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Faculty of Health Sciences

HIV Infected African Children: Gut microbiota in relation to chronic lung disease and long-term antibiotic treatment

Trym Thune Flygel

Research Group of Child and Adolescent Health, University of Tromsø / University Hospital of North Norway

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Cover graphics: The human Microbiome by *Design Cells.* Image from istockphoto.com

Table of Contents

Acknowledgements 5
Summary 7
List of papers 11
Abbreviations 13
Introduction 15
1 Human immunodeficiency virus (HIV) 15
1.1 History 15
1.2 Pathogenesis 16
1.3 The HIV pandemic in numbers 17
1.4 HIV in children and adolescents 19
1.5 Antiretroviral treatment 21
2 Chronic lung disease 23
2.1 HIV-associated chronic lung disease 23
2.1.1 Obliterative bronchiolitis and bronchiectasis 24
2.1.2 HIV and tuberculosis coinfection 25
2.2 Assessment of lung health 26
2.2.1 Spirometry 26
2.2.2 Exhaled nitric oxide 26
2.3 Biomarkers of systemic inflammation 28
2.4 Macrolide antibiotics 29
2.4.1 Macrolide antibiotics and exhaled nitric oxide 30
3 Gut microbiota 33
3.1 Development of gut microbiota 33
3.2 Gut microbiota in HIV infection 36
3.3 Pathogenesis of intestinal HIV 37
3.4 Effects of antibiotic treatment on gut microbiota 38
Aim of the thesis 41
Materials and methods 43
1 Study population 43
2 Data collection and study procedures 47
2.1 Questionnaires 47
2.2 Rectal swabs 47
2.3 Exhaled nitric oxide 47

2.4 Blood samples 48
2.5 Spirometry 48
2.6 Data storage 49
3 Laboratory methods 51
3.1 DNA extraction 51
3.2 16S real time quantitative PCR 52
3.3 16S short and long PCR 52
3.4 16S rRNA gene sequencing 54
3.5 Bioinformatics 55
4 Statistical analysis 57
4.1 Paper I 57
4.2 Paper II 58
4.3 Paper III 60
5 Ethical approvals 62
5.1 User participation 62
Results 63
1 Paper I 63
2 Paper II 67
3 Paper III 68
Discussion 73
1 Main results 73
2 Methodical and ethical considerations 81
2.1 Study designs 81
2.2 Confounding 83
2.3 Risks of intervention and safety of drug administration 83
2.4 Laboratory and data processing 84
2.5 External validity 86
Conclusions and future perspectives 87
Works cited 89
Paper I-III 111
Appendices

List of Tables

Table 1. Alpha diversity in participants with HIV, stratified by years on ART, and HIV uninfected participants ... 64
Table 2. Alpha diversity indices. Comparisons between study groups at baseline, after 48 weeks and after 72 weeks using linear mixed models ... 69
Table 3. Differentially abundant taxa between azithromycin and placebo group at 48 weeks ... 70

List of Figures

Figure 1. Life cycle of HIV and targets for antiretroviral treatment ... 16
Figure 2. Prevalence of children and adolescents, aged 0-19 years, living with HIV worldwide ... 20
Figure 3. Taxonomic classification of common gut bacteria ... 34
Figure 4. The intestinal barrier in HIV infected individuals and pathogenesis of peripheral immune activation ... 37
Figure 5. Flowchart of study participants in Paper II ... 44
Figure 6. Flowchart of study participants in Paper III ... 45
Figure 7. Venn diagram showing the overlap between the four methods used for assessing relative abundance ... 61
Figure 8. Box plot of alpha diversity indices in HIV infected and HIV uninfected participants ... 64
Figure 9. Principal coordinate plot showing beta diversity comparison between study groups using Bray-Curtis dissimilarity ... 65
Figure 10. LEfSe plot showing enriched taxa at genus level significantly different between participants with HIV and HIV uninfected individuals ... 66
Figure 11. Box plot of log transformed eNO at baseline, by experience of at least one ARE during study period ... 67
Figure 12. Box plot showing alpha diversity indices compared between azithromycin (AZM) and placebo group at baseline, 12 months (48 weeks) and 18 months (72 weeks) ... 69

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Summary

Introduction: An estimated 2.50 million children aged 0-19 years are living with HIV worldwide. HIV-associated chronic lung disease (HCLD) is the most prevalent comorbidity among children and adolescents with HIV in sub-Saharan Africa, where more than half of the population with HIV in the world reside. Obliterative bronchiolitis (OB) is found to be the most prevalent cause of HCLD in sub-Saharan Africa. The exact pathogenesis of OB is multifactorial and not completely understood. Studies indicate OB to be result of immune activation and repeated injury to the lungs causing permanent damage and excessive fibroproliferation, ultimately leading to narrowing of the airways. HIV is characterized by chronic immune activation, and $CD4^+$ T-cells are the main target for HIV infection. As the majority of $CD4^+$ T-cells reside in the gut and gut associated lymphatic tissue, HIV is also an intestinal disease. HIV causes a disrupted gut barrier and gut microbial dysbiosis, persisting even after initiation of antiretroviral therapy (ART). Several studies have linked gut microbial dysbiosis to a variety of diseases, but it is difficult to prove causality between dysbiosis and disease. The link between a disrupted gut barrier caused by HIV, gut microbiota and immune-related complications of HIV is yet to be determined.

Aim: The overall aim of the thesis was to investigate the composition of gut microbiota of HIV infected children and adolescents with HCLD, and to study the effects of macrolide antibiotic (azithromycin) treatment on gut microbial composition and the effect on local lung inflammation in HCLD.

Methods: This work was a part of the double-blinded, placebo-controlled, randomized clinical trial; BREATHE (Bronchopulmonary function in response to azithromycin treatment for chronic lung disease in HIV-infected children and adolescents). Children and adolescents aged 6-19 years with HCLD were recruited from Harare, Zimbabwe and Blantyre, Malawi. Participants were randomized to once-weekly azithromycin or placebo for one year, to investigate if it improved lung function. In addition, a control group of 103 HIV negative participants were recruited in Harare, Zimbabwe. Rectal swabs were collected at inclusion, after 48 weeks of treatment and after 72 weeks (6 months after cessation of study drug). 16S

rRNA sequencing was performed to assess diversity and composition of the gut microbiota. Exhaled nitric oxide (eNO) was measured at inclusion and after 48 weeks for a subset of participants from Harare, Zimbabwe to assess local inflammation in the lungs.

Results: In **Paper I** we demonstrated that participants with HIV had lower alpha diversity and higher beta diversity compared to participants without HIV. Prolonged ART increased richness by alpha diversity to levels comparable to that of HIV negative. We found no association between gut microbiota and airway obstruction or history of tuberculosis. *Corynebacterium*, *Finegoldia* and *Anaerococcus* were significantly enriched in participants with HIV compared to participants without HIV.

In **Paper II** we showed that higher levels of eNO served as a risk factor for developing acute respiratory exacerbations. eNO was associated with proinflammatory biomarkers involved in development of chronic lung disease and fibroproliferation. There was no effect of azithromycin on levels of eNO.

In **Paper III** we demonstrated that azithromycin reduced both richness and evenness by alpha diversity after 48 weeks of treatment. These changes were no longer statistically significant 6 months after cessation of study drug. *Eggerthella*, *Blautia* and *Dorea* was increased, whereas *Bifidobacterium* and *Campylobacter* was decreased in azithromycin group at 48 weeks. Depletion of *Campylobacter* persisted for at least 6 months after finishing treatment.

Conclusions: This work highlights the association between HIV and gut microbial dysbiosis and the effect of long-term antibiotics on lung inflammation and composition of gut microbiota. It adds knowledge to the field of HIV-associated comorbidities and can be helpful when designing new studies on gut microbiota and potential targets for microbiome-based interventions in the future.

Sammendrag

Introduksjon: Omtrent 2.5 millioner barn mellom 0 og 19 år lever med HIV i verden. HIV-assosiert kronisk lungesykdom er den vanligste følgesykdommen blant barn og ungdom med HIV i Afrika, sør for Sahara, hvor over halvparten av befolkningen med HIV bor. Bronchiolitis obliterans (OB) er den vanligste årsaken til HIV-assosiert kronisk lungesykdom i dette området. Årsaken til OB er multifaktoriell og ikke helt kjent. Studier har vist at OB kan være forårsaket av immunaktivering og gjentatt lungeskade på grunn av infeksjoner som fører til økt fibrosedannelse og til slutt forsnevring av luftveiene. HIV er karakterisert av kronisk immunaktivering og $CD4^+$ T-celler er hovedangrepspunktet for HIV-infeksjon. Siden størsteparten av $CD4^+$ T-cellene befinner seg i tarmen, kan HIV føre til en dårligere tarmbarriere og endret tarmflora, som ikke lar seg reversere av antiretroviral behandling. Flere studier har satt endring i tarmflora sammen med ulike sykdommer, men det er vanskelig å stadfeste en årsakssammenheng mellom endret tarmflora og utvikling av sykdom. Sammenhengen mellom dårlig tarmbarriere og endret tarmflora på grunn av HIV og immun-medierte følgesykdommer er ikke bevist enda.

Mål: Hovedmålet med denne avhandlingen var å undersøke sammensetningen av tarmfloraen hos barn og ungdom med HIV og HIV-assosiert kronisk lungesykdom. Samt å undersøke effekten av behandling med makrolid-antibiotika (azithromycin) på sammensetningen av tarmflora og på lokal inflammasjon i lungene ved HIV-assosiert kronisk lungesykdom.

Metoder: Avhandlingen er en del av en dobbelt-blindet, placebo-kontrollert, randomisert klinisk studie; BREATHE (Bronchopulmonary function in response to azithromycin treatment for chronic lung disease in HIV-infected children and adolescents). Barn og ungdom fra 6-19 år med HIV-assosiert kronisk lungesykdom ble rekruttert i Harare, Zimbabwe og Blantyre, Malawi. De ble randomisert til ukentlig azithromycin- eller placebo-behandling for å se om dette bedret lungefunksjonen. I tillegg ble en kontrollgruppe med 103 barn uten HIV rekruttert fra Harare, Zimbabwe. Rektale penselprøver ble samlet ved inklusjon i studien, etter 48 uker med behandling og etter 72 uker (6 måneder etter avsluttet behandling). 16S rRNA-sekvensering ble brukt for å undersøke sammensetningen av tarmfloraen. Utåndet

nitrogenoksid (NO), ble målt ved inklusjon og etter 48 uker hos en undergruppe av deltakere fra Harare, Zimbabwe for å undersøke lungeinflammasjon.

Resultater: I **Paper I** viste vi at deltakere med HIV hadde lavere alfa-diversitet og høyere beta-diversitet sammenlignet med deltakere uten HIV. Forlenget antiretroviral behandling økte alfa-diversiteten til nivåer som var sammenlignbare med de uten HIV. Vi fant ingen sammenheng mellom tarmflora og lungefunksjon eller gjennomgått tuberkulose. *Corynebacterium*, *Finegoldia* og *Anaerococcus* var signifikant høyere hos deltakere med HIV, sammenlignet med deltakere uten HIV.

I **Paper II** viste vi at høyere nivå av utåndet NO var en risikofaktor for å utvikle akutt forverring av lungefunksjon. Utåndet NO var også assosiert med proinflammatoriske biomarkører som er involvert i utviklingen av kronisk lungesykdom og fibrose. Vi så ingen effekt av azithromycin på nivå av utåndet NO.

I **Paper III** viste vi at azithromycin reduserte alfa-diversitet etter 48 uker med behandling, men at disse endringene ikke lenger var statistisk signifikant 6 måneder etter endt behandling. *Eggerthella*, *Blautia* og *Dorea* var økt, mens *Bifidobacterium* og *Campylobacter* var redusert hos de som var behandlet med azithromycin etter 48 uker. Reduksjonen i *Campylobacter* vedvarte i minst 6 måneder etter endt behandling.

Konklusjon: Arbeidet i denne avhandlingen viser sammenheng mellom HIV og endret tarmflora, og effekten av langvarig antibiotikabehandling på inflammasjon i lungene og sammensetningen av tarmfloraen. Den gir ny kunnskap om HIV og HIV-assosierte følgesykdommer og kan være nyttig når en skal designe nye studier om tarmflora og mulige behandlingsmål for tarmflora og følgesykdommer av HIV i fremtiden.

List of papers

Paper I

Flygel TT, Sovershaeva E, Claassen-Weitz S, Hjerde E, Mwaikono KS, Odland JØ, Ferrand RA, Mchugh G, Gutteberg TJ, Nicol MP, Cavanagh JP, Flægstad T. Composition of Gut Microbiota of Children and Adolescents with Perinatal Human Immunodeficiency Virus Infection Taking Antiretroviral Therapy in Zimbabwe. *J Infect Dis*. 2020;221(3):483-92. doi: 10.1093/infdis/jiz473.

Paper II

Flygel TT, Hameiri-Bowen D, Simms V, Rowland-Jones S, Ferrand RA, Bandason T, Yindom LM, Odland JØ, Cavanagh JP, Flægstad T, Sovershaeva E. Exhaled nitric oxide is associated with inflammatory biomarkers and risk of acute respiratory exacerbations in children with HIV-associated chronic lung disease. *HIV Med.* 2023;1-10. doi: 10.1111/hiv.13565.

Paper III

Flygel TT, Abotsi, Regina E, Claassen-Weitz S, Simms V, Hjerde E, Mwaikono KS, Mchugh G, Ferrand RA, Nicol MP, Cavanagh JP, Flægstad T, Sovershaeva E. Long Term Azithromycin Treatment Reduces Gut Microbial Diversity in Children and Adolescents with HIV-associated Chronic Lung Disease. *Manuscript*.

Abbreviations

Abbreviation	Full name/text/description
Adolescents	WHO: 10-19 years of age
AIDS	Acquired immunodeficiency syndrome
ARE	Acute respiratory exacerbations
ART	Antiretroviral therapy
BREATHE trial	Bronchopulmonary function in response to azithromycin treatment for chronic lung disease in HIV-infected children and adolescents
CF	Cystic fibrosis
Children	WHO: 0-14 years of age
CLD	Chronic lung disease
COPD	Chronic obstructive pulmonary disease
Co-trimoxazole	Trimetoprim-sulphametoxazole
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
eNO	Exhaled nitric oxide
FEV_1	Forced expiratory volume in 1 second
FDR	False discovery rate
FVC	Forced vital capacity
GALT	Gut associated lymphatic tissue
GI	Gastrointestinal
GOLD	Global initiative for chronic obstructive lung disease
HCLD	HIV-associated chronic lung disease
HIV	Human immunodeficiency virus
IFN-γ	Interferon gamma
iNOS	Inducible nitric oxide synthase
INSTI	Integrase strand transfer inhibitor
LEfSe	Linear discriminant effect size
LIP	Lymphoid interstitial pneumonitis
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NEC	Necrotizing enterocolitis
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
OB	Obliterative bronchiolitis
PCP	Pneumocystis jirovecii pneumonia
PCR	Polymerase chain reaction
PI	Protease inhibitor
QIIME	Quantitative insights into microbial ecology
RNA	Ribonucleic acid
SIV	Simian immunodeficiency syndrome
TB	Tuberculosis
UNAIDS	Joint United Nations programme on HIV/AIDS
VL	Viral load
WHO	World Health Organization
16S rRNA	RNA component of the small 30S subunit of the ribosome
Xpert MTB/RIF	Real-time DNA test that detects TB and rifampicin resistance

Introduction

1 Human immunodeficiency virus (HIV)

1.1 History

The first cases of what later became known as HIV infection were described by Gottlieb *et al.* among men who have sex with men (MSM) in United States of America in 1981 (1). It started with young, previously healthy patients, admitted to hospital with opportunistic infections such as *Pneumocystis jirovecii* pneumonia, and Kaposi's sarcoma, a slow growing cancer from the cell lining of lymph or blood vessels (2). Shortly after, other groups such as people with haemophilia, intravenous drug users and recipients of blood transfusions showed similar clinical manifestations (3, 4). In 1982 the centre for disease control agreed upon the acronym AIDS – Acquired Immunodeficiency Syndrome as a name for the disease (2). Within the next decade HIV/AIDS spread rapidly worldwide, not only affecting people from the groups mentioned, but throughout the general population, making it a global pandemic (5, 6).

In 1983, French clinicians had identified a new human retrovirus (7) and by 1984 a serologic test to identify HIV as the cause of AIDS was developed (8). The same year $CD4^+$ T-cells were found to be the main target of the virus (9, 10). Due to this rapid spread of AIDS, there was a research rally on the newly discovered virus, producing new knowledge and treatment options. The first treatment with zidovudine, a drug that inhibits HIV reverse transcriptase, was introduced in 1987 (11). By 1989, HIV antibodies were discovered in African monkeys, proving that HIV was a result of transmission across species of the simian immunodeficiency virus (SIV) (12). However, it was the discovery of the coreceptors CXCR4 and CCR5, as late as in 1996, that made the introduction of highly active combination antiretroviral therapy (ART) possible (13). This led to a dramatic decrease in morbidity and mortality of the disease, and made prevention of the development of AIDS possible, and helped reduce the spread of the disease (14, 15).

1.2 Pathogenesis

HIV is a retrovirus from the family Retroviridae, genus *Lentivirus* (16). The main target of HIV is $CD4^+$ T-cells (T-lymphocytes), but it can also infect monocytes, macrophages, and dendritic cells. The virus enters the human cells through binding to membrane receptors and chemokine coreceptors CCR5 or CXCR4 (17). HIV can also infect other cells that express these receptors, such as renal epithelial cells and astrocytes, causing HIV-related nephropathy and neurocognitive disorders, respectively (18, 19).

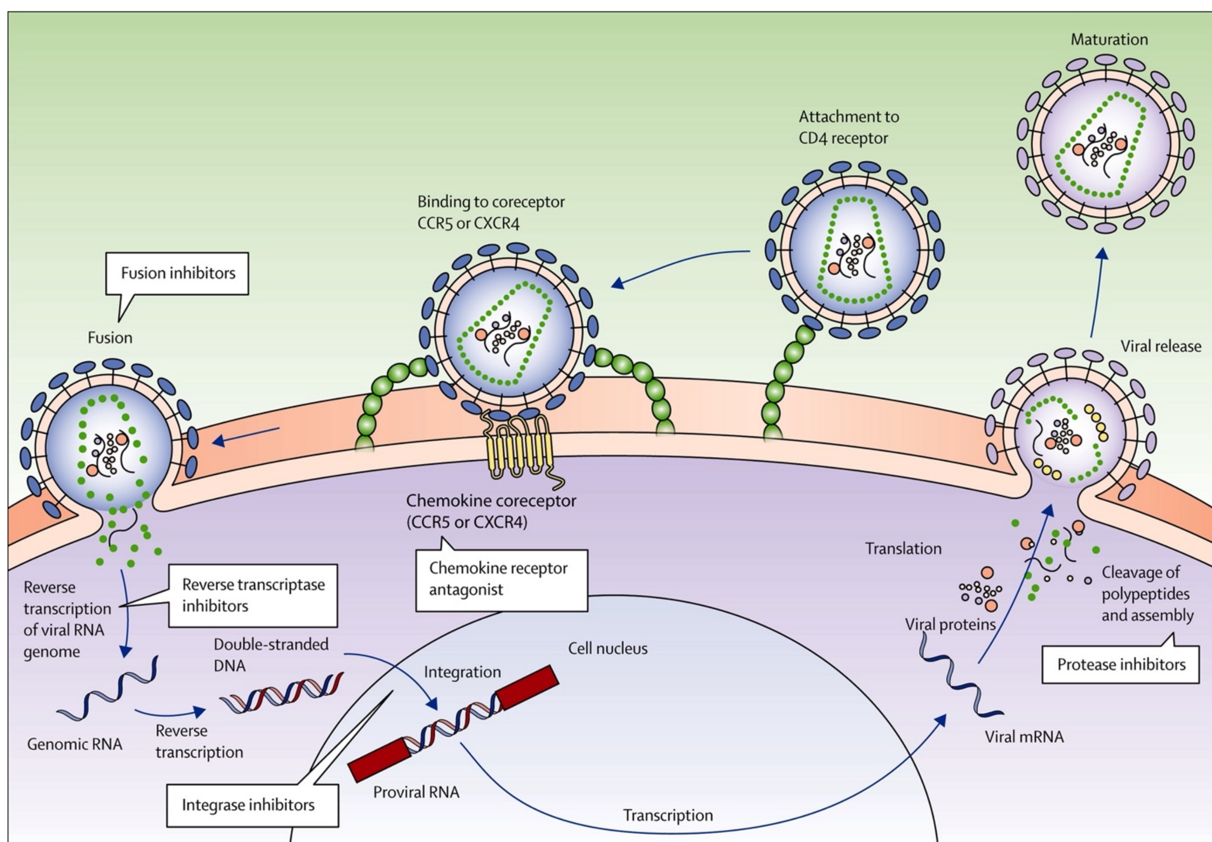


Figure 1. Life cycle of HIV and targets for antiretroviral treatment. From: Maartens, G. *et al.* 2014 (17).

