makes it superior to wet methods [101] because more parasitic eggs can be identified using concentration techniques with microscopy and thus this method is superior to the wet mount preparations [100].

1.3. Soil-Transmitted Helminths and Malaria

Human helminth and malaria form the two of the most prevalent infections that overlap extensively in their geographical distribution and usually co-exist in the same host [104]. Current findings demonstrate that STH and malaria infections are prevalent and exhibit complex interactions within the host [75,104]. Soil transmitted helminths are the most prevalent infections among poor communities in most parts of Africa [15]. Importantly their co-existence with other infections such as HIV, TB and malaria make them amenable to population-wide interventions because control programs for HIV, TB and malaria are well developed and financed [105–107] and the addition of anthelminthic therapies to such programs would be simple. However, research on helminths remains poorly funded even during the era of ARV roll out programs in Africa [15]. In most countries in Africa¸ the funding opportunities for malaria, TB and HIV/AIDs control and prevention are highly prioritised whilst this is not the case for soil transmitted helminths and this is why the latter are categorised as neglected tropical diseases [106]. The same situation exists for lymphatic filariasis, onchocerciasis, schistosomiasis, and

trachoma [108]. However, from the treatment point of view, all these NTDs can be controlled by preventive chemotherapy using currently available cost effective drugs [109]

Prospects of integrating control programs for malaria, HIV and TB with NTDs are reported in literature but are not widely applied in high endemic settings [62]. Therefore, if the control of these infections could be made community-based, there is a potential for integrated NTD and HIV/AIDs antiretroviral therapy programs to operate together with existing malaria control programs, thus utilizing the infrastructure used in the roll-out of freely provided ARTs in these settings [110,111]. The soil-transmitted helminths (STHs) are the central focus of the current study, and are each briefly described below.

1.3.1. Ascaris lumbricoides

Ascaris lumbricoides demonstrates cosmopolitan distribution [38,28]. Its highest prevalence is within tropical and subtropical regions, and is common in areas with inadequate sanitation such as refugee camps and congested communities but is highest in children of school going age and women of childbearing age [38]. Infection in humans is acquired through the ingestion of infective eggs from contaminated soil and food [38,112].

1.3.2. Trichiuris trichiura

The nematode (roundworm) *Trichiuris trichiura*, also called the 'human whipworm', causes trichuriasis in humans [113]. It is estimated that 800 million people are infected worldwide [114] Infection in humans is through ingestion of infective eggs from contaminated faecal materials; diagnosis is by identification of mature eggs from stools using microscopy, and infection can be treated by using albendazole, the major anthelminthic chemotherapy available [115,116].

1.3.3. Nectar americanus and Ancylostoma duodenale: Hookworm species

The human hookworms include the nematode species, *Ancylostoma duodenale* and *Necator americanus* [117]. A larger group of hookworms infecting animals can also invade and parasitize

humans (*A. ceylanicum*) or can penetrate the human skin, causing cutaneous larva migrans [28], but the infection does not develop any further with *A. braziliense* being an example of such an infection [118]. Occasionally *A. caninum* larvae may migrate to the human intestine, causing eosinophilia enteritis. Studies indicated that hookworms are the cause of the second most common human helminthic infection after ascariasis [40,117–121]. They are commonly distributed in areas with moist and warm climates widely in Africa, Asia and the Americas [121].

Although much improvement has been made in the diagnosis and prevention of soil transmitted helminths, many NTDs still remain severely under-treated because of lack of sufficient funding to address the disease burden [44] International organizations, and academia, as well as public–private partnerships (PPPs), have recently begun to focus more on NTD drug discovery [29,44,72,122]. Yet these efforts alone have been insufficient. Importantly, these diseases are often very dynamic and keep expanding in places where hygiene is poor [20]. This study, seeks to provide evidence to lend weight to one view in a controversy about effect of anthelminthic on disease markers of HIV progression in pregnant women on ART in a targeted versus untargeted cohort fashion.

1.4. Research Gaps

This section describes information from previous studies and the results of recent randomised control trials on the effect of deworming on HIV/AIDs immune progression markers. These studies have led to the identification of the main research questions, which I have attempted to answer in this PhD study. This section further describes programs against HIV/AIDS and initiatives to improve access to ART; mainly in rural and urban health facilities providing Antenatal care services. It provides different perspectives regarding the definition of co-infection by NTDs/helminths and HIV, reviewing studies on co–infection, including those among HIV-infected pregnant women in African antenatal settings. After a brief review of the literature on the effects of helminths infection on HIV and malaria disease progression, the author lays out a problem statement, study aims, objectives, and hypothesis of the study, and its significance.

1.4.1. Problem statement and outcome measures

The studies on the relationship between helminths and HIV/AIDS have been small in African antenatal settings (2, 83-85). The impact of malaria on anaemia and the important interplay with helminths demonstrate the need to address the interactions between HIV diseases, malaria and intestinal helminth infections in pregnant women. Earlier studies have not documented the beneficial effects of targeted versus non-targeted de-worming in pregnant women, and have not tested the hypothesis that de-worming may slow disease progression in HIV-infected patients on antiretroviral therapy with either or both of these two methods. The use of a non-targeted approach would be more pragmatic as it would not require testing for the presence of helminth infection and may therefore be more appropriate in resource-limited environments. Due to the roll out of ART in many African countries, a large number of pregnant females now receive ART. It is therefore important to study the effect of anthelminthic therapy in this group of subjects, for whom no data currently exists.

This research study comprised of a pilot study to investigate the prevalence of, and the risk factors for, helminth and malaria co-infections in HIV-positive, pregnant females receiving ART. A second study sought to elucidate the benefits of de-worming HIV infected, ARV-treated pregnant females using two different de-worming approaches: targeted and non-targeted de-worming. The examined outcomes were the impact on host viral load detection levels, CD4-cell counts, prevalence of helminths, malaria and serum haemoglobin levels.

1.4.2. Purpose of the study

The aims of this study were: to determine the prevalence and risk factors for helminth-malaria co-infections in pregnant HIV-positive females receiving ART; to investigate whether treatment of helminth infections in HIV infected pregnant women have any beneficial effect on HIV disease progression markers, such as viral load and CD4 cell counts; to determine the effects of de-worming on haemoglobin levels and body mass index; to determine the prevalence of malaria infections in the absence and presence of a helminth infection; to determine the efficacy of two de-worming intervention methods i.e. targeted and untargeted. It should be noted that the investigation of the effect of different ART regimens on the risk of helminth or malarial infection

was not an initial aim of this project, and therefore the study was not designed with this purpose in mind. However, following analysis of the data from the initial pilot study and the baseline data from the longitudinal study, it became clear that ART did have some effects, although inconsistent, on infection rates. These results therefore became quite prominent in the first two papers that were published from this PhD, and are therefore presented in the first two results chapters (chapter 3 and 4) of this thesis.

1.4.3. Study objectives

1. To determine the prevalence of helminthic-HIV co-infections among pregnant women in rural and peri-urban regions of Rwanda.

2. To determine the prevalence of malaria infection in HIV-positive pregnant females with and without helminthic infection in rural and peri-urban regions of Rwanda.

3. To determine the principal risk factors for helminth, *P. falciparum* and helminth-*P. falciparum* co-infections in HIV infected pregnant Rwandan females.

4. To determine the effect of anthelminthic therapy on HIV viral loads, CD4 cell counts and anaemia among pregnant women in the absence and presence of helminth co-infections in Rwanda.

5. To determine the effect of anthelminthics on the incidence of helminthic infections in HIV-positive pregnant Rwandan females in targeted and untargeted treatment arms over time.

1.4.4. Hypotheses tested

The hypotheses tested in this thesis were as follows:

1. Helminth-malaria dual infections and helminth infection prevalence is higher among HIV infected pregnant women on ART in Rwanda in rural compared to urban health centres.

2. Malaria–helminth co-infection and helminth infection risk negatively correlate with socio-economic status of the HIV-infected pregnant women on ART in Rwanda.

3. Deworming has a positive effect on HIV/AIDS immune progression markers i.e. CD4 counts, among HIV infected pregnant women on ART in Rwanda.

4. De-worming using a targeted or a non-targeted approach is equally effective at reducing helminth infection levels.

1.4.5. Significance of the study

The results of this study have the potential to add weight to the national health policy related to the control and treatment of helminths and other NTD infections and malaria in HIV-positive pregnant females in Rwanda. The results sheds insight on the possible benefits of untargeted de-worming (mass treatment) or targeted de-worming (diagnosis and subsequent treatment) of helminthic infections as part of routine HIV care in antenatal clinics across Rwanda.

1.5. Summary

The NTDs are a major source of morbidity and mortality in Africa. Furthermore, regions in which these diseases are prevalent also have high prevalence levels of malaria and HIV. These overlapping epidemics have severe effects in at risk populations, most particularly pregnant females. However, few studies have examined the interaction of these diseases in pregnant women in sub-Saharan Africa. The few randomized control trials of anti-helminthic therapy that have been conducted did not include HIV-infected women on ART and thus little data is available on the effect of de-worming on immune progression and anaemia in such cases. The current study will attempt to provide this data and to compare the effectiveness of a targeted anti-helminthic drug intervention to a non-targeted format.

2. Chapter 2: Materials and Methods

2.1 Introduction

This chapter describes the procedures and methods applied to conduct the baseline helminth prevalence study and the prospective interventional study to determine the effect of deworming on immune markers of HIV-1 disease progression among HIV-infected pregnant women on ART.

2.2 Description of pilot study and intervention study

The first study was carried out as a pilot study to validate the study tools and generate information on the prevalence of helminth and malaria co-infection in pregnant females receiving ART. The pilot study was conducted at three conveniently sampled PMTCT sites in the Ruhuha, Mareba (both rural sites) and Biryogo (peri-urban) districts, which are mainly rural and peri-urban settings with ANC and ARV services for HIV-infected pregnant women on ART. This study also assessed the effect of ART on the prevalence of helminth and malaria infections in HIV-infected pregnant women. Findings from the pilot study were used in the design of the longitudinal cohort study. The results from the pilot study are described in Chapter 3 of this thesis.

After obtaining data from the pilot study, we then enrolled pregnant female subjects receiving ART into a longitudinal cohort study based at the following health centres: Kamabuye, Rugarama, Kanombe, Masaka, Kicukiro, and Kacyiru. This study was split into 2 parts: a baseline study in which the prevalence of, and risk factors for, helminth and malaria infection were analysed. These data are described in Chapter 4. These same participants were then followed-up through pregnancy in an intervention study in which 2 different methods of deworming i.e. targeted and untargeted, were compared in terms of prevention of helminth infection and effects on malaria infection and CD4 counts and viral loads. Data from this study is provided in Chapter 5.

The baseline and longitudinal studies were carried out from January 2010 to December 2010 by a study team of project-recruited nurses, midwives, laboratory technicians, clinical officers and site doctors. All members of the study team were trained to use the study tools (see Appendix 3

and 4: Follow up and case report forms). The pilot and longitudinal studies will be described in detail in the following sections of this chapter.

2.2.1 Population and sample characteristics

The populations in both studies [pilot and longitudinal] consisted of HIV-positive pregnant women attending antenatal services in their second trimester. The subjects in the initial pilot study were investigated only at one-time point whilst those in the longitudinal study were visited twice during their pregnancy at 3 monthly intervals, followed by 2 visits over six months in the post-partum period. All HIV-positive women in both studies were on anti-retroviral therapy (ART) as per national PMTCT service guidelines of the Rwandan Ministry of Health, which follow WHO recommendations for pregnant women receiving highly active anti-retroviral therapy (HAART) [126].

2.2.2 Intervention and sampling procedures

Within the intervention study, in the 'targeted treatment' arm, participants who tested positive for helminths were treated with an anthelminthic (albendazole), while those found negative were not treated. In the 'untargeted treatment' arm, testing for worms was done and records captured at all visits, but all participants were de-wormed irrespective of whether or not they had a worm infection.

2.2.3 Sample size estimation

A sample size calculation was not performed for the pilot study (Chapter 3) with the sample number (328) used being based purely on logistical factors.

For the intervention study we estimated that 100 pregnant females per treatment group (N=490 per group) would be helminth infected, based on data from the pilot study. That would allow us to detect a difference in plasma viral load of 250 copies/mL. This assumes a power of 80% at a 5% significance level. The total number of pregnant women enrolled into the study was therefore 980 randomized into two groups of 490 each for the targeted deworming arm and the untargeted deworming arm.

2.2.3.1 Randomization

Within the intervention study computer-assisted modelling was used to allocate each participant randomly to the targeted or untargeted treatment arms. For this purpose, at first enrolment into the study each participant was issued with an I.D. number, which was randomly drawn. Records were kept under lock and key by the Principal Investigator in Rwanda and a copy was provided to the project supervisors in Rwanda and South Africa. The intervention was not completely blinded, as participants may have been able to recognise which subjects were receiving the white tablet (albendazole) from the study nurse. The investigators were not blinded to assignments.

2.2.4 Inclusion Criteria

These criteria were used in both the pilot and the intervention studies.

- HIV+ pregnant women in their second trimester
- Attending antenatal services or PMTCT in the selected rural or peri-urban health centres
- Aged 18-45 years
- On highly active retroviral treatments (HAART) or prophylactic ART therapies
- Ability to provide3 stool samples on three consecutive days for helminths assay

2.2.5 Exclusion criteria

These criteria were used in both the pilot and the intervention studies, except where noted.

- HIV negative pregnant women
- Below 18 years of age
- In first trimester of pregnancy or reporting at antenatal clinic late in third trimester
- Pregnant woman unable to stay within one health centre for more than six months after delivery (intervention study only)
- At enrolment not willing to sign consent to the study

2.2.6 Study visits and study data collection procedures

The study sites for the intervention study were 6 health care centres: Kamabuye, Rugarama Kanombe, Masaka, Kicukiro, and Kacyiru. These sites did not include the 3 health centres that

were used in the pilot study i,e, Mareba, Ruhuha, and Biryogo, and were strategically selected from a list of health facilities that have PMTCT/ANC/ART services and were near to lakeside areas where helminth prevalence is expected to be high among women of reproductive age.

During the intervention study participants had two antenatal visits and two post-partum visits to each participating health centre. During each visit, sampling for helminths, haemoglobin levels, CD4 counts and viral loads and malaria tests were performed. The study midwife also measured body mass index at each visit.

1. At visit 1 (V1) (baseline visit), women were screened for inclusion criteria and asked to sign consent forms. Upon enrolment in the study, each woman was given a study ID number and was physically examined by the study site doctor or a protocol- trained nurse or midwife who collected the required physical data, including age, trimester of pregnancy and medications being used by the study subjects.
2. More individual data were collected using the questionnaire forms (Appendix 3) that included the type of ART and its duration, use of supplements, TB history and results; anti-helminthic treatment history and helminth-related risk factors were also obtained in this way.
3. Laboratory specimens collected included stools for helminth testing, blood for haemoglobin, and blood slides for malaria parasites and histidine-rich protein assessments. More blood was collected according to the study protocols by PMTCT/ANC laboratory staff for CD4 cell count and viral load assays. At each visit, three stool samples were analysed for helminth-intensity determination using Kato–Katz test [128].
4. At visit 2 (V2) most women had reached their third trimester. A doctor, nurse or midwife determined the gestation stage.

With the exception of the viral load assay test (done only at V1 and V4), all other samples were collected on visits 1, 2, 3 and 4 of the study period. After delivery, the women's data were collected as described in Figure 1 of Appendix 1.

Table 2.1. Investigational schedule

No	VISITS	PARTICIPANTS	TIME BETWEEN VISITS	TESTS/VARIABLES
1	Visit 1 Baseline	Pregnant woman	3 months	Hb,CD4,vl,BS,hrp-2,epg, BMI
2	Visit 2	Pregnant woman	3 months	Hb,CD4,BS,hrp-2,epg, BMI
3	Visit 3	Mother	3 months	Hb,CD4, BS,hrp-2, epg, BMI
4	Visit 4	Mother	3 months	Hb,CD4, VL, BS,hrp-2 epg, BMI

KEY: Hb = haemoglobin level, epg = eggs per gram faeces, BS = blood slide for malaria HRP-2 = histidine rich protein-2 antigen, CD4 = cluster of differentiation-4, VL = viral load, BMI = body mass index

2.2.7 Sample collection

In the intervention study for the purpose of sample collection during the interviews at all 4 study visits participating women were supplied with labelled plastic containers, waterproof papers and applicator sticks, and were instructed to bring stool samples three times on different days to the health centre laboratory project technician for testing. Blood was also drawn from subjects for immunity markers before and after the intervention period in both study arms. These samples were taken twice during antenatal visits and twice on post-partum visits to the health centre. Samples were sent to the National Reference Laboratory in Kigali for viral load assays. A case report form (CRF) was administered in the form of a structured questionnaire to all participants (Appendix 4). Information captured included personal hygiene behaviour, age, height, weight, parity, gestational stage, level of education and HIV/AIDS treatment services received at antenatal care units, including any TB clinical history and presence or absence of any nutritional

supplements. The women were interviewed in their mother tongue, Kinyarwanda, and translated into English for data transcription forms.

2.2.8 Physical examination of the participants

Participants' social demographic data were collected using the case report form (CRF) described in Appendix 4 administered in form of a questionnaire. Physical data captured on the CRF were also entered into an Access or Excel spread sheet to enable cleaning of data before analysis.

Anthropometric indices were determined, with weight taken to the nearest 0.5 kg, using a calibrated measuring scale, height measured in meters, and body mass index calculated there-from. Body mass index (BMI) was computed as weight (kg) divided by height (m) squared, as described [129].

2.2.9 Intervention assignments

Study arm A.

- These subjects were given anthelminthic if they tested positive for helminth infection at any of the 4 study visits.
- In this targeted arm, women who tested helminth-negative did not receive anthelminthic.

Study arm B.

- This consisted of a non-targeted intervention arm; all participants in this arm received anthelminthic, irrespective of helminth-infection status, at all 4 study visits.

Treatment to subjects in both arms

- All helminth-positive women in arm A and all women in arm B received a single dose of albendazole (400mg) at all 4 study visits.
- In both arms women who had malaria were treated with anti-malarial therapy in the form of Coartem® (120mg artemether -20mg lumefantrine) at each study visits at which they tested positive for *P. falciparum*

- Iron supplements were given in accordance with the Rwanda Ministry of Health guidelines for antenatal women i.e. an iron supplement was given to all pregnant women with a haemoglobin concentration of < 8g/dl [127].

It is known that ART reduces viral replication and can reduce mother-to-child transmission of HIV by lowering plasma viral load in pregnant women and in their new-borns [130]. In this study all women enrolled were given highly active antiretroviral treatment (HAART) in accordance with the Rwandan Ministry of Health guidelines [127]. These guidelines state that pregnant mothers should be initiated on HAART irrespective of CD4 cell count levels [126]. The following regimens are available in Rwanda [126,131]:

First line treatment: AZT (Zidovudine) +3TC (Lamivudine) + Nevirapine (NVP). In case of any complications AZT is replaced with d4T (stavudine) [127]

Second line treatment: Abacavir (ABC), +Tenofovir (TDF) + Lopinavir (LOPV/r) [127]

2.2.10 Follow-up procedures

A six-month post-partum period was used to record any significant changes in mothers for each of the de-wormed and non-de-wormed arms. At each visit, information about the occurrence of symptoms, dropouts, illness and treatment, were recorded in the investigators' notes and captured on a study progress form, which was updated during each visit. Home visits by four members of the study team drawn from the list of local collaborators in Rwanda were arranged to follow up women that dropped out of the study. A database of participants' addresses was developed and used to trace them to their homes by mobile phone contact and site visit where possible, with help of community health workers.

2.2.11 Unexpected or unusual symptoms and possible risks of the study

Any adverse reaction or unusual symptom of any nature or severity which was not consistent with the study investigational protocol and not in agreement with the International Clinical Harmonization (ICH) Guidelines for Clinical Safety Data Management was treated by the study doctor in collaboration with clinical coordinators. The principal investigator regularly reported to the Institutional Review Board (IRB) in Rwanda while adhering to the existing research

principles of the Rwandan Ministry of Health treatment guidelines to safeguard the study subjects according to the principles of the Helsinki Declaration [132].

Risks There were minimal risks to subjects participating in this study. The only risk in taking part in the study could have been mild and short-lived drug reactions that could be easily treated by the research team doctor.

Potential side effects of medications used

Albendazole

Albendazole therapy during pregnancy is not associated with a significant increase in major congenital defects [133,134] but it should be avoided during the first trimester. The therapy offers beneficial effects to pregnant women in countries where intestinal helminths are endemic [135,136].

Iron supplements

There is evidence that supplementation with iron can be provided safely with anthelminthic treatment [137]. Current practice in Rwanda recommends giving an iron supplement to all pregnant mothers for anaemia if the haemoglobin concentration is < 8g/dl in second trimester, and this was applied in the current study. There were minimal side effects of iron supplementation to pregnant mothers, and the research team doctor treated any effect noted during the study.

Coartem® (artemether 120 mg plus lumefantrine 20 mg*)*

In the case of pregnant women who tested positive for malaria, Coartem was given in four doses over three days, i.e. two doses on day 1 and one dose each on day 2 and 3 [138]. Side effects of Coartem treatment in pregnant women is rare and minimal [139], consisting of nausea, vomiting and neurological disorders, and whenever any of these side effects appeared, the study doctor treated the participants.

AZT and NVP as first-line ART treatment

Side effects like diarrhoea and anaemia are related to AZT therapy and an NVP-related rash may also be encountered, and where they arose, the participants were treated by the study doctor according to guidelines of Rwandan Ministry of Health. Neonates may experience lower birth weight or congenital effects like neurological disorders. All these are rare side effects of the ART treatment regimen to mothers, and were treated by the study team doctors in accordance with Rwanda Ministry of Health guidelines for all subjects in the study [131]

2.3 Laboratory analyses

2.3.1 Laboratory stool analysis

Three slides were prepared for each stool sample collected on three different days from each participant and processed at each visit to the clinic by the Kato-Katz method as described previously [140]. Using standard methods three Kato-Katz slides were prepared from each of three stool samples collected on consecutive days, and examined within 30 minutes for *Ancylostoma duodenale* and *Necator americanus* and again the following day for the ova of *Ascaris lumbricoides* and *Trichiura trichiura* [140,141]. Eggs per gram (EPG) of stool were calculated by taking the mean of the mean values obtained for each of the three stool samples. Prevalence and incident rates as new events were measured by using methods previously described [142]. Sensitivity and specificity of different stool concentration methods were considered using standard laboratory methods [143].

The study utilized use of Kato Katz multiple stool slides taken at three consecutive days and compared the results with that of formal ether concentration technique for ensuring high sensitivity and specificity to identify helminths [143]. This rigorous approach has been recommended to yield high sensitivity and specificity for helminths prevalence [140,141].

2.3.2 CD4 and plasma viral load analysis

The CD4 cell counts were determined at enrolment using Multiset® software on a FACS Calibur XT1800i® dual-platform system (Becton Dickinson BD, San Jose, CA, USA). Plasma HIV RNA was quantified using a Gen-probe® HIV-1 viral load assay on a AmpliPrep/Cobas Taqman® (HIMCAP, Fort Lauderdale, FL, USA) [144].

2.3.3 Haemoglobin measurement

A blood drop was obtained using a finger prick device and tested for haemoglobin using a DOTmed ®, Hemocue®device [145,146]. A haemoglobin level of less than 8g/dl was defined as anaemia.

2.3.4 Malaria assessment

Asymptomatic parasitaemia was determined in all women participating in the study. Plasmodium falciparum was identified by light microscopic examination of Giemsa-stained thick and thin blood smears. Study participants were also tested with an HRP-2 rapid immunochromatographic dipstick test (Paracheck-F®, IndMED, India) for malaria antigen positivity rates [147]. This test can be applied easily in the field in epidemiological studies because it is a rapid kit method and is considered user-friendly [148-150].

2.3.5 Quality control

Quality control procedures involved review of laboratory slide results by trained experts from the National Reference Laboratory in Rwanda using existing standard operating procedures where 10% of the examined stool specimen slides for helminths diagnosis, and malaria smears were re-examined for concordance testing. Similarly, blood samples for CD4 and viral load assays were rechecked per standard operating procedures. All smears and samples were stored for review if required. In addition, a daily check of the quality of smears and samples were carried out by routine checks of study records by the principal investigator in accordance with Good Laboratory Practice (GLP) recommendations in Rwanda.

2.4 Ethical considerations

2.4.1 Ethics committees

The protocol was submitted to the Rwandan National Ethics Committee through the Kigali Health Institute and the University of the Witwatersrand's Ethics Committee, and the protocol

was approved by both organizations (see Appendix 1and 2 for Ethics Committee approval letters).

2.4.2 Conduct of study

The principal investigator of the research gave the invited participants a brief explanation of the study aims and objectives including study significance. All study participants were assured of confidentiality. Enrolment in the study adhered to the norms and declarations of Helsinki and all current amendments[132]. Procedures for the handling of study materials, collection of data and reporting of results were designed to maintain subject confidentiality and used study-coding arrangements within the requirements of good clinical practice and the Helsinki Declaration [151].

2.4.3 Information sheet and signing of consent

All information that was obtained from the participants in the study remained confidential and was recorded under an ID number specific to each participant. Paper copies of our documents were kept secure in Rwanda; the database was password-protected in a computer with the supervisor at the Kigali Health Institute in Rwanda and sent to Professor Nigel Crowther at the University of the Witwatersrand in Johannesburg, South Africa, when deemed relevant for scrutiny and at subsequent analysis stages.

2.5 Data Management and Analysis

2.5.1 Data analysis

All statistical analyses are described within the Methods section of each Results chapter.

2.5.2 Data safety and monitoring (DSM)

A local Data Safety and Monitoring (DSM) team worked with the principal investigator and included specialists with relevant skills in the fields of HIV/AIDS, parasitology, gynaecology, paediatrics, epidemiology and ethics. These people were drawn from a list of local collaborators. The monitors gave a written monitoring report to the sponsors at the Kigali Health Institute

Ethics Committee after each on-site data sheet appraisal and/or other study-related communication.

2.5.3 Direct access to data

The Permission to examine and reproduce any records and reports that are important to the evaluation of the study were given to the appropriate monitors by the principal investigator and the study supervisors. Any existing authorities, sponsor's monitors and auditors seeking direct access to data took all reasonable precautions within the constraints of the requirements of the study to maintain the confidentiality of the subjects' identities and investigator's information.

2.5.4 Data source documentation

All variables were encoded in compliance with STATA.11 (Chicago, IL, USA) software, which was used for the initial analysis of all data. Further analyses were done using Statistica version 9.1 (Stat Soft, Tulsa, OK, USA). The data from the laboratory, patient questionnaires, patients' files and physical examination of patients were first entered into Excel spreadsheets and Microsoft Access using double-entry before being entered into STATA for analysis by the investigator and the statistician.

Computer generated participant's codes were double-checked by the principal investigator and study supervisors to ensure that they correspond with the source data from the field.

2.5.5 Data collection

Trainees from the Kigali Health Institute and the National Reference Laboratory (NRL) carried out the laboratory analyses while TRAC-plus and other independent clinical collaborators involved in the study assisted in monitoring the project activities. The PI was in charge of all the project activities and supervised these together with clinical collaborators, overseeing all data collection and laboratory tests. The PI also carried out the data entry, data analysis and reporting of the study activities. However, co-investigators with appropriate medical qualifications assisted the PI in overseeing medical issues of study subjects throughout the data collection period.

2.6 Dissemination of findings

All study findings have been published and presented locally to the Rwanda Ministry of Health and to health centre authorities in Rwanda. Results were also presented as posters and abstracts at different conferences and manuscripts were published in international peer-reviewed journals as described in appendix 4 and 5 and 6 at the end of the thesis.

Chapter 3: Malaria and helminthic co-infection among HIV-positive pregnant women: prevalence and effects of antiretroviral therapy

3.1 Abstract

Introduction: The impact of malaria on anemia and the interplay with helminthes underline the importance of addressing the interactions between HIV/AIDS, malaria and intestinal helminthes infections in pregnancy. The aim of this study was to determine the prevalence of malaria–helminthes dual infections among HIV positive pregnant mothers after 12 months on ART.

Method: a cross sectional study was conducted on intestinal helminthes and malaria dual infections among HIV-positive pregnant women attending antenatal health centers in Rwanda. This was the pilot prevalence data carried out to validate study tools, before the main longitudinal study

Results: stool and malaria blood slide examinations were performed on 328 women residing in rural (n = 166) and peri-urban locations (n = 162). BMI, CD4 cell count, hemoglobin levels, type of ART and viral load of participants were assessed. Within the study group, 38% of individuals' harbored helminthes, 21% had malaria and 10% were infected with both. The most prevalent helminthes species were *Ascaris lumbricoides* (20.7%), followed by *Trichuris trichiura* (9.2%), and *Ancylostoma duodenale* and *Necator americanus* (1.2%). Helminthes infections were characterized by low hemoglobin and CD4 counts. Subjects treated with a d4T, 3TC, NVP regimen had a reduced risk of *T. trichiura* infection (OR, 0.27; 95% CIs, 0.10–0.76; $p < 0.05$) and malaria–helminthes dual infection (OR, 0.29; 95% CI, 0.11–0.75; $p < 0.05$) compared to those receiving AZT, 3TC, NVP.

Conclusion: This study shows a high prevalence of malaria and helminth co-infection among HIV-positive pregnant women in Rwanda. The differential effect of ARTs on the risk of helminth infection is of interest and should be further examined in a double-blinded placebo randomized controlled trial in a larger patient group and explore the role of drug-drug interactions.

3.2 Introduction

To summarize what has been said in earlier chapters, helminth and malaria infections have been hypothesized to be factors likely to be driving the HIV-1 epidemic in Africa (1,2). Globally, there are more than 2 billion people that are estimated to be infested with soil-transmitted helminths, with the geographical distribution of these infections overlapping considerably with regions of high HIV-1 sero-prevalence (1) and malaria endemicity (3,4). Due to their profound effect on the host immune system, which makes those infected more susceptible to HIV-1 infection (1,5), malaria and helminth infections play an important role in the pathogenesis of HIV-1 infection in Africa.

The combination of HIV, helminthic and plasmodial infections in a host create an immunologically complex profile (6) and substantially increase the risk of anemia, which is caused by all three types of infections (7). Therefore, in terms of co-infection with these diseases, pregnant women in sub-Saharan Africa represent a highly vulnerable group, particularly in light of data showing that helminth infection increases the risk of mother-to-child transmission of HIV (6).

Recent data on the prevalence of helminth infection in Rwanda for school-going children from eight districts indicated a prevalence of 64.5%. The observed prevalence was higher in rural than in urban settings (8). However, no studies have documented the prevalence of malaria and intestinal helminth dual infections among pregnant women with HIV/AIDS attending antenatal services in the context of ARV roll-out programs. Therefore, the aim of this study was to determine the baseline prevalence of helminth and malaria dual infections in HIV-1 infected pregnant women attending Rwandan health centres after ART initiation. To give some context to the results given here, a brief description is provided of the methods used to obtain them.

3.3 Methods

3.3.1 Study area and population

Enrolled participants were a sample of women attending the antenatal health centre clinics in the provinces of Ruhuha, Mareba (both rural sites) and Biryogo (peri-urban) in Rwanda. These regions are within easy reach of Kigali, where all stool and blood samples analyses were to be performed. Health facilities were chosen based on facilities that had functional PMTCT/ANC/ARV services and were located near to inland lakes, thus increasing the chance of helminth infection. One such facility was chosen randomly from each of the 3 provinces. After having given their informed consent in writing, the selected women in the second and third trimester of pregnancy were enrolled. Women were excluded if they were HIV negative, below 18 years of age, had clinical evidence of TB, and had a treatment history of anthelmintic therapy and clinical confirmation of an abnormal pregnancy. Upon enrolment, subjects were interviewed for demographic information, and the type and duration of the ARV treatment they were receiving.