HIV is an enveloped virus. The genome of HIV consists of two single stranded ribonucleic acid (RNA) molecules found in the core of the virus. Within the core you also find the enzymes reverse transcriptase and integrase. These enzymes contribute to the reverse transcription of RNA into double-stranded deoxyribonucleic acid (DNA) and integration of double-stranded HIV DNA into the human genome of the infected cell. HIV then uses the cells own mechanisms

of transcription to replicate. Between the core and the envelope, you find the enzyme protease. Protease then facilitates the assembly of transcripted HIV messenger RNA (mRNA) and viral proteins to new virus, which in turn is released from the cell as new, fully developed, infectious virus (17, 20). This knowledge has led to the development of targeted therapy using ART, that can act on any of these sites in the HIV replication cycle (Figure 1) (17).

HIV has three main routes of transmission; through sexual contact via the mucosa, through blood (percutaneous inoculation) and from mother-to-child. Mother-to-child transmission can occur either during gestation, delivery or breastfeeding (21). The use of ART during pregnancy and provided to the child immediately after delivery, together with a planned caesarean section and avoidance of breastfeeding postpartum, can reduce the rate of transmission to 1-2% (22). However, in resource-limited settings, the benefit of breastfeeding often outweighs the risks of transmission. As long as the mother is on ART and virally suppressed, and access or safety of formula feeding is restricted, breastfeeding is recommended as normal by the World Health Organization (WHO) (23).

There are two subclasses of the virus, HIV-1 and HIV-2, both being known to cause AIDS (24). Both subclasses have the same genetic arrangement, mode of replication and transmission. HIV-2 is however less aggressive, having lower rates of transmission, and mother-to-child transmission is rarely seen. The progression of HIV to AIDS is slower, and usually happens at higher $CD4^+$ T-cell counts in HIV-2. Further, HIV-2 has been shown to have lower HIV viral load (VL) and levels of immune activation compared to HIV-1. HIV-2 is mostly confined in western Africa, whereas HIV-1 is accountable for the worldwide HIV/AIDS pandemic (24, 25).

1.3 The HIV pandemic in numbers

Globally, an estimated 39 million people were living with HIV in 2022. The same year 1.3 million people got newly infected, and 630 000 people died of AIDS-related causes (26). Since the beginning of the HIV pandemic an estimated total of 85.6 million people have been infected, and 40.4 million people have died from AIDS or AIDS-related illnesses (27).

Due to the increased access to treatment, the decline of new HIV cases and AIDS-related deaths has been tremendous. In 1995, the peak year of new HIV infections, 3.2 million people got newly infected with HIV. In 2022, 1.3 million got newly infected, a 59 % reduction, and since 2010, there has been a 38% reduction of new cases (27). The biggest decline has been in high-prevalence settings, such as sub-Saharan Africa. Still, 58% of newly HIV infected in 2021 were in sub-Saharan Africa, and 20.6 million people, 54% of all people living with HIV, reside in eastern and southern Africa (28). This could be explained by lack of health services and limited access to ART (28). The reduction of AIDS-related deaths was 69% in 2022 compared to 2004, the peak year of AIDS-related deaths, with 2.0 million people dying in 2004 and 630.000 people in 2022. From 2010 the decline has been 51% (27).

Although we globally have come a long way in preventing new HIV infections and AIDS-related deaths, there is still a long way to go, reaching the end AIDS strategy of WHO and Joint United Nations Programme on HIV/AIDS (UNAIDS). In 2021 UNAIDS published the targets towards 2025. The goal is that 95% of people with HIV know their status, 95% of those who know their status are receiving ART, and 95% of those receiving ART are virally suppressed (29). By 2021, 85% of people with HIV knew their status, 88% of those knowing their status were on ART, and 92% of those on ART were virally suppressed. However, when accounting for those who do not know their status, only 75% were receiving ART and 68% were virally suppressed (27, 30). Both Malawi and Zimbabwe did however reach the previous UNAIDS 90-90-90 goals by 2020 (28).

As a part of the decline in new infections and AIDS-related deaths, people living with HIV have had a significant increase in life expectancy. In 1996 the gap in life expectancy between HIV infected and HIV uninfected people were 44.3 years. Although significantly reduced, in 2011 people living with HIV still had an estimated 11.8 years shorter life expectancy than healthy, uninfected people (31). A further decline to 7.9 years was shown in people who started ART early, before $CD4^{+}$ T-cells fall below 500 cells/μl (31). One study from South-Africa attributed 8.9 of 11.1 life-years gained in the population from 2006 to 2017 to the decline in HIV-related morality due to ART (32). However, low-income countries still represent the

largest gap in life expectancy due to HIV infection. A meta-analysis found life expectancy of HIV infected people at age 20 to increase by 43.3 years in high-income countries, and only 22.9 years in low and middle-income countries after initiating ART (33).

1.4 HIV in children and adolescents

Of people living with HIV in 2021, an estimated 2.7 million were children and adolescents, aged 0-19 years. In the same age group there were 310.000 newly HIV infected and 110.000 AIDS-related deaths in 2021 (34). It is estimated that 86% of all new HIV infections in children and adolescents occur in sub-Saharan Africa, and that 88% of all children and adolescents living with HIV reside in the same area (Figure 2) (35). Treatment programmes of prevention of mother-to-child transmission are widely established worldwide, including sub-Saharan Africa. Currently, an estimated 82 % of pregnant and breastfeeding women are receiving ART worldwide. The highest rate of 89% is seen in eastern and southern Africa. Unfortunately, this number has not increased significantly since 2014, and there is still an estimated 8.6% rate of vertical HIV transmission in eastern and southern Africa. More than 50% of new cases of HIV in children can however be explained by nonadherence to treatment among women with HIV on ART or being newly diagnosed with HIV during or just before pregnancy. This can be caused by either limited access to health services, costs of treatment, side effects, or as a consequence of stigma and discrimination (28, 35).

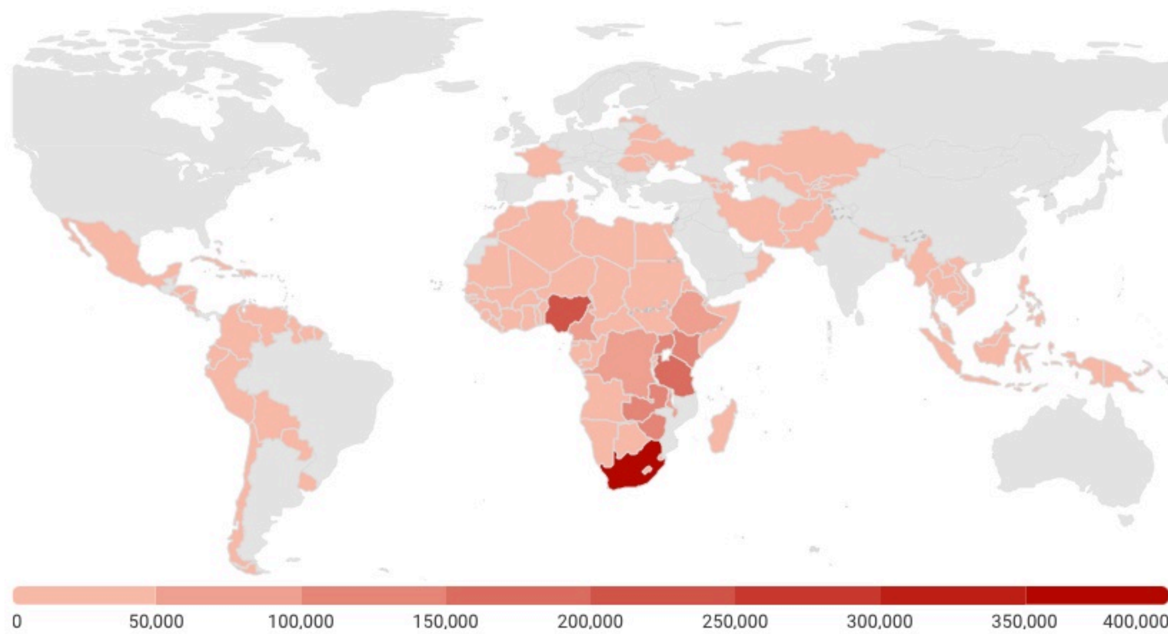


Figure 2. Prevalence of children and adolescents, aged 0-19 years, living with HIV worldwide. From: UNICEF DATA: HIV Statistics, based on UNAIDS 2022 estimates (35). *https://data.unicef.org/topic/hivaids*.

Due to the increased survival of HIV through ART programmes, a large number of children are now surviving to adolescence and adulthood with HIV. The decline in mortality among children under the age of 10 was a remarkable 75% between 2002 and 2021. However, in the same time period the decline in AIDS-related deaths in adolescents was only 21%. WHO has in fact alarmed that the number of AIDS-related deaths among adolescents are increasing, in contrary to all other age groups where mortality is decreasing. HIV is now among the top 5 causes of deaths in adolescents in the WHO African region. An arising problem is that the rate of new HIV infections among adolescent and young girls, aged 15-24 years, in eastern and southern Africa, now is 3 times higher than in men of the same age (35, 36). Further, it is estimated that more than 40% of children with HIV worldwide, do not know their status. The coverage of ART in children and adolescents is also concerning. Of children under the age of 15, only 52% were receiving ART in 2021. In adolescents 59% were receiving ART (35).

1.5 Antiretroviral treatment

As already mentioned, ART has dramatically reduced the transmission rate of HIV. HIV VL as monitoring of treatment response and detection of treatment failure has been recommended by WHO since 2013 (23, 37). Although widely implemented, in some resource limited settings the availability of HIV VL testing is limited due to high costs. $CD4^+$ T-cell count and clinical monitoring is still the recommended approach in areas with limited access to HIV VL testing, as it was prior to 2013 (23). Studies have shown that in well-treated persons with HIV, having undetectable HIV VL (< 50 copies/ml), the risk of transmission is effectively zero (38). This is also applicable for serodiscordant couples (where one part is HIV positive, and the other is HIV negative), both heterosexual and MSM, even when there are no precautionary measures taken, including the use of condoms (38, 39). In early phases of treatment, before achieving viral suppression, the risk of transmission is however not eradicated (40-42). These findings suggested that early initiation of ART in order to quickly achieve undetectable viremia serves as both an individual and population-based health and cost benefit. In 2016 WHO adjusted their recommendations for initiating ART. The new recommendations were that all people living with HIV should start ART as soon as possible after diagnosis, regardless of WHO clinical stage and at any $CD4^+$ T-cell count (43). These recommendations were continued in the newest WHO guidelines on testing, treatment and follow-up of HIV in 2021 (23).

The recommended ART regimens were also revised in the WHO 2021 guidelines. First line ART regimen now consists of combination therapy with one integrase strand transfer inhibitor (INSTI), (dolutegravir [DTG]), and two nucleoside reverse transcriptase inhibitors (NRTI). In the WHO 2021 guidelines, this regimen is recommended for both children over 4 weeks of age, adolescents and adults (23). As an alternative first line therapy, a combination of two NRTIs with one non-nucleoside reverse transcriptase inhibitor (NNRTI), (efavirenz [EFV]), can be used. Due to high prevalence of pre-treatment resistance to NNRTIs in some low- and middle-income countries, this regimen is not recommended in settings where NNRTI resistance is > 10% (23, 44). The preferred regimen with one INSTI (DTG) and two NRTI, has been found to be more effective and gave less adverse effects compared to the alternative first line regimen, also in children (45). This is especially important for children and adolescents where adherence can be poor, treatment failure is common, and there is a higher degree of multi-class ART resistance (23, 46). In addition, children and adolescents in resource-limited settings are

recommended co-trimoxazole (trimethoprim-sulfamethoxazole) prophylaxis as a part of standard HIV care (47).

Second line treatment is recommended when treatment failure on first line ART is confirmed with two subsequent measurements of detectable HIV VL (threshold for detection can vary). For those on one INSTI (DTG) + two NRTI, a change to one boosted protease inhibitor (PI) (lopinavir/ritonavir [LPV/r]) + 2 NRTI is recommended. If first line treatment consists of one NNRTI (EFV) + two NRTI, a change to one INSTI (DTG) + two NRTI is recommended for both children, adolescents and adults (23). HIV resistance testing is recommended before initiating ART and in case of treatment failure. In high-income countries this is standard of care, but in resource limited settings the availability of resistance testing is less extensive. A universal recommendation is adherence counselling and change of ART regimen following treatment failure (23). In the participants included in my PhD work, first line treatment was defined as one NNRTI and two NRTI, whereas second line treatment was defined as one PI and two NRTI as per WHO 2016 guidelines for resource limited settings with limited access to drug resistance testing (43).

2 Chronic lung disease

2.1 HIV-associated chronic lung disease

More children born with HIV grow up to adolescence and adulthood due to the increase in safe and effective ART treatment, making HIV a chronic and manageable disease. This has led to an increase in a range of chronic comorbidities among this group (48, 49). HIV-associated chronic lung disease (HCLD) is by far the most prevalent, accounting for over 50 % of mortality and morbidity among children and adolescents with HIV (50-53). These children are also at high risk of developing acute respiratory exacerbations (ARE), with respiratory infections being among the most common causes of hospitalizations in this group (54). The exact mechanism for developing HCLD is not completely understood and probably multifactorial. Among the lung diseases that could lead to reduced lung function presenting in this group are lymphoid interstitial pneumonitis (LIP), *Pneumocystis Jirovecii* pneumonia (PCP), tuberculosis (TB), obliterative bronchiolitis (OB) and bronchiectasis (51).

Before the introduction of ART, LIP was a common disease in perinatally HIV infected children (55). Clinically presenting with cough, tachypnoea, digital clubbing and a restrictive pattern on spirometry (56). LIP is associated with the development of bronchiectasis and cystic changes in the lungs with increased risk of recurrent respiratory tract infections. However, the disease responds well to ART, and the prevalence of LIP has decreased dramatically after the introduction of ART (57). PCP is a common opportunistic pathogen causing severe, rapidly progressing pneumonia, and despite rapid access to antibiotics the mortality rates of PCP are high (58). After the introduction of ART and prophylaxis by co-trimoxazole, the infection rate and mortality in children have declined significantly (56).

In addition to other underlying lung diseases and repetitive airway injury due to recurrent respiratory tract infections, an effect of HIV itself, through chronic immune activation and systemic inflammation, is thought to be involved in development of HCLD (59, 60). Global initiative for chronic obstructive lung disease (GOLD) has listed HIV as an individual risk factor for developing chronic obstructive pulmonary disease (COPD) (61). In addition, high HIV VL is associated with an increased risk of obstructive lung disease, measured by

spirometry (62). Poor immune function in children with HIV, defined by low $CD4^+$ T-cell counts, may also be associated with the development of bronchiectasis (56). This is of particular interest in young children and adolescents with the need for lifelong ART treatment and the challenges regarding virological control among this group (62).

2.1.1 Obliterative bronchiolitis and bronchiectasis

In recent years, OB and bronchiectasis are found to be the most common forms of HCLD in sub-Saharan Africa (63, 64). OB has been described as the leading cause of HCLD in children and adolescents in Malawi (65), Zimbabwe (64, 66) and South Africa (67, 68), with a prevalence of approximately 30 % (64, 66). These patients typically present with chronic respiratory symptoms including chronic cough, hypoxia, tachypnoea, breathlessness and a significantly reduced exercise tolerance (64, 65, 69). OB is irreversible, unresponsive to ART, and patients show respiratory obstruction on spirometry, with reduced FEV_1 (forced expiratory volume in one second) and FEV_1/FVC (forced vital capacity), that is not reversible by inhalation of bronchodilators (70).

OB is a severe lung disease with few treatment options available. The exact pathogenesis of the disease is unknown and poorly understood, but usually presents following a specific exposure or underlying illness (71). Various inflammatory and immunological pathways are thought to be involved in the development of the disease, yet no exact pathogenesis is known (72). The most frequently reported cases of OB are following allogenic haematopoietic stem-cell transplantation with subsequent graft-versus-host disease (73, 74), and after heart and lung transplantation with chronic allograft rejection (75-77). Other causes of OB include inhalation of toxins or air pollutants, recurrent respiratory tract infections and in association with autoimmune diseases such as rheumatoid arthritis (70, 78-80).

HIV infection is known to predispose to development of bronchiectasis (81). The cause for bronchiectasis, as in OB, is multifactorial, but is characterized by injury and subsequent neutrophilic inflammation (82). A key factor for developing HIV-associated bronchiectasis is the over-activation of the immune system seen in HIV infection (83, 84). In addition,

neutrophilic inflammation in the airways have been shown in people with HIV, and may cause excessive destruction of lung tissue, leading to development of bronchiectasis (83, 85). These mechanisms can also apply to the pathogenesis of OB. OB affects the small airways, the bronchioles, where injury and inflammation of airway epithelium and sub-epithelial structures is thought to cause excessive fibroproliferation. A poor repair response, including inadequate epithelial regrowth, can in turn lead to narrowing of the bronchioles and ultimately airflow limitation seen as respiratory obstruction on spirometry (70, 72). Dilatation and thickening of larger airways, as seen in bronchiectasis can occur with advanced stages of OB (70).

2.1.2 HIV and tuberculosis coinfection

Due to the high prevalence of HIV and TB coinfection in sub-Saharan Africa, it is also relevant to consider previous TB as a mediator of HCLD. TB is an infectious disease, caused by the *Mycobacterium tuberculosis* complex, which consists of the acid-fast bacilli *Mycobacterium bovis*, *-tuberculosis*, *-africanum* and *-microti* (86). The lungs are the main site of infection, but it can cause disease in any organ. The disease has been described for thousands of years, but Robert Koch was the first to describe *M. tuberculosis* in Germany in 1882 (87, 88). TB is today the top 13th cause of death worldwide, and second cause of death by a single infectious agent, behind covid-19, but outranking both HIV/AIDS and malaria (89). 10.6 million people were infected by TB and 1.6 million people died of TB in 2021. 187.000 of TB deaths were in people living with HIV, making TB the number one cause of death in HIV infection (89).

HIV infection gives an approximate 16 times increase in the risk of TB in untreated individuals (89). Despite a significant reduction in risk, people with HIV on ART are still at higher risk for developing TB compared to HIV uninfected individuals (90-92), this also applies to children (93). Further, children with HIV have a more rapid progression of TB, are more likely to get recurrent infections, and have poorer response to treatment (90, 93). In addition, clinical features of HIV and -TB are overlapping, especially in children, often leading to delayed diagnosis and treatment (94). Post-TB lung impairment is thought to have both inflammatory, and immunological components (95). The most common presentations of post-TB lung impairment are airway obstruction and bronchiectasis, due to injury and subsequent airway remodelling, giving an increased risk of developing COPD (96, 97). Obstructive lung function

was reported to be higher after TB-infection (97-99), and in children, history of TB was associated with lower FEV_1 in multiple studies (65, 67, 100, 101). HIV serves as an additional risk factor as HIV and TB coinfection have been associated with more severe pulmonary dysfunction than in TB infection alone (15, 95).

2.2 Assessment of lung health

2.2.1 Spirometry

There are several ways to measure and quantify lung function. The most commonly used method is spirometry. It measures volume and flow of inhaled and exhaled air as a function of time. Spirometry is the preferred measure of lung function according to American Thoracic society and European Respiratory society guidelines (102). It is used in diagnosis and grading severity of illness, as well as in evaluation of treatment response in patients with for example asthma and COPD (61, 103). The interpretation of results is dependent on various factors including age, sex, height, weight and ethnicity. Results are therefore based on reference values estimated from large population surveys (104). A lower limit of normal (LLN) value is often used as a cut-off to define airflow limitation (102). There are many parameters that can be measured, however the most commonly used are FEV_1 and FVC, and the ratio between the two (FEV_1/FVC). FEV_1 is a measure of the volume of air expired in the first second of forced expiration and is used to grade the severity of obstruction, whereas FVC measures the total volume of expired air by forced expiration. The ratio FEV_1/FVC is used to diagnose limitation of airflow (105).

2.2.2 Exhaled nitric oxide

Nitric oxide (NO) is a small molecule and neurotransmitter produced by many different cells in the body. It was previously considered as an air pollutant, but research has found it to be important in regulation of various physiological processes, especially in the respiratory tract. In small concentrations it has a beneficial effect on the airways (106, 107). When endogenously excreted in physiological concentrations in the respiratory tract it contributes to bronchodilatation, mucus secretion, ciliary motility and has an anti-inflammatory effect. In

larger concentrations it can be toxic (106). In the lungs NO is mainly produced by macrophages, but also endothelial cells, smooth muscle cells, neutrophils, and mast cells have been proven to produce NO (106). The production of NO in the lungs is mainly determined by NO synthase (NOS) enzymes, where inducible NO synthase (iNOS) is the most important (108). NOS works by converting the amino acid L-arginine to L-citrulline, generating NO in the process (109). The expression of iNOS is dependent on induction from either endogenous factors such as chemokines and cytokines or exogenous factors including bacteria, virus or allergens and air pollutants (110). Other NOS are expressed upon activation by calcium ions in endothelial and neuronal cells, and the NO produced this way is found to be more relevant in neuron signalling, vasodilation, and haemostasis inhibition (25, 109, 111).

Exhaled nitric oxide (eNO) from the lungs can easily be measured in exhaled air by non-invasive, low-cost, standardized methods (112). Due to high costs of other methods such as chemiluminescence, and that electrochemical devices yield high quality and reproducible results, this is the most used method (113). The mechanism is that it converts gas concentrations into electrical signals, showing the partial pressure of eNO as a measure of NO concentration in the exhaled air (113). eNO can be used as a marker of local airway inflammation. An elevated level of eNO is known as a marker of eosinophilic airway inflammation in asthma. eNO is suggested to be useful as a supplement to traditional methods in the diagnosing and monitoring of asthmatic disease, as well as a marker of adherence to medication, as inhaled corticosteroids reduce the expression of iNOS and thereby levels of eNO (112, 114-116).

There have been few studies to date on eNO in people with HIV infection and the results from these studies are conflicting and mainly related to TB coinfection or history of prior TB (117-119). Previous studies have shown that levels of eNO are lower in patients with active or prior TB and cystic fibrosis (CF) (109, 120). This is despite the fact that production of eNO is upregulated by inflammatory stimuli, and can contribute to killing bacteria, including *M. tuberculosis*. In fact, NO has even been suggested as a potential part of anti-TB treatment (121). The mechanism for lower levels of eNO is thought to be a result of reduced activity of iNOS, as an immune evasion mechanism of *M. tuberculosis*, which in turn leads to decreased levels of NO levels in the expired air (120). In CF, diffusion of eNO to the exhaled air is reduced by

the thick mucus, as well as a result of reduced expression of iNOS (122). A large study in well-treated adults with HIV and no history of TB or CLD from Copenhagen, Denmark did however find levels of eNO to be elevated in participants with HIV compared to healthy controls (123). This study looked at HIV infection alone. Participants had no history of TB or active TB at collection of samples and no CLD, defined as no obstruction on spirometry and absence of asthma and airway medications. These results indicate that HIV infection alone may result in elevated levels of eNO, and that active lung TB or history of TB may be the cause of lower levels of eNO seen in people living with HIV in other studies. A theory for this is the presence of HIV in alveolar macrophages despite being well-treated by ART. This can in turn lead to increased levels of proinflammatory cytokines in the lungs, and thus higher levels of iNOS stimulating the production of NO (124).

2.3 Biomarkers of systemic inflammation

Chronic lung disease is characterized by elevated levels of systemic pro-inflammatory biomarkers. Elevated levels of interleukin (IL)-8, IL-6, IL-1β, matrix metalloproteinases (MMPs), CRP and tumor necrosis factor (TNF)-α are found in children with OB compared to healthy controls (125). There have been few studies in participants with HCLD, but a recent study found several biomarkers, including MMPs to be associated with increased risk of HCLD in children and adolescents (126).

MMPs are an enzyme group of zinc-dependent endopeptidases involved in regulation of the extracellular matrix (ECM) (127). MMPs play a crucial role in ECM degradation and are involved in various receptor signalling pathways important in both inflammation and immune activation processes (127, 128). In the lungs almost every cell has been shown to produce MMPs, with varying levels of expression (128, 129). MMP-3 and MMP-7 are associated with development of lung fibrosis by increasing levels of profibrotic mediators as a response to injury (129). MMP-7 has in fact been suggested as a marker of disease progression, and even a possible therapeutic target, in idiopathic pulmonary fibrosis (129, 130). Both MMP-1, MMP-7 and MMP-10, as well as biomarkers associated with immune activation and inflammation (Interferon (IFN)-γ, CRP, IP-10) and endothelial activation and cell-adhesion (D-dimer, GCSF, FAS, e-Selectin, VCAM-1) were associated with HCLD (126). Several of these biomarkers

have previously been associated with reduced lung function in asthma, COPD, CF and HIV (126, 131). MMP-1, MMP-8 and MMP-9 were found to be associated with post-TB lung impairment (95). MMP-8 increased after ART initiation in treatment-naïve participants with HIV/TB-coinfection and was associated with reduced lung function after treatment (132).

2.4 Macrolide antibiotics

Macrolides are a group of broad-spectrum antibiotics, often used for treatment of airway infections due to its effect against a broad range of airway pathogens (133). Macrolides consists of a 12-16 atom macrolactone ring with one or more amino groups or neutral side-sugars attached (134). The mechanism of action for macrolides are by inhibiting the protein synthesis of bacteria, through binding to the 50S ribosomal subunit, and preventing mRNA translation (135-137). Azithromycin is the most studied macrolide in treating respiratory diseases. Azithromycin is a second generation, semisynthetic macrolide, consisting of a 15 atom macrolactone ring, derived from the first-generation macrolide erythromycin (135, 136). It was discovered in the 1980s and found to have a broader spectrum, be more potent and less toxic compared to its predecessor erythromycin (138, 139). Further, azithromycin has a high intracellular uptake and penetration to inflamed tissue, due to the high cellular uptake, especially in phagocytes (133, 140). The high uptake and penetration, combined with a slow hepatic excretion, results in a long half-life, which makes infrequent dosing possible, and can increase adherence to medication (136).

In addition to bacteriostatic activity against both gram-negative and gram-positive bacteria, azithromycin has anti-inflammatory properties reducing the levels of a number of pro-inflammatory cytokines, including IL-8, IL-6, MMPs, IFN-γ and TNF-α (133, 136, 141, 142). Macrolides can also directly affect the airway epithelium, by enhancing the epithelial barrier of the airways and reducing the formation of mucus in the airways (143). Thus, macrolide antibiotics are used to treat different chronic lung diseases such as asthma, CF, non-CF bronchiectasis, COPD, and OB. Studies show that macrolide treatment reduced number of exacerbations and had immunomodulatory effects in patients with these chronic lung disorders (72, 133, 136, 144). Long-term, low-dose use of macrolide antibiotics has been proven safe,

with low risk of adverse events and development of antibiotic resistance in both children and adults (145, 146). Recently (2020), the British thoracic society published guidelines on the use of long-term macrolide treatment in adults over the age of 16 with chronic lung diseases (147). The use of macrolides is primarily suggested to reduce acute respiratory exacerbations in patients with frequent exacerbations, or as prophylactic treatment to prevent development of OB in lung transplant patients (147). However, there are no current guidelines on the use of long-term macrolide treatment in children. Results from studies on the antimicrobial and immunomodulatory effects of azithromycin in chronic lung disease was the rationale for investigating whether azithromycin could be of benefit in children with HCLD that is thought to be partially driven by persistent immune activation (148).

2.4.1 Macrolide antibiotics and exhaled nitric oxide

Few studies have evaluated the effect of macrolide antibiotics on eNO, and the results are conflicting. Due to the immunomodulatory effects of macrolides, azithromycin in particular, several researchers have hypothesized that macrolide treatment could result in reduced levels of eNO. One study in rats found an effect of macrolides on iNOS causing reduced levels of eNO, suggesting a favourable effect of macrolide antibiotics on immune-mediated inflammation and injury of the lungs (149).

One randomised, controlled trial looking at the effect of either prednisolone, azithromycin, or placebo for treatment of airway remodelling in adults with severe asthma found no reduction of asthma exacerbation rate for the azithromycin group. However, they did find that both azithromycin and prednisolone gave a significant reduction in levels of eNO, where those receiving prednisolone had the most reduction in eNO (150). A reduction in frequency of respiratory exacerbations and less respiratory symptoms following azithromycin treatment have been shown in patients with non-CF bronchiectasis, but no significant reduction in levels of eNO was observed (151). In adults with treatment-resistant cough azithromycin gave no significant difference in either symptoms of coughing or in levels of eNO compared to placebo (152). A study in children with mild asthma found that a 4 week course of low-dose Clarithromycin improved both lung function, measured by FEV_1-score, and gave a significant reduction in eNO-levels (153). Another study showed that azithromycin reduced the levels of

eNO in children with refractory *Mycoplasma penumoniae* pneumonia. However, methylprednisolone together with azithromycin reduced levels of eNO and eosinophils more than azithromycin alone (154). These findings suggest there may be a favourable effect of macrolides on levels of eNO. However, the difference in pathogenesis and type of inflammation between the diseases makes comparisons difficult. Further, several of the studies to date included corticosteroid treatment that may influence the results, as corticosteroids are highly potent immunosuppressants and known to reduce levels of eNO (114, 115).

3 Gut microbiota

3.1 Development of gut microbiota

The human gut microbiota is defined by all living microorganisms that reside in the human gastrointestinal (GI) tractus. This includes bacteria, fungi, virus, and other microorganisms, where bacteria by far are the most abundant (155). In the literature, the terms microbiota and microbiome are often used interchangeably. Where microbiota is used about the organisms residing in a given environment, microbiome also includes their genetic information. In this thesis, I will use the term gut microbiota about the bacteria residing in the GI tractus. The gut microbiota is the most studied in humans and is said to host up to hundred trillion (10^{14}) microorganisms. Although not well documented, gut bacteria are claimed to make up a tenfold more cells than the human body and consist of 100 times more genetic material than the human genome (156, 157). Over the past decade there has been a huge increase in the interest for studying the gut microbiota, and its role in health and disease. This is a result of development and increased availability of next generation sequencing methods such as 16S ribosomal ribonucleic acid (rRNA) and shotgun metagenomics and whole genome sequencing (158, 159).

Colonization of the GI-tract with bacteria is thought to start as early as in utero, but it is controversial if this represents true colonization or is a feature of contamination (160). The maturation of the gut microbiota happens in early life and is found to stabilize around the age of 2-3 years (161-163). However, a complex, diverse, adult-like microbiota, may not even be reached at 5 years of age (164, 165). Development and composition of gut microbiota is dynamic throughout life, depending on a variety of factors, such as mode of delivery, geography, genetics, diet, presence of other diseases and exposure to antibiotics (161, 164, 166, 167). The diversity increases quickly over the first years of life, stabilizes, and tend to reduce with older age (168). The composition of gut microbiota is mainly made up of the three phyla Bacteroidetes (Bacteroidota), Firmicutes (Bacillota) and Proteobacteria (Pseudomonadota) (169-171). A phylum is a part of the taxonomic classification of microorganisms. The lineage goes from kingdom to phylum, followed by class, order, family, genus and finally species. In 2021 the International Committee on Systematics of Prokaryotes (ICSP) made a change in the names of several phyla (172). In this thesis I will use the old name followed by the new name

in brackets for consistency. Figure 3 shows the taxonomic classification of common gut bacteria with old phyla names.

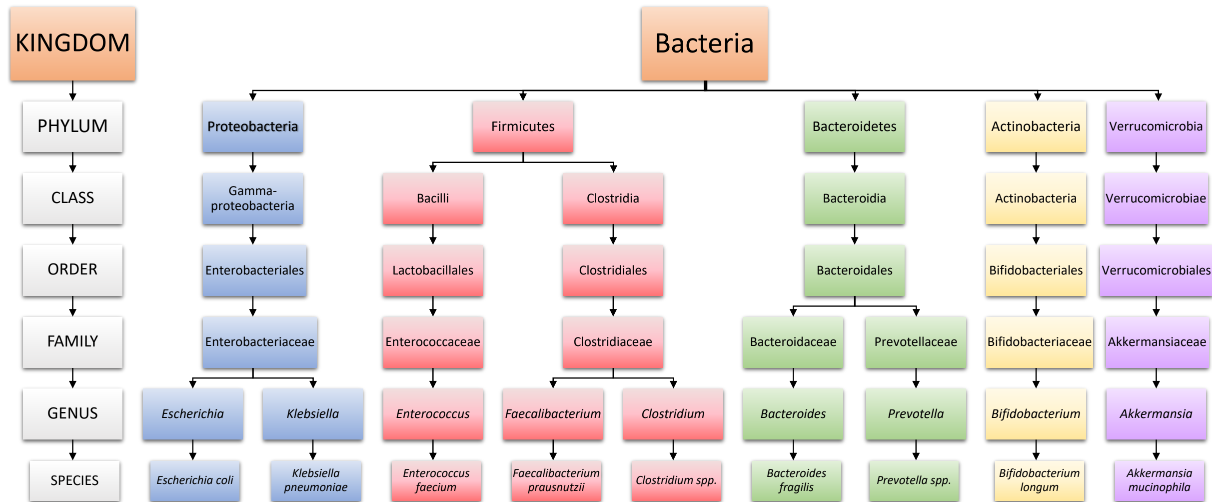


Figure 3. Taxonomic classification of common gut bacteria. Based on Rinninella, E. *et al.* 2014, and Fjalstad, J. 2018 (170, 173).

The gut microbiota is essential in maturation of the intestines and play an important role in the immune system of both children and adults. A balanced composition of the gut microbiota helps maintain structural integrity of the mucosal barrier of the GI-tract, to protect the host against pathogens and antibiotic resistant bacteria, and to regulate digestion (170). It has been shown that the development of gut associated lymphatic tissue (GALT) is present already in late stages of the first trimester (174). GALT is a key feature for the immune system, both for antigen presentation, barrier against pathogens, and it is where immune cells of the adaptive immune system undergo priming and differentiation (175).

When describing or comparing microbial diversity of the gut microbiota, alpha diversity and beta diversity are the most used measures. Alpha diversity is often referred to as "within sample

diversity", and is made up from richness (number of different species present in a sample), and evenness (the distribution of abundance of different species in a sample) (176). Beta diversity can be explained as "between sample diversity", meaning the dissimilarity in diversity of species between two or more samples within a group or ecosystem (177). For example, two people who live together, and eat the same food should have lower beta diversity than if they lived in different continents with a completely different diet. There is no consensus on what a healthy gut microbiota is. However, a low alpha diversity has been associated with various diseases, and a highly diverse microbial composition is often referred to as a healthy gut microbiota (156, 169). A recently published summary does however question this, as the evidence to support a signature gut microbiota as healthy is not sufficient. Further, research to date have not been able to establish causality that dysbiosis causes disease, or if in fact the disease is what causes dysbiosis (178).

When changes to the gut microbial composition occurs, causing an imbalance, it is referred to as gut dysbiosis. This is usually characterized by reduced diversity, overgrowth of possible pathogenic taxa, and a reduction of protective commensals (156). Gut dysbiosis has been linked to a wide range of diseases and disorders. These include obesity, type 2 diabetes, irritable bowel syndrome, inflammatory bowel disease (IBD), celiac disease, atopy, asthma, and allergy (156, 170, 179-182). A dysbiotic gut is also associated with upregulation of proinflammatory responses, suggesting a close link between dysbiosis and an unfavourable immune response (156). Recently, this immune response and alterations in gut microbiota have also been associated with development of lung diseases, emerging the term called gut-lung-axis (183). A low diversity of the gut microbiota in early years of life have been associated with the development of asthma and allergy during childhood (184, 185). Further, altered gut microbiota has been found in lung diseases such as COPD, cystic fibrosis, and asthma (183, 186). Although a complex, and not completely understood interplay, new evidence indicates that there is a potential for gut microbiota as a target for treatment in lung disease (183, 187).

3.2 Gut microbiota in HIV infection

Several studies have shown that the composition of the gut microbiota is significantly different in HIV infected individuals compared to non-infected individuals, and that these changes seem to persist even after initiation of ART (188-192). There are discrepancies in studies on the impact of ART on the gut microbiota, and to what degree it affects the dysbiotic gut community of HIV infected individuals (193). Although the overall effect seems to be that ART drives the microbiome more towards that of a healthy individual (194, 195), the composition of the gut microbiota in HIV infected individuals on ART still resembles that of ART-naive more than that of healthy, HIV uninfected individuals (190).

Multiple studies have shown a decrease of alpha-diversity and an increase in beta-diversity in HIV infected persons compared to uninfected controls (195-198). Similar findings have been shown in autoimmune diseases such as IBD and rheumatoid arthritis (199, 200). This is supporting the importance of a functioning enteric mucosal immune system to maintain a healthy gut microbiota. Furthermore, there is a link between the composition of the gut microbiota and the immune status of people with HIV, showing reduced diversity with poorer immune status and higher HIV viral loads, as well as correlations between alpha diversity and $CD4^+$ T-cell counts (196).