After informed consent was obtained, blood samples were collected for malaria microscopy, rapid malaria testing, haemoglobin level and CD4 cell count determination and viral load assays. Stool samples were obtained on three consecutive days for determination of helminthic infection.

3.3.2 Other procedures

Stool sample collection and examination from each woman involved fresh stool samples collected using an appropriate container, kept according to the national laboratory standards and then examined using microscopy by a lab technician trained for this procedure. Blood samples for malaria, viral load and CD4 samples were collected and analysed using available standard methods. The other methods used in this section of the thesis (described in detail in Chapter 2) are as follows: formal ether concentration methods for intestinal helminths identification in stools, identification of *P. falciparum* infection, measurement of serum haemoglobin levels, assessment of CD4 counts, viral load measurement (see page 25) and treatment of HIV infections (see page 23). Also described in Chapter 2 are the selection of the study sites and the sample size estimation (see pages 17-18) and inclusion and exclusion criteria (see page 19).

3.3.3 Statistical analysis

Analyses were performed using Stata® version 11.0 (College Station, TX, USA) and Statistica® version 9.1 (Stat Soft, Tulsa, OK, USA). Data included in tables and the text of this chapter are expressed as mean ± SD or median (range from 25^{th} to 75^{th} quartile). Data that were not normally distributed were log transformed to normality. Comparison of continuous data between 2 groups was carried out using the Student's non-paired T test, whilst data comparison across more than 2 groups were performed using ANOVA with paired means analysed using Turkey's test. A comparison of mean values between groups, with adjustment for possible confounding variables, was accomplished with the use of ANCOVA. Percentage levels were compared across study groups using the $\chi 2$ test. Backward, stepwise multiple regression analyses were performed to identify the principal determinants of haemoglobin levels and CD4 counts. Independent variables that were included in the initial regression model were those that correlated with the dependent variable with a significance level of $p<0.10$ in a Pearson univariate analysis. Variables were then removed one at a time based on their p-value, with the variable with the weakest p-value being removed in each round until only variables with $p<0.05$ were left in the model. Logistic regression was performed to assess the risk of infection across antiretroviral therapy (ART) regimens and data expressed as an odds ratio (OR) with 95% confidence intervals (95% CI). The categorical variable was infection/no infection whilst the independent variables were the ART regimens for which dummy variables were generated. The AZT-3TC-NVP regimen was set as the reference, with an odds ratio of 1. The logistic regression models were run with and without the confounding variable of location, i.e. peri-urban and rural, which were coded as 0 and 1 respectively.

3.4 Results

3.4.1 Participants' characteristics

The study cohort of 328 pregnant women included 166 from rural and 162 from peri-urban centres. The median [range] age of the cohort was 27.0 [8.00] years, with no significant difference between the 2 population groups. The mean BMI (± SD) of the total cohort was 25.4 ±

3.44, with women from the peri-urban group (26.7 ± 2.88) being significantly heavier than the rural population (24.1 ± 3.46; p<0.0001).

Participants were recruited to the study in the second and third trimesters of pregnancy, with 288 (87.8%) women recruited in the second and 40 (12.2%) in the third trimester. No significant differences were noted between these 2 groups for any of the study variables, except that 53.5% of women recruited in their second trimester of pregnancy originated in a rural area, compared to 30.0% recruited in their third trimester (p=0.005).

3.4.2 Prevalence of helminth and malaria infections

Table 3.1. Prevalence of malaria and helminth infections in rural, peri-urban and combined populations

VARIABLES	RURAL	PERI-URBAN	COMBINED
N (number of respondents)	166	162	328
Malaria with or without helminth (%)	29.5	12.3***	21.0
Helminth with or without malaria (%)	44.6	32.7*	37.8
Specific species : Ascaris lumbricoides (%)	23.5	17.9	20.7
Trichiura trichiura (%)	12.7	5.56*	9.15
Hookworm (%)	1.81	0.62	1.22
Asc + Trich + Hkw (%)	6.63	8.64	7.62
Both malaria and helminths (%)	15.1	4.94**	10.1
Eggs per gram of stool (EPG)	6236±6831	6491±4814	6342±6051

Data given as % except for EPG (mean±SD); mean EPG was calculated using values only from subjects with a worm infection (N=74 for rural and 53 for peri-urban); *p<0.05, **p<0.005, ***p<0.0005 versus rural group. Asc= *Ascaris lumbricoides*, Trich = *Trichiuris trichiura*, Hkw = hookworm

Table 3.1 shows that the prevalence of malarial infection with or without helminths (p<0.0005) (i.e. those cases with a malarial infection which did or did not include a helminth co-infection, in other words all cases with malaria) and of malarial-helminths dual infection (p<0.005) was significantly higher in the rural than in the peri-urban population. Helminth infections with or

without malaria (i.e. those cases with a helminth infection which did or did not include a malaria co-infection, in other words all cases with helminth infection) were also higher in the rural population ($p<0.05$), as was the prevalence of *Trichuris trichiura* infection ($p<0.05$). Cases were also detected where helminths infection was present without malaria. The prevalence of these 'helminths only' cases was 27.7% in the rural population and 27.8% in the peri-urban group. Also, malaria was detected in subjects without a helminths co-infection. The prevalence of these 'malaria only' cases was 14.5% in the rural population and 7.4% in the peri-urban group ($p<0.05$ versus rural) with an overall prevalence of 11.0% in the combined population. The EPG values did not differ between the two groups.

Table 3.2. Effects of helminth and malarial infections

VARIABLES	NO INFECTIONS	MALARIA ONLY	HELMINTHS ONLY	BOTH MALARIA & HELMINTHS
Number of subjects	168	36	91	33
Age (years)	27.0 [7.00]	27.5 [9.50]	26.0 [9.00]	28.0 [9.00]
BMI	25.7 ± 3.53*	25.5 ± 3.74	25.6 ± 3.03*	23.5 ± 3.31
Haemoglobin (g/dl)	12.3 ± 1.10** †	12.1 ± 0.95	11.9 ± 1.17	11.5 ± 1.05
Prevalence of anaemia (haemoglobin<12g/dl) (%)	34.5** †	44.4	47.2	63.6
CD4 (cells/mm3)	532 [181]** †††	533 [203]* †††	449 [131]	464 [156]

Data expressed as mean ± SD, median [interquartile range] or percentage; *$p<0.05$, **$p<0.005$ versus both malaria and helminth; †$p<0.05$, †††$p<0.0005$ versus helminth only

Table 3.2 shows that dual infection with malaria and helminths is associated with a lower BMI than in both non-infected ($p<0.05$) and helminth-only infected ($p<0.05$) subjects. Furthermore, compared to subjects with no infections, those with a malaria-helminth dual infection or an infection with helminths only are characterized by lower haemoglobin levels ($p<0.005$ and

$p<0.05$, respectively) and a higher prevalence of haemoglobin levels <12g/dL ($p<0.005$ and $p<0.05$, respectively). Subjects with malaria alone also had lower haemoglobin levels and a higher prevalence of reduced haemoglobin concentrations when compared to females with no infections; however, these differences were not statistically significant. Also, subjects with a dual infection or with a helminth-only infection had lower CD4 counts than subjects with no infection ($p<0.005$ and $p<0.0005$, respectively) and subjects with a malaria-only infection ($p<0.05$ and $p<0.0005$, respectively).

Backward, stepwise multiple regression models were developed to identify the principal determinants of haemoglobin levels and CD4 counts. The initial regression model for haemoglobin levels included the following continuous variables: helminthic egg count and body weight, and the following coded (0 or 1) variables: helminthic infection, rural/peri-urban and detectable viral load. The final model retained only 2 variables that significantly correlated with haemoglobin level: helminthic infection (beta = -0.21, $p<0.0005$) and body weight (beta = 0.13, $p<0.05$). The R-value for the final regression model was 0.27 ($p<0.0001$). The initial regression model for CD4 counts included one continuous variable (helminth egg count) and the following coded variables: helminthic infection, rural/peri-urban and antiretroviral treatment regimen. The final model retained only 1 variable that significantly correlated with haemoglobin level: helminthic infection (beta and R = -0.35, $p<0.0001$).

3.4.3 Anti-retroviral treatment regimens and their effects

Table 3.3. Comparison of anti-retroviral regimens used in rural and peri-urban populations

ANTI-RETROVIRAL REGIMENS	RURAL	PERI-URBAN
d4T-3TC-NVP (%)	63.2	0.00***
AZT-3TC-NVP (%)	19.9	3.09***
AZT-NVP (%)	0.60	95.1***
AZT (%)	12.0	1.23***
Other (%)	4.22	0.62*

*p<0.05, ***p<0.0005 versus rural group

Table 3.3 shows that there were significant differences in the antiretroviral regimens that were used at rural and peri-urban sites. Thus, over 95% of peri-urban centres used the AZT-NVP regimen. The most common (63.2%) regimen in use at rural sites was the d4T-3TC-NVP triple-combination.

Table 3.4. Comparison of BMI, haemoglobin and CD4 levels and prevalence of detectable viral load and helminthic and malaria infections across ARV treatment groups

Variables	d4T-3TC-NVP	AZT-3TC-NVP	AZT-NVP	AZT
N	105	38	155	22
Duration of ARV therapy (months)	8.62 ± 14.2	6.52 ± 9.64	8.76 ± 14.5	9.37 ± 14.9
BMI	24.3 ± 3.56***	24.0 ± 3.22***	26.9 ± 2.8	22.9 ± 2.25***
Hemoglobin (g/dl)	12.0 ± 0.87	12.1 ± 1.29	12.2 ± 1.21	12.0 ± 1.25
CD4 (cells/mm3)	490 [111]	463 [223]	500 [201]	537 [328]
Detectable viral load (%)	0.95†	10.5	0.00†††	0.00
Eggs per gram stool (EPG)	4292 ± 4881*	7344 ± 6409	7060 ± 6336	6371 ± 4902
Helminth with or without malaria (%) Specific species:	40.0†	65.8	32.2††	31.8†
Ascarislumbricoides (%)	22.9	26.3	19.3	13.6
Trichiuris trichiura (%)	8.57†	26.3	4.52†††	13.6
Hookworm species (%)	0.00	5.26	0.64	0.00
Asc + Trich + Hkw (%)	8.57	7.89	7.74	4.54
Malaria with or without helminth (%)	25.7*	39.5***	12.3	27.3
Both helminth and malaria (%)	12.4*	23.7**	5.16	9.09

Data expressed as mean ± SD or median (interquartile range) or percentage; mean EPG was calculated using values only from subjects with a worm infection (N=42 for d4T-3TC-NVP, 25 for AZT-3TC-NVP, 50 for AZT-NVP and 7 for AZT); *p<0.05, **p<0.005, ***p<0.0005 versus AZT-NVP;†p<0.05, ††p<0.005, †††p<0.0005 versus AZT-3TC-NVP; Asc = *Ascaris lumbricoides*, Trich = *Trichiuristrichiura*, Hkw = hookworm

Table 3.4 shows that duration of ARV therapy, haemoglobin levels and CD4 counts were not significantly different across the four treatment groups. However, BMI was significantly higher in those receiving AZT and NVP when compared to all other groups ($p<0.0005$ for all comparisons). Also, the prevalence of a detectable viral load (DVL) was higher in the AZT-3TC-NVP treatment group compared to both the d4T-3TC-NVP ($p<0.05$) and the AZT-NVP groups ($p<0.0005$). This pattern was reproduced in the prevalence of helminths with or without malaria ($p<0.05$ and $p<0.005$, respectively) and *Trichiura trichiura* infections ($p<0.05$ and $p<0.0005$, respectively). It was also observed that malaria with or without helminths infections was more common in the d4T-3TC-NVP and AZT-3TC-NVP treatment groups compared to the AZT-NVP group ($p<0.05$ and $p<0.0005$, respectively). The same pattern was also observed for helminthic-malaria dual infections ($p<0.05$ and $p<0.005$, respectively). The EPG value for the d4T-3TC-NVP group was significantly lower than in the AZT-NVP group ($p<0.05$), and also lower than in the AZT-3TC-NVP group, but this difference did not reach statistical significance ($p=0.08$).

Only 6 subjects (1.83%) had a DVL (>40 RNA copies/mL) and the median CD4 count in these subjects was significantly lower than in those with an undetectable viral load (UVL; 363 [114] versus 493 [173] cells/mm^3; $p<0.0001$). All the participants with a DVL tested positive for helminthic infection, whereas only 36.6% ($p<0.005$) of subjects with an undetectable viral load (UVL) were infected with helminths. Malaria was also more common in subjects with a DVL (50.0% versus 20.5%; $p=0.08$) but this difference did not reach statistical significance. The prevalence of helminthic co-infection with malaria was also more common in those with a DVL (50.0% versus 9.32%; $p<0.005$). Haemoglobin levels were lower in subjects with a DVL compared to those with an UVL (11.0 versus 12.1 g/dl; $p<0.05$). However, after adjusting for the presence of a helminths infection using ANCOVA, the p value was attenuated ($p=0.08$).

The data in Table 3.4 suggest differential effects of the ARTs on malaria and helminths infections. This was investigated further by logistic regression analysis in which the odds ratios for risk of infection for each ARV treatment regimen was calculated with and without adjustment for location (peri-urban/rural), which was the only possible confounding variable. The AZT-3TC-NVP regimen was set as the reference with an odds ratio of 1. The results of this analysis are shown in Table 3.4. The data demonstrate that the d4T, 3TC, NVP regimen reduces the risk of *Trichiura trichiura* infection and the risk of a combined malaria and helminths infection when

compared to the AZT-3TC-NVP regimen, and that adjustment for location does not cancel out this effect. This was also observed for the effect of AZT-NVP on the risk of *Trichiura trichiura* infection. However, adjusting for location did attenuate the significant risk reduction observed for this ARV regimen on both malaria and dual malaria-helminth infection. It was also observed that for the d4T-3TC-NVP regimen, after adjustment for location, the odds ratio for malaria was slightly attenuated, with this effect just missing statistical significance (p=0.06).

3.5 Discussion

The current study shows that helminths and malaria infection levels in HIV-positive pregnant women are high, and that malaria and malaria-helminths dual infections are more common in rural than in urban areas. Malaria-helminths dual infection was found to be a risk factor for reduced BMI, whilst helminths infection either on its own or in conjunction with malaria was found to be associated with lower haemoglobin and CD4 levels. The ARV regimen of d4T-3TC-NVP was found to significantly reduce the risk of *Trichiuris trichiura* and malaria-helminthic dual infection when compared to the AZT-3TC-NVP regimen, whilst AZT with NVP also lowered the risk of *Trichiuris trichiura* infection.

A prevalence rate of 37.8% for helminth infections and 21.0% for malaria indicates a high transmission intensity of these parasites among HIV-positive pregnant women in Rwanda. An earlier study of malaria prevalence conducted among HIV-positive pregnant females in Kigali, Rwanda, demonstrated that 8.0% of the study group had malaria [166]. This figure is comparable to the prevalence (12.3%) of malaria observed in our study for subjects residing in the peri-urban areas of Kigali. No studies have been conducted in Rwanda on the prevalence of helminth infections in HIV-positive pregnant females. However a study has been conducted on the prevalence of helminth infections in school children from eight districts in Rwanda where the prevalence was 64% [167], which is much lower than the 37.8% level observed in our study. This may be related to the greater recreational exposure of children to contaminated soil, as well as a reduced level of agricultural activities in pregnant females.

Our findings show lower prevalence levels for malaria and malaria-helminths co-infection, but a higher level of helminthic infection than those reported in a study of pregnant women from

Ghana [74]. In that study the prevalence of malaria, intestinal helminths and dual infection was 36.3%, 25.7% and 16.6% respectively. However, the HIV status of that population was not assessed. An investigation in Uganda among pregnant women [168] reported a prevalence level of 68% for helminthic infection and 11% for malaria, with 12% being HIV-positive. Thus, these studies show varying levels of helminths infection during pregnancy across African populations. There may be a number of reasons for this, including differences in socio-economic status and the level of helminths exposure. It should be noted, however, that in the present study all participants received ART, and we found that helminths seem to be susceptible to particular ART regimens. When comparing HIV-negative to HIV-positive pregnant females, malaria is more common in the latter group and helminth infection increases the risk of mother-to-child transmission of the virus [15].

Our findings also show that dual infection with malaria and helminths is associated with a lower BMI compared to the other 3 study groups (Table 3.2). Women with malaria-only, helminth-only, or dual infection all had lower haemoglobin levels than non-infected women, but the difference was only significant for helminth-only or dually infected subjects (Table 3.2). The dually infected subjects had the lowest haemoglobin levels. It has been observed that dual infection of malaria with helminths leads to lower haemoglobin levels when compared to malaria-only or helminth-only infected pregnant women, whilst all 3 infected groups exhibit lower haemoglobin levels compared to non-infected subjects [169]. The non-significant effect of malaria on haemoglobin levels within our study may be related to the lower number of subjects carrying only malaria, thus reducing the power of the analysis.

In subjects with a helminth-only or a malaria-helminths infection, CD4 cell counts were significantly lower than in those with malaria-only or no infection (Table 3.2). It is well established that pregnancy, and first pregnancy in particular, increases malaria susceptibility [170]. It is equally generally accepted that HIV-infected pregnant women have a significantly increased risk of malaria parasitaemia and placental malaria, more severe clinical manifestations and pregnancy outcomes [169].Our study suggests that in HIV-positive pregnant women malaria is not necessarily associated with lower CD4 cell counts. This was confirmed by a multiple regression analysis in which the presence of malaria did not associate with CD4 counts. Further support for these results comes from a study showing that CD4 counts do not differ between

HIV-positive subjects with or without malaria [169], and from the observation that the prevalence of malaria is not related to CD4 cell counts [169]. Furthermore, a prospective investigation of HIV-positive pregnant women in Kigali, Rwanda, demonstrated that HIV-positivity but not CD4 cell count was the only factor related to an increased risk of malaria [171,166]. One explanation could be the fact that HIV infection has been found to be associated with a specific cytokine dysregulation resulting from impairment of IL-12-mediated IFNγ production by intervillous blood mononuclear cells impairing the immune response against placental malaria, rather than mounting a generalized suppression of the cellular immune response against malaria [172-175]. Thus, regarding CD4 cell count level and malaria alone, there seems to be conflicting evidence blurring the otherwise clear-cut picture of the malaria-HIV interaction.

By contrast, our data also show that helminth infection is linked to lower CD4 cell counts in pregnant, HIV-positive women and that all subjects with a DVL tested positive for helminth. However, previous studies have shown that CD4 counts correlate positively with the risk of helminths infection [3,176] although others have found the opposite [178]. Reviewing the epidemiology and immunology of helminth-HIV interactions, one study concluded that there is inconsistent evidence for a beneficial effect of anthelminthic therapy on CD4 counts and viral load in HIV-1 co-infected individuals [178]. However, it could be expected that this effect would be suppressed by successful ART, reducing viremia and possibly explaining why, in some studies including ours, CD4 counts did not relate to increased helminthic infection risk. It should be noted that both the earlier studies were performed using subjects who were ART naïve, whilst our study only involved women who were already receiving ART.

It has already been proposed that ART may reduce the prevalence of helminth infection [177]. However, the study by Bachur and colleagues compared ART with non-ART in different areas, and did not control for other covariates such as education and age, which could be relevant. A major finding of the study presented here is that there is a differential effect of ART on the helminthic infection rate. Thus, the ART regimens of AZT-NVP and d4T-3TC-NVP both significantly reduced the risk of helminthic infection compared to a regimen of AZT-3TC-NVP. This effect was particularly evident for *Trichiuris trichiura*. Furthermore, the d4T-3TC-NVP regimen, but not the AZT-NVP regimen, lowered the EPG count, suggesting that the first

therapy reduces both the prevalence and intensity of helminthic infection. The reason why these particular ARV regimens reduce the risk of *Trichiuris trichiura* infection is not known and requires future *in vitro* investigations.

It was also observed that both ART regimens that reduced the risk of helminthic infection had similar but less prominent effects on malaria risk, and had lower prevalence levels of DVL than the AZT-3TC-NVP regimen. A number of in vitro studies have shown that protease inhibitors can inhibit *P. falciparum* growth [179,180]; however, similar studies have not been performed with the other antiretroviral drug classes. Clinical studies have confirmed the ability of ART to reduce malaria prevalence [128,180]. A recent study also showed that early initiation of ART was linked with reduced levels of detectable viral load in pregnancy in the context of an option B treatment program initiated in some countries including Rwanda [181].

The present study found that different ART regimens were being used in urban compared to peri-urban districts. The reasons for this difference are not known and are surprising in light of the Rwanda Ministry of Health's nationwide treatment guidelines for HIV infection. It is possible that this difference may be related to supply chain problems with the AZT-NVP regimen being more accessible to peri-urban than rural areas.

The strengths of this study reside in the screening of 3 stool samples collected from each subject on three consecutive days for the presence of helminths, and the use of a combination of two different screening techniques to increase the sensitivity of helminths and malaria diagnosis. However, this study also has some limitations. Due to its cross-sectional design, it is impossible to determine temporal direction or causality. Also, the study had no control group of HIV-negative or positive subjects not receiving ART. Additionally, the sample size for this study was relatively small and therefore it may not have been sufficiently powerful to uncover true differences or relationships within the study cohort and may also obscure or falsely reveal significant effects in the logistic and multiple regression models.

In conclusion, we found that the prevalence of malaria and helminthic infections is high in HIV-positive pregnant women in Rwanda. Helminthic infection in this population is an important risk factor for low haemoglobin and CD4 cell counts whilst malaria-helminths dual infections are characterized by a reduced BMI. Particular ART regimens seem to have positive effects on both

P. falciparum and *Trichiuris trichiura* infections, and further studies on drug-drug interaction and association needs to be undertaken in large population samples for reproducibility and further confirm these results.

The following chapter describes a cross sectional analysis of baseline data of a longitudinal study comparing targeted versus non-targeted anti-helminthic therapy in HIV-positive pregnant females receiving ART. The baseline analysis included a higher number of subjects (N=980) than the study described in the previous chapter allowing a more detailed analysis of the risk factors for helminthic infection including demographic factors and personal hygiene related with helminthic infection likely fuelling factors.

Chapter 4: THE PREVALENCE OF HELMINTH AND MALARIA INFECTION IN HIV-POSITIVE PREGNANT WOMEN ON ANTI-RETROVIRAL THERAPY IN RWANDA

4.1 Abstract

Background: Within sub-Saharan Africa, helminthic and malaria infections cause considerable morbidity in HIV-positive pregnant women and their offspring. Helminth infections are also associated with a higher risk of mother-to-child HIV transmission. The aim of this study was to determine the prevalence of, and the protective and risk factors for helminth and malaria infections in HIV-positive pregnant Rwandan women receiving anti-retroviral therapy (ART).

Methods: Pregnant females (N=980) were recruited from health centres in rural and peri-urban locations in the central and eastern provinces of Rwanda. Helminths infection was diagnosed using the Kato Katz method whilst the presence of *Plasmodium falciparum* was identified from blood smears.

Results: The prevalence of helminth infections was 34.3%, of malaria 13.3%, and of co-infections 6.6%. Helminths infection were more common in rural (43.1%) than peri-urban (18.0%; $p<0.0005$) sites. A CD4 count $\leq$ 350 cells/mm^3 was associated with a higher risk of helminth infection (odds ratio, 3.39; 95% CIs, 2.16-5.33; $p<0.0005$) and malaria (3.37 [2.11-5.38]; $p<0.0005$) whilst helminth infection was a risk factor for malaria infection and vice versa. Education and employment reduced the risk of all types of infection whilst hand washing protected against helminth infection (0.29 [0.19-0.46]; $p<0.0005$). The TDF-3TC-NVP (3.47 [2.21-5.45]; $p<0.0005$), D4T-3TC-NVP (2.47 [1.27-4.80]; $p<0.05$) and AZT-NVP (2.60 [1.33-5.08]; $p<0.05$) regimens each yielded higher helminth infection rates than the AZT-3TC-NVP regimen. Anti-retroviral therapy had no effect on the risk of malaria.

Conclusions: HIV-positive pregnant women would benefit from the scaling up of de-worming programs alongside health education and hygiene interventions. The differential effect of certain ART combinations (as observed here most strongly with AZT-3TC-NVP) possibly protecting against helminth infection warrants further investigation. The possibility of drug-drug interactions between albendazole, Coartem and ARTs deserves further investigation.

4.2 Introduction

In sub-Saharan Africa, the health burden of helminthic disease is enormous and, consequently, co-infections with malaria against a backdrop of often-high HIV sero-prevalence are numerous [182], so that combating co-infections has been identified as an important public health goal [20,75]. Consequently, the important areas of current interest are the effects of helminthic infections on immune regulation, and their possible consequences for susceptibility to other infections and immunologically mediated conditions such as allergy and autoimmune diseases [183]. Risk factors for helminthic infections depend on the route of transmission and the life cycles of the various helminth species; they are usually related to hygiene and sanitation [104,178,184] The geographical distribution of helminthic infections is largely determined by several environmental factors such as climate and the presence of stagnant water bodies [18,185,186]. In the absence of vaccinations, the only currently recommended public health intervention for soil-transmitted helminths is regular mass de-worming, particularly for high risk groups, backed up by facilitating access to clean water, improved sanitation and health education [20,23,72,187].

Pregnancy may increase susceptibility to helminths, but this is uncertain. A recent study from Gabon showed increased prevalence of helminthiasis in pregnancy [188], but a study from Thailand found no association [184]. Susceptibility and clinical outcomes are further complicated by co-infection with HIV and malaria. Malaria due to *P. falciparum*, combined with helminthic infections during pregnancy in HIV-positive women is of great concern from a public health perspective [156,188,189]. Control of *P. falciparum* infection by intermittent preventive treatment and use of insecticide-treated bed nets is of great importance, especially in primigravidae [1,94,190-192]. HIV-infected women tend to experience faster CD4 decline during and after pregnancy [193], and could therefore be even more susceptible to helminthic infection and malaria.

The objective of this cross-sectional observational cohort study was to assess the major risk factors for helminth and malaria co-infection in HIV-positive pregnant women who participated in an early anti-retroviral therapy (ART) initiation program for the prevention of mother-to-child transmission in Rwanda. We describe the prevalence of malaria and helminth infection in HIV-infected pregnant women on ART, and assess the factors that may increase or decrease rates of

both infections. This cross-sectional investigation was a baseline study for a longitudinal cohort in which future measurements during pregnancy and post-delivery would be performed to determine the effect of anthelminthic intervention on helminth and *P. falciparum* infection rates and HIV disease progression.

4.3 Methods

4.3.1 Study population and procedures

Ethical approval of the methodology was obtained from the Rwanda National Ethics Committee and the Ethics Committee (Human Research) of the University of the Witwatersrand Medical School, Johannesburg, South Africa.

The participants in the study were recruited between 02 January 2010 and 29 December 2010 from women accessing antenatal care and ART services at rural and peri-urban health centres in the central and eastern provinces of Rwanda. After giving written informed consent, women in the second trimester of pregnancy were enrolled at their fourth, fifth or sixth month of gestation. In addition, women were enrolled if they lived within walking distance of the study areas, and if they planned to deliver at the health centre registered for the study. Enrolment criteria were: HIV infection; pregnancy in the second trimester; use of ART; and willingness to provide three stool samples on consecutive days. Women were excluded if they were diagnosed with tuberculosis, or if they had taken any anti-helminthic drugs at any time prior to entry into the study. Those who had been enrolled in other research projects during the study period were also excluded. Upon enrolment, participants were interviewed to obtain demographic and socio-economic information pertaining to the relevant environmental risk factors for helminthic and malaria infections, (questionnaire used is attached as Appendix 3). Subsequently, participants provided blood and stool samples before being treated in accordance with the study protocol.

4.3.2. Other procedures

The other methods used in this section of the thesis are as follows: intestinal helminthic identification in stools; identification of *P. falciparum* infection; measurement of serum haemoglobin levels; assessment of CD4 counts; viral load measurement; treatment of helminthic, *P. falciparum* and HIV infections. All these methods are described in detail in Chapter 2 of this thesis.

4.3.3. Statistical analysis of data

Data analysis was performed using Stata® version 11.0 (College Station, TX, USA) and Statistica® version 9.1 (StatSoft, Tulsa, OK, USA). Data that were not normally distributed were log-transformed to normality before analysis. Differences between percentage values were assessed using the χ^2 test.

Backward stepwise multiple logistic regression analysis was used to determine the principal factors associated with helminth and malaria infections and helminthic-malaria co-infections. Models were constructed for infection with each individual helminthic species, i.e. *Ascaris lumbricoides*, *Trichiuris trichiura* and hookworms, and a combined model for infection with any helminthic species. The independent variables included in the initial logistic regression models were, for helminthic infections: location, month of year, ART, number of pregnancies (gravidity), education, employment, water source, use of shoes, hand washing, dietary supplement use, viral load detection, presence/absence of malaria, age, height, gestational stage and CD4 counts. Effect modification between ART regimens and each of the other independent variables was assessed by including an interaction term in the model for any helminth infection. With malaria as the dependent dichotomous variable, the same list of independent variables was used. The presence/absence of helminthic infection was included as an additional independent variable, whilst presence/absence of malaria was removed. With helminthic-malaria co-infection as the dependent variable, both malaria and helminthic presence/absence were removed from the model. In all the logistic regression models, the independent variable with the highest p-value was removed until only variables with $p<0.05$ were left in the model.

Backward stepwise multiple regression analysis was used to identify the principal determinants of faecal helminthic egg counts and blood haemoglobin levels. Univariate analyses were initially performed and any variable with $p<0.50$ was included as an independent variable in the multiple regression models. The same procedure as described for the logistic regression models was then followed.

4.4 RESULTS

4.4.1 Prevalence of helminthic infections and malaria

The data in Table 4.1 show that, in the presence or absence of malaria, infection with any helminth species occurred in 336 (34.3%) of the participating sample, being significantly ($p<0.0005$) more common in rural than in peri-urban communities. Infection with helminths in the absence of malaria showed a similar trend, occurring in 36.5% of rural and in 11.3% of peri-urban subjects ($p<0.0005$).

Table 4.1. Prevalence of helminth and malaria infections in rural and peri-urban areas

VARIABLES		RURAL	PERI-URBAN	COMBINED
N		635	345	980
Helminth with or without malaria		43.1	18.0***	34.3
Helminth species:	*Ascaris lumbricoides*	26.1	11.0***	20.8
	Trichiuris trichiura	8.66	4.35**	7.14
	Hookworm	8.35	2.61***	6.33
Malaria with or without helminths		11.0	17.4*	13.3
Helminths with malaria		6.61	6.67	6.63

All data expressed as a percentage; *$p<0.05$, **$p<0.005$, ***$p<0.0005$ vs. rural

Infection with each of the three-helminth species also occurred more often in the rural than in the peri-urban sample, with *A. lumbricoides* being the most common. The presence of a malaria infection (in the presence or absence of a helminth) was more frequent in peri-urban than in rural subjects ($p<0.05$). This trend was mirrored by malaria-only infections, with a prevalence of 4.41% in rural and 10.7% in peri-urban women ($p<0.0005$). The prevalence of helminth-malaria co-infection was similar in both environments (Table 4.1).

4.4.2 Prevalence of helminthic infections and malaria in different population sub-groups

Helminthic infections of any type, asymptomatic malaria or co-infections were all less prevalent in subjects receiving AZT-3TC-NVP when compared to those taking d4T-3TC-NVP ($p<0.005$) (see Table 4.2). Treatment with AZT-3TC-NVP was also associated with a lower prevalence of malaria co-infection when compared to AZT-NVP, and TDF-3TC-NVP therapy. The latter

therapy, on the other hand, was associated with a lower prevalence of asymptomatic malaria compared to the administration of AZT-NVP.

Table 4.2. Prevalence of helminth and malarial infections in relation to various risk factors

VARIABLES		N	HELMINTH (%)	MALARIA (%)	CO-INFECTION (%)
ART	AZT-3TC-NVP	299	27.4**	6.35**††††	2.67*††††
	AZT-NVP	126	32.5	24.6**	14.3**
	d4T-3TC-NVP	461	39.7	14.5	6.72
	TDF-3TC-NVP	94	31.9	13.8†	8.51‡
Months	Jan-Feb	262	27.9	24.4	12.2
	Mar-May	718	36.6*	9.19***	4.60***
Age	≤ 30 years	545	37.4	10.3	5.50
	> 30 years	435	30.3*	17.0**	8.05
Gravidity	1	225	42.2	16.0	10.2
	2-5	755	31.9**	12.4	5.56*
Gestational stage	4 months	505	33.5	22.0	11.3
	5-6 months	476	35.2	4.00***	1.68***
Education	Some	480	21.6	10.6	2.80
	None	500	47.5***	16.0*	10.6***
Employment	Yes	181	28.3	10.9	3.63
	No	799	60.8***	23.8***	19.9***
Water	Piped	215	13.0	16.7	3.26
	River	765	40.3***	12.3	7.58*
Shoe wearing	Yes	380	30.5	16.3	8.16
	No	600	36.7*	11.3*	5.67
Hand washing	Yes	695	29.3	17.7	8.63
	No	285	46.3***	2.46***	1.75***
Diet supplements	Yes	281	28.1	21.3	11.7
	No	699	36.8*	10.0***	4.58***
Viral load	Detectable	90	70.0	17.8	14.4
	No detectable	890	30.7***	12.8	5.84**
CD4	≤ 350 cells/mm3	209	62.7	27.7	20.6
	> 350 cells/mm3	771	26.6***	9.34***	2.85***
Malaria	Present	130	50.0	-	-
	Absent	850	31.9***	-	-
Helminths	Present	336	-	19.3	-
	Absent	644	-	10.1***	-

All data expressed as percentages *p<0.05, **p<0.005, ***p<0.0005; For ART: *p<0.05, **p<0.005 vs. d4T-3TC-NVP; †p<0.05, †††p<0.0005 vs. AZT-NVP; ‡p<0.05 vs. AZT-3TC-NVP

Compared to malaria or co-infection rates, a number of other factors had the opposite effect on helminthic infection. Thus helminthic infections were more common, but malaria and consequently co-infections with HIV were less common in the women who were tested between March and May compared to those tested in January or February. The same pattern was observed

in women who did not wear shoes compared to those who did, and in women who did not regularly wash their hands or take dietary supplements compared to those that did (Table 4.2).

Pregnant women who were older than 30 years at testing had a lower prevalence of helminthic infection, but higher levels of asymptomatic malaria compared to women who were 30 years or younger. Primigravidae had higher prevalence's for all 3 infection types compared to women who had had more than one previous pregnancy, whilst women who presented for testing at an earlier stage of their pregnancy (4 months) had higher prevalence levels of malaria and co-infections compared to those who were tested at a later stage (5-6 months) (Table 4.2).

Study participants who were unemployed and subjects with no formal education had a higher prevalence of helminthic infections, malaria and co-infections compared to subjects who were employed, and educated, respectively. Women who used water directly from a river rather than piped water for their water needs had a higher prevalence of both helminthic infection and co-infection, but a lower prevalence of malaria, although this last comparison did not reach statistical significance (p=0.09). A detectable viral load and a CD4 count $\leq$ 350 cells/mm^3 were both associated with higher levels of all infections. Subjects with asymptomatic malaria had a higher prevalence of helminthic infections, and vice versa (Table 4.2).

4.4.3 Identification of risk and protective factors for helminth infections

Table 4.3 gives the results of multiple logistic regression analyses to identify risk and protective factors for helminth infections. With regard to ART, the d4T-3TC-NVP regimen groups exhibited higher prevalence's of infection with *A. lumbricoides* and hookworm compared to AZT-3TC-NVP. The same applied with the AZT-NVP and TDF-3TC-NVP regimens regarding *T. trichiura* prevalence when compared to the AZT-3TC-NVP therapy. No interaction was observed between ART regimens and any of the other independent variables within the multivariable model for any helminth infection.

Table 4.3. Multiple logistic regression analyses to identify risk factors for helminth infections

INDEPENDENT VARIABLES		ODDS RATIOS FOR ASCARIS INFECTION	ODDS RATIOS FOR TRICHIURA INFECTION	ODDS RATIOS FOR HOOKWORM INFECTION	ODDS RATIO FOR ANY HELMINTH INFECTION
ART†	d4T-3TC-NVP	2.59 (1.79-3.75)***	-	2.19 (1.27-3.79)**	3.47 (2.21-5.45)***
	AZT-NVP	-	4.65 (2.41-8.96)***	-	2.60 (1.33-5.08)*
	TDF-3TC-NVP	-	3.57 (1.69-7.57)**	-	2.47 (1.27-4.80)*
Months:	Jan-Feb vs. Mar-May	-	-	0.32 (0.13-0.77) *	-
Age:	>30 vs. **≤30 yrs.**	-	-	-	0.66 (0.47-0.94)*
No. of pregnancies	>1 vs. **1**	-	-	-	0.59 (0.39-0.87)*
Location:	Urban vs. **Rural**	0.52 (0.33-0.82)**	-	0.32 (0.15-0.66)**	0.41 (0.27-0.62)***
Education:	Yes vs. **No**	0.41 (0.28-0.59)***	-	-	0.39 (0.28-0.55)***
Employment:	Yes vs. **No**	0.47 (0.31-0.73)**	0.14 (0.08-0.27)***	-	0.23 (0.15-0.36)***
Water:	Piped vs. **River**	0.30 (0.16-0.53)***	-	-	0.23 (0.14-0.38)***
Hand washing:	Yes vs. **No**	0.52 (0.33-0.80)**	0.20 (0.10-0.40)***	-	0.29 (0.19-0.46)***
Detectable viral load:	Yes vs. **No**	1.95 (1.11-3.42)*	-		2.42 (1.29-4.55)*
CD4:	≤350 vs.**>350 cells/mm^3**	2.12 (1.38-3.23)**	2.27 (1.32-3.90)**	3.03 (1.75-5.26)***	3.39 (2.16-5.33)***
Malaria:	Infected vs. **Not**	-	-	-	2.13 (1.27-3.59)**

†As compared to AZT-3TC-NVP; data are odds ratios (95% confidence intervals); reference group for each variable is shown in bold in column 2; odds ratios are not given for variables that had no significant effect and were removed from regression model; the following variables did not significantly affect risk for any of the above infections: gestational stage, wearing shoes, use of dietary supplements and height; each of the helminth infections were used as the dependent variable in multivariable regression models adjusted for all the variables shown in each column. More details on how each model was developed are given in section 4.3.3; *p<0.05, **p<0.005, ***p<0.0005.

There were lower rates of hookworm infestation in subjects who were screened for infections during January and February compared to those screened later in the year, whilst subjects who were older than 30 years, or who were multigravidae had a lower risk of any helminthic infection when compared, respectively, to those 30 and younger, or primigravidae (Table 4.3).

Subjects who were residents of a peri-urban location had a lower risk of *A. lumbricoides*, hookworm or any helminthic infection compared to those from a rural environment. Educated study participants and those who used piped water were at a lower risk of *A. lumbricoides* or any helminth infection compared, respectively, to subjects with no formal education and those who used river water. Furthermore, pregnant women who were employed or who regularly washed their hands were at a lower risk for *A. lumbricoides, T. trichiura* or any helminth infection compared to subjects who, respectively, were employed or who did not wash their hands regularly (Table 4.3).

Pregnant women who had a detectable HIV viral load compared to those who did not, were at a higher risk for *A. lumbricoides*, and subjects with a CD4 count at, or below 350 cells/mm^3 were at a higher risk for all types of helminthic infections compared to those with CD4 counts above 350 cells/mm^3. The presence of malaria was associated with a higher risk of any helminthic infection (Table 4.3).

4.4.4 Identification of risk and protective factors for malaria and co-infections

The risk of malaria was higher in the months of January and February than in the months from March to May. Risk was also higher in older women but lower in those in the third trimester of their pregnancy. This latter trend was also mirrored by risk of co-infection. Co-infection risk was also reduced in subjects with some formal education and in those who were employed. Pregnant women who used piped rather than water directly from a river ran a higher risk of malaria. Helminthic infection was associated with a higher risk of malaria, whilst low CD4 counts were also linked to a higher risk of malaria and co-infection. Interestingly, women who regularly washed their hands ran a higher risk of both malaria and co-infections (Table 4.4).

Table 4.4. Multiple logistic regression analyses to identify risk factors for malaria infection and helminth-malaria co-infection

VARIABLES		ODDS RATIOS FOR MALARIA INFECTION	ODDS RATIOS FOR HELMINTH-MALARIA CO-INFECTION
Months:	Jan-Feb vs. Mar-May	1.70 (1.08-2.68)*	-
Age:	>30 vs. ≤30 years	1.76 (1.15-2.69)*	-
Gestational stage:	5-6 vs. 4 months	0.17 (0.10-0.29)***	0.16 (0.07-0.35)***
Education:	Yes vs. No	-	0.32 (0.17-0.63)**
Employment:	Yes vs. No	-	0.26 (0.14-0.49)***
Water:	Piped vs. River	1.76 (1.04-2.97)*	-
Hand washing:	Yes vs. No	5.81 (2.51-13.5)***	2.96 (1.07-8.19)*
Helminth infection:	Yes vs. No	2.42 (1.51-3.89)***	-
CD4:	≤350 vs.>350 cells/mm^3	3.37 (2.11-5.38)***	7.13 (3.95-12.9)***

Data are odds ratios (95% confidence intervals); odds ratios are not given for variables that had no significant effect and were removed from regression model; The following variables did not significantly affect risk for any of the above infections: location, ART, viral load, number of pregnancies, wearing shoes, use of dietary supplements and height; *p<0.05, **p<0.005, ***p<0.0005.

4.4.5 Identification of the principal determinants of faecal helminth egg count and haemoglobin level

Backward stepwise multiple regression analysis demonstrated that faecal helminth egg counts were highest in women who were multigravidas, who did not wear shoes and who had low CD4 counts (Table 4.5). Haemoglobin levels were lowest in women who had helminth or malaria infections, who had low CD4 counts and who were at a more advanced (5 or 6 months compared to 4 months) gestational stage.

Table 4.5. Multiple regression models for determinants of helminth egg count and haemoglobin level

MODEL NUMBER	DEPENDENT VARIABLE	INDEPENDENT VARIABLES	BETA VALUE (P- VALUE)	R FOR MODEL (P-VALUE)
1	Egg count (log)	No. of pregnancies	0.17 (0.001)	0.28 (<0.0005)
		Use of shoes	-0.13 (0.01)	
		CD4 count (log)	-0.54 (0.03)	
2	Haemoglobin level	Helminths	-0.67 (<0.0005)	0.34 (<0.0005)
		Malaria	-0.31 (0.01)	
		CD4 count (log)	1.37 (<0.0005)	
		Gestational stage	-0.19 (0.02)	

Coding for number of pregnancies: primigravida - 1, multigravida - 2; Coding for use of shoes: wear shoes - 1, do not wear shoes - 0; Coding for helminth or malaria: infected - 1, no infection - 0; Coding for gestational stage: 4 months - 1, 5 or 6 months - 2

4.5 Discussion

In this study, we determined the prevalence and identified protective and risk factors of helminthic, malaria and co-infections in HIV-infected pregnant women on ART in Rwanda. We found that helminthic infection was more prevalent in rural than in peri-urban settings. Poor education and unemployment were risk factors for both helminthic and *P. falciparum* infection, whilst hand washing protected against worm infections. Treatment of HIV with AZT-3TC-NVP was associated with a lower prevalence of helminthic infections. A CD4 count of ≤350 cells/mm^3 was associated with higher levels of all infections. Multiple linear regression analysis demonstrated that helminth egg counts (EPG) were highest in women who were multigravidae and haemoglobin levels were lowest in women who had helminthic or malarial infections.

The prevalence of helminth infection was higher among rural than peri-urban participants, the most prevalent helminth species being *A. lumbricoides* followed by *T. trichiura* and hookworm species (*Ancylostoma duodenale* and *Necator americanus*) being the least common. This agrees with previous findings from the same location [188]. Our results are further supported by findings from an earlier study in the region, which indicated that *A. lumbricoides* and *T. trichiura* were more commonly found in Rwanda and Burundi than in most other East African countries [194]

Our findings also show lower prevalence levels for malaria and malaria-helminthic co-infection than previously reported for pregnant women in Ghana, but higher rates of helminthic infections [74]. A study in Uganda [168,186,195] reported that the prevalence of helminth infection among pregnant women there was 68% and for malaria was 11%; however, only 12% of those women were HIV-infected. Not surprisingly, such results indicate that there are varying prevalence levels of helminthic infection during pregnancy across East African populations. It should be noted that in the Ghana study the HIV status of the participants was not known, whilst in our study all participants were HIV-infected and receiving ART.

An earlier study conducted in Kigali, Rwanda, of malaria prevalence in HIV-positive pregnant women reported that 8.0% of the study group had malaria [167]. It is well documented that pregnant women living in malaria-endemic areas have an increased risk of *P. falciparum* infection during pregnancy, but this usually remains asymptomatic. In the current study we found seasonal fluctuation, with the prevalence of asymptomatic malaria being higher in subjects tested in the months of January and February compared to those tested in March through to May. This may be due to the higher rainfall in January and February leading to greater numbers of mosquitoes.

In the current study, we also found that pregnant women who were older than 30 years at the time of testing had a lower prevalence of helminthic infection, but higher levels of malaria compared to younger females. The helminth data is supported by a previous study from Uganda [196]. However, most studies show that malaria is also more common in younger, pregnant women [197] and similarly this difference may be related to a number of factors, including lifestyle and socio-cultural differences across the population groups included in these studies.

Little data exists on the relationship between number of previous pregnancies and the risk of helminthiasis. One study shows no effect of pregnancy number on the risk of helminthic infection [75] whilst a second study demonstrates a higher risk of hookworm infection but a lower risk of *A. lumbricoides* infection in primigravidae compared to multigravida women [198]. The data from the current study suggest that primigravid females have a higher prevalence and risk of helminth infection compared to multigravida women. This is an important finding and suggests that de-worming programs should target such individuals. Our data also show a higher faecal egg count in women who have already had pregnancies compared to women in their first pregnancy. Thus, although multigravida women are at a lower risk of helminthiasis than primigravidae, when they do acquire a helminthic infection they have a higher intensity of infection than primigravid women.

Women who presented for testing at an earlier stage of their pregnancy (4 months) had a higher prevalence of malaria and helminth-malaria co-infection than those at a later stage of pregnancy (5-6 months). This result is supported by findings from previous African studies [198]. Education and employment acted as protective factors against both helminthic infection and helminth-malaria co-infection. Earlier studies showed similar associations [22] suggesting that

socio-economic status is a strong modulator of disease risk. Helminth infection was shown to be more prevalent in subjects who did not wash their hands. Other studies [198] have shown that the risk of helminth infection is reduced in subjects who regularly wash their hands, more so in those who use soap [199]. Thus, simple changes in hygiene practice would be important for reducing the prevalence of helminth infection. In our analysis, however, hand washing was statistically significantly associated with an increased risk of malaria and consequently helminth-malaria co-infection. This finding is surprising and difficult to understand. Also to be noted is the finding that the use of piped compared to open river water reduced the risk of helminthic infection, but seemed to increase the risk for malaria. Whilst improved access to clean water is known to reduce the risk for helminthic infection [200], the greater risk of malaria associated with hand washing and piped water is an unusual finding, with little data available in the literature to confirm these associations. We believe that we are dealing with a confounder, although it is apparently difficult to understand its nature, and neither an elevated social status nor local vector behaviour and distribution offer any clue to understanding this result. It may also be speculated that this involves standpipes, not in-door plumbing, and that stagnant pools are accumulating around the standpipes thus attracting malaria vectors and risks increment. Further investigation is required to fully understand these results.

Helminth egg counts were highest in multigravidas who did not regularly wear shoes and who had low CD4 counts (Table 4. 5). Haemoglobin levels were lowest in women who had helminth or malarial infections or both, who had low CD4 counts and whose gestational stage was 5 or 6 months. Based on the distinct mechanisms by which helminths and malaria affect haemoglobin levels, it can be speculated that their combined presence might interact to enhance the risk of anaemia when intensity is moderately higher than in light worm infection intensities.

The relationship between helminthic infection, intensity and anaemia has been described in several settings in Africa as well as in South East Asia [104,201]. Although the women in our study group were all on ART, with some having received nutritional supplements as part of their antenatal care package, previous regional studies also reported lower haemoglobin levels associated with high prevalence's of helminths and malaria [75]. Our findings are further supported by other studies [19,23], which report that pregnant women are known to exhibit fluctuating CD4 levels in pregnancy, which might expose them to a higher prevalence of

helminthic infection, leading to maternal anaemia. This could be explained by the fact that the immune system is impaired during pregnancy and therefore HIV positive pregnant women who live in highly helminthic and malaria endemic areas in sub-Saharan Africa are likely to be at increased risk for helminthic-malaria co-infections.

In the present study the risk of helminthic infection was higher in women with a reduced CD4 cell count, and in subjects with a detectable viral load. This is in agreement with previous studies conducted in pregnant women in Uganda [195] and Rwanda [202], where CD4 counts correlated negatively with the risk of helminthic infection. However, another study [74] has found the opposite, although this investigation was not carried out in pregnant women. Webb *et al.* [203] reviewed the epidemiology and immunology of helminthic–HIV interactions, and concluded that the data were too inconsistent to postulate a beneficial effect for anti-helminthic therapy on CD4 counts and viral load in HIV-1 co-infected individuals.

With regard to malaria, we found that a low CD4 count was associated with an increased risk of *P. falciparum* infection. This contrasted with findings from a very similar study performed in Rwanda, where no such association was found [166,202]. The discrepancy may be related to the lower power of the earlier investigation, as there is clear evidence from a number of studies that, particularly in pregnant women, HIV does lead to more malaria episodes [94,204].

The prevalence of helminthic infection was higher in subjects with malaria, and *vice versa*. A study conducted in Ghana on pregnant women also showed that helminthic infection increased the risk of malaria [74]. It is thought that helminthic infections have a number of effects on the immune system that lead to increased susceptibility to malaria [103,157].

In our study sample all subjects were taking ART irrespective of CD4 count, as prescribed by the current Rwandan Ministry of Health guidelines for the prevention of mother-to-child transmission of HIV [16]. Helminthic infections of any type, malaria, or helminthic-malaria co-infections were all less prevalent in subjects receiving AZT-3TC-NVP compared to the other three ART regimens (Table 4 2). These effects remained significant for helminthic infections after adjusting for confounding variables in a logistic regression analysis (Table 4.3). However, the protective effect of AZT-3TC-NVP for malaria was not sustained in the logistic regression model (Table 4.4). An earlier study conducted among pregnant Rwandan women also

demonstrated that specific ART regimens seemed to reduce helminthic prevalence, but had less effect on malaria [205]. That study demonstrated that the AZT-3TC-NVP regimen was the least protective compared to the other therapies, i.e. AZT-NVP, d4T-3TC-NVP and AZT [205].

This finding contradicts data from the current study and may be related to a much smaller sample size (N=328) in the earlier study compared to the current study (N=980). Whilst these findings suggest a possible anti-helminthic effect of certain ART combinations, there was no non-ART control 'arm' in either study, as neither study was designed to detect the effect of ART on the risk of helminthic infections. This finding of possible anti-helminthic effects of certain ART regimens does warrant further investigation.

Although the ART-induced reconstitution of cellular immunity would probably be the main factor for reducing helminthic infections among HIV patients, earlier *in vitro* and *in vivo* investigations have indicated that HIV treatment, especially with protease inhibitors (PIs), could have a direct effect by killing off parasites, including malaria [179]. It had already been suggested that ART without PIs might reduce the prevalence of helminthic infection [206,202]. Thus, ART itself might have contributed to the decline in helminth prevalence, asymptomatic malaria or co-infection seen in our study. We hypothesize that the anti-mitochondrial toxicity of ART compounds may play a direct role here, a hypothesis for which support needs to be tested in future field trials, designed to address this question. The strengths of the current study lie in the screening of a large number of women from eight health centres catering to women of all socio-economic classes; the screening for the presence of helminths of three stool samples on three consecutive days; and the combination of two different screening techniques to increase the sensitivity of helminth diagnosis. However, our study also has limitations. Methodologically its cross-sectional design makes it impossible to determine temporal causality, including the inability of multivariate models to adjust for all confounding factors. The Kato Katz method used to determine the number of helminth eggs could have underestimated the proportion of women with light hookworm infection. The study had no control group of HIV-positive subjects not receiving ART.

In conclusion, we found that the prevalence of helminthic infections, malaria, and co-infections are common among HIV-positive pregnant women on ART in Rwanda. Helminthic and malarial infections in this population are important risk factors for low haemoglobin levels. Subjects with

low CD4 counts were at higher risk of infections, and helminthic infection is a risk factor for malaria. Education and employment were independent protective factors for helminthic infection and malaria, whilst hand washing reduced the risk only for helminthic infections. The possible anthelminthic effect of some ART combinations and probably the drug-drug interactions warrants further study.

5 Chapter 5: Effect of de-worming on markers of disease progression in HIV-1 infected pregnant women on ART: a longitudinal observational cohort study

5.1 Abstract

Background: De-worming individuals with HIV infection who are also receiving ART may be of benefit, particularly in pregnant females. Therefore, the aim of this study was to determine the efficacy of targeted versus non-targeted anti-helminth therapy. The study outcomes were to measure the effects of anthelminthic therapy on *Plasmodium falciparum* infection, helminth infection haemoglobin levels, CD4 counts and viral load levels in pregnant, HIV-positive females receiving ARTs.

Methods: A cohort of 980 HIV-infected pregnant women receiving ART were tested for helminth infection, malaria, CD4 counts, and haemoglobin levels at two visits during pregnancy and two visits postnatally, within 12 weeks between each visit. The women were given anti-malarial drugs whenever they tested positive for malaria, and were randomised to either a targeted (n=467) or non-targeted (n=513) anthelminthic intervention with albendazole therapy.

Results: No significant differences were noted between the targeted and non-targeted albendazole treatment arms for any of the variables measured at each study visit except for CD4 counts, which were lower ($P<0.05$) in the latter compared to the former group at the final visit. Albendazole therapy was associated with favourable changes in haemoglobin levels, CD4 counts and viral loads, particularly in those subjects with helminthic infections. However, co-therapy of albendazole with anti-malarial (Coartem: artemether-lumefantrine) increased the risk of *P. falciparum* infection when compared to no therapy (OR [95% CIs] =5.51 [1.99-15.3]; $p<0.005$).

Conclusions: Anti-helminthic therapy reduces detectable viral loads, and increases CD4 counts and haemoglobin levels in subjects with helminth infections receiving ART. However, albendazole therapy used in conjunction with Coartem is associated with an increased risk of malarias but further studies are required to confirm this data to rule out drug –drug interaction and other factors.

5.2 Introduction

Public health strategies such as preventive chemotherapy aim to reduce the burden of helminthic diseases and are pertinent in Africa where over 22 million people are estimated to be co-infected with helminths and HIV-1 [207]. Co-infection commonly affects the natural history and disease progression of both helminthic infections and HIV [4,156,169]. However, the effects of interaction between the two infections are still little explored. Findings from observational studies and randomized control trials (RCTs) have shown impaired immune response to HIV in individuals with helminthic co-infection [13,94], suggesting that helminths play a role in immunity attenuation [35], HIV pathogenesis and disease progression [1,13,94,208–210]. Furthermore, findings from randomised control trials suggest that treatment of helminth co-infection might delay HIV disease progression [5,8,211,212]. The immune interplay between helminthic infections and HIV is complex, and there are different hypotheses about the influence of these infections on each other, the most described influence being the Th2 bias induced by helminthic infections, suppressing Th1 responses specific to HIV, thereby leading to more rapid HIV progression [13,94,213]. Acquisition of HIV was found to be positively correlated with female urogenital schistosomiasis [20,214] but a RCT showed in contrast no benefit of de-worming on the prevention of mother-to-child transmission of HIV [168,211]. Other systematic reviews described the effect of anti-helminthic treatment on markers for HIV disease progression, with lower increases in HIV viral loads and increases in CD4 counts being reported [1,79,203]. However, results from a more recent study did not suggest a beneficial role for de-worming in HIV-positive subjects who were not eligible for anti-retroviral therapy (ART) [211]. However, other plausible reasons have linked malaria and helminths in HIV infected individuals [75], and the dual interactions consistently exhibit a high degree of prevalence of co-infection with HIV [215]. Findings suggest that helminths can affect HIV/AIDS, and malaria disease progression [216–222]. Data suggests that a combination of immunological, epidemiological, and clinical factors can contribute to these interactions and leads to a worsening prognosis for people affected by HIV/AIDS and malaria [156,223]. Studies suggest that helminths skew the immune response towards Th2 cells characterized by higher levels of interleukins IL-4, IL5, and IL13 [35,223].Together these studies highlight the impact of the high prevalence of these NTDs combined with their immunological effects on human hosts co-infected with HIV, which presents opportunities to design new strategies for the combied control of these diseases.

A recent study, conducted within districts of Rwanda where helminthic infection is endemic, showed a high rate of HIV and helminthic co-infection (37.8%) in pregnant females receiving ART [224]. During pregnancy such co-infection increases the risk of both anaemia and mother-to-child transmission of HIV [156]. However, there is a paucity of data from African settings to give insights into whether HIV-infected pregnant women in ante- and postnatal settings receiving ART may benefit from de-worming. The aim of the current study was to determine the effect of de-worming among HIV-infected pregnant Rwandan women receiving ART on markers of HIV disease progression and haemoglobin levels. A further aim was to determine the efficiency of de-worming using albendazole (ABZ) in targeted versus non-targeted treatment arms.

5.3 Methods

5.3.1 Study population

Pregnant women from rural and peri-urban areas were recruited at local antenatal clinics. Inclusion criteria were HIV infection; pregnancy in the second trimester; use of ART and willingness to provide three stool samples on consecutive days. Women were excluded if they were diagnosed with tuberculosis, or if they had taken any anti-helminthic drugs at any time prior to entry into the study. Measurements were taken at two visits during pregnancy and two visits postnatally, with 12 weeks between each visit. Ethical approval was obtained from the Rwanda National Ethics Committee (RNEC) and the Ethics Committee (Human Research) of the University of the Witwatersrand Faculty of Health Science in Johannesburg, South Africa.

5.3.2 Therapies

With computer-generated random ID numbers, patients were assigned to a targeted de-worming treatment arm (n=550) with 400 mg albendazole (ABZ) given at any study visit if participants were helminthic positive, or an untargeted de-worming treatment arm (n=550) where all women were given 400 mg ABZ at each visit irrespective of their helminthic status. If they tested positive for *P. falciparum* at any of the 4 study visits, the women received Coartem (120mg artemether and 20mg lumefantrine per tablet). At all of the 4 visits to the antenatal clinics research assistants observed the taking of the all the study drugs by the study participants. All women received nevirapine for the prevention of mother-to-child HIV transmission and

subsequent combination ART, irrespective of CD4 cell levels − in accordance with the latest Rwandan Ministry of Health treatment guidelines of 2010 [225].

5.3.3 Measurements taken at each study visit

We measured CD4 cell counts and serum haemoglobin levels at every visit, as were weight and height for the calculation of BMI. Viral loads were measured at baseline and at the final visit. Due to ART, viral loads were low and were therefore recorded as a categorical variable, being either above or below the detectable limit of 40 copies per mL. The presences of helminthic (*Ascaris lumbricoides, Trichiuris trichiura* and hookworms) and *P. falciparum* infections were assessed at every study visit, irrespective of whether they were in the targeted or untargeted ABZ treatment cohort. The methods used for all these measurements are described in Chapter 2 of this thesis, and a related publication [226] and the STROBE guidelines for observational cohort studies were followed (http://www.strobe-statement.org).

5.3.4 Study sub-groups

Initial analysis involved the comparison of the targeted with the non-targeted treatment arm. Taking advantage of the study format, we also performed a sub-group analysis in which participants were divided into 3 groups based on the frequency of the ABZ treatment. These groups consisted of:

1. A control group (H-all ABZ-all) in which all subjects were helminth-free and received no ABZ throughout the study;
2. A group in which all subjects were helminth-free but did receive ABZ throughout the study (H-all ABZ+all);
3. A group in which all participants were helminth-infected at baseline and received ABZ therapy, either at all visits or only at visits where helminth infection was detected (H+ABZ+).

These groups allowed us to determine the effect of ABZ therapy in the absence of a helminth infection (H-all ABZ+all) and to observe the effect of untreated helminth infection at baseline and the subsequent effects of ABZ therapy (H+ABZ+).

5.3.5 Statistical analyses

Variables that were not normally distributed were log-transformed to normality before analysis, and are expressed in medians [interquartile range] in the tables. Normally-distributed data are expressed as mean ± SD. Comparisons between the targeted and non-targeted groups at each time point were made using Students t-test for continuous variables, whilst within-group comparisons were made using a paired Students t- test with Bonferroni correction for multiple testing. Comparisons at each time point for the sub-group analysis were performed using ANOVA followed by a Turkeys' *post hoc* test. Categorical variables were analysed using the χ^2 test. Logistic regression was used to determine the effects of the ABZ and Coartem therapies on the risk of *P. falciparum* infection at visit 4 by dividing the cohort into 4 groups based on therapies received at visit 3: those who received neither treatment (reference group); those who received only ABZ; those who received only Coartem; those who received ABZ and Coartem.