The reported changes of the gut microbiota in HIV infection are not only decreased diversity, but also a shift in the gut microbial composition towards a more unfavourable gut microbiota (201, 202). This includes decreased abundance of what is considered as commensal bacteria with anti-inflammatory features and a corresponding increased abundance of bacteria that are found to be pro-inflammatory (190). These include genera such as *Pseudomonas*, *Enterobacteriaceae*, *Campylobacter* and *Acinetobacter* (201-203). Increased abundance of *Prevotella* has also been associated with increased levels of T-cell activation in people with HIV (192). Of note, one study did however show that the increased levels of *Prevotella* previously reported in people with HIV was in fact linked to sexual orientation, rather than HIV infection itself (204). In addition to the gut microbial changes due to HIV infection alone, the effects of both long- and short-term antibiotic treatment in this group are not well established and therefore of interest, as antibiotics are frequently prescribed in this group (205).

3.3 Pathogenesis of intestinal HIV

The GI tract plays an important role in HIV infection. The largest reservoir of $CD4^+$ T-cells in the human body resides in the GI-tract and GALT. $CD4^+$ T-cells presenting the chemokine receptor CCR5 are highly represented in the gut (206). This makes the GI tract one of the main sites for depletion of $CD4^+$ T-cells in HIV infection (207). The depletion of $CD4^+$ T-cells happens at early stages of HIV infection and seems to persist even after introduction of ART (207). The $CD4^+$ T-cell subsets TH-17 and -22 are especially targeted (208). These subsets are involved in defence mechanisms and maintenance of the epithelial gut barrier (189, 207, 209). In addition, the release of proinflammatory cytokines such as IFN-γ, IL-6 and IL-10 as a response to HIV infection can increase the depletion of T-cells in the lamina propria of the gut (208). This leads to damages on the gut mucosal barrier, including a loss of intestinal integrity and apoptosis of enterocytes (210).

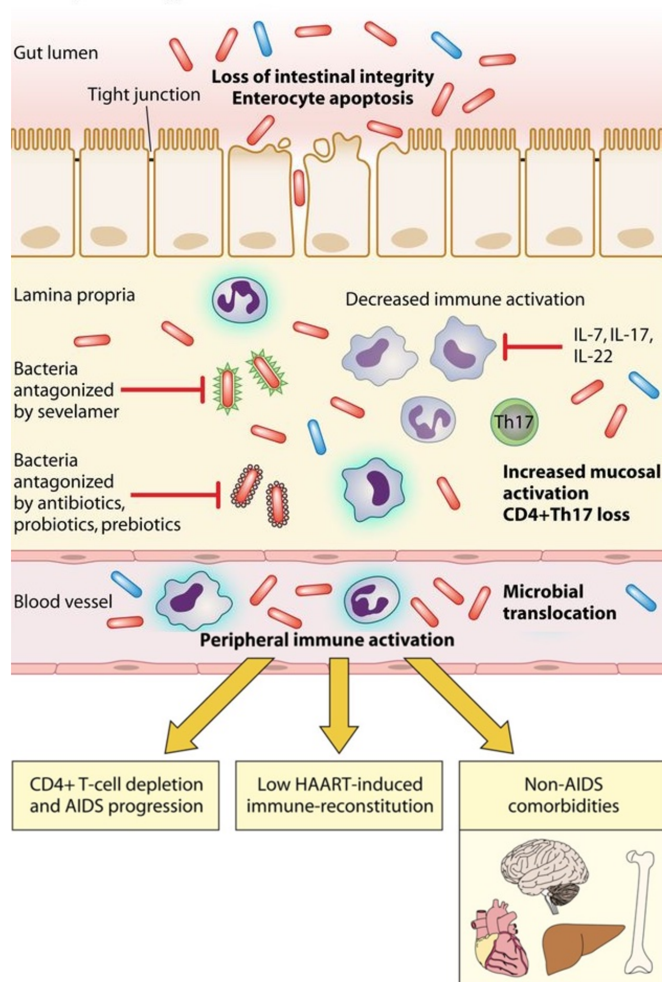


Figure 4. The intestinal barrier in HIV infected individuals and pathogenesis of peripheral immune activation. Modified from: Marchetti, G. *et al.* 2013 (209).

Further, translocation of bacteria from the gut lumen to the systemic circulation, through a default lamina propria, due to a reduced enteric mucosal immune function, makes chronic gut inflammation a characteristic of HIV infection. The bacterial translocation, and local gut inflammation can further result in a microbial gut dysbiosis, which in turn can exacerbate and help to maintain the systemic immune activation seen in HIV (84, 201, 210). This is illustrated in Figure 4. Furthermore, studies have shown a significant correlation between abundance of specific taxa, such as *Enterobacteriaceae* and systemic markers of inflammation, including

IFN-γ and IL-1β (188). *Fusobacterium* has been associated with low $CD4^+$ T-cell count and poorer immune recovery following ART (211).

Data are limited in children and adolescents. Available evidence suggest children are presenting a smaller reservoir of virus in GALT, and there has been shown lower levels of microbial translocation and chronic immune activation, compared to adults (212, 213). This is suggested to be partially explained by early initiation of ART at an acute phase of infection, that can limit the intestinal damage of HIV (214). However, when not properly treated, HIV infection can lead to enteropathy characterized by villous atrophy, crypt hyperplasia and epithelial damage causing microbial translocation, as mentioned (210, 215). The clinical presentation involves diarrhoea and malabsorption of nutrients, bile acids and proteins that ultimately can lead to failure to thrive in children (216, 217). The most effective treatment to date is nutritional support and early ART initiation (218).

3.4 Effects of antibiotic treatment on gut microbiota

The use of antibiotics is essential in the fight against bacterial infections. However, as with all other drugs, it comes with side effects. One of the most important side effects of antibiotics is the disruption of gut homeostasis, that ultimately can lead to gut microbial dysbiosis (219-221). The use of antibiotics has been shown to reduce both the overall diversity, as well as causing a decreased abundance of specific taxa, including protective commensals (222). Antibiotics could also affect both gene expression, protein activity and the overall metabolism of the gut microbiota (223). In addition to depleting beneficial bacteria, allowing potentially pathogenic taxa to thrive, antibiotics can also potentiate the emergence of antibiotic resistance (224). WHO is in fact calling antibiotic resistance one of the world's biggest threats against global health (225). Repeated antibiotic treatments, as well as the use of broad-spectrum antibiotics increases the risk of both dysbiosis and development of resistance. It is also suggested that children could be more susceptible to gut microbial changes. For the smallest children prolonged antibiotic use has been shown to increase the risk of severe illnesses, including necrotizing enterocolitis (NEC) and invasive fungal infections, and even death (226). Further, the changes in the gut microbiota can persist for years after treatment and may predispose to other adverse health

outcomes including obesity, diabetes and asthma, by promoting growth of potentially pathogenic taxa (227-230).

Antibiotic use, macrolides in particular, have been shown to significantly reduce the richness of gut microbiota in children when compared to children who did not receive antibiotics (228). This finding was consistent throughout several studies (227, 231, 232). One study did find richness estimates to be reduced for up to two years following exposure to macrolide antibiotics (227). The concentration of macrolides in stool after oral administration is found to be high, implicating a not irrelevant impact on the gut microbial composition (233, 234). For alpha diversity indices, including Shannon and Simpson index, a meta-analysis found that use of azithromycin significantly reduced the overall alpha diversity (228). This analysis included data from 4 different studies, all confirming a reduced alpha diversity after azithromycin exposure (229, 231, 232, 235). With regards to beta diversity, results are more conflicting. Where one study found beta diversity to be unchanged after azithromycin treatment (235), another found it to be reduced (231). A third study found lower beta diversity in children treated with any antibiotic. Of note, this study looked at overall antibiotic exposure, not differentiating between different classes of antibiotics (236).

Macrolides have been described to affect the abundance of a variety of phyla and genera. The genera *Bifidobacterium, Clostridium* and *Lactobacillus* and the phylum Actinobacteria (Actinomycetota) as well as *Campylobacter* from Epsilonbacteraeota (Campylobacterota) were found to be decreased after exposure to macrolides (227, 237-240). One study even found a 50-fold reduction in *Bifidobacterium* after a three-day course of azithromycin (231). *Bifidobacterium* is found to be sensitive to a range of different antibiotic classes, including macrolides, meaning several antibiotics could cause alterations in the abundance of *Bifidobacterium* (241). Abundance of the genera *Bacteroides*, *Clostridium* and the phylum Proteobacteria (Pseudomonadota) were found to be increased in one study (227). However, in a systematic review, four studies found both *Bacteroides* and *Clostridium* to be decreased following exposure to erythromycin or clarithromycin (222). Decreased levels of *Enterococcus* and *E. coli* was described in several studies (222, 237-240, 242), whereas *Klebsiella*, *Eggerthella*, *Blautia*, *Dorea* and *Pseudomonas* were found to be increased (222, 237, 240).

Other genera including *Enterobacteriaceae*, *Streptococcus*, *Micrococcus* and *Staphylococcus* were observed both to be increased and decreased in different studies (222). An Indian study in infants found Proteobacteria (Pseudomonadota) and *E. coli* to be reduced following a three-day course of azithromycin (232). Of note, the latter study was done in infants, suggesting that both age and study setting, as well as baseline abundance of bacteria could be of relevance to the effect of antibiotics on specific bacteria (232). Although some discrepancy between studies, the reported changes in gut microbiota after macrolide exposure could lead to a depletion of commensals and an increase in possible pathogens. This reduction in colonization resistance, the ability of a healthy microbiota to inhibit and prevent overgrowth of potential pathogens (243), could in turn increase systemic immune activation and contribute to chronic inflammation (219).

Most studies reported that levels of aerobic bacteria were normalised to pre-antibiotic levels after 3 weeks upon cessation of macrolides, whereas abundance of anaerobic bacteria seemed to persist longer (222). Of note, the use of the macrolides erythromycin and clarithromycin led to increased abundance of candida in two studies (238, 242). While the abundance of *Bifidobacterium*, *Bacteroides* and macrolide resistance genes returned to normal levels within one year, the genera *Collinsella, Lactobacillus* and *Anaerostipes* was reported to remain reduced even two years following macrolide exposure in Finnish children (227). The effect of long-term azithromycin therapy on gut microbiota in children and adolescents with HIV is therefore of particular interest, as a disrupted gut microbiota already have been reported in this group, irrespective of antibiotic exposure (195, 244-247).

Aim of the thesis

The overall aim of the thesis was to investigate the composition of gut microbiota of HIV infected children and adolescents with HIV-associated chronic lung disease (HCLD), and to study the effects of macrolide antibiotic (azithromycin) treatment on gut microbial composition and effect on lung inflammation in HCLD.

Paper I (Gut microbiota - Baseline)

The aim of this study was to investigate the gut microbiota in HIV infected and HIV uninfected children in Harare, Zimbabwe and to evaluate the association between gut microbial composition and clinical and laboratory parameters of HIV (duration of ART, $CD4^{+}$ T-cell count, HIV viral load) and HCLD.

Paper II (Exhaled nitric oxide in HIV-associated chronic lung disease)

The aim of this study was to evaluate the effect of long-term azithromycin treatment on levels of exhaled nitric oxide (eNO), as a marker of local inflammation in the lungs, in children with HCLD. Further, we wanted to investigate the association between eNO and acute respiratory exacerbations in children with HCLD. In addition, association between eNO and plasma soluble biomarkers of systemic inflammation was estimated.

Paper III (Gut microbiota – Follow-up)

Antibiotic treatment is known to cause changes in the gut microbiota, not only targeting pathogens, but also commensals residing in the gut. We wanted to investigate and describe the changes in microbial diversity and composition of the gut microbiota of HIV infected children and adolescents receiving long-term (48 weeks) antibiotic treatment with the macrolide azithromycin. Further we wanted to see if the observed changes persisted 6 months after cessation of antibiotic therapy.

Materials and methods

1 Study population

This thesis was based on data from the Bronchopulmonary Function in Response to Azithromycin Treatment for Chronic Lung Disease in HIV-infected Children (BREATHE) trial. The BREATHE trial was a double-blind, placebo-controlled randomized clinical trial conducted in Harare, Zimbabwe and Blantyre, Malawi between 2016 and 2019 (BREATHE trial, clinicaltrials.gov, identifier NCT02426112). Inclusion criteria and detailed study protocol have been published (148). In short, the BREATHE trial investigated the effect of once-weekly azithromycin for one year (48 weeks) on HIV-associated chronic lung disease in children and adolescents. Inclusion criteria were age between 6 to 19 years, perinatally acquired HIV, taking ART for at least 6 months, having chronic lung disease (defined as FEV_1 *Z* score < -1.0 measured by spirometry) with no reversibility (<12% improvement in FEV_1 after 200 μg salbutamol inhaled using a spacer), no history or symptoms of active TB or any acute respiratory tract infection. All participants were screened for TB using Xpert MTB/RIF assay upon enrolment and diagnosis of active TB led to exclusion. Participants were randomized 1:1 using block randomization stratified by trial site.

The primary hypothesis of the BREATHE trial was that azithromycin would prevent worsening or improve lung function and reduce the prevalence of acute respiratory exacerbations (AREs) among children and adolescents with HCLD. The rationale for testing this is the inflammatory nature of HCLD, thought to be OB or Bronchiectasis, and that azithromycin has shown both anti-infective as well as anti-inflammatory and immunomodulating effects. A total of 347 children and adolescents with HCLD were recruited into the trial. The main trial results are already published, showing no effects on lung function, but a significantly lower incidence of ARE among participants receiving azithromycin (248). The experimental design and use of antibiotics has led to several sub-studies, including respiratory microbiome (234, 249), immunology (126, 250) and gut microbiota (195).

For **Paper I** only participants from Harare, Zimbabwe was included. The analysis was done when only baseline samples were available. 149 participants with HCLD were included. A control group, as part of the BREATHE trial, was recruited from the same area. This group consisted of HIV infected children with normal lung function, no active TB, no history of chronic respiratory symptoms or heart disease, and MRC dyspnoea score < 2. 28 participants were enrolled in the control group at the time of the study. Additionally, a group of 103 HIV negative, healthy, participants was recruited from the same catchment area in Harare, Zimbabwe as participants with HIV.

For **Paper II** only participants from Harare, Zimbabwe were included. Measurement of eNO started after the first 68 participants were enrolled in the study. Thereafter, 173 consecutively enrolled and randomized participants delivered eNO samples at inclusion. One participant had missing eNO at baseline and was therefore excluded. 86 participants were in the azithromycin group and 86 in the placebo group. 15 participants in the azithromycin group and 19 participants in the placebo group were LTFU, withdrew or did not provide eNO at 48 weeks, leaving 71 and 67 participants with follow-up samples, respectively (Figure 5).

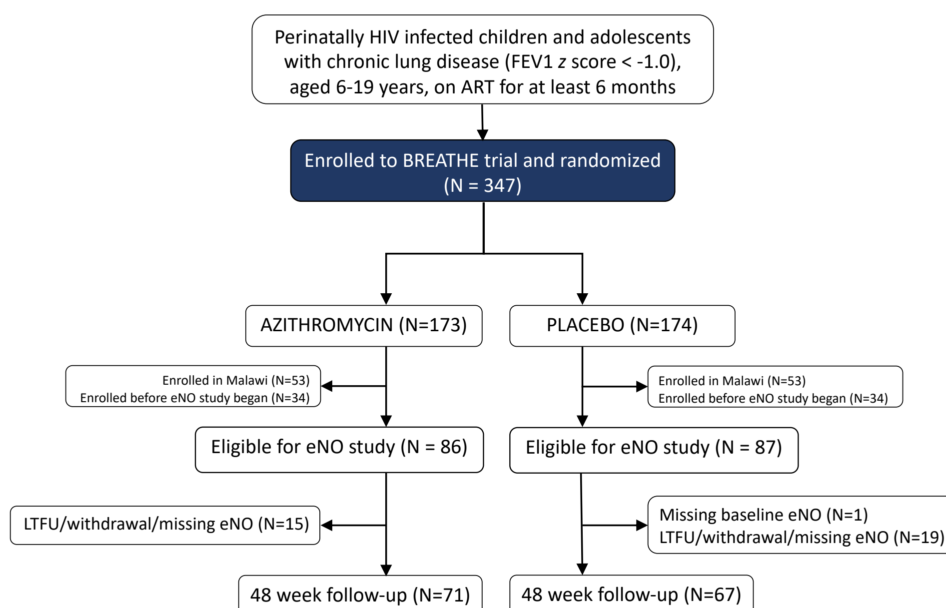


Figure 5. Flowchart of study participants in Paper II.

FEV_1, forced expiratory volume in 1 sec; ART, antiretroviral therapy; eNO, exhaled nitric oxide; LTFU, lost to follow-up.

For **Paper III** all participants from the BREATHE trial in both Harare, Zimbabwe and Blantyre, Malawi was included. Participants delivered rectal swabs at inclusion, after 48 weeks (upon finishing treatment), and after 72 weeks (6 months after cessation of study drug). In the azithromycin group 14 participants were LTFU, withdrew or died before 48 weeks, and 27 before 72 weeks. In addition, 5 samples were excluded due to low read count (< 4000 reads/sample), leaving 156 participants at 48 weeks and 126 participants at 72 weeks. In the placebo group 17 participants were LTFU, withdrew or died before 48 weeks, and 39 before 72 weeks, leaving 157 participants at 48 weeks and 118 participants at 72 weeks (Figure 6).

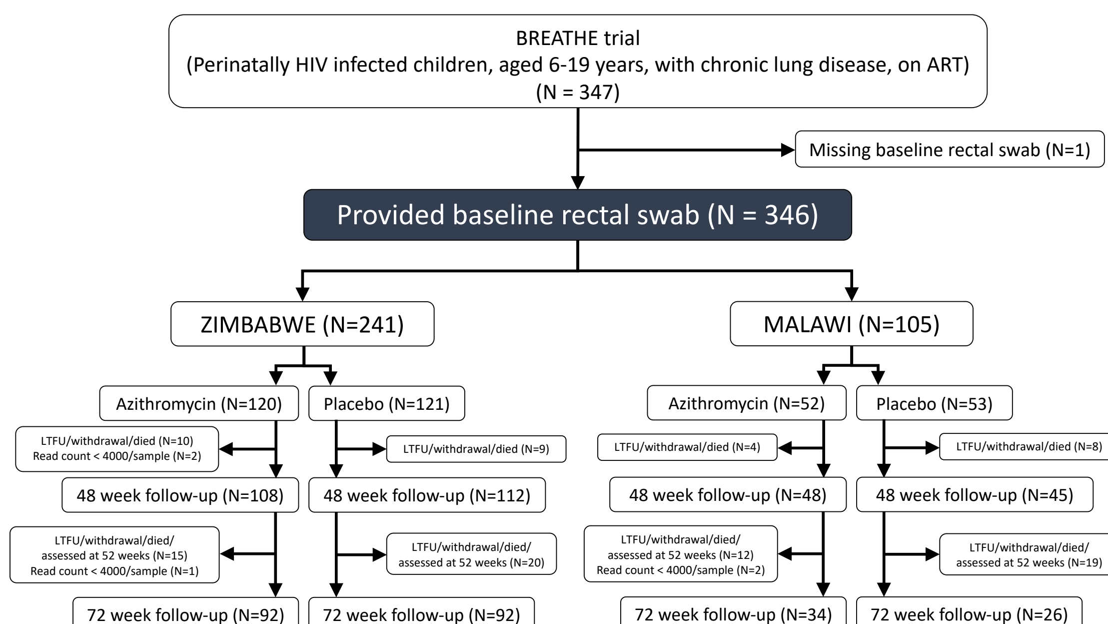


Figure 6. Flowchart of study participants in Paper III.
ART, antiretroviral therapy; LTFU, lost to follow-up.