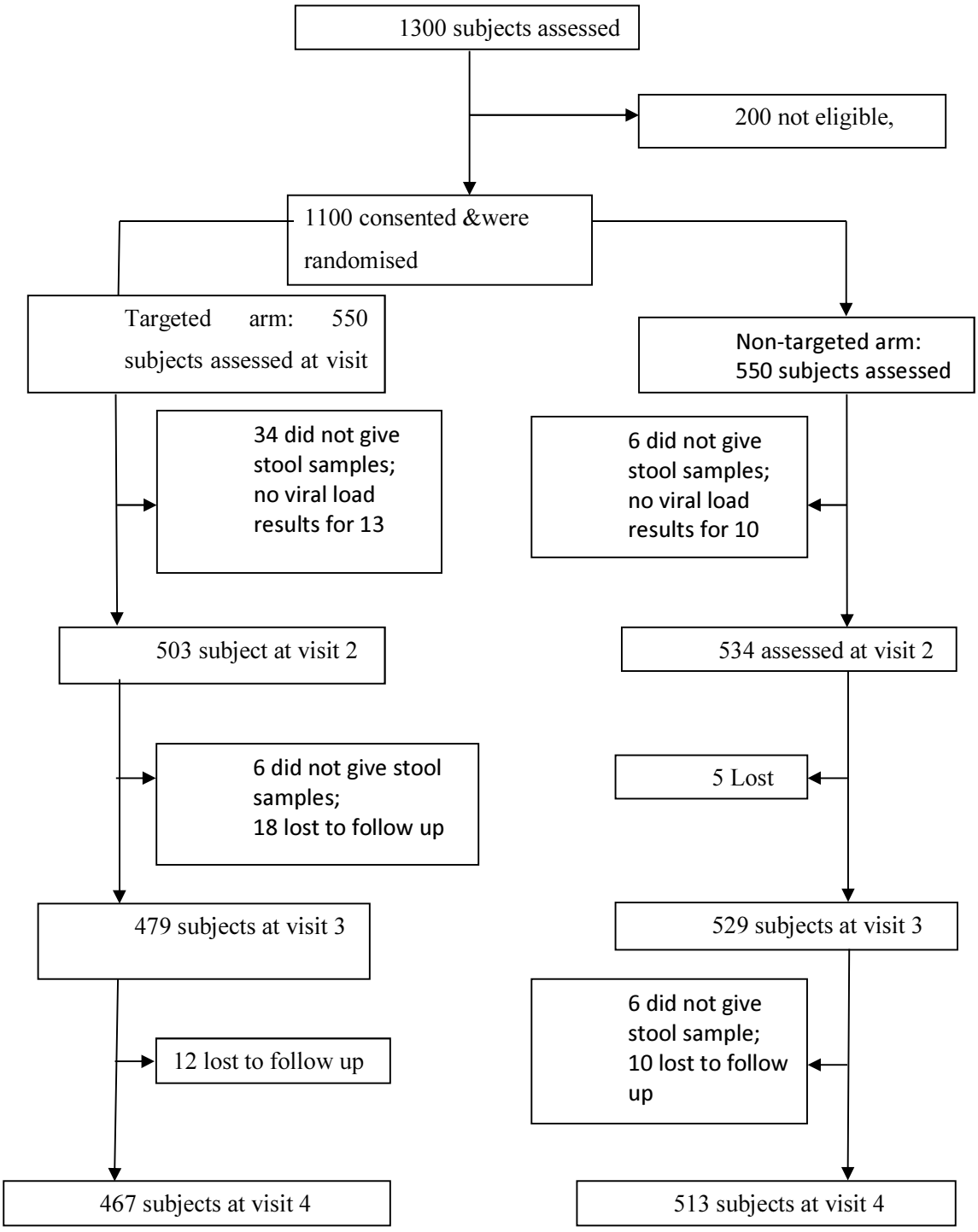
Backward, stepwise multivariable linear regression analysis was used to determine the correlates of percentage changes in haemoglobin levels and CD4 counts between visit 1 and visit 2. The reason for using this time period was that it covers the outcome of treatment of more long-term helminthic or *P. falciparum* infections than would be observed at other periods in the study, and would give a more accurate picture of response to therapy in subjects who had not previously been treated with anti-helminthic medication. The initial regression models included variables that correlated ($p<0.50$) in univariate correlation analyses with each of the 2 outcome variables. All statistical analyses were performed using Statistica version 9.1 software (StatSoft, Tulsa, OK, USA).

5.4 Results

5.4.1 Recruitment and loss to follow up

A total of 1300 pregnant women were enrolled and assessed for eligibility. However, 200 participants were excluded because they did not meet the study inclusion criteria. The resulting 1100 eligible participants were 1:1 equally randomized to targeted and non-targeted treatment arms. A total of 121 subjects (12.3%) did not complete the study due to various reasons (see Fig 5.1) and were excluded from the final analysis. Extra details on sample size estimation is given in Chapter 2, page 18, section 2.2.3.

Figure 5.1. Study flow diagram

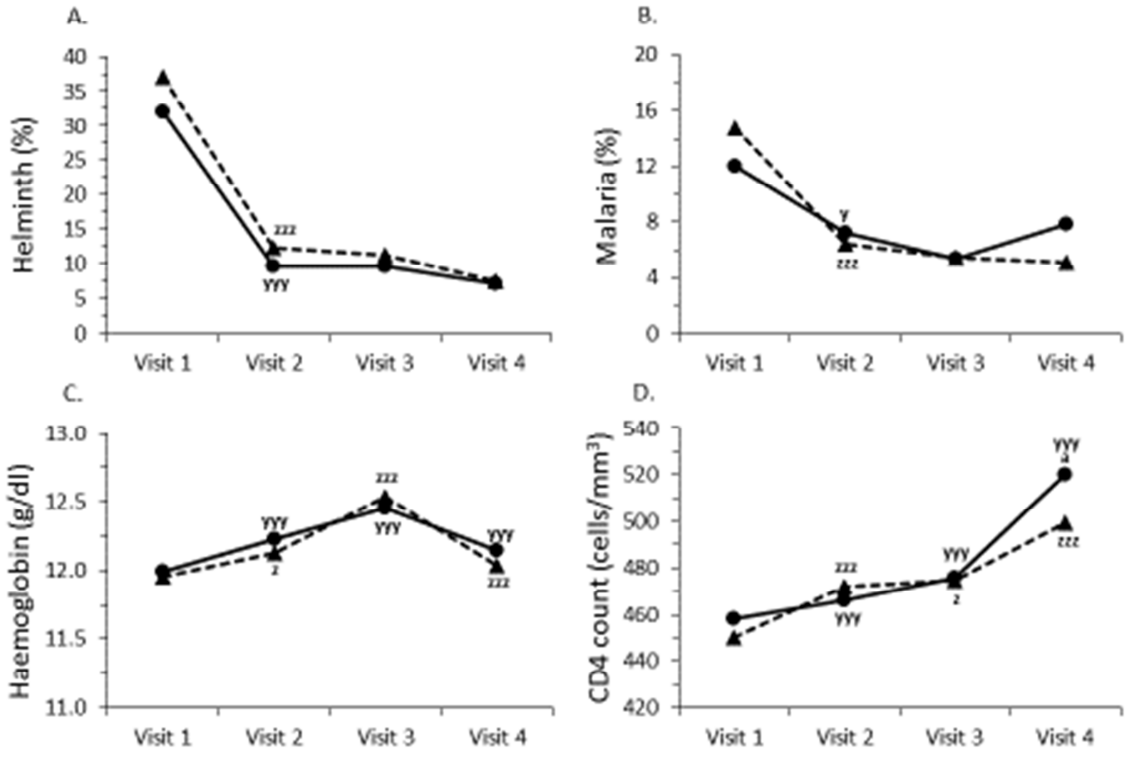


5.4.2 Helminths, malaria and hemoglobin in targeted and non-targeted groups

At baseline the only differences between the 2 groups were that the targeted had less subjects receiving AZT-3TC-NVP therapy than the non-targeted group (27.4 vs 33.3%; p=0.04) whilst the non-targeted group had less subjects receiving AZT-NVP therapy (10.7 vs 15.2; p=0.04). In addition more subjects in the targeted group had malaria-helminth co-infections (see below).

The data in Fig 5.2A shows that the prevalence of helminthic infection in the targeted and non-targeted study arms was similar at all-time points. In both groups helminthic infection dropped dramatically ($p<0.0005$) by visit 2 from that at visit 1 for both comparisons, with a shallower decrease by visit 4. The prevalence levels of individual helminthic infections at baseline for the total cohort (N=980) were as follows: *A. lumbricoides*, 20.8%; *T. Trichiura*, 7.14%; hookworm species, 6.33%. The prevalence of *P. falciparum* infection was similar at all-time points for the two treatment arms, with a significant fall in prevalence by visit 2 in both arms. However, there was a tendency for the infection rate to be higher at visit 4 in the non-targeted arm (7.8% vs. 5.1%; p=0.09) (Fig 5.2B). The baseline prevalence of helminthic co-infection with *P. falciparum* was significantly higher in the targeted (8.60%) compared to the non-targeted group (4.90%; $p<0.05$), but for both groups no co-infection was detected at any of the subsequent visits.

Figure 5.2. Comparison of targeted (hashed line) versus non-targeted (solid line) treatment arms on helminth infection (A), *P. falciparum* infection (B), serum haemoglobin level (C) and CD4 count (D)



Data expressed as % for helminth and *P. falciparum* infections and as means for haemoglobin levels. The SD values were not included to aid clarity; $^{y}p<0.05$, $^{yyy}p<0.0005$ vs. preceding visit of non-targeted arm; $^{z}p<0.05$, $^{zzz}p<0.0005$ vs. preceding visit of targeted arm.

Haemoglobin levels were similar in both arms at all study visits (Fig 5.2C), rising significantly from visit 1 to visit 2 in both arms, and peaking by visit 3. Thereafter, levels fell significantly ($p<0.0005$ for both comparisons) for visit 4.

5.4.3 Baseline comparison of 3 study sub-groups

The results in Table 5.1 show that at baseline (visit 1) no significant differences across the 3 study groups were noted for age, gestation or BMI. The prevalence of multigravidity was higher in the H-all ABZ+all group when compared to the H+ABZ+ group ($p<0.05$). Baseline haemoglobin levels and CD4 counts, and the prevalence of detectable viral loads and malaria are also shown in Fig 3 and are discussed in the following section. The use of the 4 different ART

regimens did differ across the 3 study groups with the AZT-3TC-NVP regimen being more common in the H-all ABZ-all and H-all ABZ+all groups. The D4T-3TC-NVP regimen was found less often in the H-all ABZ-all and H-all ABZ+all groups when compared to the H+ABZ+group. No differences were noted for the use of the TDF-3TC-NVP or AZT-NVP regimens. The prevalence of some level of formal education, employment and peri urban residence was all lower in the H + ABZ + compared to the other 2 groups.

Table 5.1. Baseline characteristics of study groups

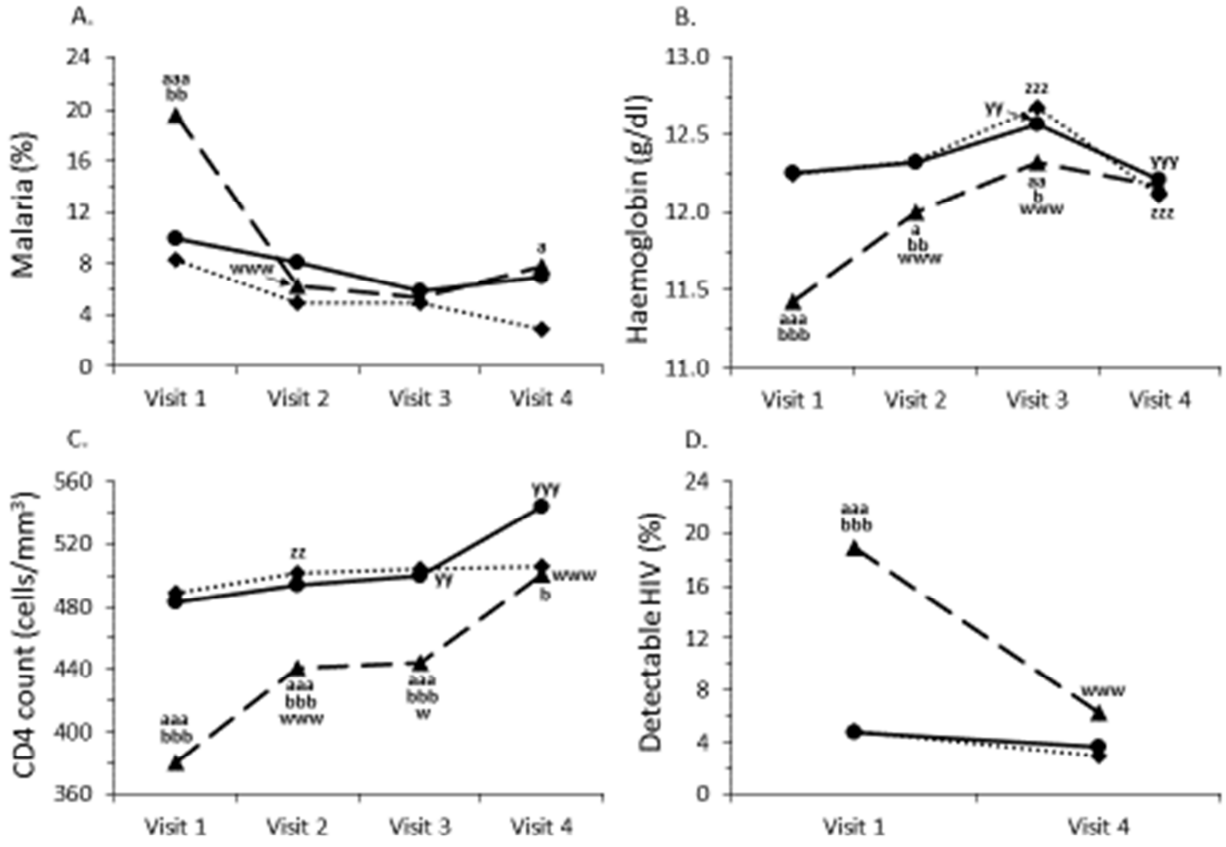
VARIABLES	**Subject groups**		
	H-all ABZ-all	**H-all ABZ + all**	**H+ABZ+**
N	204	272	334
Age (years)	30.5 ± 4.62	30.3 ± 4.78	29.9 ± 4.73
Gestation (months)	4.58 ± 0.72	4.65 ± 0.74	4.64 ± 0.72
Gravidity >1 (%)	77.9	80.9*	71.6
BMI	25.4 ± 3.27	25.2 ± 3.32	25.3 ± 3.28
Haemoglobin (g/dl)	12.2 ± 1.18***	12.3 ± 1.22***	11.4 ± 1.35
CD4 (cells/mm^3)	489 [173]***	483 [182]***	380 [112]
Detectable HIV (%)	4.90***	4.78***	18.9
ART regimen (%):			
AZT-3TC-NVP	35.3*	39.7***	24.2
AZT-NVP	11.8	9.55	12.3
D4T-3TC-NVP	42.1**	42.3**	54.8
TDF-3TC-NVP	10.8	8.45	8.68
Malaria (%)	8.33***	9.93**	19.5
Education (%)	60.8***	57.0***	32.0
Employment (%)	88.2***	90.1***	67.1
Peri-urban (%)	46.1***	39.0***	18.6

Data given as %, mean ± SD or median [interquartile range]; H-all ABZ-all = helminth free and not treated with ABZ at any visits; H-all ABZ+all = helminth free and treated with ABZ at all visits; H+ABZ+ = infected with helminth at baseline and treated with ABZ; $^{***}p<0.0005$, $^{**}p<0.005$, $^{*}p<0.05$ vs H+ABZ+

5.4.4 Longitudinal changes in malaria, haemoglobin, detectable viral load and CD4 counts in 3 study sub-groups

The study variables at each visit for the 3 treatment sub-groups are shown in Fig 5.3. Helminth infection by definition was 100% in the H+ABZ+ group and 0.00% in all other groups at baseline and remained at 0.00% at all subsequent visits for the H-all ABZ-all and H-all ABZ+all groups. Within the H+ABZ+ group helminth infection fell to 5.99% at visit 2 ($p<0.0005$ vs. visit 1), rose to 10.8% at visit 3 ($p<0.05$ vs. visit 2) and fell to 7.78% at visit 4 ($p=0.10$ vs. visit 3). Malaria was significantly more prevalent at the baseline visit for the H+ABZ+ group when compared to each of the 2 other groups ($p<0.05$ and $p<0.0005$ for the 2 comparisons) (Fig 5.3A). However, at visit 2 *P. falciparum* detection fell significantly in the former group ($p<0.0005$) to levels comparable to that of the other 2 groups, and remained so at visit 3. The prevalence of *P. falciparum* positivity rose slightly by visit 4 in both groups receiving ABZ therapy, but fell in the group not receiving ABZ (H-all ABZ-all), such that this group had a significantly ($p<0.05$) lower level of infection compared to the H+ABZ+ group at the final visit. In order to determine whether the rise in *P. falciparum* infection at visit 4 in those receiving ABZ therapy was due to an interaction between ABZ and Coartem therapy, a logistic regression analysis was performed. This demonstrated that subjects receiving both ABZ and Coartem at visit 3 had an odds ratio [95% CIs] for *P. falciparum* infection at visit 4 of 5.51 [1.99-15.3] ($p<0.005$) versus subjects receiving neither therapy. Subjects receiving Coartem only or ABZ only had odds ratios of 3.48 [0.94-12.9] ($p=0.06$) and 1.68 [0.93-3.03] ($p=0.08$), respectively. Addition of possible confounders to the logistic regression models i.e. gravidity, gestational age at baseline, employment status and ART regimen did not negate this effect. Haemoglobin levels were significantly lower in the H+ABZ+ group at baseline when compared to the 2 other sub-groups ($p<0.0005$ for both comparisons; Fig 5.3B) but rose significantly at visit 2 ($p<0.0005$) and visit 3 ($p<0.0005$). Within the remaining 2 study groups, haemoglobin levels did not rise significantly between visits 1 and 2 but did so between visits 2 and 3 ($p<0.005$ and $p<0.0005$ for the 2 comparisons). Haemoglobin levels fell significantly ($p<0.0005$ for both comparisons) between visits 3 and 4 in the H-all ABZ-all and H-all ABZ+all groups, but not in the H+ABZ+ group.

Figure 5.3: Sub-group analysis of malaria prevalence (A), haemoglobin levels (B), CD4 counts (C) and prevalence of detectable viral loads (D) across study visits



Data expressed as % for malaria and detectable viral load and as means for haemoglobin levels and medians for CD4 counts. The SD and IQR values were excluded to aid clarity. *Small dashes with diamond markers*: H-all ABZ-all = helminth free and not treated with ABZ at any visits; *solid line with circle markers*: H-all ABZ+all = helminth free and treated with ABZ at all visits; *Large dashes with triangle markers*: H+ABZ+ = infected with helminth at baseline and treated with ABZ; $^{a}p<0.05$, $^{aa}p<0.005$, $^{aaa}p<0.0005$ H+ABZ+ vs H-all ABZ-all; $^{b}p<0.05$, $^{bb}p<0.005$, $^{bbb}p<0.0005$, H+ABZ+ vs H-all ABZ+all; $^{w}p<0.05$, $^{www}p<0.0005$ vs preceding visit within H+ABZ+ group; $^{yy}p<0.005$, $^{yyy}p<0.0005$ vs preceding visit within H-all ABZ+all group; $^{zz}p<0.005$, $^{zzz}p<0.0005$ vs preceding visit within H-all ABZ-all group.

The CD4 counts were significantly lower in the H+ABZ+ group at baseline when compared to the 2 other sub-groups ($p<0.0005$ for both comparisons (Fig 5.3C). The CD4 counts rose significantly ($p<0.0005$) within this group from visit 1 to 2 and ($p<0.05$) from visit 2 to visit 3,

but at both visits the CD4 levels were still significantly ($p<0.0005$ for all 3 comparisons) lower when compared to the 2 other sub-groups. However, a large ($p<0.0005$) increase in CD4 counts from visit 3 to visit 4 brought levels to within the range observed in the other groups. There was a shallow but significant rise in CD4 counts from visit 1 to visit 3 in the H-all ABZ-all and H-all ABZ+all groups. The CD4 counts rose significantly ($p<0.0005$) from visit 3 to visit 4 in the H-all ABZ+all group leaving levels significantly ($p<0.05$) higher than in the H+ABZ+ group.

The prevalence of detectable viral levels was significantly ($p<0.0005$ for both comparisons) higher in the H+ABZ+ group at baseline when compared to the 2 other groups, but fell significantly ($p<0.0005$) by visit 4 (Fig 5.3D). The prevalence of detectable viral loads did not change between the first and final visits for the 2 other study groups.

5.4.5 Multivariable linear regression analyses

The results of multivariable, backward, stepwise linear regression analyses for determining the correlates of percentage change in CD4 counts and percentage changes in haemoglobin level are shown in Table 5.2. These data show that, over the time period from visit 1 to visit 2, loss of a *P. falciparum* infection was associated with an increase in the percentage change in CD4 counts by 8.64% ($p<0.0005$) whilst gain of an infection can reduce CD4 counts by 9.81% ($p<0.0005$) when compared to subjects with no change in infection status between these 2 study visits. Similar results were found for *A. lumbricoides* infections, with a loss of infection being associated with an increase in the percentage change in CD4 counts of 6.74% ($p<0.0005$) and a gain in infection leading to a fall in CD4 counts by 7.37% ($p=0.003$). Loss of a *T. trichiura* or a hookworm infection caused an increase in CD4 counts of 5.28% ($p=0.02$) and 5.94% ($p=0.02$), respectively. Subjects in whom a detectable viral load was observed at visit 1 had a 25.0% increase in CD4 counts ($p<0.0005$) when compared to subjects with no detectable viral load. The independent variables within the model for percentage change in CD4 counts explained 22.0% ($p<0.0005$) of the variance in the dependent variable

Table 5.2. Backward, stepwise multivariable regression models for % change in CD4 counts and haemoglobin levels from visit 1 to visit 4

DEPENDENT VARIABLES	INDEPENDENT VARIABLES WITH UNSTANDARDIZED BETA COEFFICIENT (P VALUE)		ADJUSTED R^2 FOR FULL MODEL (P VALUE)
Percentage change in CD4 count	Loss of malaria infection	8.64 (<0.0005)	0.22 (<0.0005)
	Gain in malaria infection	-9.81 (<0.0005)	
	Loss of *Ascaris* infection	6.74 (<0.0005)	
	Gain in *Ascaris* infection	-7.37 (0.003)	
	Loss of *Trichuris* infection	5.28 (0.02)	
	Loss of hookworm infection	5.94 (0.02)	
	Detectable viral load at visit 1	25.0 (<0.0005)	
Percentage change in haemoglobin level	Peri-urban vs. rural	-1.90 (0.01)	0.09 (<0.0005)
	Employment	2.13 (0.02)	
	Gestational age	2.08 (0.002)	
	Gain of malaria infection	-3.82 (0.009)	
	Loss of *Ascaris* infection	4.93 (<0.0005)	
	Loss of *Trichuris* infection	2.93 (0.03)	
	Loss of hookworm infection	8.96 (<0.0005)	

Variable coding: loss or gain of any helminth or *P. falciparum* infection was coded as -1 and +1 respectively and compared against no change in infection status (coded as 0); presence of a detectable viral load at visit 1 was coded as 1 whilst an undetectable viral load was coded as 0; periurban coded as 1 and urban as 0; employed coded as 1 and unemployed as0; gestational age of 5 or 6 months coded as 1 and gestational age of 4 months coded as 0

Table 5.2 shows that periurban compared to urban-dwelling subjects had a 1.90% haemoglobin decline (p=0.01) between the study visits. Employed women had a 2.13% haemoglobin increase

compared to unemployed women (p=0.02). Malaria between study visits 1 and 2 was associated with a 3.82% haemoglobin decrease (p=0.009). Successful treatment of *A. lumbricoides, T. trichiura* or hookworm infections were associated with 4.93% (p<0.0005), 2.93% (p=0.03) and a 8.96% (p<0.0005) haemoglobin gain.

5.5 Discussion

The present study is the first to determine the effect of ABZ therapy on CD4 cell counts, viral loads and haemoglobin levels in HIV-infected pregnant women, who were also receiving ART and anti-malarial medications. Favourable changes were observed in all these variables over a period of 1 year, particularly in those subjects who had a helminthic infection at the baseline visit. The anti-helminthic therapy was administered in either a targeted or non-targeted fashion, and both these treatment arms were equally effective. However, *P. falciparum* infection levels at the final study visit were found to be higher in subjects receiving both ABZ and anti-malarial therapy (artemether with lumefantrine).

This study was conducted in regions of Rwanda where helminthic infection is endemic, and under such conditions non-targeted anti-helminthic therapy may be more cost effective than a test-then-treat procedure. However, it is important to determine whether non-targeted therapy is efficacious and safe. The results from the present study suggest that this is the case, with the prevalence of helminth infection being comparable at all-time points in the two treatment arms, and the absence of any participant withdrawal due to adverse reactions to the ABZ therapy.

Studies investigating the effect of deworming on CD4 cell counts and viral loads in HIV-positive patients have been analysed in a systematic review, which demonstrated a significant and beneficial effect [3,60,227]. Data from the current study support these findings and demonstrate that, in pregnant females receiving ABZ therapy, CD4 counts increase and the prevalence of detectable viral loads fall in the presence of concurrent ART. Thus, ABZ therapy significantly augments ART in improving immune function and blocking viral replication. Furthermore, haemoglobin levels also increase after initiation of anti-helminthic therapy. A recent study has shown that anti-helminthic therapy in HIV-positive pregnant females reduced viral load, but the effect was not statistically significant [207]. However, this study involved only 1 dose of ABZ or praziquantel, which was administered at baseline (second or third trimester of pregnancy), and

measurement of changes in viral load, but not CD4 counts, at 6 weeks' post-treatment and at delivery. Furthermore, no participants were receiving ART at recruitment but were given single dose intrapartum and neonatal NVP to reduce mother-to-child transmission. Another study has also shown that, in HIV-positive subjects not receiving ART, ABZ therapy had no effect on HIV-associated disease progression [168]. However, as stated above, a meta-analysis of 3 randomized controlled trials has shown benefits of anthelminthic therapy in HIV-positive subjects [89]. These contradictory data may result from differences in study format and methodology, including treatment of different helminth species, variable sample sizes, differences in study duration and frequency of anti-helminthic therapy, presence of different parallel therapies (e.g. for HIV or *P. falciparum* infection), variations in the prevalence of helminthic infections, and differences in population ages and genders.

The current study demonstrates that increases in CD4 counts occur with the removal of each of the 3-helminth species measured, i.e. *A. lumbricoides, T. trichiura* and hookworm. The effects were significant, with the loss of each helminth infection causing a 5-7% increase in CD4 counts over a 3-month period. One other study has shown that treatment of *A. lumbricoides* with ABZ does improve CD4 counts [228,229] but these findings, or those for treatment of *T. trichiura* and hookworm observed in our study, are not supported by other reports [230]. It is interesting to note that none of these other studies included subjects receiving ART. It is therefore possible that ABZ therapy has a more significant effect on the immune system responsiveness of HIV-infected subjects in the presence of ART. However, it must be noted that one reported study [72] did demonstrate a positive effect of ABZ therapy in subjects not receiving ART. More studies are therefore required to compare the effect of ABZ on HIV-associated disease progression in helminthic-infected, HIV-positive subjects receiving or not receiving ART.

An interesting observation from the present study is that CD4 counts were higher at visit 4 in the non-targeted than in the targeted ABZ-treatment arm. The sub-group analysis demonstrated that this was due to a significant increase of CD4 counts between visit 3 and visit 4 in the subjects receiving ABZ therapy in the absence of any detectable helminthic infection. This suggests that ABZ may have an effect on CD4 counts that is independent of its ability to clear a worm infection. Another possible explanation for these results is that there may have been helminths present that were below the limit of detection of the stool microscopy, or that other parasites that

we were not set up to detect were being cleared by the ABZ therapy. A previous study of HIV-infected pregnant women demonstrated that viral loads fell in subjects treated with ABZ irrespective of the presence of a helminthic infection [55,214].These data also suggest a direct effect of ABZ on immune function in HIV-infected subjects, but the mechanisms involved are not known. However, it is interesting to note that ABZ does have non-helminthic effects, as is shown by its ability to bind mammalian tubulin [231,232] and to inhibit vascular endothelial growth factor (VEGF) production [231].

Our study further confirms the higher prevalence of *P. falciparum* in subjects carrying a helminth infection [156]. Data from our investigation also shows that, at the final study visit, the prevalence of *P. falciparum* infection was higher in the non-targeted than in the targeted arm. The data from the sub-study analysis confirmed that this was due to a significantly higher prevalence of malaria in subjects receiving both ABZ and Coartem, suggesting a drug-drug interaction. An *in vitro* study has shown that exposing a human hepatoma cell line, HepG2, to ABZ does induce increased expression of cytochrome P450 enzymes [233]. Such enzymes are known to be responsible for the hepatic metabolism of anti-malarial drugs such as artemether and lumefantrine [234–236], which are the active constituents of Coartem. Thus, there is some experimental evidence suggesting that ABZ may lead to increased hepatic metabolism of the anti-malarial agents used in this study. However, pharmacokinetic investigations are required to confirm these results.

Treatment with ABZ led to increases in serum haemoglobin levels, particularly in subjects carrying helminthic infections. Linear multiple regression analyses demonstrated that removal of helminthic and *P. falciparum* infections independently led to increases in serum haemoglobin levels. Both these types of parasitic infection are known to cause anaemia [230,237]. Haemoglobin levels increased postpartum, reaching a maximum at 3 months, and then falling to a nadir at 6 months. A previous study has demonstrated rising haemoglobin levels postnatally, but no decline in levels was observed at 6 months [75,168].

The current study had several strengths. It was a randomized, longitudinal, controlled cohort study carried out at multiple sites in Rwanda. Compliance with the study treatments was documented by direct observation by study staff. Retention of participants in the study was high and did not differ between study groups. Furthermore, the objective outcome measures were

systematically assessed and documented. Limitations of the study were the absence of a placebo arm, and neither participants nor study staffs were masked to treatment allocations. Also, there was no control group without ART or an HIV-negative group. The study had other design weaknesses, such as a lack of data collection on concomitant treatments such as cotrimoxazole. Also, adverse events to the drug therapy that were not severe enough to cause participants to withdraw from the study, were not recorded. Data on the duration of ART was not collected and therefore could not be adjusted for in the multivariable regression model for changes in CD4 levels across pregnancy. The study was not developed to investigate drug-drug interactions, and therefore such effects could not be confirmed. In addition, the analysis of the 3 sub-groups was not planned before initiation of the study and therefore no power analyses were performed. It is therefore possible that this sub-group analysis was not powered to detect more subtle differences between the groups. However, despite this, significant and important differences were noted.

Our findings suggest that ABZ treatment of HIV-positive pregnant women is of benefit, without a need for screening for helminth infection. Thus, within regions where helminths are endemic, a non-targeted intervention may be more efficient than a targeted approach. However, the suggestion of a drug-drug interaction between ABZ and Coartem must be further investigated in pharmacokinetic studies. The present study shows the beneficial effects of ABZ therapy on viral loads, CD4 counts and serum haemoglobin levels, especially in women who carry helminth infections. However, there is some evidence that the effect on CD4 counts may not be entirely due to helminthic clearance, and requires further investigation. The very significant effect of ABZ therapy on CD4 counts and viral loads on subjects already receiving ART is an important finding, particularly in the light of results from previous studies where no beneficial effect of ABZ was observed in HIV-positive subjects not receiving ART [58,85,176,238]. Future studies must therefore compare the effect of ABZ on immune reconstitution in helminths-infected, HIV-positive participants receiving or not receiving ART. Furthermore, a cost benefit analysis of targeted versus untargeted helminth treatment programmes is warranted in a large controlled study. The equal effectiveness of these methods in reducing helminth infection rates has been shown in the current study however, it is essential to determine whether there is any cost benefit for one of the treatment methods, particularly in light of the poor resource settings in which such programmes would be initiated.

Chapter 6: Discussion, Limitations, Conclusions, Recommendations and Future work

6.1 Introduction

This chapter summarizes the results of the whole study by highlighting the key points in a consolidated table, with a summary of the results presented in chapters three, four and five of the thesis. There is a further discussion of the study limitations encountered, the findings from each set of objectives, implications for key findings, conclusions drawn from the findings, and suggestions for future studies

6.2 Hypotheses

Hypotheses: The hypotheses from this thesis tested were as follows:

I. Helminth-malaria dual infection and helminth infection prevalence is higher among HIV infected pregnant women on ART in Rwanda in rural compared to urban health centres.

II. Malaria–helminth co-infection and helminth infection risk negatively correlate with socio-economic status of the HIV-infected pregnant women on ART in Rwanda.

III. Deworming has a positive effect on HIV /AIDS immune progression markers among HIV infected pregnant women on ART in Rwanda.

IV. De-worming using a targeted or a non-targeted approach is equally effective at reducing helminth infection levels.

6.3 Summaries and consolidation of findings reported herein

Table 6.1. Consolidation of findings

OBJECTIVE OF THE STUDY	CHAPT.	EVIDENCE FROM THE RESULTS
Determining the prevalence of helminth-malaria co-infection among HIV+ pregnant women in rural and peri-urban settings in Rwanda and effects of antiretroviral therapy.	3	38% of individuals harboured helminths, 21% had malaria; and 10% were co-infected. Prevalence of helminths and malaria was greater in the rural than in peri-urban populations. The most prevalent helminth species were *Ascaris lumbricoides* (20.7%), followed by *Trichuris trichiura* (9.2%), and *Ancylostoma duodenale* and *Necator americanus* (1.2%). Helminth infections were characterized by low haemoglobin and CD4 counts. In multivariate analysis subjects treated with a d4T-3TC-NVP regimen had a reduced risk of *Trichuris trichiura* infection (OR, 0.27; 95% CIs, 0.10-0.76; $p<0.05$) and malaria-helminth dual infection (OR, 0.29; 95% CI, 0.11-0.75; $p<0.05$) compared to those receiving AZT-3TC-NVP
Describing helminthic and malaria infection levels and risk and protective factors in HIV-infected pregnant women on antiretroviral therapy in Rwanda	4	Helminth infection was more prevalent in rural than peri-urban settings. Poor education and unemployment were risk factors for both helminth and *P. falciparum* infection, whilst hand washing protected against worm infections. In multivariate logistic regression analysis of data, HIV treatment with AZT-3TC-NVP was associated with a lower risk of helminthic infections. A CD4 count $\leq$ 350 cells/mm^3 was associated

OBJECTIVE OF THE STUDY	CHAPT.	EVIDENCE FROM THE RESULTS
		with higher levels of helminthic and malaria infections. Multiple linear regression analysis demonstrated that helminth egg counts (EPG) were highest in multigravid women. Haemoglobin levels were lowest in women who had helminthic or malaria infections.
The effect of deworming on immune markers of disease progression among HIV-infected pregnant women on ART. A longitudinal study of two anti-helminth treatment arms: targeted and untargeted	5	Anti-helminthic therapy showed a significant effect on: helminthic prevalence rates, Hb levels, CD4 cell counts, and viral load detection. The prevalence levels of individual helminthic infections at baseline for the total cohort (N=980) were as follows: *A. lumbricoides*, 20.8%; *T. Trichiura*, 7.14%; hookworm species, 6.33%. The prevalence of *P. falciparum* infection was similar at all time-points for the two treatment arms, with a significant fall in prevalence by visit 2 in both arms. However, there was a tendency for the infection rate to be higher at visit 4 in the non-targeted arm: 7.8% vs. 5.1% ($p=0.09$). The prevalence of helminthic co-infection with *P. falciparum* was significantly ($p<0.05$) higher in the targeted (8.60%) compared to the non-targeted group (4.90%) at baseline, but in both groups no co-infection was detected at any of the subsequent visits. Haemoglobin levels were similar in both arms at all study visits, rising significantly from visit 1 to visit 2 in both arms and peaking by visit 3.

OBJECTIVE OF THE STUDY	CHAPT.	EVIDENCE FROM THE RESULTS
		Thereafter, levels fell significantly for visit 4. The CD4 counts rose significantly ($p<0.0005$) from visit 3 to visit 4 in the H-all ABZ+all group leaving levels significantly ($p<0.05$) higher than in the H+ABZ+ group. The loss of a *P. falciparum* infection or any type of helminth infection between study visits 1 and 2 was associated with a significant increase in CD4 counts and haemoglobin levels (for worm infections only).

6.4 Malaria and helminthic co-infection among HIV-positive pregnant women: prevalence and effects of antiretroviral therapy

6.4.1 The preliminary prevalence study

The aim of the pilot study was to determine the prevalence of helminths and malaria dual infection in the study population of HIV infected pregnant women on antiretroviral therapy. The pilot study was to guide validate study tools before commencing the large longitudinal study. We found that the prevalence of helminthic and malaria co-infections is high among HIV-positive pregnant women in rural than urban population settings in Rwanda. Earlier study indicated that malaria and helminth infections are co-endemic and constitute disease burdens of major public health importance [152,239]. The study results have indicated that helminthic infection in this population is an important risk factor for low haemoglobin and CD4 cell counts as presented in chapter 3, whilst malaria-helminthic dual infections are characterized by a reduced BMI. The high burden of parasitic infections with high co-infections sited in pregnant women in Rwandan population, have implications for more immunological studies needed. This study further adds on existing body of knowledge for the overlapping co-endemicity nature of these parasites with HIV-1 and is common in most other East and central African countries where helminths and malaria co-exists [59,70,239,240]. Ladner et al in a prospective study in Rwanda documented the association between malaria and maternal HIV infection and concluded the increased risk of malaria occurrence in all HIV-infected women [167]. In our study both helminths and malaria

were common in rural than peri urban settings similar to another study conducted in eight districts among school going children for prevalence of helminths. Our results are in agreement with data from Rwanda secondly education students [19], which showed that intestinal parasites of public health relevance are prevalent among students attending tertiary schools. The importance of living and eating in hygienic environments as well as drinking safe water is important and all efforts need to be strengthened in children and in adults leaving in poor resource settings [241,242]. This implies that there is a clear need to strengthen the deployment of existing malaria and HIV prevention and intervention measures for pregnant women as reported by many other studies on the burden of co infection of these ailments with HIV in poor communities [243–247]. Early systematic review commends improved hygiene as important tools to combat helminthic infections among risky population groups [248]. The overlapping nature of helminths, malaria among HIV infected pregnant women calls for strategies of control in similar settings [59,239]. This part of thesis data concludes that co-infection of helminth and malaria was common among pregnant women taking antiretroviral drugs mainly from rural than urban settings. Of particular interest, certain ART regimens seem to have positive effects on both *Plasmodium falciparum* and *Trichiura trichiura* infections, and further studies need to be undertaken in large prospective cohorts and trials to confirm and further specify these results

6.5 Helminthic infection and malaria in HIV-infected pregnant women on anti-retroviral therapy in Rwanda

6.5.1 The prevalence, risk and protective factors study

The intervention data of this study aimed to determine the actual helminth and malaria infection rates among HIV infected pregnant women on antiretroviral therapy. This is presented in chapter 4 of the thesis as earlier described. Thus the findings were in agreement with the preliminary prevalence study showing that co infection rates was higher among women dwelling in rural than per -urban settings [205]. This clearly indicates that co-infection with diseases of poverty can severely affect the poorest rural population and therefore the strategy to control these infections should purely involve use of existing anthelminthic drugs that are effective as reported in most existing literature [72,92,108,200,249,250]. Like in most other studies, in this study use of anthelmintic therapy is highly recommended in such populations [200,251] but because re infection typically occurs more frequently, in this thesis it is further recommended that integrated

control approaches should utilise health education tools to interrupt transmission of these ailments as reported in similar studies [45]. This study furthermore indicated that hand washing and improved sanitation reduced infection rates that concurs with earlier studies recommending improved sanitation to reduce helminth infections [20,45,92,200,248,252]. Co-infection of helminth and malaria prevalence and risk of infection with each was common in this study but like most other studies the effect of helminth co infection on malaria in human still remain not conclusive [75,104,253]. However, more studies are required to find out how interaction of helminth or malaria could contribute to increased prevalence of malaria infection or vice versa in populations where co-endemicity is common as earlier reported in evaluation study to measure the association [76,188,254].

The findings on effect of antiretroviral drugs on malaria and helminthic infection rates were found very interesting. Whilst there is a limit to the interpretation of the findings, they warrant further investigation. Although the ART-induced reconstitution of cellular immunity would probably be the main factor for reducing helminthic infections among HIV patients [78,94], previous *in vitro* and *in vivo* investigations indicated that HIV treatment, especially with protease inhibitors (PIs), could have a direct effect in killing off parasites, including those causing malaria [179]. It has been proposed that ART without PIs may reduce the prevalence of helminthic infection [255]. Thus, ART itself might have contributed to the decline in the helminth prevalence, asymptomatic malaria or co-infection seen in our study as previously reported in other studies [70,125,239]. We hypothesize that the anti-mitochondrial toxicity of ART compounds may play a direct role here, a hypothesis for which support needs to be gained from further in the field trials, designed to address this question.

To summarize, the findings from this study lead the author to suggest that HIV-positive pregnant women would benefit from the scaling-up of de-worming programs alongside health education and hygiene interventions. But further studies need to be conducted regarding the findings on the differential effect of certain ART combinations, as observed most strongly with AZT-3TC-NVP, possibly protecting against helminthic infection and rule out possible drug–drug interactions

6.6 Effect of deworming on immune markers of HIV-1 − disease progression among pregnant women on ART: targeted versus untargeted longitudinal groups

6.6.1 Implications for STHs management and control policies at local and national levels

Deworming can be a helpful tool in control programs targeting helminths, malaria co infections in HIV/AIDs patients on ART. In this study we have shown that immune markers of HIV disease progression such as viral load and CD4 Cell levels can be boosted with anthelminthic use along with specific ARTs and have also shown positive impact on haemoglobin levels. Previous studies among pregnant women had indicated similar trends suggestive of significant benefits in population subgroups but with varying results in other groups of different settings [123].However, there are biological reasons to believe existence of such benefits and this has been earlier reported [123,125,200]

Our results have shown that certain ART combination therapies have significant effect on prevalence of helminths and malaria helminth co infection rates. Implying that in places where co infection is common, selection of ART combination is important to control helminth co infection while deworming of pregnant women is encouraged. Several investigators have indicated that deworming is of potential benefit to the pregnant women [13,75]. In ART eligible Pregnant HIV-infected women, ART helps in virological suppression [256–259]. Specifically, AZT-3TC-NVP, AZT-3TC-LPV-r, and AZT-3TC-ABC have been shown to decrease MTCT [260] and more benefits have been suggested by new ART guidelines suggesting a switch to earlier ART initiation by world health organisation guidelines [261–264] and this approach supports innovative efforts towards total viral suppression [262]. In many countries aiming towards the goal of eliminating mother-to-child transmission of HIV [265–267] and our study results were generated among women who initiated ART early and benefits for deworming was found very significantly beneficial on Viral load, CD4 and hemoglobin levels. Future studies should explore the role of ART combination therapies in the management of haemoglobin levels to control the effects of anaemia in helminthic-malaria co infections in other longitudinal studies specific for this purpose. This would elucidate the immunological mechanism associated with increase in CD4 levels and viral load suppression and combined effects of worm removal.

The results of this thesis have the potential to inform the local and national health policy managers to strengthen the use of anthelminthic during pregnancy, and to extrapolate it to other services such as those for non-pregnant women. Targeting the control and treatment of HIV-1 co-infections with helminths, malaria and other NTDs in Rwanda and link it to existing antiretroviral therapy programs.

It can be suggested that the relationship between helminths with malaria is particularly very important strategy that further supports disease integration. Therefore, combined control programs are likely to explore more opportunities to enhance control efforts and diagnostics central to this joint approaches to fight a global war against these parasites targeting poor population as reported by other studies [250]. The results presented in chapter 5 of this thesis shed more light on whether mass treatment (untargeted-deworming) or diagnosis and subsequent treatment of helminthic infections (targeted deworming) should be part of routine HIV care within antenatal and prevention of mother to child transmission (PMTCT) services.

This thesis has ground reasons to incorporate periodic deworming, using albendazole, into existing ANC and PMTCT programs as a single package that is less costly and more effective for resource-limited communities where co infection with HIV-1co-exists. The benefits of this intervention approach would improve haemoglobin levels and thus the control of anaemia, which is commonly associated with both malaria and helminths during and after pregnancy, and would improve immunological response to ART. The fact that certain ART combination therapies seemed to reduce the prevalence of helminth infection in the co-infected populations suggests that, in areas where co-infection is common, selection and prescription of particular ART combinations could be considered along with use of albendazole.

The timing of deworming is also an important consideration during the implementation programs. In our study, albendazole was given to women every 12 weeks and re-infection rates were reduced considerably, which implies that deworming is relevant and more effective when given routinely but with repetitive dosage after about 12 weeks. The effect of anthelminthic on HIV viral load detection and CD4 cell count levels that rose when subjects were given albendazole without helminthic infections is indicative that anthelminthic actually can have an effect on subjects' immunity independent of their anti-helminthic effect.

6.7 Strength and weakness of the study

While conducting the pilot primary prevalence study, the conclusions were striking. However, the sample size used to test the effect of ART on specific helminth infections and malaria was relatively small so that the effect of sample size might have reduced the significance of the conclusions.

In the second study the baseline intervention data again suggested an effect of ART on helminth infection but the ART regimen that was most effective in the pilot study was not so in the baseline study. This might imply a mass effect of ART compounds on helminth infections, which would call for further longitudinal studies to specifically analyse these effects for reproducibility.

The current study was not primarily designed to look for the effect of ART on helminth prevalence rates and malaria infection. Certain ART combinations showed a differential effect on helminthic infections and, specifically in the study reported in chapter 3, where the d4T-3TC-NVP and the AZT-NVP regimens both had a significant effect on the risk of helminth infection when compared to the reference ART regimen (AZT-3TC-NVP). These results were in stark contrast to those reported in chapter 4 where the AZT-3TC-NVP regimen had the strongest effect on helminth infections.

The unexpected differences observed between these two studies may be due to differences in sample size, or may be due to other uncontrolled confounding factors. Thus, in the social demographic case report form used, described in chapter 2 we did not capture the different drugs which the women were using or had used, particularly the reported prophylactic combinations of routine drugs such as cotrimoxazole [51,125,128,216], which is very commonly used in PMTCT routine programs, and which might have differential effects on the level of opportunistic infections such as malaria in HIV-infected individuals that has been reported in several other studies [51,70,239]. This is a hypothesis, which needs to be explored further and a limitation to this analysis. However, the data provided here is important and opens up ideas for future studies regarding the role and effect of ART combinations on helminthic and malaria co-infections in HIV infected populations.

The influence of confounders and spatial factors that might have affected our results cannot be ruled out, given the lack of specific control groups and failure to consider independent effect of

cotrimoxazole on malaria and helminths in our setting. Subjects in the study were recruited via antenatal care (ANC) routine settings and health centres with PMTCT services in rural and peri-urban community facilities, and was randomly assigned to two groups, 'targeted' and 'untargeted' intervention arms. These may nonetheless be biased, which would affect the generalizability of findings beyond the study population.

The HIV-infected pregnant women represented Rwandan rural and peri-urban residents utilizing PMTCT/ANC services, and did not include those in the first trimester of pregnancy or even include recent migrant users of the free ANC/PMTCT services existing in Rwanda. Therefore, the sample may not be sufficiently representative of contemporary Rwandans

It can also be argued that few rural and peri-urban sites were sampled, due to logistical problems at the time the study was conducted. Thus the study did not sample women living in all parts of the country. The thesis findings therefore cover only specific sub-populations within Rwanda, and it remains speculative whether these data on prevalence can be extrapolated to other areas. However, these data highlight the importance of conducting similar studies in different settings and different population groups

Two of the studies were cross-sectional in nature, so that we cannot prove causality for any association described in those studies. Within the longitudinal study, all immune markers of disease progression, like CD4 cell counts, viral loads and haemoglobin levels were measured at intervals of 12 weeks over a period of one year. This is still too short a time for following up measures of association and predictors of this nature, and thus may not be indicative as markers for long-term status. It would have been better to take measurements for at least two years or longer, but lack of funding was a limitation to elucidate this hypothesis.

Further limitations of the study were the absence of a placebo arm, and neither participants nor study staffs were masked to treatment allocations. Also, there was no control group without ART or an HIV-negative group. Additionally, the Kato Katz method used to determine the number of helminths eggs could have underestimated the proportion of women with light hookworm infection.

Our intervention study cohort had several strengths. It was a randomized, controlled observational cohort study carried out at a number of sites in Rwanda. Compliance with study

treatment was documented by direct observation by study staff. Compliance with treatment was shown by a dramatic fall in helminths infections from the first to the final visit. Retention of participants in the study was high and did not significantly differ between study groups. Furthermore, the objective outcome measures were systematically assessed and documented. Compliance with the study treatments was documented by direct observation by study staff. Retention of participants in the study was high and did not differ between study groups. Furthermore, the objective outcome measures were systematically assessed and documented. Additional strengths of this study are the screening of 3 stool samples on consecutive days for the presence of helminths, and the use of a combination of two different screening techniques to increase the sensitivity of helminths and malaria diagnosis. The strengths of this study also included the screening of a large number of women from health centres, which cater for women of all socio-economic classes.

6.8 General conclusions and recommendations from the thesis

6.8.1 The prevalence and effect of antiretroviral therapy at baseline

This study shows a high prevalence of malaria and helminthic infection among HIV-positive pregnant women in Rwanda.

Helminthic and malaria infection during pregnancy in HIV-infected individuals may lead to serious consequences for mother and baby. It is possible that such findings can inform policy-makers in better planning for interventions and analysis of the effect of infections in health outcomes. This study showed that more infections occurred in rural than in peri-urban settings. It is important that future interventions should be targeted to rural communities rather than urban settings, with health education and sensitization programs to promote individual hygiene in the rural settings. Antenatal care service interventions for malaria, HIV/ AIDs and helminth control should continue to be promoted in these settings, and women coming from rural settings with higher helminthic infection rates should be provided with treatment opportunities with available drugs and should also be offered health education to enhance self-hygiene practices in their communities.

The information obtained in these studies can be used to plan longer-term interventions, target higher-risk women and integrate interventions for co-infections in existing health infrastructures

the better to mitigate the impact of infections in pregnancy. The findings demonstrate that demographic and socioeconomic risk factors may play a role in co-infection exposure. Our findings can be used to guide public health interventions and also identify potential confounders to be considered during further analysis of the effects on health outcomes of these infections in pregnancy. We recommend that future interventions to control HIV and malaria in ANC settings should be integrated with deworming to control and treat helminthic co-infections in pregnancy, targeting mostly rural settings and peri-urban locations, while utilizing ANC/PMTCT infrastructure. This can be extrapolated to non-HIV pregnant women in similar settings.

6.8.2 The differential effect of ARTs on the risk of helminthic infection

In areas endemic of helminthic infection, tuberculosis (TB), malaria and HIV are to a large extent overlapping [59,239]. This is in agreement with a study in Ethiopia, on co-infection involving the 'big three' diseases (HIV, malaria, TB) were conducted in which a third of smear-positive TB patients were infected by helminths. This implies that co-infection by HIV, TB and malaria is likely to carry helminths along. In the same study, threat of worm infection declined during TB treatment in HIV+/TB co-infected patients, whereas no decline was seen in HIV−/TB group. This implies that, when co infection is treated, the intervention gain is likely to be two- or three–fold.

6.8.3 Prevalence, risk and protective factors

ART without PIs may reduce the prevalence of helminthic infection. Thus, ART itself might contribute to the decline in helminth prevalence, asymptomatic malaria or co-infection, as seen in our study. Although immune reconstitution might be praying a role [94], we hypothesize that the anti-mitochondrial toxicity of ART compounds may play a direct role here. The possible anti-helminthic effect of some ART combinations on helminths and malaria warrants further study.

This data leads us to suggest that the prevalence of helminthic infections, malaria and co-infections is common in HIV-positive pregnant women on ART in Rwanda. Helminthic and malaria infection in this population are important risk factors for low haemoglobin levels. Subjects with low CD4 counts were at higher risk of infection, and helminthic infection is a risk

factor for malaria. Education and employment were independent protective factors for helminthic infection and malaria, whilst hand washing reduced the risk only for helminthic infections.

In addition to the obvious recommendation to emphasize continuous health education and individual hygiene practice, there are increased opportunities for generating income, in the making and selling of soap to improve hygiene and to reduce helminthic transmission; this could also extend to malaria-control devices such as insecticide-treated nets (ITN).

Particularly with regard to the conclusions related to the indications of deworming during pregnancy for women infected with HIV and on ART, we recommend measuring haemoglobin as a key indicator of maternal and neonatal health. This provides a rationale for having PMTCT services integrated into maternal, newborn and child health services, as a cost-effective approach

6.8.4 Effect of deworming on markers of disease progression

Life saved with availability of ART is commendable but HIV patients often get opportunistic infection, which deteriorates the health of infected patients. In sub Saharan Africa, co-infection with HIV and helminths is very common. Deworming with albendazole has been recommended very effective even in pregnant women. Our findings demonstrated that Deworming with albendazole (400mg) in HIV-infected pregnant women on antiretroviral therapy demonstrates a significant positive impact on HIV viral load between visit 1 and visit 4. This has important implications for the control of anaemia in pregnancy, which is very common in helminthic and malaria infections and may lead to problems for both the mother and the baby. Additionally, this again points out a vital implication on viral load as a best surrogate marker for disease progression and prognosis, which should be used while monitoring of HIV patients on ARTs

We recommend that the treatment of these co-infections should be integrated into the routine HIV CD4 cell count testing programme, and HIV viral load detection profiles may be used as surrogate markers of co-infection in the national health programmes where resources are limited.

Further studies are recommended to examine the effect of specific ART combinations on helminths so as to select ART regimens during helminthic co-infections. There may be important alternative approaches for delaying HIV /AIDs disease progression in the course of applying de-

worming methods. Further studies are also suggested to rule out role of drug-drug interaction in such cases

6.9 Suggestions for further research

The findings in the first paper show that the study population has a moderately high prevalence of helminth-malaria co-infections. Further studies are warranted, using different populations to monitor the immunological response to co-infection and to its treatment.

The beneficial effect of anthelminthic treatment on hemoglobin levels, viral load detection rates and CD4 cell trajectories observed in the third paper warrants further studies in controlled, randomized clinical trials for different population groups.

The observed benefits of de-worming on immune markers of HIV disease progression in the targeted and untargeted de-worming arms is impressive, and the analysis of cost-effectiveness is recommended to be carried out to determine which of these therapies has the best cost-benefit outcomes.

The mechanisms by which certain ART combinations affect helminth infection levels calls for further work to confirm these results. In addition, possible drug-drug interactions between albendazole and Coartem must be analysed in future studies.

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Appendix 1: Rwanda ethical committee letter

KIGALI HEALTH INSTITUTE

B.P. 3286 Kigali, RWANDA
Tel: +(250) 572172; +250 571788
Fax: +(250) 571787
Website: http://www.khi.ac.rw
E-mail: info@khi.ac.rw

7th September 2009

Institutional Review Board

Mr Emil Ivan
Principal Investigator

Dear Mr Emil Ivan

RE: YOUR APPLICATION FOR THE ETHICS CLEARANCE

Reference is made to your application for ethics clearance for the study entitled **"Effects of de-worming and prevalence of helminthes, malaria, and HIV-1 co-infections in pregnant women in selected Rwandan rural health Centers where helminthes, malaria and HIV-1 co-exist: A prospective targeted versus untargeted cohort study"**.

You will be pleased to learn that the ethics clearance has been offered for your study after receiving your revised proposal which did incorporate all the components proposed by the reviewers.

You shall be required to submit the progress report and any other major changes made in the proposal during the implementation stage. At the end of the survey the Institutional Review Board shall also require to be given a final report of the study.

I wish you success in this important study.

Kigali Health Institute K.H.I Faculty of Community Health Development

Mrs MUKAMANA Donatilla
For Chairperson, KHI Institutional Review Board

CC:

- Vice Rector, Academics and Research, KHI
- Rector, KHI
- Members of IRB
- Chairperson, National Ethics Committee

Appendix 2: Wits ethical committee letter

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Mr Ivan Emil

CLEARANCE CERTIFICATE	**M090705**
PROJECT	The benefits of De-Worming and Prevalence of Helminths and Human Immunodeficiency Virus-1 Coinfections in Pregnant Women attending Antenatal Services in Selected Rwandan health Centres. A Prospective Targeted VS Untragated...
INVESTIGATORS	Mr Ivan Emil.
DEPARTMENT	Chemical pathology and Medical Microbiology
DATE CONSIDERED	09.07.31
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 2009/09/28 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof N Crowther

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

Appendix 3: Questionnaire (CRF)

A) Health centre Information : District Health centre : G.I.S Information /PMTCT/ANC

1. Health centre Name:

2. Health centre Identification code Number :Enter Identification code for each participant

3. Name of the I/C of the Medical officer in the Health centre ,his/her code Number

I.D code participant									
Dr Code									

4. Province where the hospital is located

Province									
District									

5. Date of ANC visit by the participant: DDD/MMM/YYY

Visit 1	Visit 2	Visit 3	Visit 4	Visit 5					

6. B) Geographical coordinates of the health Facility Location

Degrees of arrival	North	South	East	West	Central				
Degrees of departure									
Altitude									
Type of soil									

7. **C) Information related to Hygiene and Sanitation for Socio Demographic characteristics Data**

7.1 Do you have tap water at home compound or shelter house? (1)=Yes____, (0) =No. ____

7.2 other water source around=2_____

8. Do you use river water as source for drinking and other domestic use at home?

8.1 Drinking? (1)=Yes ____ (0)= No_____

8.2 Domestic use? (1)=Yes _____ (0)= No_____

8.3 If yes do you fetch water from the river yourself? (1)=Yes____ (0)/=No____

8.4 Do you normally put on shoes while at home? (1)=Yes___ (0) =No____

8.5 Do you wash your hands after using toilet at home? Tick one: (1)= Often (2)= Not often____ (0)= not at all

8.6 What type of fertilizers do you use in the gardens at home? = (1) Cow dung_= (2) human faeces= (3) organic fertilizers= (0) none of these

8.7 Are there flushing toilets at home? (1)=Yes (2) = No

8.8 Type of drinking water? Boiled water? =1 not boiled=0 (1)/ (0) Yes/No Unboiled water? (1)/ (0) Yes/no? Bottled water (1)/ (0) Yes/No_____

8.9 How often do you go gardening, fishing and bathing in water ponds or lakes? (1)Often____ (2) Not often-___ (0) Not at all

D) **Information Related To Participant's Immunity For Laboratory Data:**

9. Is this your first pregnancy? (1)Yes___ (0) No___ if No its Number 1=0 2=1, 3=2, 4=3, 5th=4 Circle corrects one.

9.1. How many times have you attended the antenatal care during this pregnancy? (1)1st _, (2)2nd __, (3)3rd__ (4) More than 3_ (0)4 never

9.2. When did you last have a de-worming service at antenatal care PMTCT, VCT in the last three months? ____

(1) one month ago (2) two month ago (3) three month ago (4) six months ago (5) one year ago (6) not at all

9.3. Do you know your HIV status? (1)Yes___, (0) No___, (2) don't know, (3)/ (4) Want to now test Yes/no____

9.4. Do you know your CD4 count? (1)/ (0) Yes/No. Do you have evidence? (1)/ (0) Yes/no___ the new CD4 count is___

9.5. Viral load? (1)Yes _____or (2) No_____ if yes how many____? Where and when did you last have it measured? _____

9.6. If No, do you want the viral load or CD4 determined now? (1)Yes_____, (0) No_____

9.7. How old is your pregnancy in terms of weeks or trimester? Number of weeks_____or, Don't Know=3

9.8. Or the TRIMESTER 1STTREMESTER=0_____2ND TRIMESTER=1_____3RD TRIMESTER=2_____

9.9. If Don't know, do you want the weeks of pregnancy determined By Doctor/Midwife Examination? By Medical examination? Yes=1no=0 ___, by Ultra sound machine by a gynecologist? Yes=1 no=0

E) Information Related To Physical Examination And Medication History:

10. Number of weeks of pregnancy determined is: _____

10.1. Do you want your body weight and height determined? (1)Yes____ (0) No_____

10.2. If Yes, Your body weight determined is_____ (Kgs), Height is_____Metres squared.

10.3 When were you born? _____Your age in year's is_____

10.4. Do you know how to read and write? (1)/ (0) Yes/No: If yes, tick the rates as: Poor=0, Fair=1, Good=2 very good=3.

10.5. What is your sexual intercourse behaviour? Protected=1 or (0) un protect=0? If (1)= yes; (2) often, (3) not often, (4) always

10.6 Do you receive Nutritional supplements at antenatal /PMTCT services? Yes= (1) no= (0) Yes /No

If yes give name of supplements

10.7. Are you taking any anti- TB medications? (1)/(0) Yes/No? If Yes which ones and since when did you start taking the medication? Which TB test did you last have? Sputa test=1 serology test=0, please show me the TB test results if any?

10.8. Are you already on ARVs? No/yes (0)/ (1)

10.9. If yes how long have you been taking ARV? Less than three months, Three months, six or one year (0), (1),(2),(3)

10.10. What type of ARVs are you taking at the moment, first line=0, second line=1

F) Information Related To Nutrition and Food Supplements

10.11. What type of food did you eat for the last three days? List them for three days

10.12. What type of food did other members of your house hold eat for the last three days? List them for the last three days.

Appendix 4: Follow up form (FU)

SUBJECT TEL: _____SUBJECT ID:

Helminths-HIV STUDY Follow-Up Form

FOLLOW UP VISIT #1:

Date: ___/__/___Age__ Trimester__

Physicals: Weight/ kgs: _____

Height /m^2: _____BMI: _____

Compliant With Meds: ___Yes ___No

Type of ARV__ Duration on ARV___

Malaria Smear: Positive: ___Yes ___No

HRP-2 Positive: Yes -----No__Stool Smear: positive: Yes__ No__ epg ____Worm type_____$CD4_1$:___$Viralload_1$:_____Hb_1/gdl_____

FOLLOW UPVISIT #2:

Date: ___/_____/_____

Physicals:

Weight/ kgs: _____

Height/m^2:_____

BMI: _____Compliant With Meds: ___Yes ___No

Malaria Smear: Positive: ___Yes ___No__HRP-2 positive: Yes__ No__

Stool smear positive: Yes__/No___ _ epg___

Worm type_____$CD4_2$:____Hb_2/ gdl____

FOLLOW UP POST TERM VISIT#3:

Date: ___/_____/_____

Physicals:

Weight/Kgs: _____

Height/m^2: _____

BMI: _____Compliant With Meds: ___Yes ___No

Malaria Smear:

Positive: ___Yes ___No

HRP-2 positive: Yes__ No___

Stool Smear: Positive: Yes__ No___ epg___Worm type $CD4_3$: HB_3/gdl

FOLLOW UP POST TERM VISIT #4:

Date: ___/_____/_____

Physicals: _____

Weight/Kgs: _____

Height/m2: _____

BMI: _____Compliant With Meds: ___Yes ___No

Malaria Smear:

Positive: ___Yes ___No__

HRP-2 Positive: Yes___ No__

Stool Smear: Yes__ No__ epg__Worm type___$CD4_4$:___/Viral $load_2$: Hb_4/gdl

Appendix 5: Published paper with Acta Tropica

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Malaria and helminthic co-infection among HIV-positive pregnant women: Prevalence and effects of antiretroviral therapy

Emil Ivan[a], Nigel J. Crowther[b], Aniceth T. Rucogoza[a], Lawrence O. Osuwat[a], Elizaphane Munyazesa[c], Eugene Mutimura[d], Kato J. Njunwa[a], Kakoma J.B. Zambezi[e], Martin P. Grobusch[f,g,h,*]

[a] Department of Biomedical Laboratory Sciences, Kigali Health Institute, P.O. Box 3286, Kigali, Rwanda
[b] Department of Chemical Pathology, University of the Witwatersrand Medical School, National Health Laboratory Services, 7 York Road, Parktown 2193, Johannesburg, South Africa
[c] Department of Quality Assurance and Quality Improvement, National Reference Laboratory, P.O. Box 4668, Rwanda Biomedical Center, Kigali, Rwanda
[d] Women Equity in Access to Care and Treatment (WE-ACTx), P.O. Box 5141, Kigali, Rwanda
[e] School of Public Health, National University of Rwanda, P.O. Box 4558, Kigali, Rwanda
[f] Department of Infectious Diseases, University of the Witwatersrand Medical School, National Health Laboratory Services, 7 York Road, Parktown 2193, Johannesburg, South Africa
[g] Institute of Tropical Medicine, University of Tübingen, Wilhelmstr. 27, 72074 Tübingen, Germany
[h] Center of Tropical Medicine and Travel Medicine, Division of Internal Medicine, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1100 DE Amsterdam, The Netherlands

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ABSTRACT

The impact of malaria on anemia and the interplay with helminths underline the importance of addressing the interactions between HIV/AIDS, malaria and intestinal helminth infections in pregnancy. The aim of this study was to determine the prevalence of malaria–helminth dual infections among HIV positive pregnant mothers after 12 months of ART. A cross sectional study was conducted on intestinal helminths and malaria dual infections among HIV-positive pregnant women attending antenatal health centers in Rwanda. Stool and malaria blood slide examinations were performed on 328 women residing in rural ($n = 166$) and peri-urban locations ($n = 162$). BMI, CD4 cell count, hemoglobin levels, type of ART and viral load of participants were assessed. Within the study group, 38% of individuals harbored helminths, 21% had malaria and 10% were infected with both. The most prevalent helminth species were *Ascaris lumbricoides* (20.7%), followed by *Trichuris trichiura* (9.2%), and *Ancylostoma duodenale* and *Necator americanus* (1.2%). Helminth infections were characterized by low hemoglobin and CD4 counts. Subjects treated with a d4T, 3TC, NVP regimen had a reduced risk of *T. trichiura* infection (OR, 0.27; 95% CIs, 0.10–0.76; $p < 0.05$) and malaria–helminth dual infection (OR, 0.29; 95% CI, 0.11–0.75; $p < 0.05$) compared to those receiving AZT, 3TC, NVP. This study shows a high prevalence of malaria and helminth infection among HIV-positive pregnant women in Rwanda. The differential effect of ARTs on the risk of helminth infection is of interest and should be examined prospectively in larger patient groups.