2 Data collection and study procedures

2.1 Questionnaires

All study participants completed detailed, standardized, questionnaires regarding socioeconomic and demographic data, as well as clinical history. Details on HIV diagnosis and treatment, and both self-reported and physician-diagnosed lung diseases, respiratory symptoms and atopic disorders, including rhinitis and atopic dermatitis and asthma were recorded.

2.2 Rectal swabs

Rectal swabs were collected from all trial participants at enrolment and served as baseline samples. New rectal swabs were collected after 48 weeks of treatment with study drug, and after 72 weeks, 6 months after cessation of study drug. Rectal swabs were obtained by study nurses, inserting the swab approximately 2-3 cm inside the rectum. All samples had to be visibly stained with faeces before immediately being preserved in 1,5 ml of transport medium PrimeStore® MTM (Langhorn, Bethesda, Maryland, USA), put directly on ice for maximum one hour, and then frozen at minus 80 °C before shipment on dry ice to the laboratory at Division of Medical Microbiology, University of Cape Town (UCT), South Africa for further processing.

2.3 Exhaled nitric oxide

Levels of eNO was measured by an electrochemical analyser (NIOX VERO, Circassia, UK) according to American Thoracic Society (ATS) guidelines and shown in parts per billion (ppb) (112). The machine uses an electrochemical sensor that converts gas concentrations to electrical signals that are in direct proportion to the partial pressure of NO expired (113). The range of detection is between 5 ppb to 300 ppb. Calibration and quality control of the device was done according to manufacturer's instructions. All measurements of eNO were done between 8.00 AM and 2.00 PM, before spirometry. Participants had to sit for 5 minutes before testing.

Repeated exhalations of eNO were made to obtain at least two measurements that agreed within 10 %. Up to six attempts were made. The mean eNO value was calculated from the two measurements with least difference between them. Where the difference between 3 measurements were the same, we calculated the mean of all 3 measurements (118).

2.4 Blood samples

Blood samples were collected from participants at inclusion, after 48 weeks and after 72 weeks. The blood was tested for full blood count, HIV VL and $CD4^+$ T-cell count. HIV VL was measured using Gene Xpert assay (Xpert® HIV-1 Viral Load; Cepheid, Sunnyvale, CA, USA), with a lower limit of detection of 40 copies/ml. $CD4^+$ T-cell count was measured using a Pima™ Analyser (Alere, Orlando, FL, USA).

Plasma soluble biomarkers were measured from cryopreserved plasma stored at minus 80 °C before analysis, using Luminex multiplex bead assay on a MagPix® instrument according to manufacturer's protocol (Luminex technology, Hertogenbosch, Netherlands). Plasma samples collected from heparinized blood were thawed on their first use, diluted appropriately, and the levels of biomarkers assessed immediately in duplicates on a single MagPix® instrument. Measurements falling outside the standard curve were repeated at appropriate dilutions. Biomarkers that still fell below the lower limit of detection upon repeating were classified as undetectable and assigned half the minimum value measured for the specific biomarker (126).

2.5 Spirometry

Spirometry was performed using the EasyOne™ portable spirometer (ndd Medical Technologies Inc., Andover, MA, USA). The tests were executed by trained research staff certified in performing spirometry, and according to the ATS guidelines (103). Up to eight forced exhalations were made and the highest values for FEV_1 and FVC were recorded. FEV_1 and FVC *Z* score was then calculated using the Global Lung Initiative reference (251).

Participants with a FEV_1 *Z* score lower than -1.0 were given 200 µg of salbutamol, inhaled via spacer, and repeated spirometry was performed after 15 minutes to measure reversibility. The highest pre- and post-inhalation values for FEV_1 and FVC were used.

2.6 Data storage

Electronic record forms (for questionnaires) were collected on Google Nexus tablets (Google, Mountain View, California, USA) with OpenDataKit software. Paper forms were used for data collection of clinical tests. Data from the paper forms were extracted using CARDIFF TELEFORM character optical mark recognition software (version 10.9). Data were stored in Microsoft Access database (Microsoft, Redmond, Washington, USA) (148).

3 Laboratory methods

For **Paper I and III** the same 16S rRNA library preparation and Illumina Miseq sequencing was performed at Department of Medical Microbiology at University of Cape Town and Centre for Proteomic and Genomic Reasearch (CPGR) in Cape Town, South Africa. A detailed description as follows has already been published as supplementary materials in **Paper I** (195).

3.1 DNA extraction

Preliminary experiments done at UCT found that manual extraction of DNA from low biomass rectal samples yielded better quality of DNA, compared to automatic methods, and that is why the DNA isolation protocol was based on manual extraction (Masters project, not published). We used the Zymo Research Quick-DNA™ Fecal/Soil Microbe Microprep kit (Irvine, California, USA) manual extraction kit due to previous optimization protocols done in our laboratory. After performing some test extractions, and according to UCT protocol for swab samples, we decided on using an aliquot of 400 μl of each sample to get the concentrations of DNA needed for further analysis.

Extraction of nucleic acid from the rectal swabs was done using the Zymo Research Quick-DNA™ Fecal/Soil Microbe Microprep kit (Irvine, California, USA) with minor modifications to the protocol. A 400 μl aliquot of each sample was mixed with 400 μl of BashingBead™ Buffer in a ZR BashingBead™ Lysis Tube. Mechanical lysis (bead beating) was performed using the TissueLyser LT™ (QIAGEN) set to 50 Hz for 5 minutes. Then 500 μl of supernatant was transferred to Zymo-Spin™ III-F Filter (Irvine, California, USA) and centrifuged at 8000 x g for 1 minute. Further chemical lysis was done by adding Genomic Lysis Buffer (Zymo Research, Irvine, California, USA). All other procedures were done according to the manufacturers protocol.

3.2 16S real time quantitative PCR

After extractions of nucleic acids, we performed a real-time quantitative polymerase chain reaction (qPCR) on the extracted product to see if the DNA was giving amplicons of good quality for further analysis, and to establish the total bacterial load of the samples as previously described (252). The PCR reaction consisted of 15 µl SensiFAST™ Probe No-ROX (catalogue no. BIO-86020, Bioline, London, UK), 1 µl of forward primer 16S-F1 (5′-CGA AAG CGT GGG GAG CAA A-3′) at 10µM , 1 µl of reverse primer 16S-R1 (5′-GTT CGT ACT CCC CAG GCG G-3′) at 10 µM, 1 µl of the probe 16S-P1 (FAM-ATT AGA TAC CCT GGT AGT CCA-MGB) at 5 µM, 2,5 µl of DNA template and 9,5 µl MilliQ water, giving a total volume of 30 µl per sample (252). We used a set of 7 bacterial DNA standards by Zymo Research (Irvine, California, USA) with 10-fold dilutions, and 1 non-template control (Femto Bacterial DNA quantification, catalogue no. ZR E2006-2) as a standard curve. The amplifications were done using a BioRad C1000™ thermal cycler with CFX96™ Real-Time system (Hercules, California, USA), using the previously described protocol: 50 °C for 2 min, 95 °C for 5 min, and 45 repetitive cycles denaturation at 95 °C for 15 s and annealing at 60 °C for 60 s.

All sample concentrations were also measured by spectrophotometry, using the NanoDrop™ ND100 (Thermo Fisher Scientific, Massachusetts, USA), for quantification and purity assessment, with varying results. Using 1,5 µl of each sample, most of the samples gave low NanoDrop-concentrations (< 5ng/ml), but all samples gave good amplifications by real time qPCR, with most samples having a CQ-value of < 28. In comparison MilliQ water gave an average CQ-value of 34 within the 5 runs.

3.3 16S short and long PCR

We performed two sets of PCRs targeting the hypervariable V4 region of the 16S rRNA gene using the primers 515F (5’ GTGCCAGCHGCYGCGGT 3’) and 806R (3’ TAATCTWTGGG NNCATCAGG 5’), according to previously described protocols (253, 254). The first PCR run,

aiming to amplify the V4 region using target-only primers, consisted of 12.5 µl of 2X MyTaq™ HS Mix (Bioline, London, UK), 2 µl of the forward and reverse primers at concentrations of 10 µM each, 0,75 µl dimethyl sulfoxide (catalogue no D2650, Sigma-Aldrich®, Missouri, USA) and 4 µl template, made to a final volume of 25.25 µl using PCR-grade water (Thermo Fisher Scientific Inc., Massachusetts, USA). Amplifications were done under the following conditions: Denaturation at 95 °C for 3 min, 10 repetitive cycles of amplification at 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 s, with a final extension at 72 °C for 5 min.

The second run of PCR used 4 µl of the amplified V4 product from the first run as template and the primers 515Fmod4_SM_12N(-15N) (5’AATGATACGGCGACCACCGAGATCTAC ACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNNNGTGCCAGCHGCYGC GGT 3’) and 806Rmod1_SM_12N(-15N) (5’CAAGCAGAAGACGGCATACGAGAT ACGAGACTGATTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNN NNNGGACTACNNGGGTWTCTAAT 3’) to further amplify the V4 product from run one. The long, modified primers also include the needed sequencing adapters, primer region and 12-15 staggered random nucleotides (NNNNNNNNNNNN) serving as a spacer. To reduce non-specific binding risks and introduction of additional PCR bias when adding extra components to the primers, the PCR were performed using this two-step approach (255): Golay barcodes were incorporated in the reverse primer 860R_SMod_long (12 underlined bases) to identify each sample individually (253), with the modifications of adding 20 cycles to the amplification step (254).

Agencourt® AMPure® XP PCR Purification kit (Beckman Coulter, California, USA) was used to clean the amplicons as previously described (254). Modified from manufacturers protocol with a 0.65:1 ratio of Agencourt® AMPure® XP solution to PCR products in step 2. Using a 2% agarose gel electrophoresis at 110V for 90 min, we verified the PCR products, and amplicons were quantified using QuantiFluor® dsDNA System quantification kit on Promega Glomax®-Multi Detection system (Promega, Wisconsin, USA). Determined by the calculations from the quantification, we pooled the samples at 120 ng, followed by purification using a 1:1 ratio of

Agencourt® AMPure® XP solution. The pooled and purified samples were again quantified using the Qubit™ dsDNA BR Assay Kit (Thermo Fischer Scientific Inc., Massachusetts, USA), and run on a 1,6% agarose gel electrophoresis for 30 min at 35V, 45 min at 40V, 3 hours at 70V, and 60 min at 50V. The products were excised from the gel and purified using QIAquick Gel Extraction Kit (50) (QIAGEN, Hilden, Germany) with slight modifications as described by Claassen *et al.* (254). Modifications included incubation of sample for 5 min at 37 ˚C at step 10, and heating of elution buffer, Tris-EDTA (pH 8.0) at 60-70 ˚C at step 13.

3.4 16S rRNA gene sequencing

The final 16S library from rectal swabs at 50 µl was measured to a concentration of 49,2 ng/ µl using the Qubit™ dsDNA BR Assay Kit (Thermo Fischer Scientific Inc., Massachusetts, USA). KAPA qPCR quantification kit (KAPA Biosystems, Massachusetts, USA) and Agilend DNA 1000 kit (Agilent Technologies, California, USA) were used to quantify and size the library. The library was then diluted to 4 nM using Buffer EB (QIAGEN) and denatured using 0,2 N NaOH and finally diluted to a concentration of 6 pM using HT1 buffer, before being sequenced on the Illumina Miseq® using the Miseq® Reagent v3 kit, 600 cycles (Illumina, California, USA), adding a 25% PhiX library at 6 pM as internal control, as per manufacturer's instructions (256). This step was done at the Centre for Proteomic and Genomic Research (CPGR) in Cape Town, South Africa.

Sequencing controls used in the run included two no-template water controls and two PrimeStore® controls, one of each spiked with *Mycobacterium smegmatis*. ZymoBIOMICS microbial community standard and ZymoBIOMICS microbial community DNA standards (Zymo Research, Irvine, California, USA) were used, as well as four randomly selected biological samples that were run in duplicates to check reproducibility and control for technical variations within the single sequencing run. Both PrimeStore® and ZymoBIOMICS microbial community standard went through the entire extraction protocol, to control for contamination in all steps of the library preparations.

3.5 Bioinformatics

For **Paper I** the pre-processing of sequence reads was done using the H3ABioNet 16S rDNA diversity analysis package (https://github.com/h3abionet/h3abionet16S) (254), with the exception that the taxonomy of representative reads was assigned using the SILVA version 132 database (257). The raw sequence files have been submitted to the European Nucleotide Archive (ENA) under the accession number PREJB32077.

Controls, including the ZymoBIOMICS microbial community DNA standards, and biological samples were analysed separately. The average sequence reads of each operational taxonomic unit (OTU) detected in the spiked controls (except from the DNA these controls were spiked with) were calculated and subtracted from the biological samples. In the biological replicates the number of reads between the two replicates varied (+/- 20%), but the taxonomic profiles were completely overlapping. In addition, the replicated samples were separated into four clusters both after alpha and beta diversity analysis, confirming technical reproducibility of the run (195).

Since we had more samples, and the development of bioinformatics tools are continuously evolving, we used another method for pre-processing and decontamination for **Paper III.** Sequence data were pre-processed and taxonomically classified using the DADA2 pipeline (258) and the Decontam R package (259). Figures and statistical analysis were done using the phyloseq (260) and microViz package (261) in R version 4.2.2 (R studio, version 2022.02.2 build 485). Merged sequence read pairs passing default DADA2 quality filtering using the pool="pseudo"-option were decontaminated using the Decontam R package. Before removing any amplicon sequence variant (ASV) or samples, our data had 6875 unique ASVs. The Decontam outputs showed 264 taxa marked as potential contaminants. These taxa were removed from biological samples, after checking these against biological samples and negative controls from the sequencing. After quality control steps, we removed samples with less than

4000 reads (N=5). Only ASVs classified as bacteria were kept and ASVs with 0 reads (N=1361) were removed, leaving 5250 unique ASVs for downstream analysis. For relative abundance analysis further filtering was done, removing all ASVs with less than 10 sequences in total and those appearing in less than 2 samples (N = 3 272). This was done to explore the most relevant ASVs, and to reduce noise from very low abundant ASVs. Bacteria were classified at the lowest assigned taxonomic level.

4 Statistical analysis

4.1 Paper I

Statistical analyses were performed in STATA 15 (StataCorp LLC, College Station, TX, USA) and R statistical software (http://r-project.org/). Characteristics between study groups were compared using Fishers exact test for categorical parameters and Kruskal-Wallis or Wilcoxon rank-sum test for continuous parameters.

For alpha diversity (within sample diversity), richness of taxa within a single sample was represented by the number of OTUs and Chao1 index, a ratio estimation of richness, with a correction factor for the observed number of species (262). Richness and evenness were characterized by Shannon index (263). Alpha diversity measures were calculated at a sampling depth of 4000 reads to include 95% of samples. Alpha diversity indices between groups were compared using Wilcoxon rank-sum test. P-values were corrected for multiple testing using false discovery rate (FDR). Spearman rank correlation with Bonferroni correction was used to assess the association between alpha diversity and continuous parameters (264). A linear regression model was fitted to estimate the association between HIV status and alpha diversity indices. Body mass index (BMI), age, and sex were adjusted for a priori. An interaction term between HIV status and the use of antibiotics the previous 3 months before enrolment (co-trimoxazole prophylaxis for HIV infected participants) was included in the model to determine whether antibiotics modify the effect of HIV status on alpha diversity. The association between alpha diversity and other participant characteristics was further evaluated in regression analysis stratified by HIV status and adjusted for BMI, age, and sex. A two-tailed significance level of 0.05 was used.

For beta diversity (between sample diversity), Bray-Curtis dissimilarity index was used (265). Sampling depth was set to 2000 reads to include 99% of samples. Beta diversity comparisons were explored using Principal Coordinate plots, generated by the *stats* package in R (version 3.4.4) Comparisons were made using Wilcoxon rank-sum test and Kruskal-Wallis test, the latter

in cases where more than two groups were compared. The same groups were compared using Permutational multivariate analysis of variance (PERMANOVA) in QIIME2 (version 2018.4) (266), with number of permutations set to 999. P-values were adjusted for multiple testing using the Benjamini-Hochberg method (267).

To assess relative abundance, linear discriminant analysis effect size (LEfSe) was used (268). Default settings was used, with alpha values for the statistical test set to 0.05. To reduce the number of markers, the effect size threshold was set to 1.0 for the plots. Relative abundance comparisons plots were generated using the MicrobiomeAnalyst web-based software tool with standard feature filtering (269). Heatmaps for comparing relative abundance of taxa between groups were generated using only the taxa found to be significantly different by LEfSe-comparisons. The average fraction of each taxa was calculated from all samples within each group. The data were transformed to fractional abundance using Phyloseq (260) before performing LEfSe analysis. All P-values reported were corrected for multiple testing using FDR (195).

4.2 Paper II

Statistical analyses were performed using R studio version 2022.02.2, build 485, with R version 4.2.0, and IBM SPSS version 28.0.0. Characteristics between study participants were compared using Wilcoxon rank sum test for continuous parameters and Fisher exact test for categorical parameters. Data are shown as median with interquartile range (IQR 25%-75%) or geometric means with 95% confidence intervals (CI). The distribution of eNO values were not normally distributed. Therefore, they were log transformed using the natural logarithm to approximate normality for statistical analysis, and back transformed to geometric means with 95% CI for presentations. Weight-for-age and height-for-age Z scores were calculated using British 1990 Growth Reference Curves (270). Participants with Z scores lower than -2.0 were characterized as stunted or underweight. When comparing levels of eNO between trial groups students T-test was used. To test differences between time points paired T-test was used on the log transformed values of eNO. Plots were made using the *ggplot2* package in R studio (271).

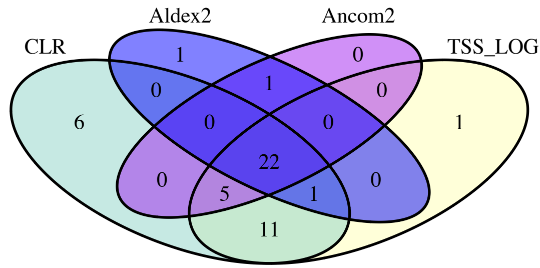
A generalized linear regression model (GLM) was used to describe the effect of azithromycin on levels of eNO. GLM was adjusted for age, sex, history of TB and baseline levels of eNO. The same model was used to determine whether there was an association between baseline levels of eNO and having at least one ARE during the study period. Adjustments were made for trial group, age, sex, and previous history of TB. There were no significant interactions to include in the model. The association between eNO and markers of systemic inflammation was estimated in linear regression analysis. We chose not to include haemoglobin or neutrophils in the GLM, as we know haemoglobin in strongly correlated to age, and that the difference in haemoglobin is explained by age. There was no significant difference in cases of anaemia between groups. We used log transformed values for MMPs and the other biomarkers to approximate normality and fit of the model. Biomarkers included were found to be associated with either HCLD or response to azithromycin treatment in the same cohort (250).