1. Introduction

Helminth and malaria infections have been hypothesized to be factors likely to be driving the HIV-1 epidemic in Africa (Harms and Feldmeier, 2002; Slutsker and Marston, 2007). Globally, there are more than 2 billion people that are estimated to be infected with soil-transmitted helminths, with the geographical distribution of these infections overlapping considerably with regions of high HIV-1 sero-prevalence and malaria endemicity (Fincham et al., 2003; Hotez and Kamath, 2009). Malaria and helminth infections play a role in the pathogenesis of HIV-1 infection in Africa, due to their profound effects on the host immune system, which makes those infected more susceptible to HIV-1 infection (Harms and Feldmeier, 2002; Korenromp et al., 2005).

The combination of HIV, helminthic and plasmodial infection in the host creates an immunologically complex profile (Gallagher et al., 2005) and substantially increases the risk of anemia, which is caused by all three types of infections (Laufer et al., 2006). Therefore, in terms of co-infection with these diseases, pregnant women in sub-Saharan Africa represent a highly vulnerable group, particularly in light of data showing that helminth infection increases the risk of mother-to-child transmission of HIV (Gallagher et al., 2005).

* Corresponding author at: Center of Tropical Medicine and Travel Medicine, Division of Internal Medicine, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1100 DE Amsterdam, The Netherlands. Tel.: +31 205 66 2097; fax: +31 697 2286.
E-mail addresses: emil.ivank@gmail.com (E. Ivan), nigel.crowther@nhls.ac.za (N.J. Crowther), munyazesa@hotmail.com (E. Munyazesa), eugene.mutimura@gmail.com (E. Mutimura), jbkakoma@nur.ac.rw (K.J.B. Zambezi), m.p.grobusch@amc.uva.nl (M.P. Grobusch).

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Recent data on the prevalence of helminth infection in Rwanda for school-going children from eight districts indicated a prevalence of 64.5%. The observed prevalence was higher in rural than in urban settings (Mupfasoni et al., 2009). However, no studies have documented the prevalence of malaria and intestinal helminth dual infections among pregnant women with HIV/AIDS attending antenatal services in the setup of ARV roll-out programs. Therefore, the aim of this study was to determine the baseline prevalence of helminth and malaria dual infections in HIV-1 infected pregnant women attending Rwandan health centers after ART initiation.

2. Materials and methods

2.1. Study area and population

Participants were enrolled among women attending the antenatal health center clinics in the provinces of Ruhuha, Mareba and Biryogo. After having given written informed consent, women in the second and third trimester of pregnancy were enrolled into the study. Women were excluded if they were HIV negative, below 18 years of age, had clinical evidence of TB, and had a treatment history of antihelminthic therapy and clinical confirmation of an abnormal pregnancy. On enrolment, subjects were interviewed for demographic information, and type and duration of ARV treatment.

After informed consent was obtained, blood samples were collected for malaria microscopy, rapid malaria testing, hemoglobin level and CD4 cell count determination, and viral load assays. Stool samples were obtained on three consecutive days for determination of helminth infection.

2.2. Laboratory tests

Intestinal helminths were identified microscopically by the Kato–Katz method (Katz et al., 1972; Bukusuba et al., 2004). Three Kato–Katz slides were prepared from each of three stool samples collected on consecutive days, and examined within 30 min for *Ancylostoma duodenale* and *Necator americanus* and again the following day for ova of *Ascaris lumbricoides* and *Trichuris trichiura*. Eggs per gram (EPG) of stool were calculated by taking the mean of the mean values obtained for each of the three stool samples. Malaria antigen HRP-2 (Orchid Bio Medicals Goa, India) was identified using a rapid serial testing algorithm, as previously described (Grobusch et al., 2003). *Plasmodium* spp. parasitemia was identified by examination of thick and thin blood films. Hemoglobin levels were measured using a Rapid HemoCue® kit (HemoCue AB, Angelholm, Sweden). The CD4 cell counts were determined at enrolment using multiset® software on a FACS Calibur/Sysmex XT1800i® dual-platform system (Becton Dickinson BD, San Jose, CA, USA). Plasma HIV RNA was quantified using a Gen-probe® HIV-1 viral load assay on a AmpliPrep/Cobras Taqman® (HIMCAP Fort Lauderdale, FL, USA), detecting <40 copies/mL.

2.3. Treatment of infections

Helminth infections were treated according to study protocol and in accordance with recommendations of the Rwanda Ministry of Health. All women who tested positive for malaria were treated with artemether-lumefantrine (Coartem® Dispersible, Novartis, Garden City, NY, USA). All women were receiving anti-retroviral therapy (ART) and the duration of therapy was determined by entry on the patient treatment card and cross checking with records in the patient's file. The study was approved both by the Kigali Health Institute's Ethical Review Committee and the University of the Witwatersrand's Medical Ethical Review Committee.

2.4. Statistical analysis

Analyses were performed using Stata® version 11.0 (College Station, TX, USA) and Statistica® version 9.1 (StatSoft, Tulsa, OK, USA). Data were expressed in tables and text as mean ± SD or median [interquartile range]. Data that were not normally distributed were log transformed to normality. Comparison of continuous data between 2 groups was carried out using a Student non-paired *t* test, whilst data comparison across more than 2 groups were performed using ANOVA with paired means analyzed using Tukey's test. Comparisons of mean values between groups, with adjustment for possible confounding variables, was accomplished with the use of ANCOVA. Percentage levels were compared across study groups using the χ^2 test. Backward, stepwise multiple regression analyses were performed to identify the principle determinants of hemoglobin levels and CD4 counts. Independent variables that were included in the initial regression model were those that correlated with the dependent variable with $p < 0.10$ in a Pearson univariate analysis. Variables were then removed one at a time based on their *p*-value, with the variable with the weakest *p*-value being removed in each round until only variables with a $p < 0.05$ were left in the model. Logistic regression was performed to assess the risk of infection across antiretroviral therapy (ART) regimens and data expressed as an odds ratio (OR) with 95% confidence intervals (95% CI). The categorical variable was infection/no infection whilst the independent variables were the ART regimens for which dummy variables were generated. The AZT, 3TC, NVP regimen was set as the reference, with an odds ratio of 1. The logistic regression models were run with and without the confounding variable location, i.e. peri-urban and rural, which were coded as 0 and 1, respectively.

3. Results

3.1. Participants' characteristics

The study cohort of 328 pregnant women included 166 from rural and 162 from peri-urban centers. The median [interquartile range] age of the cohort was 27.0 [8] years with no significant difference in age between the 2 population groups. The mean BMI (±SD) of the total cohort was 25.4 ± 3.44 with women from the peri-urban (26.7 ± 2.88) group being significantly heavier than the rural (24.1 ± 3.46; $p < 0.0001$) population. Study participants were recruited to the study in the second and third trimesters of pregnancy with 288 (87.8%) women recruited in the second and 40 (12.2%) in the third trimester. No significant differences were noted between these 2 groups for any of the study variables except that 53.5% of women recruited in the second trimester of pregnancy originated from the rural area compared to 30.0% in the third trimester ($p = 0.005$).

3.2. Prevalence of helminth and malaria infections

Table 1 shows that the prevalence of malarial (with or without helminth) infection ($p < 0.0005$) and of malarial-helminth dual infection ($p < 0.005$) was significantly higher in the rural than peri-urban population. Helminth (with or without malaria) infections were also higher in the former population but this difference did not reach statistical significance. However, the prevalence of *T. trichiura* infection was significantly higher ($p < 0.05$) in the rural population. Cases were also detected where helminth infection was present without malaria. The prevalence of these 'helminth only' cases was 27.7% in the rural population and 27.8% in the peri-urban group. Also, malaria was detected in subjects without a helminth co-infection. The prevalence of these 'malaria only' cases was 14.5%

Table 1
Prevalence of malaria and helminth infections in rural, peri-urban and combined populations.

Variables	Rural	Peri-urban	Combined
N number	166	162	328
Malaria with or without helminth (%)	29.5	12.3***	21.0
Helminth with or without malaria (%)	44.6	32.7	37.8
Specific species			
Ascaris lumbricoides (%)	23.5	17.9	20.7
Trichuris trichiura (%)	12.7	5.56*	9.15
Hookworm (%)	1.81	0.62	1.22
Asc + Trich + Hkw (%)	6.63	8.64	7.62
Both malaria and helminths (%)	15.1	4.94**	10.1
Eggs per gram of stool (EPG)	6236 ± 6831	6491 ± 4814	6342 ± 6051

Data given as % except for EPG (mean ± SD); mean EPG was calculated using values only from subjects with a worm infection (*N* = 74 for rural and 53 for peri-urban); Asc, *Ascaris lumbricoides*; Trich, *Trichuris trichiura*; Hkw, hookworm.
* $p < 0.05$ versus rural group.
** $p < 0.005$ versus rural group.
*** $p < 0.0005$ versus rural group.

in the rural population and 7.4% ($p < 0.05$ versus rural) in the peri-urban group with an overall prevalence of 11.0% in the combined population. The EPG values did not differ between the two groups.

3.3. Effects of helminth and malarial infections

Table 2 shows that dual infection with malaria and helminths is associated with a lower BMI than in both non-infected ($p < 0.05$) and helminth-only infected ($p < 0.05$) subjects. Furthermore, in comparison to subjects with no infections, those with a malaria–helminth dual infection or an infection with helminths only are characterized by lower hemoglobin levels ($p < 0.005$ and $p < 0.05$, respectively) and a higher prevalence of hemoglobin levels <12 g/dL ($p < 0.005$ and $p < 0.05$, respectively). Subjects with malaria alone also had lower hemoglobin levels and a higher prevalence of reduced hemoglobin concentrations when compared to females with no infections; however, these differences were not statistically significant. Also, subjects with a dual infection or with a helminth-only infection had lower CD4 counts than subjects with no infection ($p < 0.005$ and $p < 0.0005$, respectively) and subjects with a malaria-only infection ($p < 0.05$ and $p < 0.0005$, respectively).

Backward, stepwise multiple regression models were developed to identify the principal determinants of hemoglobin levels and CD4 counts. The initial regression model for hemoglobin levels included the following continuous variables: helminth egg count and body weight, and the following coded (0 or 1) variables: Helminth infection, rural/peri urban and detectable viral load. The final model retained only 2 variables that significantly correlated with hemoglobin level: helminth infection (beta = −0.21; $p < 0.0005$) and body weight (beta = 0.13; $p < 0.05$). *R* for the final regression model was 0.27 ($p < 0.0001$). The initial regression model for CD4 counts included one continuous variable (helminth egg count) and the following coded variables: helminth infection, rural/peri urban and antiretroviral treatment regimen. The final model retained only 1 variable that significantly correlated with hemoglobin level: helminth infection (beta and $R = -0.35$; $p < 0.0001$).

Table 3
Comparison of anti-retroviral regimens used in rural and peri-urban populations.

Anti-retroviral regimens	Rural	Peri-urban
D4T, 3TC, NVP (%)	63.2	0.00***
AZT, 3TC, NVP (%)	19.9	3.09***
AZT, NVP (%)	0.60	95.1***
AZT (%)	12.0	1.23***
Other (%)	4.22	0.62*

* $p < 0.05$ versus rural group.
*** $p < 0.0005$ versus rural group.

3.4. Antiretroviral treatment regimens and their effects

Table 3 shows that there were significant differences in the antiretroviral regimens that were used at rural and peri-urban sites. Thus, over 95% of peri-urban centers used the AZT, NVP regimen. The most common regimen in use at rural sites (63.2%) was the d4T, 3TC, NVP triple-combination.

Table 4 shows that duration of ARV therapy, hemoglobin levels and CD4 counts were not significantly different across the 4 treatment groups. However, BMI was significantly higher in those receiving AZT and NVP when compared against all other groups ($p < 0.0005$ for all comparisons). Also, the prevalence of a detectable viral load (DVL) was higher in the AZT, 3TC, NVP treatment group compared to both the d4T, 3TC, NVP ($p < 0.05$) and the AZT, NVP groups ($p < 0.0005$). This pattern was reproduced in the prevalence of helminth (with or without malaria) ($p < 0.05$ and $p < 0.005$, respectively) and *T. trichiura* infections ($p < 0.05$ and $p < 0.0005$, respectively). It was also observed that malaria (with or without helminth infections) was more common in the d4T, 3TC, NVP and AZT, 3TC, NVP treatment groups compared to the AZT, NVP group ($p < 0.05$ and $p < 0.0005$, respectively). The same pattern was also observed for helminth–malaria dual infections ($p < 0.05$ and $p < 0.005$, respectively). The EPG value for the d4T, 3TC, NVP group was significantly lower than in the AZT, NVP group ($p < 0.05$) and also lower than in the AZT, 3TC, NVP group, but this difference did not reach statistical significance ($p = 0.08$).

Only 6 subjects (1.83%) had a DVL (>40 RNA copies/mL) and the median CD4 count in these subjects was significantly lower than in those with an undetectable viral load (UVL; 363 [114] cells/mm^3 versus 493 [173] cells/mm^3; $p < 0.0001$). All the participants with a DVL tested positive for helminth infection, whereas only 36.6% ($p < 0.005$) of subjects with an undetectable viral load (UVL) were

Table 2
Comparison of age, BMI, hemoglobin and CD4 levels across infection sub-groups.

Variables	No infections	Malaria only	Helminths only	Both malaria and helminths
N number	168	36	91	33
Age (years)	27.0 [7.00]	27.5 [9.50]	26.0 [9.00]	28.0 [9.00]
BMI	25.7 ± 3.53*	25.5 ± 3.74	25.6 ± 3.03*	23.5 ± 3.31
Hemoglobin (g/dL)	12.3 ± 1.10**,†	12.1 ± 0.95	11.9 ± 1.17	11.5 ± 1.05
Prevalence of hemoglobin <12 g/dL (%)	34.5**,†	44.4	47.2	63.6
CD4 (cells/mm^3)	532 [181]**,†††	533 [203]*,†††	449 [131]	464 [156]

Data expressed as mean ± SD, median [interquartile range] or percentage.
* $p < 0.05$ versus both malaria and helminth.
** $p < 0.005$ versus both malaria and helminth.
† $p < 0.05$ versus helminth only.
††† $p < 0.0005$ versus helminth only.

Table 4
Comparison of BMI, hemoglobin and CD4 levels and prevalence of detectable viral load and helminth and malaria infections across ARV treatment groups.

Variables	d4T, 3TC, NVP	AZT, 3TC, NVP	AZT, NVP	AZT
N number	105	38	155	22
Duration of ARV therapy (months)	8.62 ± 14.2	6.52 ± 9.64	8.76 ± 14.5	9.37 ± 14.9
BMI	24.3 ± 3.56***	24.0 ± 3.22***	26.9 ± 2.8	22.9 ± 2.25***
Hemoglobin (g/dL)	12.0 ± 0.87	12.1 ± 1.29	12.2 ± 1.21	12.0 ± 1.25
CD4 (cells/mm^3)	490 [111]	463 [223]	500 [201]	537 [328]
Detectable viral load (%)	0.95†	10.5	0.00†††	0.00
Eggs per gram stool (EPG)	4292 ± 4881*	7344 ± 6409	7060 ± 6336	6371 ± 4902
Helminth with or without malaria (%)	40.0†	65.8	32.2††	31.8†
Specific species				
Ascaris lumbricoides (%)	22.9	26.3	19.3	13.6
Trichuris trichiura (%)	8.57†	26.3	4.52†††	13.6
Hookworm species (%)	0.00	5.26	0.64	0.00
Asc + Trich + Hkw (%)	8.57	7.89	7.74	4.54
Malaria with or without helminth (%)	25.7*	39.5***	12.3	27.3
Both helminth and malaria (%)	12.4*	23.7**	5.16	9.09

Data expressed as mean ± SD or median [interquartile range] or percentage; mean EPG was calculated using values only from subjects with a worm infection (N = 42 for d4T, 3TC, NVP, 25 for AZT, 3TC, NVP, 50 for AZT, NVP and 7 for AZT); Asc, *Ascaris lumbricoides*; Trich, *Trichuris trichiura*; Hkw, hookworm.
* $p < 0.05$ versus AZT, NVP.
** $p < 0.005$ versus AZT, NVP.
*** $p < 0.0005$ versus AZT, NVP.
† $p < 0.05$ versus AZT, 3TC, NVP.
†† $p < 0.005$ versus AZT, 3TC, NVP.
††† $p < 0.0005$ versus AZT, 3TC, NVP.

Table 5
Odds ratios for risk of infection within each ARV regimen with and without adjustment for location (peri-urban or rural).

Infections	AZT, 3TC, NVP	d4T, 3TC, NVP	AZT, NVP	AZT
Malaria	1.00 (–)	0.53	0.21	0.57
	1.00 (–)	(0.24–1.17)	(0.09–0.48)***	(0.18–1.81)
		0.47 (0.21–1.05)	*0.58 (0.09–3.55)*	*0.55 (0.09–2.88)*
Trichuris trichiura	1.00 (–)	0.26	0.13	0.44
	1.00 (–)	(0.10–0.71)*	(0.05–0.38)***	(0.11–1.83)
		0.27	*0.11*	*0.45 (0.11–1.85)*
		(0.10–0.76)*	*(0.02–0.64)**	
Malaria with helminth	1.00 (–)	0.35	0.13	0.39
	1.00 (–)	(0.14–0.86)*	(0.05–0.36)***	(0.09–1.59)
		0.29	*0.96 (0.01–67.6)*	*0.36 (0.09–1.51)*
		(0.11–0.75)*		

Data expressed as odds ratio (95% confidence intervals); data in normal text is unadjusted for location, whilst data in italics is adjusted for location; N number for all the models is 328.
* $p < 0.05$ versus AZT, 3TC, NVP.
*** $p < 0.0005$ versus AZT, 3TC, NVP.

infected with helminths. Malaria was also more common in subjects with a DVL (50.0% versus 20.5%; $p = 0.08$) but this difference did not reach statistical significance. The prevalence of helminth co-infection with malaria was also more common in those with a DVL (50.0% versus 9.32%; $p < 0.005$). Hemoglobin levels were lower in subjects with a DVL in comparison to those with an UVL (11.0 g/dL versus 12.1 g/dL; $p < 0.05$). However, after adjusting for the presence of a helminth infection using ANCOVA, the p-value was attenuated ($p = 0.08$).

The data in Table 4 suggests differential effects of the ARTs on malaria and helminth infections. This was investigated further by logistic regression analysis in which the odds ratios for risk of infection for each ARV treatment regimen was calculated with and without adjustment for location (peri-urban/rural), which was the only possible confounding variable. The AZT, 3TC, NVP regimen was set as the reference odds ratio of 1. The results of this analysis are shown in Table 5. The data demonstrate that the d4T, 3TC, NVP regimen reduces the risk of *T. trichiura* infection and the risk of a combined malaria and helminth infection when compared to the AZT, 3TC, NVP regimen, and that adjustment for location does not level-out this effect. This was also observed for the effect of AZT, NVP on the risk of *T. trichiura* infection. However, adjusting for location did indeed level-out the significant risk reduction observed for this ARV regimen on both malaria and dual malaria–helminth infection. It was also observed that for the d4T, 3TC, NVP regimen, after adjustment for location, the odds ratio for malaria was slightly attenuated, with this effect just missing statistical significance ($p = 0.06$).

4. Discussion

The current study shows that helminth and malaria infection levels in HIV-positive pregnant women are high and that malaria and malaria–helminth dual infection are more common in rural than urban areas. Malaria–helminth dual infection was found to be a risk factor for reduced BMI, whilst helminth infection either on its own or in conjunction with malaria was found to lead to lower hemoglobin and CD4 levels. The ARV regimen of d4T, 3TC and NVP was found to significantly reduce the risk of *T. trichiura* and malaria–helminth dual infection when compared to the AZT, 3TC, NVP regimen, whilst AZT with NVP also lowered the risk of *T. trichiura* infection.

A prevalence rate of 37.8% for helminth infections and 21.0% for malaria indicates a high transmission intensity of these parasites among HIV-positive pregnant women in Rwanda. A study of malaria prevalence conducted in HIV-positive pregnant females in Kigali, Rwanda demonstrated that 8.0% of the study group had malaria (Ladner et al., 2002). This figure is comparable to the

prevalence of malaria observed in our study (12.3%) for subjects residing in the peri-urban areas of Kigali. The prevalence of helminth infection in our study was lower than that reported in a study of school children in Rwanda with a prevalence of 64% from eight surveyed districts (Mupfasoni et al., 2009). This may be related to the greater recreational exposure of children to contaminated soil, and a reduced level of agricultural activities in pregnant females. A previous study has shown that soil-transmitted helminthiasis is more common in children than adults (Knopp et al., 2010). Our findings show lower prevalence levels for malaria and malaria–helminth co-infection but a higher level of helminth infection than those reported in a study of pregnant women from Ghana (Yatich et al., 2009). In that study the prevalence of malaria, intestinal helminth and dual infection was 36.3%, 25.7%, and 16.6%, respectively. However, the HIV status of this population was not assessed. An investigation in Uganda among pregnant women (Ndibazza et al., 2010) reported a prevalence level of 68% for helminth infection and 11% for malaria, with 12% being HIV-positive. Thus, these studies show varying levels of helminth infection during pregnancy across African populations. There may be a number of reasons for this including differences in socio-economic status and the level of helminth exposure. However, it should be noted that in the present study all participants received ART, and we show that helminths seem to be susceptible to particular ART regimens.

Our findings show that dual infection with malaria and helminths is associated with a lower BMI compared to the other 3 study groups (Table 2). Females with malaria-only, helminth-only, or dual infection all had lower hemoglobin levels than non-infected females, but the difference was only significant for helminth-only or dually infected subjects (Table 2). The dually infected subjects had the lowest hemoglobin levels. It has been observed that dual infection of malaria with helminths leads to lower hemoglobin levels when compared to malaria-only or helminth-only infected pregnant females, whilst all 3 infected groups exhibit lower hemoglobin levels compared to non-infected subjects (Yatich et al., 2009). The non-significant effect of malaria on hemoglobin levels within our study may be related to the lower number of subjects carrying only malaria, thus reducing the power of the analysis.

In subjects with a helminth-only or a malaria–helminth infection, CD4 cell counts were significantly lower than in those with malaria-only or no infection (Table 2). It is well-established textbook knowledge that pregnancy, and first pregnancy in particular, increases malaria susceptibility. It is equally generally accepted that HIV-infected pregnant women have a significantly increased risk of malaria parasitemia and placental malaria, more severe clinical manifestations and pregnancy outcomes (Ayisi et al., 2003; Van Eijk et al., 2003; Ticconi et al., 2003; Ter Kuile et al., 2004). Our study suggests that in HIV-positive pregnant females, malaria is not necessarily associated with lower CD4 cell counts. This was confirmed in a multiple regression analysis in which the presence of malaria did not associate with CD4 counts. Further support for these results comes from a study showing that CD4 counts do not differ between HIV-positive subjects with or without malaria (Onyenekwe et al., 2008), and from the observation that the prevalence of malaria is not related to CD4 cell counts (Laufer et al., 2006). Furthermore, a prospective investigation of HIV-positive, pregnant females in Kigali, Rwanda, demonstrated that HIV-positivity but not CD4 cell count was the only factor related to an increased risk of malaria (Ladner et al., 2002). One explanation could be the fact that HIV infection has been found to be associated with a specific cytokine dysregulation resulting from impairment of IL-12-mediated IFNγ production by intervillous blood mononuclear cells impairing the immune response against placental malaria, rather than mounting a generalized suppression of the cellular immune response against malaria (Ned et al., 2005). Thus, regarding CD4 cell count level and malaria alone, there seems to be conflicting evidence blurring the otherwise clear-cut picture of the malaria–HIV interaction.

In contrast, our data also show that helminth infection is linked to lower CD4 cell counts in pregnant, HIV-positive women and that all subjects with a DVL tested positive for helminths. However, previous studies have shown that CD4 counts correlate positively with the risk of helminth infection (Walson et al., 2010; Woodburn et al., 2009), but others have found the opposite (Asma et al., 2011). Reviewing the epidemiology and immunology of helminth–HIV interactions, Webb et al. (2012) concluded that there is inconsistent evidence for a beneficial effect of at least anthelminthic therapy on CD4 counts and viral load in HIV-1 co-infected individuals. However, this effect would be expected to be suppressed by successful ART reducing viremia, possibly explaining why in some studies including ours CD4 counts were not relating to increased helminth infection risk. It should be noted that both these studies were performed in subjects who were ART naïve whilst our study only involved females who were receiving ART.

It has been described before that ART may reduce the prevalence of helminth infection (Bachur et al., 2008). However, the study by Bachur and colleagues did compare ART/non-ART in different areas and did not control for other covariates such as education and age, which could be relevant. A major finding of the study presented here is that there is a differential effect of ART on the infection rate. Thus, the ART regimens of AZT, NVP and d4T, 3TC, NVP both significantly reduced the risk of helminth infection compared to a regimen of AZT, 3TC, NVP. This effect was particularly evident for *T. trichiura*. Furthermore, the d4T, 3TC, NVP regimen, but not the AZT, NVP regimen, lowered the EPG count, suggesting that the former therapy reduces both the prevalence and intensity of helminth infection. It was also observed that both ART regimens that reduced the risk of helminth infection had similar but less prominent effects on malaria risk, and had lower prevalence levels of DVL than the AZT, 3TC, NVP regimen. A number of in vitro studies have shown that protease inhibitors can inhibit *Plasmodium falciparum* growth (Mishra et al., 2010; Nathoo et al., 2003; Parker et al., 2004; Skinner-Adams et al., 2004); however, similar studies have not been performed with the other antiretroviral drug classes. Clinical studies have confirmed the ability of ART to reduce malaria prevalence (Losina et al., 2007; Mermin et al., 2006a,b).

The strengths of this study are the screening of 3 stool samples on consecutive days for the presence of helminths, and the use of a combination of two different screening techniques to increase the sensitivity of helminth and malaria diagnosis. However, this study also has some limitations. Due to its cross-sectional design, it is impossible to determine temporal direction or causality. Also, the study had no control group of HIV-positive subjects not receiving ART. Additionally, *N* for this study is relatively small and therefore it may not be sufficiently powered to uncover true differences or relationships within the study cohort and may also obscure or falsely reveal significant effects in the logistic and multiple regression models.

In conclusion, we found that the prevalence of malaria and helminth infections is high in HIV-positive pregnant women in Rwanda. Helminth infection in this population is an important risk factor for low hemoglobin and CD4 cell counts whilst malaria–helminth dual infections are characterized by a reduced BMI. Particular ART regimens seem to have positive effects on both *P. falciparum* and *T. trichiura* infections, and further studies need to be undertaken in large prospective cohorts to confirm and further specify these results.

Disclosure

None of the authors has any conflict of interest to declare.

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Appendix 6: Published paper with PLOS NTDs

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Helminthic Infections Rates and Malaria in HIV-Infected Pregnant Women on Anti-Retroviral Therapy in Rwanda

Emil Ivan[1,2,3], Nigel J. Crowther[3], Eugene Mutimura[4], Lawrence Obado Osuwat[5], Saskia Janssen[6,7], Martin P. Grobusch[6,7]*

1 Kigali Health Institute Department of Biomedical Laboratory Sciences, Kigali, Rwanda, **2** Rwanda Biomedical Centre National Reference Laboratory, Department of Laboratory Network, Kigali, Rwanda, **3** Department of Chemical Pathology, University of the Witwatersrand Medical School, National Health Laboratory Services, Parktown, Johannesburg, South Africa, **4** Women Equity in Access to Care and Treatment (WE-ACTx), Kigali, Rwanda and Regional Alliance for Sustainable Development (RASD Rwanda), Kigali, Rwanda, **5** Department of Medical Laboratory Science, School of Health Sciences, Mount Kenya University, Kigali, Rwanda, **6** Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany, **7** Center of Tropical Medicine and Travel Medicine, Division of Internal Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Abstract

Background: Within sub-Saharan Africa, helminth and malaria infections cause considerable morbidity in HIV-positive pregnant women and their offspring. Helminth infections are also associated with a higher risk of mother-to-child HIV transmission. The aim of this study was to determine the prevalence of, and the protective and risk factors for helminth and malaria infections in pregnant HIV-positive Rwandan women receiving anti-retroviral therapy (ART).

Methodology and principle findings: Pregnant females (n = 980) were recruited from health centres in rural and peri-urban locations in the central and eastern provinces of Rwanda. Helminth infection was diagnosed using the Kato Katz method whilst the presence of *Plasmodium falciparum* was identified from blood smears. The prevalence of helminth infections was 34.3%; of malaria 13.3%, and of co-infections 6.6%. Helminth infections were more common in rural (43.1%) than peri-urban (18.0%; $p<0.0005$) sites. A CD4 count ≤350 cells/mm^3 was associated with a higher risk of helminth infections (odds ratio, 3.39; 95% CIs, 2.16–5.33; $p<0.0005$) and malaria (3.37 [2.11–5.38]; $p<0.0005$) whilst helminth infection was a risk factor for malaria infection and vice versa. Education and employment reduced the risk of all types of infection whilst hand washing protected against helminth infection (0.29 [0.19–0.46]; $p<0.0005$);). The TDF-3TC-NVP (3.47 [2.21–5.45]; $p<0.0005$), D4T-3TC-NVP (2.47 [1.27–4.80]; $p<0.05$) and AZT-NVP (2.60 [1.33–5.08]; $p<0.05$) regimens each yielded higher helminth infection rates than the AZT-3TC-NVP regimen. Anti-retroviral therapy had no effect on the risk of malaria.

Conclusion/significance: HIV-positive pregnant women would benefit from the scaling up of de-worming programs alongside health education and hygiene interventions. The differential effect of certain ART combinations (as observed here most strongly with AZT-3TC-NVP) possibly protecting against helminth infection warrants further investigation.