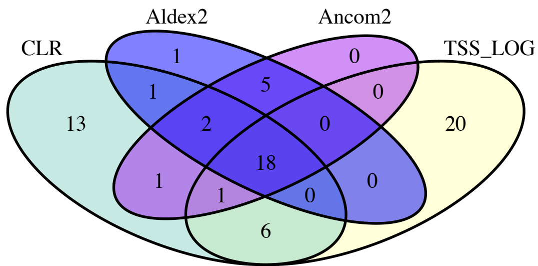
As history of TB previously have been associated with lower levels of eNO (118), a subgroup analysis comparing levels of eNO between participants treated for TB and not was done. Biomarkers and MMPs included in regression analyses were chosen based on previously confirmed association to HCLD or response to azithromycin treatment in the same group of participants (250, 272).

4.3 Paper III

Statistical analysis was performed using R version 4.2.2 (http://www.r-project.org/). Characteristics between study groups were compared using Students t-test for continuous parameters, and Chi square test for categorical parameters. Alpha diversity was described by richness estimates using number of observed ASVs and Chao1 index (262). Richness and evenness (taking the relative abundance into account) was described by Shannon index (263). Alpha diversity was calculated at a sequencing depth of 4000 reads per sample, this included 99% of samples. A linear mixed model was fitted using the *lme4* and *lmerTest* package in R (273) to investigate the effect of azithromycin on alpha diversity. Study site (Zimbabwe vs. Malawi) was set as random effect, and age at enrolment, sex, ever being treated for TB before enrolment, duration of ART by group (6 months-2 years, 2-4 years, 4-6 years and more than 6 years), use of cotrimoxazole, season of sampling (dry vs. wet), being stunted (HAZ < -2.0) and baseline and 48 week values of the relevant index tested was set as fixed effects. We did not detect any relevant interaction-terms. When comparing study sites at baseline a generalized linear model (GLM) was used, including all covariates significantly different between the two study sites. Plots were made using the *ggplot2* package in R version 4.2.2 (271).

We used four methods for assessing the difference in relative abundance between study groups and time points, ANCOM2 (274), Aldex2 (275), Maaslin2 with total sum scaling and log transformation and Maaslin2 with centered log transformation after applying a pseudo count of one (276). We then compared the overlap between the different methods as showed in Figure 7. We chose to report from Aldex2, as this method has shown most consistent results across different studies (277), serves high precision and low false discovery rate when the sample size is sufficient (278), and had the most overlap with the three other methods.





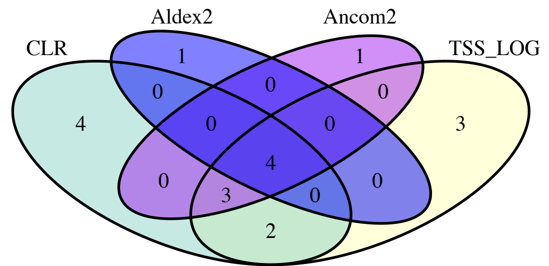


Figure 7. Venn diagram showing the overlap between the four methods used for assessing relative abundance: Aldex2, ANCOM2, Maaslin2 with TSS_LOG (total sum scaling and log transformation) and CLR (centered log transformation).

5 Ethical approvals

The study was approved by the London School of Hygiene and Tropical Medicine Ethics Committee (ref. 8818); the Harare Central Hospital Ethics Committee, the Medical Research Council of Zimbabwe (MRCZ/A/1946); College of Medicine Research Ethics Committee Malawi (P.04/15/1719); University of Cape Town Human Research Ethics Committee (754/2015), and Regional Committee for Medical and Health Research Ethics in Norway (REC North) (2015/1650). Clinical trial and importation of drugs were approved by Medicines Control Authority of Zimbabwe (B/279/5/14/2016) and Pharmacy, Medicines and Poisons Board Malawi (PMPB/CTRC/III/76). Written and informed consent by guardian and assent by participants aged under 18 were obtained. Those over 18 years of age gave independent consent. Biobanking was performed locally, and the consent included a storage period for maximum ten years and allowed shipment of biological samples to University of Cape Town for laboratory analysis. All person-identifiable information were to be anonymously stored, and only researchers involved in the project locally would have access to personified information in case of adverse events (148).

5.1 User participation

The children and adolescents participating in the project are defined as users together with their families. In sub-Saharan Africa, the role of user organizations is not strong, and participants are most likely less involved in user participation compared to a European setting. To involve the users more, participants in the study were included in focus groups through the BREATHE trial, where the projects for my thesis were sub studies. Families had regular contact with outpatient clinics where sampling was done, and they had ongoing discussions with local project coordinators throughout follow-up about advantages and possible problems with participating in the project. Further, they got comprehensive information about the study, procedures and the voluntary participant agreement for the study, where they at any stage of the study could withdraw.

Results

1 Paper I

149 participants with HCLD, 28 participants with HIV, but without diagnosis of CLD, and 103 HIV uninfected controls from Harare, Zimbabwe were enrolled and included in this study. Participants with HIV were older (15.6 years, interquartile range [IQR] = 12.8–17.7 vs 9.9 years, IQR = 7.4–12.7, $P < 0.001$), more likely to be stunted or underweight (stunted 41% vs 5%, $P < 0.001$, underweight 45% vs 5%, $P < .001$) and have diarrheal episodes 3 months prior to enrolment (11% vs. 3%, $P = 0.03$) compared to HIV uninfected participants. 89% of participants with HIV were taking co-trimoxazole prophylaxis as recommended in WHO guidelines.

For alpha diversity, a significantly higher richness was observed in HIV uninfected participants compared to participants with HIV. There was no difference in Shannon index (Figure 8). The difference remained significant in linear regression analysis, adjusted for BMI, age and sex ($P = 0.02$ for OTUs, $P = 0.001$ for Chao1). The use of antibiotics prior to enrolment did not change the significant effect of HIV infection on richness estimates. There was no difference in alpha diversity in participants with HIV, with or without CLD. Participants with HIV and suppressed VL (< 1000 copies/ml) had a tendency towards higher richness compared to virally unsuppressed participants (OTUs median 192.5 [IQR, 145.5-228.5] vs. 176 [IQR, 138-220], $P = 0.18$; Chao1 median 259.3 [IQR, 201.2-302.1] vs 233.2 [IQR, 174-276], $P = 0.05$). $CD4^+$ T-cell count had no effect on alpha diversity.

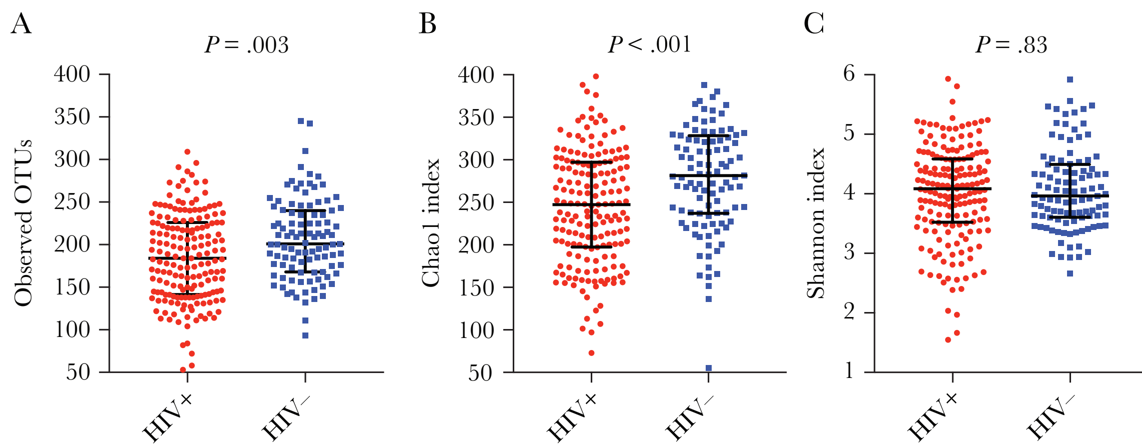


Figure 8. Box plot of alpha diversity indices in HIV infected and HIV uninfected participants. Midline shows median and error bars IQR. P-value obtained using Wilcoxon rank sum test.
OTUs, operational taxonomic units; IQR, interquartile range.

When looking at participants with HIV and the duration of ART, we split the participants into three groups based on duration of ART (< 5 years [N = 53]; 5-10 years [N = 100]; ≥ 10 years [N = 23]). Participants who had been longer time on ART had a richer gut microbiota, and those on ART for ≥ 10 years had an alpha diversity similar to HIV uninfected participants (Table 1). This association remained significant in linear regression analysis adjusted for age, sex and BMI.

Table 1. Alpha diversity in participants with HIV, stratified by years on ART, and HIV uninfected participants.

Alpha diversity indices	HIV+, <5 Years on ART (N = 53) [Median (IQR)]	HIV+, 5–10 Years on ART (N = 100) [Median (IQR)]	HIV+, ≥10 Years on ART (N = 23) [Median (IQR)]	HIV− Group (N = 103) [Median (IQR)]	HIV+, <5 Years on ART vs HIV− (*P* Values*)	HIV+, 5–10 Years on ART vs HIV− (*P* Values*)	HIV+, ≥10 Years on ART vs HIV− (*P* Values*)
Observed OTUs	176 (138–214)	186.5 (143–223.5)	204 (162–242)	201 (168–240)	.001	.10	.28
Chao1	229.4 (175.0–277.9)	249.6 (200.2–299.6)	268.9 (224.4–306)	281.3 (237.2–328.4)	<.001	.02	.08
Shannon index	4.03 (3.48–4.39)	4.12 (3.52–4.58)	4.23 (3.82–4.84)	4.0 (3.6–4.5)	.20	.75	.86

ART, antiretroviral therapy; IQR interquartile range; OTUs, operational taxonomic units.

Participants with HIV had significantly higher beta diversity, by Bray-Curtis dissimilarity, compared to HIV uninfected ($P < 0.01$) (Figure 9). Participants with HCLD had higher beta diversity compared to both participants with HIV without CLD ($P = 0.03$) and HIV negative participants ($P < 0.01$). There was no association between beta diversity and VL, CD4$^+$ T-cell count or duration of ART. Unweighted UniFrac analysis showed similar results.

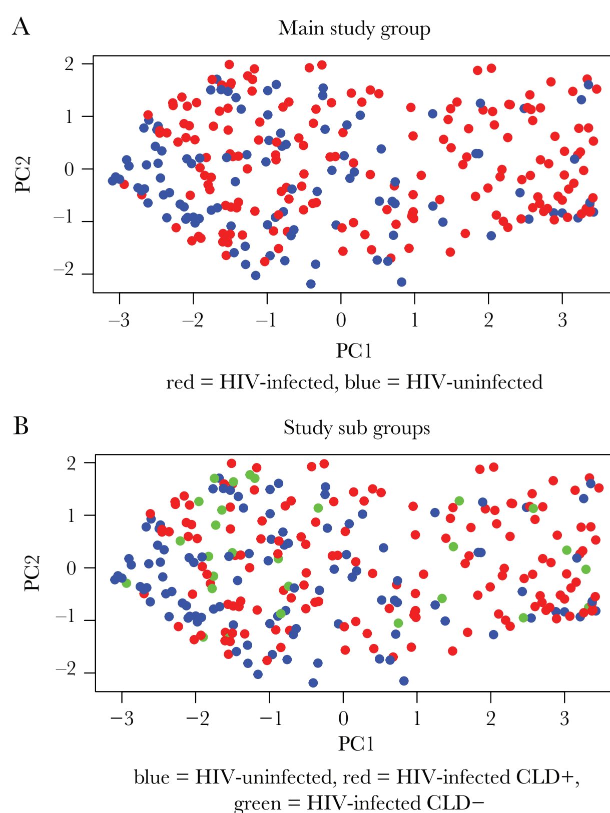


Figure 9. Principal coordinate plot showing beta diversity comparison between study groups using Bray-Curtis dissimilarity. (A) comparing participants with and without HIV, (B) comparing participants with HIV with and without CLD and HIV uninfected.

99.8% of the bacteria present in the samples consisted of the phyla Firmicutes (Bacillota) [43.9 %], Bacteroidetes (Bacteroidota) [33.9 %], Epsilonbacteraeota (Campylobacterota) [9 %], Proteobacteria (Pseudomonadota) [7.7 %] and Actinobacteria (Actinomycetota) [5.3 %]. At phylum level participants with HIV had lower abundance of Epsilonbacteraeota (Campylobacterota) [7 vs. 13 %, $P < 0.01$], and Bacteroidetes (Bacteroidota) [32 vs. 38 %, $P < 0.01$] compared to HIV uninfected participants. At genus level participants with HIV, compared to HIV uninfected participants, had enriched *Corynebacterium* ($P < 0.01$), *Lawsonella* ($P < 0.01$) and *Collinsella* ($P = 0.04$), from Actinobacteria (Actinomycetota) phylum and *Finegoldia* ($P < 0.01$), *Anaerococcus* ($P < 0.01$), *Erysipelotrichaceae* ($P = 0.02$) and *Lachnoclostridium* ($P = 0.04$) from Firmicutes (Bacillota) phylum. HIV uninfected participants, compared to participants with HIV, had enriched *Campylobacter* ($P < 0.01$), phylum Epsilonbacteraeota (Campylobacterota), *Porphyromonas* ($P < 0.01$) and *Prevotella* ($P = 0.03$), phylum Bacteroidetes (Bacteroidota) and *Ruminococcaceae* ($P < 0.01$), *Fastidiospila* ($P < 0.01$),

Fournierella (*P* < 0.01), *W5053* (*P* < 0.01), *Eubacterium coprostanoligenes_group* (*P* < 0.01), *Murdochiella* (*P* < 0.01) and *Coprococcus* (*P* = 0.02), phylum Firmicutes (Bacillota) (Figure 10).

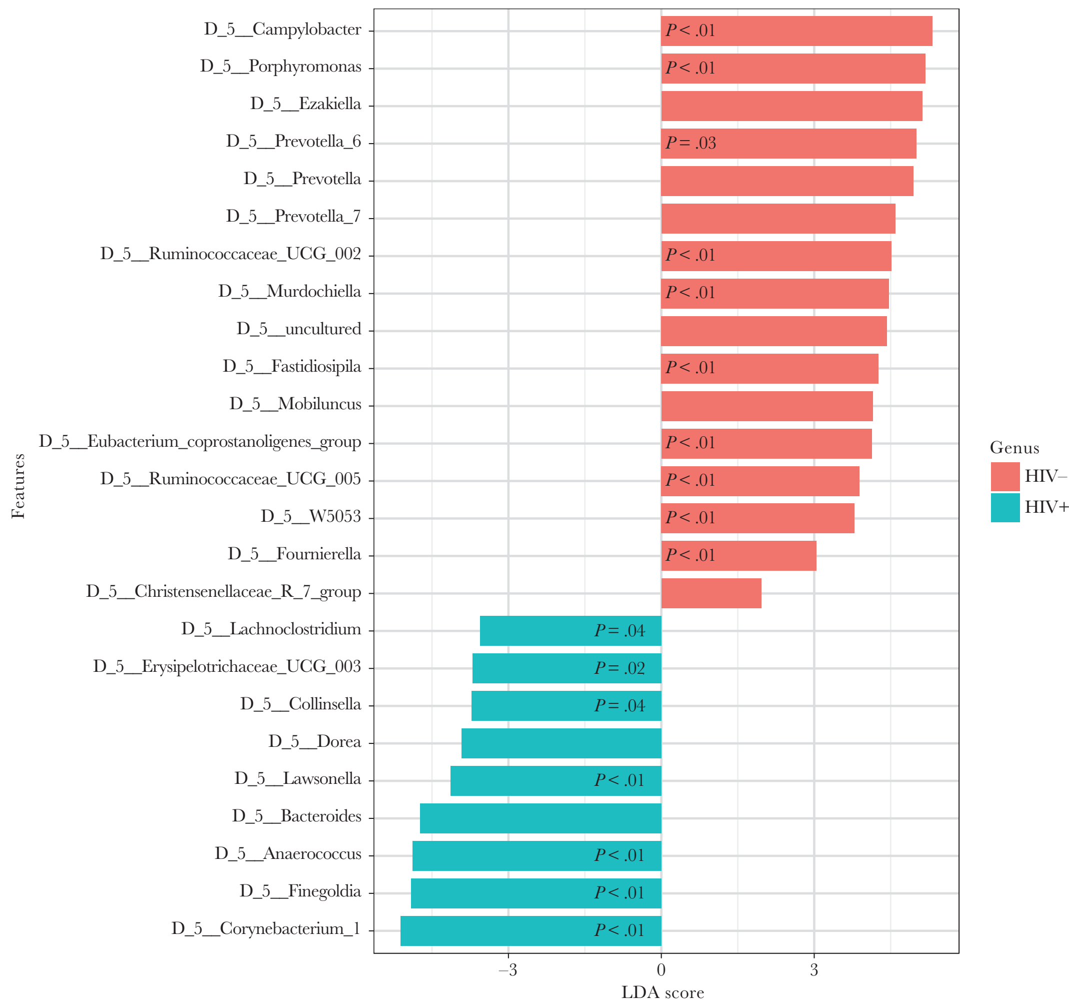


Figure 10. LEfSe plot showing enriched taxa at genus level significantly different between participants with HIV (blue) and HIV uninfected individuals (red). Only taxa meeting a significant level of 0.05 and effect size threshold of 1.0 are shown. P-values are adjusted for FDR.
LEfSe, linear discriminant effect size; FDR, false discovery rate.

In participants with HIV, those with CLD had higher abundance of the genus *Faecalibacterium* (*P* = 0.05), phylum Firmicutes (Bacillota) compared to participants without CLD. Participants with no CLD compared to participants with CLD had higher abundance of *W5053* (*P* < 0.01),

phylm Firmicutes (Bacillota) and *Prevotella* (P = 0.05), phylum Bacteroidetes (Bacteroidota). There were no significant differences between participants being virally suppressed (VL < 1000 copies/ml) and not. Participants with $CD4^+$ T-cell count ≤ 400 cells/mm^3 had higher abundance of Enterobacterioaceae (P = 0.02) and Burkholderiaceae (P = 0.04) at family level, and those with $CD4^+$ T-cell count > 400 cells/mm^3 had enriched Succinivibrionaceae. Finally, the longer participants had been on ART, the fewer taxa were significantly different when comparing participants with and without HIV. Genera such as *Bacteroides*, *Prevotella*, *Porphyromonas*, *Blautia*, and *Roseburia* were similarly abundant in HIV uninfected participants and participants with HIV who had been on ART ≥10 years.

2 Paper II

173 participants with HCLD were included in this study. One participant had missing eNO at baseline and was therefore excluded. 86 participants were from the placebo group, 19 of whom were LTFU, withdrew or did not provide eNO at 48 weeks, leaving 67 with follow-up samples. 86 participants were from the azithromycin group, of whom 15 were LTFU, withdrew or did not provide eNO at 48 weeks, leaving 71 with follow-up samples. There were no significant differences in baseline characteristics between placebo and azithromycin group.