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* E-mail: m.p.grobusch@amc.uva.nl

Introduction

Globally, the most common nematode species that cause soil-transmitted helminthic diseases are *Ascaris lumbricoides*, *Trichuris trichiura*, and the hookworm species *Necator americanus and Ancylostoma duodenale* [1,2,3]. Although morbidity due to helminths can be controlled by delivering preventive chemotherapy with antihelminthic medicines, elimination and finally eradication will not be achieved until affected populations have access to effective sanitation, sewage treatment and waste disposal; which remains a common problem in most rural African settings. In most of sub-Saharan Africa, the health burden of helminthic disease is enormous [4]. Co-infections with malaria and HIV are numerous and important causes of morbidity and mortality. Combating co-infections has been identified as an important public health goal [5]. Important areas of current research interests are the effects of helminth infections on immune regulation and their possible consequences for susceptibility to other infections and immunologically mediated conditions such as allergy and autoimmune diseases [6].

The immunological interplay between helminth infections and HIV is complex, and there are different hypotheses on the influence of the infections on each other; the most important being the Th2 bias induced by helminth infections, suppressing Th1 responses specific to HIV; thus leading to more rapid HIV progression [7,8]. HIV acquisition was positively correlated with female urogenital schistosomiasis [9] but in contrast, a randomized controlled trial (RCT) showed no benefit of deworming on prevention from mother to child transmission of HIV [10]. A few systematic reviews have been published describing the effect of anti-helminthic treatment on markers for HIV disease progression showing inconsistently beneficial effects of anti-helminthic treatment; lower increases in HIV viral loads and increases in CD4 counts have been reported [11–13]. However, results from a

Author Summary

There is an overlap in the worldwide distribution of intestinal worms (helminths), malaria and HIV. Co-infections with helminth and malaria parasites cause a significant problem in the host, particularly in the presence of HIV infection. The aim of this study was to assess the prevalence of intestinal worm and malaria infection and co-infections and the associated risk factors among HIV-positive pregnant women that attended rural and peri-urban health centers in Rwanda. Our findings indicate that intestinal worms were more common among HIV-infected pregnant women in the rural than peri-urban settings. HIV-positive pregnant women who had lower CD4 cell counts were more at risk of being infected by intestinal worms and malaria. Malaria also increased the risk of being infected by intestinal worms and vice versa. Socio-economic factors such as lack of education and unemployment were among the risk factors for intestinal worm infections and malaria. Hand washing was found to reduce the risk for worm infections; whilst one particular ART combination (AZT-3TC-NVP) led to a reduced rate of helminth infections when compared to others.

recent RCT did not suggest a beneficial role of empric deworming to delay HIV progression [14].

Risk factors for helminth infections depend on the route of transmission and the life cycles of the various helminth species; they are usually related to hygiene and sanitation [15]. The geographical distribution of helminth infections is largely influenced by several environmental factors such as climate and presence of stagnant water bodies [16]. In the absence of vaccinations, the only currently recommended public health intervention for soil transmitted helminths is regular mass de-worming, particularly for high risk groups; backed up by facilitating access to clean water, improved sanitation and health education [17].

Pregnancy may increase susceptibility to helminths, but this is uncertain. A recent study from Gabon showed increased prevalence in pregnancy [18], but a study from Thailand found no association [19]. Susceptibility and clinical outcomes are further complicated by co-infection with HIV and malaria. Malaria in pregnancy due to *Plasmodium falciparum*, combined with helminthic infections in HIV-positive women is of great concern from a public health perspective [20,21]. Control of *P. falciparum* infection by intermittent preventive treatment and use of insecticide-treated bed nets is of high importance especially in primigravidae [22,23]. HIV-infected women tend to experience faster CD4 decline during and after pregnancy [24], and could therefore be even more susceptible to helminth infection and malaria.

This prospective cohort study was designed to assess the major risk factors for helminth and malaria co-infection in HIV-positive pregnant women who participated in an early anti-retroviral therapy (ART) initiation program for the prevention of mother-to-child transmission in Rwanda. We describe the baseline prevalence of malaria and helminth infection in HIV-infected pregnant women on ART, and assess the factors that may increase or decrease rates of both infections.

Methods

Ethics Statement

Ethical approval was obtained from the Rwanda National Ethics Committee and the Ethics Committee (Human Research) of the University of the Witwatersrand Medical School, Johannesburg, South Africa. All subjects provided written informed consent at enrolment. Subjects who could only provide oral consent were asked to give thumb marks using indelible ink on the consent form, in case they were illiterate; in accordance with the IRB oral standard ethical consent guidelines of the two ethical committees and in accordance with Helsinki declarations.

Study Population and Procedures

The study participants were recruited amongst women accessing antenatal care and ART services at rural and peri-urban health centers in the central and eastern provinces of Rwanda, between 02 January 2010 and 29 February 2011. After giving written informed consent, women in the second trimester of pregnancy were enrolled at their fourth, fifth or sixth month of gestation. Additionally, women were enrolled if they lived within walking distance from the study areas, and if they planned to deliver at the registered study health center. Enrolment criteria were HIV infection; pregnancy (in the second trimester); use of ART; and willingness to provide three stool samples on consecutive days. Women were excluded if they were diagnosed with tuberculosis, or if they had taken any anti-helminthic drugs at any time point prior to entry into the study. Those who had been enrolled in other research projects during the study period were also excluded. Participants provided blood and stool samples before being treated in accordance with the study protocol.

Collection of Demographic, Socio-economic and Pregnancy-Related Data

On enrolment, participants were interviewed to obtain demographic and socio-economic information pertaining to the relevant environmental risk factors for helminth infections and malaria. Subjects were asked whether they had attended school, whether they were employed, their source of water (river or piped), if they wore shoes, whether they washed their hands after using the toilet, and whether they used dietary supplements. Study participants were also asked about their number of previous pregnancies.

Diagnosis and Treatment of Infections

Intestinal helminths were identified by the Kato Katz method [22,23]. Three Kato Katz slides were prepared from each stool sample, and then examined within 30 minutes for hookworm species. The same specimens were again examined the following day for ova of other soil transmitted helminths with the formol ether concentration method. Eggs per gram (EPG) of stool were calculated by taking the mean of the mean values obtained for each of the three stool samples. In all study women asymptomatic parasitaemia was determined. *Plasmodium falciparum* was identified by light microscopic examination of Giemsa stained thick and thin blood smears, and screening for malaria was also performed by detection of the *P. falciparum* histidine rich protein 2 (HRP-2) antigen using a rapid diagnostic test kit (Biotec Laboratories Ltd., UK). In the case of discordant results, expert light microscopy results were considered as gold standard. Participants received treatment for helminth infections and malaria in accordance with the study protocol. Artemether/lumefantrine (120/20 mg) was given as standard falciparum malaria treatment, administered orally in four doses for three days. Deworming was performed every 12 weeks with 400 mg albendazole given to women with helminth infections only/not to those negative in the 'targeted treatment'arm; whereas all women received 400 mg albendazole irrespective of infection status in the 'untargeted treatment'arm.

Women received nevirapine for prevention of mother-to-child HIV transmission and subsequent combination ART, irrespective of CD4 cell levels in accordance with the latest (2010) Rwandan Ministry of Health treatment guidelines [25].

Statistical Analysis

Data analysis was performed using Stata version 11.0 (College Station, TX, USA) and Statistica version 9.1 (StatSoft, Tulsa, OK, USA). Data that was not normally distributed was log transformed to normality before analysis.

The prevalence of helminth, malaria and helminth-malaria co-infections were determined in population sub-groups e.g. in employed and unemployed subjects, and differences between the groups were assessed using the χ^2 test.

Backward, stepwise multiple logistic regression analysis was used to determine the principal factors associated with helminth and malaria infections and helminth-malaria co-infections. Models were constructed for infection with each individual helminth species, i.e. *A. lumbricoides*, *T. trichiura* and hookworms, and a combined model for infection with any helminth species. The independent variables included in the initial logistic regression models were, for helminth infections: location, month of year, ART, gravidity, education, employment, water source, use of shoes, hand washing, dietary supplement use, HIV viral load, presence/absence of malaria, age, height, gestational age and CD4 counts. With malaria as the dependent, dichotomous variable, the same list of independent variables was used. The presence/absence of helminth infection was included as an additional independent variable whilst presence/absence of malaria was removed. With co-infection (helminth-malaria infection) as the dependent variable, both malaria and helminth presence/absence were removed from the model. In all the logistic regression models, the independent variable with the highest p-value was removed at iteration until only variables with a $p<0.05$ were left in the model.

Backward, stepwise multiple regression analysis was used to identify the principal determinants of fecal helminth egg counts and blood hemoglobin levels. Univariate analyses were initially performed, and any variable with $p<0.50$ was included as an independent variable in the multiple regression models. The same procedure as described for the logistic regression models was then followed.

A sample size calculation was not performed for this study. N was chosen based on logistical factors, taking into account future follow-up studies. However, if one performs a post-study sample size calculation based on the logistic regression model for identifying the principal determinants of helminth infection and using the equation $N = (10*k)/p$ [26], where k is the number of co-variables ($k = 18$) and p is the frequency of helminth infections ($p = 0.34$), we obtain a minimum N of 529, which is far below the actual N of 980.

Results

Prevalence of Helminth Infections and Malaria

The data in Table 1 show that infection with any helminth species (in the presence or absence of malaria) occured in 336 participants (34.3% of the population investigated), being significantly ($p<0.0005$) more common in rural than in peri-urban communities. Infection with helminths in the absence of malaria showed a similar trend, occurring in 36.5% of rural, and in 11.3% of peri-urban subjects ($p<0.0005$). Infection with each of the three helminth species also occured more often in the rural than the peri-urban population, with *A. lumbricoides* being the most commonest. The presence of a malaria infection (in the presence or absence of helminths) was more frequent in peri-urban than in rural subjects ($p<0.05$). This trend was mirrored by malaria-only infections, with a prevalence of 4.39% in rural and 10.7% in peri-urban females ($p<0.0005$). The prevalence of helminth-malaria co-infection was similar in both environments (Table 1).

Prevalence of Helminth Infections and Malaria in Different Population Sub-Groups

The prevalence of helminth, malaria and helminth-malaria co-infections were calculated for different population sub-groups (Table 2). Helminth infections of any type, asymptomatic malaria or co-infections were all less prevalent in subjects receiving AZT-3TC-NVP when compared to those taking d4T-3TC-NVP ($p<0.005$). Treatment with AZT-3TC-NVP was also associated with a lower prevalence of malaria or co-infection when compared to AZT-NVP therapy, and a lower prevalence of co-infection compared to TDF-3TC-NVP. The latter therapy was associated with a lower prevalence of asymptomatic malaria compared to subjects receiving AZT-NVP.

A number of factors had the opposite effect on helminth infection compared to malaria or co-infection rates. Thus, helminth infections were more common, but malaria and consequently co-infections less common in females who were tested in March–May compared to those tested in January or February. This same pattern was observed for females who did

Table 1. Prevalence of helminth and malarial infections in rural and peri-urban populations.

Variables		Rural	Peri-urban	Combined
n		635	345	980
Helminth infection		43.1 (39.3–47.0)	18.0 (13.9–22.0)***	34.3 (31.3–37.3)
Specific species:	*Ascaris lumbricoides*	26.1 (22.7–29.6)	11.0 (7.69–14.3)***	20.8 (18.3–23.4)
	Trichuris trichiura	8.66 (6.47–10.8)	4.35 (2.18–6.51)**	7.14 (5.53–8.76)
	Hookworm	8.35 (6.19–10.5)	2.61 (0.92–4.30)***	6.33 (4.80–7.85)
Malaria infection		11.0 (8.58–13.5)	17.4 (13.4–21.4)*	13.3 (11.1–15.4)
Helminth-malaria co-infection		6.61 (4.68–8.55)	6.67 (4.02–9.31)	6.63 (5.07–8.19)

All data expressed as percentage (95% confidence intervals);
*p<0.05,
**p<0.005,
***p<0.0005 vs rural.
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Table 2. Prevalence of helminth and malarial infections in relation to various risk factors.

Variables		*n*	Helminth (%)	Malaria (%)	Co-infection (%)
ART:	*AZT-3TC-NVP NNNNNVP*	299	27.4**	6.35**†††	2.67†††
	AZT-NVP	126	32.5	24.6**	14.3**
	d4T-3TC-NVP	461	39.7	14.5	6.72
	TDF-3TC-NVP	94	31.9	13.8†	8.51‡
Months:	*Jan–Feb*	262	27.9	24.4	12.2
	Mar–May	718	36.6*	9.19***	4.60***
Age:	*≤30 years*	545	37.4	10.3	5.50
	>30 years	435	30.3*	17.0**	8.05
Gravidity:	*1*	225	42.2	16.0	10.2
	2–5	755	31.9**	12.4	5.56*
Gestation:	*4 months*	505	33.5	22.0	11.3
	5–6 months	476	35.2	4.00***	1.68***
Education:	*Some*	480	21.6	10.6	2.80
	None	500	47.5***	16.0*	10.6***
Employment:	*Yes*	181	28.3	10.9	3.63
	No	799	60.8***	23.8***	19.9***
Water:	*Piped*	215	13.0	16.7	3.26
	River	765	40.3***	12.3	7.58*
Shoe wearing:	*Yes*	380	30.5	16.3	8.16
	No	600	36.7*	11.3*	5.67
Hand washing:	*Yes*	695	29.3	17.7	8.63
	No	285	46.3***	2.46***	1.75***
Diet supplements:	*Yes*	281	28.1	21.3	11.7
	No	699	36.8*	10.0***	4.58***
Viral load:	*Detectable*	90	70.0	17.8	14.4
	Not detectable	890	30.7***	12.8	5.84**
CD4:	*≤350 cells/mm^2*	209	62.7	27.7	20.6
	>350 cells/mm^2	771	26.6***	9.34***	2.85***
Malaria:	*Present*	130	50.0	-	-
	Absent	850	31.9***	-	-
Helminths:	*Present*	336	-	19.3	-
	Absent	644	-	10.1***	-

All data expressed as percentage; *p<0.05, **p<0.005, ***p<0.0005 vs other sub-group of same variable.
For ART: *p<0.05, **p<0.005 vs d4T-3TC-NVP;
†p<0.05,
†††p<0.0005 vs AZT-NVP;
‡p<0.05 vs AZT-3TC-NVP.
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not wear shoes compared to those who did, and in females who did not regularly wash their hands or take dietary supplements when compared to those that did (Table 2).

Pregnant females who were older than 30 years at testing had a lower prevalence of helminth infections but higher levels of asymptomatic malaria and co-infections than those females who were 30 years or younger. Primigravidae had higher prevalences for all three infection types compared to females who had more than one previous pregnancy, whilst females who presented for testing at an earlier stage of their pregnancy (4 months) had higher prevalence levels of malaria and co-infections compared to those at a later stage of pregnancy (5–6 months) (Table 2). If this latter group was divided into 5 and 6 months of gestation, the prevalence of malaria was not significantly different between them (4.20% vs 3.52% respectively).

Study participants who were unemployed and subjects with no formal education had a higher prevalence of helminth infections, malaria and co-infections compared to subjects with employment and the uneducated, respectively. Women who used river surface rather than piped water had a higher prevalence of both helminth infection and co-infection but a lower prevalence of malaria, although this last comparison did not reach statistical significance ($p = 0.09$). A detectable viral load and a CD4 count ≤350 cells/mm^3 were both associated with higher levels of all infections. Subjects with asymptomatic malaria had a higher prevalence of helminth infections, and vice versa (see Table 2).

Identification of Risk and Protective Factors for Helminth Infections

Table 3 depicts the results of multiple logistic regression analyses to identify risk and protective factors for helminth infections. With regard to ART, the d4T-3TC-NVP regimen groups exhibited higher prevalences of infection with *A. lumbricoides* and hookworm compared to AZT-3TC-NVP. The same applied with the AZT-NVP and TDF-3TC-NVP regimens regarding *T. trichiura* prevalence when compared to the AZT-3TC-NVP therapy.

There were lower rates of hookworm infestation in subjects who were screened for infections during January and February compared to those screened later in the year, whilst subjects who were older than 30 years, or who were multigravid had a lower risk of any helminth infection when compared, respectively, to those 30 and younger, or primigravidae.

Subjects who were residents of a peri-urban location had a lower risk of *A. lumbricoides*, hookworm or any helminth infection in comparison to those from a rural environment. Educated study participants and those who used piped water were at a lower risk of *A. lumbricoides* or any helminth infection when compared, respectively, to subjects with no formal education and who used river water. Furthermore, pregnant women who were employed or who regularly washed their hands were at a lower risk for *A. lumbricoides*, *T. trichiura* or any helminth infection in comparison to subjects who, were employed or did not wash their hands regularly, respectively.

Pregnant females who had a detectable viral load when compared to those who did not, were at a higher risk for *A. lumbricoides*, and subjects with a CD4 count at or below 350 cells/mm^3 were at a higher risk for all kinds of helminth infections when compared to those with CD4 counts above 350 cells/mm^3. The presence of malaria was associated with a higher risk of any helminth infection.

Identification of Risk and Protective Factors for Malaria and Co-Infections

The risk for malaria was higher in the months of January and February than from March to May (Table 4). Risk was also higher in older females but lower in those in the third trimester. This latter trend was also mirrored by risk for co-infection. Co-infection risk was also reduced in subjects with some formal education and in those with employment. Pregnant females who used piped rather than river surface water had a higher risk of malaria. Helminth infection was associated with a higher risk for malaria, whilst low CD4 counts were linked to a higher risk of malaria and co-infection. Interestingly, and being difficult to interpret, women who regularly washed their hands had a higher risk of both malaria and co-infections.

Identification of the Principal Determinants of Fecal Helminth Egg Count and Hemoglobin Level

Backward, stepwise multiple regression analysis demonstrated that fecal helminth egg counts were highest in females who were multigravid; who did not wear shoes and who had low CD4 counts (Table 5). Hemoglobin levels were lowest in females who had helminth or malaria infections, who had low CD4 counts and who had a higher (5 or 6 months compared to 4 months) gestational age.

Discussion

In this study population, we determined the prevalence and identified protective and risk factors of helminth, malaria and co-infections in HIV-infected pregnant women on ART in Rwanda. We found that helminth infection was more prevalent in rural

Table 3. Multiple logistic regression analyses to identify risk factors for helminth infections.

Variables		Odds ratios for *Ascaris* infection	Odds ratios for *Trichuris* infection	Odds ratios for hookworm infection	Odds ratio for any helminth infection
ART*	***d4T-3TC-NVP***	2.59 (1.79–3.75)***	-	2.19 (1.27–3.79)**	3.47 (2.21–5.45)***
	AZT-NVP	-	4.65 (2.41–8.96)***	-	2.60 (1.33–5.08)*
	TDF-3TC-NVP	-	3.57 (1.69–7.57)**	-	2.47 (1.27–4.80)*
Months:	***Jan–Feb vs Mar–May***	-	-	0.32 (0.13–0.77)*	-
Age:	***>30 vs ≤30 yrs***	-	-	-	0.66 (0.47–0.94)*
Gravidity:	***>1 vs 1***	-	-	-	0.59 (0.39–0.87)*
Location:	***Periurban vs Rural***	0.52 (0.33–0.82)**	-	0.32 (0.15–0.66)**	0.41 (0.27–0.62)***
Education:	***Yes vs No***	0.41 (0.28–0.59)***	-	-	0.39 (0.28–0.55)***
Employment:	***Yes vs No***	0.47 (0.31–0.73)**	0.14 (0.08–0.27)***	-	0.23 (0.15–0.36)***
Water:	***Piped vs River***	0.30 (0.16–0.53)***	-	-	0.23 (0.14–0.38)***
Hand washing:	***Yes vs No***	0.52 (0.33–0.80)**	0.20 (0.10–0.40)***	-	0.29 (0.19–0.46)***
Detectable viral load:	***Yes vs No***	1.95 (1.11–3.42)*	-		2.42 (1.29–4.55)*
CD4:	***≤350 vs >350 cells/mm³***	2.12 (1.38–3.23)**	2.27 (1.32–3.90)**	3.03 (1.75–5.26)***	3.39 (2.16–5.33)***
Malaria:	***Infected vs Not***	-	-	-	2.13 (1.27–3.59)**

*as compared to AZT-3TC-NVP;
data are odds ratios (95% confidence intervals);
odds ratios for ARTs are in comparison to therapy with AZT-3TC-NVP;
odds ratios are not given for variables that had no significant effect and were removed from regression model;
the following variables did not significantly affect risk for any of the above infections: gestational age, wearing shoes, use of dietary supplements and height;
*p<0.05,
**p<0.005,
***p<0.0005.
doi:10.1371/journal.pntd.0002380.t003

Table 4. Multiple logistic regression analyses to identify risk factors for malaria infection and helminth-malaria co-infection.

Variables		Odds ratios for malaria infection	Odds ratios for helminth-malaria co-infection
Months:	*Jan–Feb vs Mar–May*	1.70 (1.08–2.68)*	-
Age:	*>30 vs ≤30 years*	1.76 (1.15–2.69)*	-
Gestation:	*5–6 vs 4 months*	0.17 (0.10–0.29)***	0.16 (0.07–0.35)***
Education:	*Yes vs No*	-	0.32 (0.17–0.63)**
Employment:	*Yes vs No*	-	0.26 (0.14–0.49)***
Water:	*Piped vs River*	1.76 (1.04–2.97)*	-
Hand washing:	*Yes vs No*	5.81 (2.51–13.5)***	2.96 (1.07–8.19)*
Helminth infection:	*Yes vs No*	2.42 (1.51–3.89)***	-
CD4:	*≤350 vs >350 cells/mm³*	3.37 (2.11–5.38)***	7.13 (3.95–12.9)***

Data are odds ratios (95% confidence intervals); odds ratios are not given for variables that had no significant effect and were removed from regression model; the following variables did not significantly affect risk for any of the above infections: location, ART, viral load, gravidity, wearing shoes, use of dietary supplements and height;
*p<0.05,
**p<0.005,
***p<0.0005.
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than peri-urban settings. Poor education and unemployment were risk factors for both helminth and *P. falciparum* infection, whilst hand washing protected against worm infections. HIV treatment with AZT-3TC-NVP was associated with a lower prevalence of helminth infections. A CD4 count ≤350 cells/mm^3 was associated with higher levels of all infections. Multiple linear regression analysis demonstrated that helminth egg counts (EPG) were highest in females who were multigravid and hemoglobin levels were lowest in females who had helminth or malaria infections.

The prevalence of helminth infection was higher among the rural than peri-urban populations. Whilst we did not notice general differences between women recruited at the various health centers, this is best explained by variations in lifestyle between both settings. The most prevalent species were *A. lumbricoides* followed by *T. trichiura* and hook worm species (*A. duodenale and N. americanus*). This is in agreement with previous findings from the same location [21]. Our results are further supported by findings from an earlier study in the region which indicated that *A. lumbricoides* and *T. trichiura* were more commonly found in Rwanda and Burundi than in most other East African countries [27]. Our findings show lower prevalence levels for malaria and malaria-helminth co-infection than previously reported for pregnant females in Ghana but higher rates of helminth infections [28]. A study in Uganda [29] reported that the prevalence of helminth infection among pregnant women was 68% and malaria was 11%; however, only 12% of the women were HIV infected. These results indicate (not surprisingly) that there are varying prevalence levels of helminth infection during pregnancy across East African populations. It should be noted that in the study in Ghana the HIV status of the participants was not known, whilst in our study all participants were HIV-infected and receiving ART.

An earlier study of malaria prevalence conducted in HIV-positive pregnant females in Kigali, Rwanda, demonstrated that 8.0% of the study group had malaria [21]. It is well documented that pregnant women living in malaria endemic areas have an increased risk of *P. falciparum* infection during pregnancy but this usually remains asymptomatic. In the current study, we found seasonal fluctuation, with the prevalence of asymptomatic malaria being higher in subjects tested in the months of January–February than those tested in March–May.

In the current study, we report that pregnant females who were older than 30 years at the time of testing had a lower prevalence of

Table 5. Multiple regression models for determinants of helminth egg count and hemoglobin level.

Model number	Dependent variable	Independent variables	Beta value (p value)	R for model (p-value)
1	Egg count (log)	Gravidity[a]	0.17 (0.001)	0.28 (<0.0005)
		Use of shoes[b]	−0.13 (0.01)	
		CD4 count (log)	−0.54 (0.03)	
2	Hemoglobin level	Helminth[c]	−0.67 (<0.0005)	0.34 (<0.0005)
		Malaria[c]	−0.31 (0.01)	
		CD4 count (log)	1.37 (<0.0005)	
		Gestational age[d]	−0.19 (0.02)	

[a]Gravidity coding: primigravida - 1, multigravida - 2;
[b]Coding for use of shoes: wear shoes - 1, do not wear shoes - 0;
[c]Coding for helminth or malaria: infected - 1, no infection - 0;
[d]Coding for gestational age: 4 months - 1, 5 or 6 months – 2.
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helminth infections but higher levels of malaria than younger females. The helminth data is supported by a previous study from Uganda [30]; however, most studies show that malaria is also more common in younger, pregnant females [31,32]. This difference may be related to a number of factors including lifestyle and socio-cultural differences across the population groups included in these studies.

Little data exists on the relationship between gravidity and the risk of helminthiasis. One study shows no effect of gravidity on the risk of helminth infection [28] whilst a second study demonstrates a higher risk of hookworm infection but a lower risk of *A. lumbricoides* infection in primigravid compared to multigravid females [32]. The data from the current study shows that primigravid females have a higher prevalence and risk of helminth infection compared to multigravid females. This is an important finding and suggests that de-worming programs should target such individuals. Our data also shows a higher fecal egg count in multigravid compared to primigravid females. Thus, although multigravid females are at a lower risk of helminth infections than primigravidae, when they do acquire a helminth infection they have a higher intensity of infection than primigravid females.

Females who presented for testing at an earlier stage of their pregnancy (4 months) had a higher prevalence of malaria and helminth-malaria co-infection than those at a later stage of pregnancy (5–6 months). This data is supported by findings from previous African studies [31,33].

Education and employment acted as protective factors against both helminth infection and helminth-malaria co-infection. Previous studies have shown similar associations [28,30], suggesting that socio-economic status is a strong modulator of disease risk.

Helminth infection was shown to be more prevalent in subjects who did not wash their hands. Studies have shown that the risk of helminth infection is reduced in subjects who regularly wash their hands, more so in those who use soap [34,35]. Thus, simple changes in hygiene practices would be important for reducing the prevalence of helminth infections. In our analysis, however, hand washing was statistically significantly associated with an increased risk of malaria and – consequently - helminth-malaria co-infection. This finding is surprising and difficult to understand. Of note, the use of piped compared to river water reduced the risk of helminth infection but seemed to increase the risk for malaria. Whilst improved access to water is known to reduce the risk for helminth infection [36] the possible reasons for a greater risk of malaria associated with hand washing and piped water are not known, with little data available in the literature to confirm these associations. We believe that we are dealing here with a confounder, although it is apparently difficult to understand its nature, and neither an elevated social status nor local vector behavior or distribution offers any clue to understand this observation. However, one possible explanation is that stand pipes for the collection of water may have been situated in areas more suitable for mosquito breeding, or that puddle formation around stand pipes created favourable breeding conditions.

Helminth egg counts were highest in multigravidae who did not wear shoes regularly and who had low CD4 counts (Table 5). Hemoglobin levels were lowest in females who had helminth or malaria infections, who had low CD4 counts and who had a gestational age of 5 or 6 months. Based on the distinct mechanisms by which helminth and malaria affect hemoglobin levels, it can be speculated that their combined presence might interact to enhance the risk of anemia when intensity is moderately higher than in light worm intensities. The relationship between helminth infection, intensity and anemia has been described in several settings in Africa as well as in South East Asia [37]. Although women in our study group were all on ART with some having received nutritional supplements as part of their antenatal care package, previous regional studies also reported lower hemoglobin levels to be associated with high prevalence of helminth and malaria [38]. Our findings are further supported by other studies [17,39] which report that pregnant women are known to exhibit fluctuating CD4 levels in pregnancy, which might expose them to higher helminth infection prevalence leading to maternal anemia. This could be explained by the fact that during pregnancy the immune system is impaired; therefore, HIV positive pregnant women who live in highly endemic areas in sub-Saharan Africa are likely to be at increased risk for helminth-malaria co-infections.

In the present study the risk of helminth infection was higher in females with a reduced CD4 cell count, and in subjects with a detectable viral load. This is in agreement with previous studies conducted in pregnant females in Uganda [38] and Rwanda [21] where CD4 counts correlated negatively with the risk of helminth infection. However, another study has found the opposite [38], although this investigation was not carried out in pregnant females. Webb et al. [40] reviewed the epidemiology and immunology of helminth–HIV interactions, and concluded that there is inconsistent data support to postulate a beneficial effect of anti-helminthic therapy on CD4 counts and viral load in HIV-1 co-infected individuals. With regard to malaria, we found that a low CD4 count was associated with an increased number of malaria episodes. This is in contrast with data from a very similar study performed in Rwanda, where no such an association was found [21]. This discrepancy may be related to the lower power of the earlier investigation. There is clear evidence from a number of studies that HIV does lead to more, and to more severe malaria episodes, particularly in pregnant women [41].

The prevalence of helminth infection was increased in subjects with malaria, and *vice versa*. A study conducted in Ghana on pregnant females also showed that helminth infection increased the risk of malaria [28]. It is thought that helminth infections have a number of effects on the immune system that leads to increased susceptibility to malaria [39,42].

In our study population all subjects were taking ART irrespective of CD4 counts, as prescribed by the new (2010) Rwandan Ministry of Health guidelines for the prevention of mother to child transmission of HIV [25]. Helminth infections of any type, malaria or co-infection, were all less prevalent in subjects receiving AZT-3TC-NVP when compared to the other three ART regimens (Table 2). These effects remained significant for helminth infections after adjusting for confounding variables in a logistic regression analysis (Table 3). However, the protective effect of AZT-3TC-NVP for malaria was not sustained in the logistic regression model (Table 4). A previous study performed in pregnant Rwanda females also demonstrated that specific ART regimens seemed to reduce helminths prevalence but had less effect on malaria [21]; in this study the AZT-3TC-NVP regimen was the least protective compared with the other therapies i.e. AZT-NVP, d4T-3TC-NVP and AZT. This may be related to a much smaller sample size (n = 328) compared to the current study (n = 980), and the lack of data for confounding variables. Whilst these findings suggest a possible anti-helminthic effect of (certain) ART combinations, there was no non-ART control arm in both studies, as they were not designed to detect ART effects on helminth infections in the first place. Whilst there is therefore a limit to the interpretation of this finding, it warrants further investigation. Although the ART-induced reconstitution of cellular immunity would probably be the main factor for reducing helminth infections among HIV patients, previous *in vitro* and *in vivo* investigations indicated that HIV treatment, especially with protease inhibitors (PIs), could have a direct effect in killing of parasites

including malaria [43]. It has been described before that ART without PIs may reduce the prevalence of helminth infection [21,44]. Thus, ART itself might have contributed to the decline in helminth prevalence, asymptomatic malaria or co-infection seen in our study. We hypothesize that anti-mitochondrial toxicity of ART compounds may play a direct role here, a hypothesis for which support seems to accrue in a currently ongoing field trial in Gabon, designed to address this question (MP Grobusch and S Janssen, unpublished data).

The strengths of this study are the screening of a large number of women from eight health centers which caters for women of all socio-economic classes; the screening of three stool samples on three consecutive days for the presence of helminths; the use of a combination of two different screening techniques to increase the sensitivity of helminth diagnosis. However, our study has limitations. Methodologically, its cross-sectional design makes it impossible to determine temporal causality, including the inability of multivariate models to adjust for all confounding factors. All participants in the study were women and as hemoglobin levels differ between men and women, our findings cannot be extrapolated to men. The Kato Katz method used to determine the number of helminth eggs could have underestimated the proportion of women with light hookworm infection. The study had no control group of HIV-positive subjects not receiving ART.