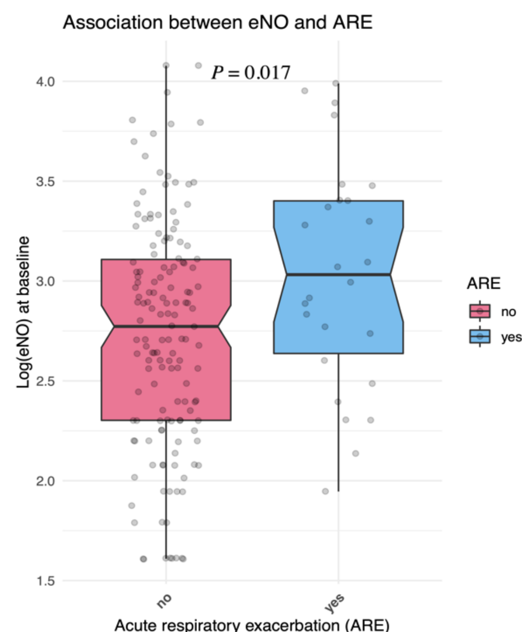


Figure 11. Box plot of log transformed eNO at baseline, by experience of at least one ARE during study period. P-value obtained by T-test. *eNO, exhaled nitric oxide; ARE, acute respiratory exacerbations.*

Participants that experienced at least one ARE during the study period had significantly higher levels of eNO at baseline (geometric mean 20.8, 95% CI [16.5-26.1]), compared to those who did not experience ARE (geometric mean 15.4, 95% CI [14.0-16.9]) (P = 0.017) (Figure 11). The difference remained significant in both adjusted linear regression (geometric mean ratio 1.35,

95% CI [1.07-17], $P = 0.013$), and in adjusted GLM (geometric mean ratio 1.13, 95% CI [1.03-1.24], $P = 0.015$).

MMP-3 ($P = 0.02$), MMP-7 ($P = 0.02$) and MMP-10 ($P < 0.01$) were significantly associated with higher levels of eNO at baseline in multivariable linear regression adjusted for age, sex and history of TB. Surprisingly, IFN-γ was associated with lower levels of eNO at baseline ($P = 0.04$). There were no other biomarkers significantly associated with levels of eNO. Age was, as expected, associated with higher levels of eNO at baseline ($P = 0.03$). History of TB was associated with lower levels of eNO ($P < 0.01$), as previously described in the same cohort (118). We could not find evidence to suggest that azithromycin affects levels of eNO after 48 weeks of treatment, by GLM (geometric mean ratio 0.86, 95% CI [0.72-1.03], $P = 0.103$).

3 Paper III

In total 347 participants were recruited into the BREATHE trial. One participant had missing rectal swab at baseline, and was therefore excluded, leaving 346 participants eligible for this study. 241 participants from Zimbabwe and 105 participants from Malawi. 172 participants were randomized to azithromycin and 174 to placebo. 73.3 % of participants in azithromycin group and 67.8% in placebo group completed the 72 week follow-up.

There were no statistically significant differences in alpha diversity between study groups or study sites at baseline. Total bacterial load (16S rRNA gene copy number) was higher in samples from Zimbabwean participants compared to Malawian at all three timepoints. Both observed ASVs (coefficient -23.98 [95% CI -37.81 – -10.15], $P < 0.001$), Chao1 index (coefficient -25.64 [95% CI -38.67 – -11.61], $P < 0.001$) and Shannon index (coefficient -0.21 [95% CI -0.35 – -0.07], $P = 0.004$) were significantly lower in the azithromycin group at 48 weeks in linear mixed models (Figure 12, Table 2). At 72 weeks, 6 months after cessation of study drug, there was still a tendency towards lower richness in the azithromycin group; observed ASVs (coefficient -7.03 [95% CI -21.98 – 7.92], $P = 0.37$), Chao1 index (coefficient

-6.90 [95% CI -22.15 – 8.34], *P* = 0.39) and Shannon index (coefficient -0.09 [95% CI -0.25 – 0.07], *P* = 0.30). However, this difference was not statistically significant.

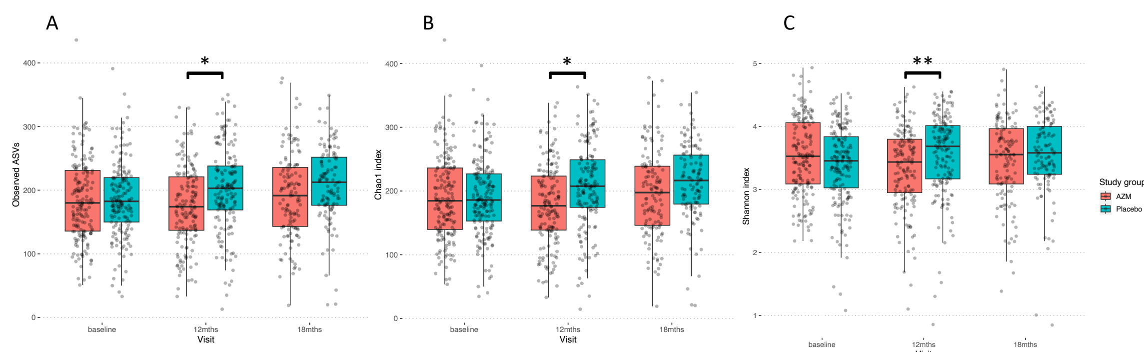


Figure 12. Box plot showing alpha diversity indices compared between azithromycin (AZM) and placebo group at baseline, 12 months (48 weeks) and 18 months (72 weeks).
A: Observed ASVs, B: Chao1 index, C: Shannon index. *P < 0.001, **P = 0.004.

Table 2. Alpha diversity indices. Comparisons between study groups at baseline, after 48 weeks and after 72 weeks using linear mixed models.

Alpha diversity	Total (N = 895)								
	Baseline (N = 346)			48 weeks (N = 313)			72 weeks (N = 236)[+]		
	Azithromycin (N = 172)	Placebo (N = 174)	P-value*	Azithromycin (N = 156)	Placebo (N = 157)	P-value*	Azithromycin (N = 120)	Placebo (N = 116)	P-value*
	Coefficient (95% CI)			Coefficient (95% CI)			Coefficient (95% CI)		
Observed ASVs	0.36 (-12.67, 13.39)	Ref.	0.958	-23.98 (-37.81, -10.15)	Ref.	<0.001	-7.03 (-21.98, 7.92)	Ref.	0.370
Chao1	0.07 (-13.19, 13.34)	Ref.	0.991	-25.64 (-38.67, -11.61)	Ref.	<0.001	-6.90 (-22.15, 8.34)	Ref.	0.388
Shannon	0.13 (-0.01, 0.26)	Ref.	0.056	-0.21 (-0.35, -0.07)	Ref.	0.004	-0.09 (-0.25, 0.07)	Ref.	0.299
Total bacterial load (16S copy-number, log-transformed)	-0.13 (-0.55, 0.29)	Ref.	0.547	-0.70 (-1.17, -0.23)	Ref.	0.004	0.08 (-0.41, 0.56)	Ref.	0.745

Fixed effects: Age at enrolment, sex, ever being treated for TB before enrolment, duration of ART by group, use of cotrimoxazole, season of sampling, being stunted (HAZ < -2.0) and baseline/48 week values of the relevant index tested. Random effect: Study site (Zimbabwe/Malawi).
**Analysis performed using lmer function from the lme4 package in R version 4.2.2. P-values obtained from the afex package.*
[+]8 samples at 72 weeks had missing values from 48 weeks and were therefore excluded from analysis

98.9% of the bacteria present in the samples consisted of the phyla Firmicutes (Bacillota) [50.3%], Bacteroidetes (Bacteroidota) [32.8 %], Actinobacteria (Actinomycetota) [6.0 %], Proteobacteria (Pseudomonadota) [5.2 %], Epsilonbacteraeota (Campylobacterota) [4.3 %] and Fusobacteria (Fusobacteriota) [1.0 %]. There were no significant differences between study groups at baseline. Significant differences in relative abundance between azithromycin and placebo group after 48 weeks of treatment is shown in Table 3.

Table 3. Differentially abundant taxa between azithromycin and placebo group at 48 weeks

Phylum	Genus	Effect size*	Difference (Between)	Difference (Within)	P-value**
Proteobacteria	Parasutterella	-0.44	-3.17	6.54	<0.001
Firmicutes	Dorea	-0.26	-1.03	3.05	<0.001
Firmicutes	Blautia	-0.25	-0.92	2.93	<0.001
Firmicutes	Flavonifractor	-0.24	-1.42	5.61	0.016
Firmicutes	Anaerostipes	-0.23	-1.45	5.48	0.010
Actinobacteriota	Eggerthella	-0.22	-1.33	5.34	0.020
Firmicutes	Fusicatenibacter	-0.22	-1.18	4.48	0.005
Firmicutes	Lachnoclostridium	-0.22	-1.03	4.03	0.002
Firmicutes	Lachnospiraceae UCG-004	-0.18	-0.87	4.00	0.012
Firmicutes	Coprococcus	-0.17	-0.86	4.24	0.018
Firmicutes	[Eubacterium] hallii group	-0.14	-0.71	4.13	0.045
Fusobacteriota	Fusobacterium	0.21	1.56	7.09	0.026
Proteobacteria	Escherichia-Shigella	0.21	1.57	7.03	0.020
Firmicutes	Fenollaria	0.22	1.91	7.98	0.023
Bacteroidota	Porphyromonas	0.22	1.64	6.93	0.015
Cyanobacteria	Gastranaerophilales (Order)	0.22	1.33	5.57	0.028
Firmicutes	Clostridium sensu stricto 1	0.22	1.43	5.81	0.015
Firmicutes	Christensenellaceae R-7 group	0.23	1.38	5.50	0.008
Firmicutes	Fastidiosipila	0.25	1.84	6.84	0.011
Firmicutes	Mitsuokella	0.27	1.72	5.87	0.008
Firmicutes	Bacilli_RF39 (Order)	0.33	2.05	5.60	<0.001
Desulfobacterota	Desulfovibrio	0.34	2.19	5.72	<0.001
Actinobacteriota	Bifidobacterium	0.36	2.71	7.12	<0.001
Firmicutes	Negativicoccus	0.36	2.72	7.02	<0.001
Firmicutes	Clostridia UCG-014 (Order)	0.57	3.37	5.42	<0.001
Campylobacterota	Campylobacter	0.77	5.72	6.87	<0.001
Proteobacteria	Sutterella	0.80	5.22	5.72	<0.001

** Negative effect size indicates higher abundance in azithromycin group and positive effect size indicates higher abundance in the placebo group.*

*** Wilcoxon test with FDR set to 0.05 using the Benjamini-Hochberg method.*

Genera differentially abundant between azithromycin and placebo group at 48 weeks were highly concordant with those differentially abundant between baseline and 48 weeks within the azithromycin group in Zimbabwean participants (22 of the 25 genera, all with the same direction). At 72 weeks, 5 genera remained significantly different between the azithromycin and placebo group; *Lachnospiraceae UCG-004* (effect size -0.21, P = 0.031) Firmicutes (Bacillota) phylum, and *Parasutterella* (effect size -0.47, P < 0.001), Proteobacteria (Pseudomonadota) phylum were enriched in the azithromycin group. Whereas *Sutterella* (effect size 0.51, P < 0.001), Proteobacteria (Pseudomonadota) phylum, *Campylobacter* (effect size 0.35, P < 0.001), Epsilonbacteraeota (Campylobacterota) phylum and *Fastidiosipila* (effect size 0.30, P = 0.033), Firmicutes (Bacillota) phylum were depleted in the azithromycin group.

In the azithromycin group, 4 genera were significantly enriched at 72 weeks compared to 48 weeks (Supplementary table 6); *Escherichia-Shigella* (effect size 0.26, P = 0.028), Proteobacteria (Pseudomonadota) phylum, *Negativicoccus* (effect size 0.28, P = 0.018) and Clostridia UCG-014 (Order) (effect size 0.34, P = 0.006), Firmicutes (Bacillota) phylum and *Campylobacter* (effect size 0.45, P < 0.001), Epsilonbacteraeota (Campylobacterota) phylum. There were no significant differences in relative abundance between 48 and 72 weeks in the placebo group.

Discussion

1 Main results

In **Paper I** we showed that gut microbiota in children with HIV on ART was less diverse than in children without HIV. Children who had been taking ART for 10 years or more, had a more diverse microbiota resembling that of children without HIV. Our results suggested that prolonged ART may minimize differences in gut microbiota between children with and without HIV (195). Our findings were supported by a number of other studies in adults showing reduced alpha diversity and increased beta diversity (194, 196, 279), and that these changes could persist despite ART (5, 6, 10, 38, 39). Previous studies have found similar alpha diversity profiles in people with HIV on ART and people without HIV (188, 194). Lozupone *et al.* also found that individuals with longer ART duration showed closer resemblance to individuals without HIV than to subjects with untreated HIV infection (191). These studies support our findings that long-term ART may help restore an HIV-associated dysbiotic gut microbiota and has later been confirmed in newer publications (280-282). Other studies have found a negative impact of ART on gut microbiota diversity (196, 283). However, this was investigating the effect of ART initiation, with a short follow up period (196). Our study is one of the largest investigating the gut microbiota and HIV. We had only participants on ART, with a minimum duration of 1 year, and alpha diversity was lower in those on ART for less than 10 years compared to participants without HIV. Still, the impact of ART on gut microbiota is poorly understood and further studies with longitudinal follow-up are needed to fully understand the effects of ART on gut microbiota in HIV.

In **Paper III** we found alpha diversity, both richness and evenness, to be significantly lower at 48 weeks in participants treated with azithromycin compared to those receiving placebo. This is in line with previous studies investigating the effects of macrolide antibiotic exposure in both children and adults (227-229, 231, 232). In our study, alpha diversity measures almost returned to baseline levels 6 months after cessation of antibiotic therapy. This is supported by Wei *et al.* who observed no long-term effects of azithromycin on alpha or beta diversity or on relative abundance after a 3-day course of azithromycin compared to placebo for acute asthmatic

exacerbations in young children (231). Another study did however observe a long-term reduction in richness that remained significantly lower up to two years after macrolide exposure (227). A meta-analysis of antibiotic exposure and gut microbiota in children found macrolides to be associated with reduced alpha diversity and that macrolides reduced richness for twice as long as penicillin (228).

Oldenburg *et al.* found azithromycin to significantly reduce alpha diversity, whereas placebo, amoxicillin and cotrimoxazole had no effect on alpha diversity of the gut microbiota (229). Several other studies underline this, showing no difference in alpha diversity in people with HIV taking cotrimoxazole or not (198, 202, 284). This is in line with our findings in **Paper I**, where we found no significant effect of cotrimoxazole on the gut microbiota (195), and the effect of azithromycin shown in **Paper III**. However, since approximately 90% of participants with HIV received cotrimoxazole prophylaxis as per WHO guidelines (47), it is not possible to completely tease apart the effect of HIV from that of cotrimoxazole. Although, recent evidence suggests cotrimoxazole does not affect global gut microbial composition, but rather specific inflammatory pathways in people with HIV (285).

Data on relative abundance are more conflicting. HIV infected, ART treated adults in Nigeria had higher abundance of *Finegoldia* and *Anaerococcus* (283), consistent with our findings in **Paper I**. However, in the same study *Campylobacter* was significantly enriched in participants with HIV, whereas we found enriched *Campylobacter* in participants without HIV. In **Paper III** we found relative abundance of *Campylobacter* to be significantly reduced after 48 weeks of azithromycin treatment compared to placebo. The reduced abundance of *Campylobacter* was still present at 72 weeks, 6 months after ended treatment. A large trial of mass distribution of twice-yearly azithromycin for trachoma control and childhood mortality in Niger, Tanzania, and Malawi by Doan *et al.*, also showed a significant reduction in *Campylobacter* among those treated with azithromycin in a subset of participants from Niger (286, 287). The same was found in another study by Parker *et al.* in India (232). *Campylobacter* is known to cause diarrheal disease among children, and azithromycin is characterized as first-line treatment (288). Further, Doan *et al.* found macrolide resistance to be 7.5 times higher in participants that had received

azithromycin compared to those receiving placebo and remained higher 6 months after the last administration of azithromycin (289). In addition, azithromycin treatment caused an increase in non-macrolide resistance, including beta-lactams, aminoglycosides, trimethoprim, and metronidazole (289). Colleagues have previously demonstrated an increase in azithromycin, tetracycline, and clindamycin resistance in *Staphylococcus aureus* from sputum samples, persisting 6 months after ended azithromycin treatment in BREATHE participants (234). The increase in resistance is of concern for future treatment of gastrointestinal *Campylobacter* infections, as the rate of fluoroquinolone resistance among *Campylobacter* species already are high and macrolide resistance is increasing (290). This could in turn lead to reduced treatment options and increased morbidity and mortality of *Campylobacter* infections in the future.

Korpela *et al.* found macrolide exposure to lead to a significant increase in *Eggerthella*, *Blautia* and *Dorea,* and a decrease in *Bifidobacterium* (227). Choo *et al.* also found *Bifidobacterium* to be depleted and an increase in *Eggerthella* and *Coprococcus* following both azithromycin and erythromycin exposure among healthy adults (291). This was in line with our results from **Paper III**. Wei *et al.* also found a decrease in *Bifidobacterium* following azithromycin treatment but observed no long-term effects on relative abundance (231). Korpela *et al.* found a decrease in *Anaerostipes* that remained decreased for up to two years after macrolide exposure, whereas we found *Anaerostipes* to be increased after 48 weeks of azithromycin treatment. The difference in *Anaerostipes* between azithromycin and placebo group disappeared at 72 weeks, 6 months after ended treatment. In line with Doan *et al.*, an increase in macrolide resistance was also observed by Korpela *et al.*, that declined to baseline levels 6-12 months after ended treatment (227). A systematic review found the macrolide Clarithromycin to cause a transient reduction in *Enterobacteria* and *Clostridium*, whereas *Lactobacilli* and *Bifidobacteria* were depleted for a longer time (220). In **Paper III**, the decrease in *Bifidobacterium* was transient, and the difference between azithromycin and placebo group disappeared at 72 weeks. *Bifidobacterium* is characterized as a probiotic, with beneficial traits, including maintaining microbial balance and inhibiting growth of potential pathogens (241). It is most likely highly susceptible to macrolides, which can explain the depletion (241). *Eggerthella* has been associated with an increased risk of autoimmune diseases such as ulcerative colitis and an increase in gut microbial translocation (292). *Blautia* and *Dorea*

have been associated with IBD, metabolic disease and multiple sclerosis and can be proinflammatory, leading to increased chronic inflammation (293, 294). This reduction in protective commensals and enrichment of proinflammatory taxa seen after azithromycin treatment could possibly increase systemic immune activation. The effect of gut microbial dysbiosis on immune activation and chronic inflammation, and what implications this can have on HIV and progression of HCLD needs further investigation.

Proteobacteria has been shown to be enriched in ART naive people with HIV (192, 196, 203), but only one study showed similar findings in ART treated individuals (188). We found enriched Proteobacteria in participants with HIV in **Paper I**, but this was not statistically significant. Other studies have found a significantly lower microbiome diversity in those with more severe HIV status (196, 204, 295). In our participants we could not observe an association between gut microbiota and virological or immunological markers (HIV VL and $CD4^+$ T-cell count). We did find that relative abundance of *Enterobacteriacea* and *Burkholderiaceae* was enriched in those with low $CD4^+$ T-cell counts (≤400 cells/mm^3) in **Paper I**. *Enterobacteriaceae* may cause gastrointestinal and urinary tract infections in HIV infected children (295) and *Burkholderiaceae* includes species known to cause severe lung infections in patients with cystic fibrosis (296). This indicates that poor immune status could be related to pathogen bacteria. However, clinical significance is unclear, and causality cannot be proven. Findings of previous studies may have been affected by sample size and ART duration. A longitudinal study with repeated measurements of VL, $CD4^+$ T-cell count, and microbiome profiles is needed to uncover the relationship between these parameters.