In conclusion, we found that the prevalence of helminth infections, malaria and co-infections is common in HIV-positive pregnant women on ART in Rwanda. Helminth and malaria infection in this population are important risk factors for low hemoglobin levels. Subjects with low CD4 counts were at higher risk of infections and helminth infection is a risk factor for malaria. Education and employment were independent protective factors for helminth infection and malaria, whilst hand washing reduced the risk only for helminth infections. The possible antihelminthic effect of some ART combinations warrants further studies.

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Author Contributions

Conceived and designed the experiments: EI MPG NJC EM LOO. Performed the experiments: EI. Analyzed the data: EI NJC EM LOO SJ MPG. Contributed reagents/materials/analysis tools: EI NJC EM LOO SJ MPG. Wrote the paper: EI NJC EM LOO SJ MPG.

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Appendix 7: Published paper with Clin Infect Dis

MAJOR ARTICLE HIV/AIDS

Effect of Deworming on Disease Progression Markers in HIV-1–Infected Pregnant Women on Antiretroviral Therapy: A Longitudinal Observational Study From Rwanda

Emil Ivan,[1,2] Nigel J. Crowther,[2] Eugene Mutimura,[3] Aniceth Rucogoza,[1] Saskia Janssen,[4] Kato K. Njunwa,[1] and Martin P. Grobusch[4]

[1]College of Medicine and Health Sciences, Department of Biomedical Laboratory Sciences, University of Rwanda, Kigali; [2]Department of Chemical Pathology, University of the Witwatersrand Medical School, National Health Laboratory Services, Johannesburg, South Africa; [3]Rwanda Alliance for Sustainable Development, Kigali; and [4]Center of Tropical Medicine and Travel Medicine, Department of Infectious Diseases, Division of Internal Medicine, Academic Medical Center, University of Amsterdam, The Netherlands

Background. Deworming human immunodeficiency virus (HIV)-infected individuals on antiretroviral therapy (ART) may be beneficial, particularly during pregnancy. We determined the efficacy of targeted and nontargeted antihelminth therapy and its effects on *Plasmodium falciparum* infection status, hemoglobin levels, CD4 counts, and viral load in pregnant, HIV-positive women receiving ART.

Methods. Nine hundred eighty HIV-infected pregnant women receiving ART were examined at 2 visits during pregnancy and 2 postpartum visits within 12 weeks. Women were given antimalarials when malaria-positive whereas albendazole was given in a targeted (n = 467; treatment when helminth stool screening was positive) or nontargeted (n = 513; treatment at all time points, with stool screening) fashion.

Results. No significant differences were noted between targeted and nontargeted albendazole treatments for the variables measured at each study visit except for CD4 counts, which were lower ($P < .05$) in the latter group at the final visit. Albendazole therapy was associated with favorable changes in subjects' hemoglobin levels, CD4 counts, and viral loads, particularly with helminth infections.

Conclusions. Antihelminthic therapy reduces detectable viral load, and increases CD4 counts and hemoglobin levels in pregnant HIV-infected women with helminth coinfections receiving ART.

Keywords. Rwanda; HIV; pregnancy; malaria; helminths.

Infection with soil-transmitted helminths remains highly prevalent in vast areas of the world. The geographical distribution of these infections overlaps considerably with regions of high human immunodeficiency virus type 1 (HIV-1) seroprevalence [1, 2]. In Africa, >22 million people are estimated to be coinfected with helminths and HIV-1 [3]. Effective chemotherapy remains the mainstay of helminth burden–reducing strategies. However, strategies differ widely.

Malaria and helminth infections play a role in the pathogenesis of HIV, as both infections contribute to HIV disease progression [4, 5]. Furthermore, there are important clinical implications as these infections contribute to anemia [6–8] and other disorders, but uncertainty remains with regard to disease interactions. HIV acquisition was found to be positively correlated with female urogenital schistosomiasis [7, 9]. However, 2 randomized controlled trials [10, 11] showed no benefit of deworming on prevention of mother-to-child HIV transmission.

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Correspondence: Martin P. Grobusch, MD, PhD, Center of Tropical Medicine and Travel Medicine, Department of Infectious Diseases, Division of Internal Medicine, Academic Medical Center, University of Amsterdam, PO Box 22700, Meibergdreef 9, 1100 DE Amsterdam, The Netherlands (m.p.grobusch@amc.uva.nl).

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The immunological interplay between helminth infections and HIV is complex. Several hypotheses describe the influence of these infections on each other. One hypothesis is that T-helper cell 2 bias is induced by helminth infection, suppressing the T-helper cell 1 response to HIV [12]. However, clinical data on this topic are limited and contradictory. Thus, observational studies demonstrated impaired immune responses to HIV in helminth-coinfected individuals [11, 13], and randomized controlled trials suggested that treatment of helminth coinfection delayed HIV disease progression [3, 14]. However, systematic reviews reported lower effects of antihelminthic treatment on HIV disease progression [15, 16], and a recent study demonstrated no benefits from deworming antiretroviral therapy (ART)-naive, HIV-positive subjects [11].

Studies from Rwanda showed high rates of HIV/helminth coinfections in pregnant women receiving ART [17, 18]. During pregnancy, coinfections increased anemia risk and mother-to-child HIV transmission [19], whereas treatment of helminth infections reduced HIV RNA load [13]. However, there is little evidence regarding benefits of deworming in HIV-infected pregnant women receiving ART. We determined the effect of deworming HIV-infected pregnant Rwandan women receiving ART on markers of HIV disease progression and hemoglobin levels, and the efficiency of deworming in targeted vs nontargeted treatment modes.

MATERIALS AND METHODS

Study Population

Pregnant women were recruited at antenatal clinics. Inclusion criteria were HIV infection, pregnancy in the second trimester, and use of ART. Women were excluded if diagnosed with tuberculosis, or if they had taken antihelminthic drugs at any time point before study entry. Measurements were taken at 2 visits during pregnancy and 2 visits after birth, all 12 weeks apart. Ethical approval was obtained from the Rwanda National Ethics Committee and the Ethics Committee of the University of the Witwatersrand's Faculty of Health Sciences, Johannesburg, South Africa.

Therapies

Patients were randomly assigned to targeted deworming (n = 550), with 400 mg albendazole (ABZ) (ASPARTEC-400, Dwarkesh Pharmaceuticals, Ahmedabad, India) given at any study visit if participants were helminth positive, or to untargeted deworming (n = 550), where all women were given 400 mg ABZ at each visit irrespective of helminth infection status. If testing positive for *Plasmodium falciparum* at any study visit, they were treated with artemether-lumefantrine (AL) (Coartem). On all study visits, research assistants confirmed the intake of all drugs. Women received nevirapine (NVP) for prevention of mother-to-child HIV transmission and subsequent combination ART, irrespective of CD4 cell levels, in accordance with the Rwandan Ministry of Health guidelines [20].

Measurements Taken at Each Visit

Weight and height were measured at each visit, as were CD4 cell counts and hemoglobin levels. Viral loads (VLs) were measured at baseline and at the final visit. Due to ART use, VLs were low and were recorded as either above or below the detectable limit of 40 copies/mL. The presence of helminth (*Ascaris lumbricoides*, *Trichuris trichiura*, and hookworms) and *P. falciparum* infections were assessed on each visit [18].

Laboratory Tests

Intestinal helminths were identified by the Kato-Katz method [21]. Three Kato-Katz slides were prepared from each of 3 stool samples collected on consecutive days, and examined within 30 minutes for *Ancylostoma duodenale* and *Necator americanus*, and the following day for ova of *A. lumbricoides* and *T. trichiura*. Eggs per gram of stool were then calculated. Malaria antigen Histidine-Rich Protein-2 (Orchid Bio Medicals, Goa, India) was identified using a rapid serial testing algorithm [22]. *Plasmodium* species parasitemia was identified by examination of thick and thin blood films. Hemoglobin levels were measured using a Rapid HemoCue kit (HemoCue AB, Angelholm, Sweden). The CD4 counts were determined using a FACSCalibur/Sysmex XT1800i system (BD, San Jose, California). Plasma HIV RNA was quantified using a Gen-Probe HIV-1 VL assay on a AmpliPrep/COBAS TaqMan (HIMCAP, Fort Lauderdale, Florida).

Study Subgroups

The initial analysis compared the targeted group with the nontargeted group. A subgroup analysis was performed in which participants were divided into 3 groups based on the ABZ treatment: (1) a comparator group in which all subjects were helminth free and received no ABZ throughout the study (H^-ABZ^-); (2) a group in which all subjects were helminth-free but received ABZ throughout the study (H^-ABZ^+); and (3) a group in which all participants were helminth-infected at baseline and received ABZ therapy either at all visits or only at visits where helminth infection was detected ($H^{+/-}ABZ^{+/-}$).

Statistical Analyses

Skewed variables were log-transformed to normality and expressed as median (interquartile range) in the tables. Normally distributed data were expressed as mean ± SD. Continuous variables were compared between targeted and nontargeted groups using Student *t* test or analysis of variance (ANOVA)/analysis of covariance (ANCOVA) if adjustment for confounding variables was required, whereas within-group comparisons were performed using a paired Student *t* test with Bonferroni correction. Comparisons across subgroups were performed using ANOVA with Tukey post hoc test. Categorical variables were analyzed

using the χ^2 test or Fisher exact test. Logistic regression was used to determine effects of therapy on risk of *P. falciparum* infection at visit 4 by dividing the cohort into 4 groups based on therapies received at visit 3: those who received neither treatment; those who received only ABZ; those who received only AL; and those who received ABZ and AL. Backward, stepwise multivariate regression analysis was used to determine the correlates of percentage changes in hemoglobin levels and CD4 counts between visits 1 and 2. This time period shows the outcome of treatment of more long-term helminthic or *P. falciparum* infections compared with other periods in the study in subjects who had not previously been dewormed. The initial regression models included variables that correlated with each of the 2 outcome variables at $P < .50$ in univariate correlation analyses. All statistical analyses were performed using Statistica version 9.1 (StatSoft, Tulsa, Oklahoma).

RESULTS

Study Population

In total, 1300 pregnant women were screened for eligibility. Two hundred participants were not eligible for the following reasons: 100 subjects were enrolled in other studies; 50 were to deliver at facilities situated outside the study area; 40 did

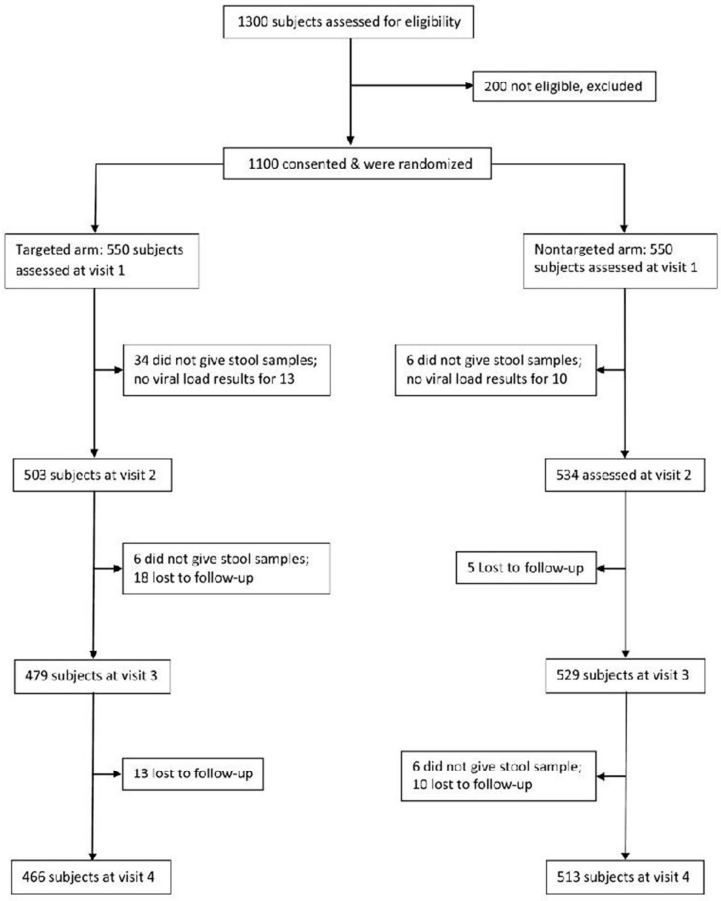

Figure 1. Study profile for subjects in targeted and nontargeted treatment groups.

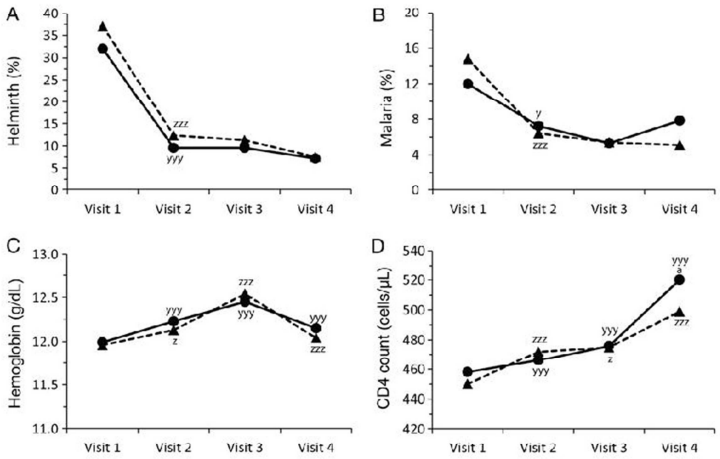

Figure 2. Comparison of targeted (dashed line) vs nontargeted (solid line) albendazole treatment on helminth infection (*A*), *Plasmodium falciparum* infection (*B*), serum hemoglobin levels (*C*), and CD4 counts (*D*). $^{a}P < .05$ vs targeted arm; $^{y}P < .05$, $^{yyy}P < .0005$ vs preceding visit of nontargeted arm; $^{z}P < .05$, $^{zzz}P < .0005$ vs preceding visit of targeted arm. Data expressed as percentage for helminth and *P. falciparum* infections and as means for hemoglobin levels and medians for CD4 counts. For clarity, the SD and interquartile ranges were not included. Abbreviation: SD, standard deviation.

not wish to participate; 10 were receiving antihelminthic therapy. The remaining 1100 participants were randomized to targeted and nontargeted treatment groups, of whom 120 (12.3%) did not complete the study due to various reasons (Figure 1). These were excluded from the final analysis, leaving a total patient population of 980.

Helminths, Malaria, and Hemoglobin in Targeted and Nontargeted Groups

The prevalence of helminth infection was similar at all time points in the targeted and nontargeted groups (Figure 2*A*). In both groups, helminth infections dropped dramatically by visit 2 ($P < .0005$ vs visit 1 for both comparisons), with a more shallow decrease by visit 4. Prevalence levels of helminth infections at baseline for the total cohort were as follows: *A. lumbricoides*, 20.8%; *T. trichiura*, 7.1%; hookworm species, 6.3%. The prevalence of *P. falciparum* infection was similar at all time points for the 2 treatment arms, with a significant prevalence decline at visit 2. There was a tendency for the infection rate to be higher at visit 4 with nontargeted ABZ administration (7.8% vs 5.1%; $P = .09$; Figure 2*B*). The prevalence of helminth coinfection with *P. falciparum* was significantly higher in the targeted (8.6%) than in the nontargeted group (4.9%; $P < .05$) at baseline, but in both groups no coinfection was detected at any of the subsequent visits.

Hemoglobin levels were similar in both arms at all study visits (Figure 2*C*), rising significantly from visit 1 to visit 2 in both arms and peaking by visit 3. Thereafter, levels fell significantly ($P < .0005$) by visit 4.

CD4 Counts and Detectable VL in Targeted and Nontargeted Groups

Within both treatment groups, CD4 counts rose significantly, reaching a peak at visit 4, where levels were significantly higher in the nontargeted vs the targeted group ($P < .05$; Figure 2). This difference between the 2 treatment groups was analyzed using ANOVA with treatment mode as the grouping variable and level of CD4 counts at visit 4 as the dependent variable ($F = 5.03$; $P = .025$). Adjusting for possible confounders in an ANCOVA (eg, ART, frequency of detectable VL, AL usage, age, education, employment, gravidity, and gestational age) did not attenuate this effect. However, adjusting for ABZ therapy in the absence of helminth infection did weaken this effect significantly ($F = 0.10$, $P = .77$). Adjustment for ABZ therapy with helminth infection had minimal effect ($F = 4.90$, $P = .027$).

The prevalence of detectable VL dropped from 9.0% at visit 1% to 4.7% at visit 4 ($P < .005$) in the nontargeted group and from 9.4% at visit 1 to 3.6% at visit 4 in the targeted group ($P < .0005$). No significant differences were noted between treatment modes.

Baseline Comparison of Study Subgroups

At visit 1, no significant differences across the 3 study groups were noted for age, gestation, or body mass index (Table 1).

Table 1. Baseline Characteristics of Study Subgroups

Variables	Subject Groups		
	H^-ABZ^- [a]	H^-ABZ^+ [b]	$H^{+/-}ABZ^{+/-}$ [c]
No. of subjects	204	272	334
Age, y	30.5 ± 4.62	30.3 ± 4.78	29.9 ± 4.73
Gestation, mo	4.58 ± 0.72	4.65 ± 0.74	4.64 ± 0.72
Gravidity >1, %	77.9	80.9*	71.6
BMI, kg/m^2	25.4 ± 3.27	25.2 ± 3.32	25.3 ± 3.28
Hemoglobin, g/dL	12.2 ± 1.18***	12.3 ± 1.22***	11.4 ± 1.35
CD4, cells/μL	489 [173]***	483 [182]***	380 [112]
Detectable HIV, %	4.90***	4.78***	18.9
ART regimen, %			
ZDV, 3TC, NVP	35.3*	39.7***	24.2
ZDV, NVP	11.8	9.55	12.3
d4T, 3TC, NVP	42.1**	42.3**	54.8
TDF, 3TC, NVP	10.8	8.45	8.68
Malaria, %	8.33***	9.93**	19.5
Education, %	60.8***	57.0***	32.0
Employment, %	88.2***	90.1***	67.1
Periurban, %	46.1***	39.0***	18.6

Data given as %, mean ± SD, or median (interquartile range).

Abbreviations: 3TC, lamivudine; ABZ, albendazole; ART, antiretroviral therapy; BMI, body mass index; d4T, stavudine; HIV, human immunodeficiency virus; NVP, nevirapine; SD, standard deviation; TDF, tenofovir; ZDV, zidovudine.

[a] Helminth free and not treated with ABZ at any visits.

[b] Helminth free and treated with ABZ at all visits.

[c] Infected with helminth at baseline but helminth free at other visits and treated with ABZ at baseline.

*P < .05, **P < .005, ***P < .0005, vs $H^{+/-}ABZ^{+/-}$.

The prevalence of multigravidity was higher in the H^-ABZ^+ group compared with the $H^{+/-}ABZ^{+/-}$ group (P < .05). The use of the zidovudine/lamivudine (3TC)/NVP ART regimen was more common in the H^-ABZ^- and H^-ABZ^+ groups. The stavudine/3TC/NVP regimen was found less often in the H^-ABZ^- and H^-ABZ^+ groups when compared to the $H^{+/-}BZ^{+/-}$ group. No differences were noted for the tenofovir/3TC/NVP regimen.

Longitudinal Changes in Malaria, Hemoglobin, Detectable VL, and CD4 Counts in 3 Study Subgroups

The study variables at each visit for the 3 treatment subgroups are given in Figure 3. Helminth infection was 100% by definition in the $H^{+/-}ABZ^{+/-}$ group and 0 in all other groups at baseline, remaining as such at all subsequent visits for the H^-ABZ^- and H^-ABZ^+ groups. Within the $H^{+/-}ABZ^{+/-}$ group, helminth infection fell to 6% at visit 2 (P < .0005 vs visit 1), rose to 10.8% at visit 3 (P < .05 vs visit 2), and fell to 7.8% at visit 4 (P = .10 vs visit 3). Malaria was more prevalent at baseline for the $H^{+/-}ABZ^{+/-}$ group than for each of the 2 other groups (P < .05 and P < .0005; Figure 3*A*). However, at visit 2, *P. falciparum* detection fell significantly in the $H^{+/-}ABZ^{+/-}$ group (P < .0005) to levels comparable to that of the other 2 groups, and remained so at visit 3. The prevalence of *P. falciparum* positivity rose slightly by visit 4 in both groups receiving ABZ therapy, but fell in the group not receiving ABZ (H^-ABZ^-), such that this group had a significantly (P < .05) lower level of infection compared to the $H^{+/-}ABZ^{+/-}$ group at visit 4.

Hemoglobin levels were significantly lower in the $H^{+/-}ABZ^{+/-}$ group at baseline compared with the 2 other subgroups (P < .0005; Figure 3*B*) but rose significantly at visit 2 (P < .0005) and visit 3 (P < .0005). Within the remaining 2 study groups, hemoglobin levels did not rise significantly between visits 1 and 2 (P < .005) but did so between visits 2 and 3 (P < .0005). Hemoglobin levels fell significantly (P < .0005) between visits 3 and 4 in the H^-ABZ^- and H^-ABZ^+ groups, but not in the $H^{+/-}ABZ^{+/-}$ group.

CD4 counts were significantly lower in the $H^{+/-}ABZ^{+/-}$ group at baseline when compared with the 2 other subgroups (P < .0005; Figure 3*C*). CD4 counts rose significantly within this group from visit 1 to visit 2 (P < .0005) and from visit 2 to visit 3 (P < .05). However, at both visits CD4 counts were still significantly lower compared with the 2 other subgroups (P < .0005 for all comparisons). A large increase in CD4 counts from visit 3 to visit 4 (P < .0005) brought levels to within the range observed in the other groups. There was a shallow but significant rise in CD4 counts from visit 1 to visit 3 in the H^-ABZ^- and H^-ABZ^+ groups. CD4 counts rose significantly from visit 3 to visit 4 in the H^-ABZ^+ group (P < .0005), leaving levels significantly higher than in the $H^{+/-}ABZ^{+/-}$ group (P < .05), confirming the observations shown in Figure 2*D*.

The prevalence of detectable VL was significantly higher in the $H^{+/-}ABZ^{+/-}$ group at baseline compared with the other 2 groups (P < .0005 for both comparisons), but fell significantly by visit 4 (P < .0005; Figure 3*D*). The prevalence of detectable VLs did not change between the first and final visits for the 2 other study groups.

Multivariable Linear Regression Analyses

Backward, stepwise linear regression analyses for determining the correlates of percentage change in CD4 counts and percentage change in hemoglobin levels demonstrated that from visit 1 to visit 2, malaria treatment was associated with a CD4 count increase of 8.6% (P < .0005; Table 2), whereas malaria at the later time point reduced CD4 counts by 9.8% (P < .0005) compared with subjects with no change in malaria status between these 2 study visits. Results for *A. lumbricoides* infections were similar, with treatment being associated with a 6.7% CD4 count increase (P < .0005) and a later-acquired infection leading to a 7.4% CD4 count decline (P = .003). Treating *T. trichiura* or hookworm infections increased CD4 counts by 5.3% (P = .02) and 5.9% (P = .02), respectively. Subjects with detectable VL at visit 1 underwent a 25% CD4 count increase (P < .0005) compared with subjects with undetectable VL.

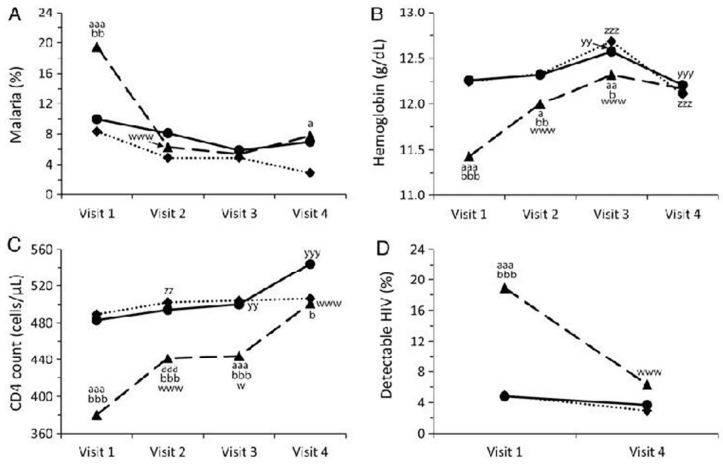

Figure 3. Substudy analysis of 3 subject groups for *P. falciparum* infection (*A*), serum hemoglobin (Hb) levels (*B*), CD4 counts (*C*), and detectable viral load (*D*). Study groups are as follows: H^-ABZ^- (helminth free and not treated with albendazole [ABZ] at any visits; small dashes with diamond markers); H^-ABZ^+ (helminth free and treated with ABZ at all visits; solid line with circle markers); $H^{+/-}ABZ^{+/-}$ (infected with helminth at baseline but helminth free at other visits and treated with ABZ at baseline; large dashes with triangle markers). $^{a}P < .05$, $^{aa}P < .005$, $^{aaa}P < .0005$ for $H^{+/-}ABZ^{+/-}$ vs H^-ABZ^-; $^{b}P < .05$, $^{bb}P < .005$, $^{bbb}P < .0005$ for $H^{+/-}ABZ^{+/-}$ vs H^-ABZ^+; $^{w}P < .05$, $^{www}P < .0005$ vs preceding visit within $H^{+/-}ABZ^{+/-}$ group; $^{yy}P < .005$, $^{yyy}P < .0005$ vs preceding visit within H^-ABZ^+ group; $^{zz}P < .005$, $^{zzz}P < .0005$ vs preceding visit within H^-ABZ^- group. Data expressed as percentages for malaria and for detectable viral load and as means for Hb levels and medians for CD4 counts. The SD and interquartile ranges were excluded for clarity. Abbreviations: HIV, human immunodeficiency virus; *P. falciparum*, *Plasmodium falciparum*; SD, standard deviation.

Table 2. Backward, Stepwise Multivariable Regression Models for Percentage of Change in CD4 Counts and Hemoglobin Levels From Visit 1 to Visit 2

Dependent Variable	Independent Variables With Unstandardized β Coefficient (*P* Value)	Adjusted R^2 for Full Model (*P* Value)
Percentage change in CD4 count	Loss of malaria infection, 8.64 (<.0005) Gain in malaria infection, −9.81 (<.0005) Loss of *Ascaris* infection, 6.74 (<.0005) Gain in *Ascaris* infection, −7.37 (.003) Loss of *Trichuris* infection, 5.28 (.02) Loss of hookworm infection, 5.94 (.02) Detectable viral load at visit 1, 25.0 (<.0005)	0.22 (<.0005)
Percentage change in hemoglobin level	Periurban vs rural, −1.90 (.01) Employment, 2.13 (.02) Gestational age, 2.08 (.002) Gain in malaria infection, −3.82 (.009) Loss of *Ascaris* infection, 4.93 (<.0005) Loss of *Trichuris* infection, 2.93 (.03) Loss of hookworm infection, 8.96 (<.0005)	0.09 (<.0005)

Variable coding: loss or gain of any helminth or *Plasmodium falciparum* infection was coded as −1 and 1, respectively, and compared against no change in infection status (coded as 0); presence of a detectable viral load at visit 1 was coded as 1 whereas an undetectable viral load was coded as 0; periurban was coded as 1 and urban as 0; employed was coded as 1 and unemployed as 0; gestational age of 5 or 6 months was coded as 1 and gestational age of 4 months was coded as 0.

Periurban subjects had a 1.9% hemoglobin decline ($P = .01$) compared with urban-dwelling subjects between the study visits. Employed women had a 2.1% hemoglobin increase compared with unemployed women ($P = .02$). Malaria between study visits 1 and 2 was associated with a 3.8% hemoglobin decrease ($P = .009$). Successful treatment of *A. lumbricoides*, *T. trichiura*, or hookworm infections was associated with a 4.9% ($P < .0005$), 2.93% ($P = .03$), and 8.96% ($P < .0005$) increase in hemoglobin.

DISCUSSION

The present study is the first to systematically determine the effect of ABZ therapy on CD4 counts, VL, and hemoglobin in HIV-infected pregnant women on ART and antimalarial medications. Favorable changes were observed in all these variables over a period of 1 year in subjects who had helminth infections at baseline visit. ABZ was administered in either targeted or nontargeted fashion, and both these treatment modes were equally effective. This suggests that in regions endemic for geohelminths, nontargeted anthelminthic therapy may be more cost-effective than a test-then-treat procedure.

The current study demonstrates that in pregnant women receiving ABZ therapy, CD4 counts increase, whereas detectable VLs decrease in the presence of concurrent ART. Thus, ABZ therapy significantly augments ART in improving immune function and blocking viral replication. Furthermore, hemoglobin levels increase after deworming.

Previous studies investigating the effect of deworming on CD4 counts and VL in HIV-positive patients have been analyzed in systematic reviews [3, 11], which demonstrated statistically significant beneficial effects. A recent study from Uganda showed that deworming HIV-positive pregnant women reduced VL, but the effect was statistically nonsignificant [13]. However, this study involved only 1 dose of ABZ or praziquantel, which was administered at baseline. VLs were measured, but not CD4 counts, at 6 weeks posttreatment at delivery. Furthermore, no participants were receiving ART at recruitment but were given single-dose intrapartum and neonatal NVP to reduce mother-to-child transmission. The Uganda study showed that in HIV-infected subjects not receiving ART, ABZ therapy had no effect on HIV-associated disease progression [13]. However, a meta-analysis of 3 randomized controlled trials showed that anthelminthic therapy benefited HIV-infected subjects [3]. Contradictory data may result from differences in study methodology, including treatment of different helminth species, variable sample sizes, differences in study duration, frequency of antihelminthic therapy, presence of different parallel therapies (eg, for HIV or *P. falciparum* infection), variations in the prevalence of helminth infections, and differences in population demography.

Our findings demonstrate that increases in CD4 counts occur with removal of each of the 3 different helminth species. Thus, the loss of each helminth infection caused a 5.0%–7.0% increase in CD4 counts over a 3-month period. One study has shown that treating *A. lumbricoides* with ABZ does improve CD4 counts [3]; however, these findings, and those for treatment of *T. trichiura* and hookworm observed in our study, are not supported by other studies [4, 11, 15, 16, 23]. None of these studies included subjects receiving ART. It is therefore possible that ABZ therapy has a more significant effect on the immune system responsiveness of HIV-infected subjects in the presence of ART.

The current study has several strengths. Compliance with the study treatments was documented by direct observation by study staff. Retention of participants in the study was high and did not differ between groups. Outcome measures were systematically assessed. The limitation of the study is that, in a setting where women received anthelminthics during pregnancy routinely, the design fell short of that of a placebo-controlled, randomized, double-blind clinical trial, which would have been preferable over the quasi-observational approach we undertook. Furthermore, the study was not developed to investigate drug–drug interactions and therefore such effects could not be confirmed.

Our findings suggest that ABZ treatment of HIV-positive pregnant women is of benefit, without a need for screening for helminth infection. Thus, within regions where geohelminths are endemic, nontargeted intervention may be more efficient than targeted approach. The present study shows beneficial effects of ABZ therapy on VL, CD4 counts, and hemoglobin levels, particularly in women who harbor helminth infections. However, there is some evidence that the effect on CD4 counts may not be due to helminth clearance, thus requiring further investigation.

Notes

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Author contributions. E. I., M. P. G., and N. J. C. conceived of the study; E. I. and A. R. coordinated the field work; E. I., N. J. C., E. M., A. R., K. K. N., S. J., and M. P. G. analyzed the data; and E. I. wrote the first draft. All authors contributed to the writing and approved the final version of the paper.

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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