Evidence suggests gut microbiota to be involved in maintaining lung health, and that alterations in gut microbiota can be observed in patients with lung diseases (183, 185, 187). In addition, a dysbiotic gut microbiota, and microbial translocation can lead to chronic inflammation and increase the risk of non-infectious HIV-related complications, including heart and lung disease (183, 201, 297, 298). In **Paper I** we could not observe any difference in alpha diversity between participants with and without HCLD. We did however find *Faecalibacterium* to be enriched in

participants with HCLD. *Faecalibacterium* has been regarded as a protective commensal, and depletion have been linked to the development of IBD and asthma, and low levels have been shown in patients with cystic fibrosis (187, 299). However, increased levels of the species *Faecalibacterium prausnitzii* have been shown in the gut of children with untreated Crohn's disease (300), and another study showed increased levels of *Faecalibacterium* in the gut microbiota of patients with active TB (301). In our participants from **Paper I**, 32 % had been treated for TB prior to enrolment. The interplay between HIV infection, gut microbiota, and chronic lung disease is complicated and the results published this far are contradicting. This may be explained by difference in age, geographic location, sample size, comorbidities and study intervention and length of follow-up between studies.

In **Paper II**, we found that participants who experienced at least one ARE during the study period, had higher levels of eNO (272). We know that levels of eNO are elevated during asthmatic exacerbations (112), but evidence are limited and conflicting with regards to other chronic lung diseases. In COPD, one study showed elevated levels of eNO during an episode of exacerbation, where those with viral infection had the highest increase in eNO (302). This was supported by a meta-analysis, although they could not prove a statistically significant increase (303). However, another study found levels of eNO to be increased only in participants with asthmatic exacerbations (304). Another study of annual measurements of eNO in people with severe asthma, showed that higher levels of eNO and large variation in eNO was associated with developing ARE (305). Together this supports that increased airway inflammation is an important factor in ARE, and the potential role of eNO in monitoring disease and in prediction of ARE. However, the evidence to support use of eNO as a risk factor for ARE in other diseases than asthma is limited. Our results suggest that the use of eNO in children with HCLD could be of interest, but further studies are needed.

In **Paper II** we hypothesised that azithromycin would lead to lower eNO-levels, because of its antimicrobial and immunomodulatory properties. However, we did not find any effect of azithromycin treatment on levels of eNO after 48 weeks of treatment (272). Previous studies on azithromycin treatment and levels of eNO are conflicting. In line with our results, one study

in participants with non-CF bronchiectasis and another on treatment-resistant cough, found no effect of azithromycin on levels of eNO (151, 152). Other studies in participants with asthma have shown a reduction in levels of eNO following azithromycin and clarithromycin treatment (150, 153). However, both these studies were done in participants with asthmatic disease, which is characterized by type 2 inflammation and eosinophilic activity, that eNO is most strongly linked to (306). In addition, they also included treatment with corticosteroids, which are highly potent immunosuppressants, in addition to macrolides. Further investigation on the use of corticosteroids for improvement of lung function and reduced inflammation in HCLD could be of interest. However, it warrants special caution, due to the increased risk of infection, especially in a virally non-suppressed population. In addition, comparisons are difficult due to the difference in pathogenesis between CLDs. Our participants had HCLD, and few had a history of asthma or atopic disease.

HCLD is thought to be a result of both local and systemic inflammation. Elevated systemic levels of the pro-inflammatory cytokine IFN-γ have been shown to be associated with having HCLD, but was not associated with reduced lung function in participants from the BREATHE trial (126). Previous studies have shown that IFN-γ induces NO-production through activation of iNOS in macrophages (307). In **Paper II**, we did however find a negative association between IFN-γ and eNO in children with HCLD (272). Previous studies have shown that lower levels of IFN-γ were associated with increased risk of pulmonary fibrosis following COVID-19 infection (308). Other studies have found lung tissue injury promoted by IFN-γ-mediated release of MMPs to be linked to the development of COPD (309), and that IFN-γ-producing $CD8^+$ T-cells correlated positively with levels of eNO in participants with COPD (310). With our participants most likely having bronchiectasis or OB, one could think that this negative association of IFN-γ on eNO could be explained by the fibrotic component of HCLD. However, this is speculative, and evidence is lacking. eNO could potentially be used as a marker of lung injury in COPD, but the exact signalling pathway and role of IFN-γ in lung tissue injury and development of HCLD need further investigation.

Further, we found a positive association between MMP-3, -7 and -10 and levels of eNO in **Paper II**. Levels of MMPs in bronchoalveolar lavage and blood samples of patients with COPD and pulmonary fibrosis have been described as elevated (129, 311, 312). This may indicate that MMPs could have a mediating role in the development of CLD. MMP-3, -7 and -10 were found to be elevated in adults with COPD and were associated with radiological markers of small airway disease and severity of emphysema (311). MMP-10 was most strongly linked to emphysema, and in **Paper II**, MMP-10 had the strongest association to elevated levels of eNO, suggesting that these participants may be at higher risk of CLD progression in the future. Previous studies found MMP-3 and MMP-7 to be associated with development of lung fibrosis through increasing levels of profibrotic mediators (129). MMP-7 has been significantly associated with mortality and disease progression of pulmonary fibrosis and has in fact been suggested as a biomarker for progression and disease activity in idiopathic pulmonary fibrosis (312). This highlights the potential role of MMPs in airway remodelling, an important part in the pathogenesis of COPD, lung fibrosis and HCLD (311, 313). Our findings of an association between these MMPs and higher levels of eNO in **Paper II** can indicate higher levels of local inflammation in participants with a higher degree of airway remodelling.

2 Methodical and ethical considerations

2.1 Study designs

The work of this thesis is comprised of three studies, all performed in children and adolescents with HIV and chronic lung disease in sub-Saharan Africa. When selecting the study population, selection bias can become an issue. Selection bias occurs when the inclusion criteria causes the group studied to be systematically different from those excluded or from the general population (314). The thesis is based on data from a large randomized, placebo-controlled trial, BREATHE, with some differences in study design between the three papers. The participants were recruited from central HIV outpatient clinics in Harare, Zimbabwe and Blantyre, Malawi and had a demographic profile in accordance with the HIV profile of the country, reducing the risk of selection bias (148).

Paper I was a cross-sectional study. This design allows estimation of prevalence and to study associations between multiple outcomes. However, data on both outcome/disease and exposure are collected at the same time, and therefore does not allow for conclusions on causality, meaning you cannot differentiate between cause and effect (315). The exploratory nature of cross-sectional studies allows for screening of hypothesis and can be applied to a range of different populations and outcomes. However, as exposure and outcome are measured at the same time, it can lead to inclusion bias as participants who develop an outcome and for instance dies before the study is performed will not be included (316). Associations observed in observatory studies can often be used to generate hypothesis and research questions for longitudinal cohort studies or randomized controlled trials (315). The exploratory nature and use of secondary data in **Paper I** made a cross-sectional design most fitting.

Data from a randomized controlled trial was the background for **Paper II** and **Paper III**. Randomized controlled trials are considered the gold standard within research for determining the efficacy of a specified treatment (317). This study design allows for a controlled environment with highly specified participants to investigate the question in hand, usually the

effect of a treatment. The inclusion and exclusion criteria of participants allows to reduce confounding factors and randomization minimizes the differences between study groups. However, strict inclusion criteria could lead to low generalizability of the results (318). When the study is blinded, this prevents both participants and researchers to influence outcomes based on what treatment they are receiving. On the other hand, randomized controlled trials are both expensive and time consuming, and for some diseases or treatments the experimental design could be unethical. As an example, it would be highly unethical today to administer placebo to people with HIV instead of ART, if you wanted to investigate the effect of a new HIV drug, although this has been done earlier (319). This is because we now know that ART prevents development of AIDS and reduces the rate of transmission of HIV.

It is also preferred that a defined hypothesis or end point of the study is defined a priori. This is so that statistical power can be calculated for the effect size hypothesized (the difference between groups one tries to detect) to reduce the risk of type II error (318). Power calculations are however rare in microbiome studies. This is partially due to challenges of calculating proper effect sizes for statistical power analysis because of the compositional nature of microbiome data, and that the clinically relevant difference in variables is currently hard to define (320, 321). It was therefore not done any power calculations for the microbiome studies in **Paper I** and **Paper III**. However, the sample sizes in our studies are large when comparing to other microbiome studies to date. For the primary outcome of the BREATHE trial a power analysis was performed and showed that 300 participants were required to detect a 0.32 SD change in FEV_1 with 80% statistical power at a $P < 0.05$ (248). When doing secondary analysis from a randomized controlled trial the risk of spurious associations or results could be an issue due to multiple testing (322). However, in **Paper II** and **Paper III** we investigated different outcomes than in the primary study, and these hypotheses were also pre-defined in the study protocol, sampling was done accordingly, and p-values were adjusted for multiple testing.

2.2 Confounding

Finally, the role of confounding factors needs to be addressed properly for both study designs, especially for observational studies. This is to rule out other plausible explanations of the results, making sure this is not caused by spurious associations by other factors or variables (316, 323). This can be done by stratification or multivariate statistical analysis, where confounders are included in multivariate regression models (324). In **Paper I** age, sex and BMI were potential confounders and were adjusted for. In **Paper II** adjustments were made for age, sex, history of TB and baseline levels of eNO, and for investigating ARE, trial group was added to the model. In **Paper III** a mixed model was fitted, including study site as a random effect, and age, sex, history of TB, duration of ART by group, use of cotrimoxazole, season of sampling, being stunted (HAZ < -2.0) and baseline and 48-week values of the relevant index tested was set as fixed effects. In addition to these confounders, there are several other parameters that could be of relevance for gut microbiota and eNO that were not assessed in this study. In fact, evidence suggests a set of different covariates, including stool consistency (by Bristol stool chart), diet and medication needs to be accounted for in future microbiome studies (325). For **Paper I** and **Paper III** information about housing, diet, stool consistency and level of education could also impact gut microbiota. Genetic activity of eNO synthase and environmental factors such as air pollution could impact levels of eNO in **Paper II**.

2.3 Risks of intervention and safety of drug administration

The risks and potentially negative outcomes related to the study drug azithromycin in **Paper II** and **Paper III** were considered relatively low. Azithromycin is a broadly used, and well-studied antibiotic, with few severe side effects (144, 326). All participants were followed closely and controlled for known severe side effects such as prolonged QT-time, rashes and *Clostridium difficile* infection at each visit. Participants experiencing adverse events thought to be related to the study drug were excluded from further participation in the trial (248). All participants also continued their standard HIV care throughout the study period. However, the use of antibiotics presents a risk for developing resistance and dysbiosis, and therefore the sub-studies

on both gut microbiota and respiratory microbiome was performed to gain knowledge and estimate these effects.

2.4 Laboratory and data processing

For **Paper I** and **Paper III** rectal swabs were used to assess the gut microbiota. Rectal swabs are low biomass samples and may therefore not be optimal in profiling the gut microbiota. Although faecal samples generally are thought to be representative of the gut microbiota, studies have shown that composition and abundance of different bacteria vary along the GI tract, depending on the availability of oxygen, nutrients, antimicrobial peptides and physical features such as acidity (327). It is also proven that composition of the gut microbiota may even vary between locations within the same faecal sample, showing that reproducibility of especially low abundant microbes is not always possible to capture (328). However, the general opinion in microbiome research today is that faecal samples mirrors the gut microbiota well, until new and better methods for non-invasive sampling are implemented (329). Previous studies have shown that the use of rectal swabs yield good quality of data and can be comparable to solid stool samples for profiling and comparing the gut microbiota (330, 331).

16S rRNA sequencing of the V4 hypervariable region was used to assess the gut microbiota in **Paper I** and **Paper III**. The use of 16S sequencing has been the main method in assessing microbiome for years (332, 333). The field of microbiome research has developed rapidly over the last decades, with increased availability of sequencing methods. This is partially due to the decline in costs over time, reduced complexity of bioinformatic procedures and increased availability of good reference databases such as Greengenes (334) and SILVA (257, 335) for 16S (332, 336). However, there are several limitations to this method. Sequencing of sub-regions of the 16S rRNA gene gives limited resolution, and only allows for taxonomic comparisons down to the genus level (158, 333). With newer methods such as shotgun whole genome sequencing, the information you could get from the data are not only limited to descriptive taxonomy but includes functional genes and level of expression of these (336-338). In addition the sequencing depth allows for a higher resolution including taxonomic levels

down to not only species but even bacterial strain (338). Further, shotgun whole genome sequencing is found to be more sensitive to changes in the microbiome when looking at abundance of genera and for low abundant microbes in experimental conditions (336, 337). However, this is a relatively new method, where gene databases are still not developed to the degree that they are for 16S sequencing. In addition, the costs and resources needed for whole genome sequencing are still substantially higher compared to 16S, making its availability limited (337, 338).

Multiple methods of data collection and measurement were used in this thesis. The reliability of procedures needs to be good, and errors or poor accuracy in instruments or measures can lead to misclassifications and measurement bias (339). In **Paper II** eNO was measured using a NIOX VERO portable device. Measurements were done according to ATS guidelines (112), and with the same instrument for all participants. The gold standard for measurement of eNO is chemiluminescence, however, the method we used of electrochemical analysis have been shown to have better reproducibility and reliability compared to chemiluminescence (340, 341). For **Paper I**, **II** and **III** FEV_1 *Z* score was used instead of predicted values. This was due to its advantage in eliminating biases of height, age, sex and ethnicity by taking underlying distribution of lung function into account when classifying into categories of severity of lung impairment compared to predicted values (105). HIV VL was measured using Gene Xpert assay that have shown good precision on plasma samples and correlates well with reference assays (342, 343). Plasma soluble biomarkers in **Paper II** were measured using Luminex multiplex bead assay on a MagPix instrument. Evidence show good reproducibility and high comparability to other similar methods of detecting antibodies and biomarkers (344).

In **Paper II** we used log transformed values of eNO and some of the plasma soluble biomarkers for analysis. Log transforming the data to achieve approximate normality, makes the data more suitable for statistical tests where normality is needed. Back transforming into geometric means that can be used in analysis is a well-documented method, and gives both possibility for statistical testing that require normality, and serves as a better measure for central tendencies,

as the either very high or very low values of the tail of the data are given less value and thus the statistics are less likely to be distorted (345).

Finally, the use of questionnaires and self-reported information is a common approach for collecting information but can be a source of information and recall bias. It is known that self-reporting can be a less reliable source of information compared to medical records (339). We used questionnaires for **Paper I**, **II** and **III**. An introduction of recall bias could be an issue as almost all participants had chronic lung disease, defined by measurements of spirometry, and were asked about exposure or treatment of TB in the past. To minimize the risk of recall bias, medical records could have been obtained to verify previous treatment for TB, but this was not feasible in our studies.

2.5 External validity

External validity refers to the generalizability of results from a study, and whether these can be applied to the general population or a specific group of interest (346). Considering the limitation mentioned in this thesis, we do believe that our results can be generalised to children and adolescents with HIV in southern Africa. As mentioned, our population had a similar demographic profile as in the countries where they were recruited and the burden of HCLD in this region is high. We know that both gut microbiota and eNO are influenced by age, ethnicity, geography, and diet (347) which can further limit the generalizability to this geographic region.

Conclusions and future perspectives

The overall aim of the thesis was to investigate the composition of gut microbiota of HIV infected children and adolescents with HCLD, and to study the effects of azithromycin treatment on gut microbial composition and effect on lung inflammation in HCLD. It is previously described that azithromycin did not improve lung function of children and adolescents with HCLD, but that it reduced the rate of ARE (248). Our studies show that HIV alters gut microbiota, and that azithromycin significantly affects both diversity and composition of the gut microbiota in children with HCLD. Further, eNO, as a measure of local lung inflammation was associated with having ARE and proinflammatory biomarkers in blood in the same particpants.

In **Paper I** we demonstrated that HIV alters the gut microbiota but that prolonged use of ART can help restore the gut microbiota towards that of HIV uninfected. As expected, we showed in **Paper III** that antibiotic treatment had an effect on both diversity and composition of the gut microbiota. For future studies, new methods such as full length 16S rRNA gene sequencing or shotgun whole genome sequencing should be considered to capture not only taxonomic differences, but also functional microbiome characteristics and rates of antimicrobial resistance (333, 337). Further studies on the impact of gut microbial dysbiosis on the progression of HIV and HIV-related comorbidities are needed to better understand the pathogenesis and to investigate whether microbiome-based interventions can serve as treatment options for this group. Finally, the use of antibiotics for other purposes than necessary clinical interventions need to be well founded, as we don't fully know what implications a possible negative effect of antibiotic treatment on gut microbiota can lead to. In addition, it is important to reduce the risk of further dysbiosis and development of antibiotic resistance, especially in a population with HIV, as this group is prone to infections needing effective antibiotic treatment.

HCLD is associated with chronic inflammation in the airways and in **Paper II** we found that higher levels of eNO served as a risk factor for developing ARE. eNO was also associated with proinflammatory MMPs involved in pathogenesis of CLD and airway remodelling. We found

no effect of azithromycin on levels of eNO. It is unclear what role eNO can play in monitoring or management of chronic lung disease in this population group. Due to the high prevalence of chronic lung disease among children and adolescents with HIV in sub-Saharan Africa, it is however warranted that management and monitoring of lung function in this group is implemented as a part of routine HIV care to improve health and reduce risks of long-term complications. The methods for measuring eNO are easy and can be cost-effective, making it a potential supplement to today's HIV care in resource-limited settings when availability improves. Further studies are needed to investigate the potential role measurement of eNO can have in monitoring of HIV-associated chronic lung disease.

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Paper I

The Journal of Infectious Diseases

MAJOR ARTICLE

Composition of Gut Microbiota of Children and Adolescents With Perinatal Human Immunodeficiency Virus Infection Taking Antiretroviral Therapy in Zimbabwe

Trym T. Flygel,[1,2] Evgeniya Sovershaeva,[1,3] Shantelle Claassen-Weitz,[4] Erik Hjerde,[5] Kilaza S. Mwaikono,[6] Jon Ø Odland,[3,7] Rashida A. Ferrand,[8,9] Grace Mchugh,[8] Tore J. Gutteberg,[10] Mark P. Nicol,[4,11] Jorunn P. Cavanagh,[1,2] and Trond Flægstad[1,2]; and the BREATHE Study Team

[1]Paediatric Research Group, Department of Clinical Medicine, Faculty of Health Sciences, UiT - The Arctic University of Norway, Tromsø, Norway, [2]Department of Paediatrics, University Hospital of North Norway, Tromsø, Norway, [3]Department of Community Medicine, Faculty of Health Sciences, UiT- The Arctic University of Norway, Tromsø, Norway, [4]Division of Medical Microbiology, Department of Pathology, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa, [5]Department of Chemistry, Norstruct, UiT - The Arctic University of Norway, Tromsø, Norway, [6]Computational Biology Division, Department of Integrative Biomedical Sciences, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa, [7]Department of Public Health and Nursing, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology, Trondheim, Norway, [8]Biomedial Research and Training Institute, Harare, Zimbabwe, [9]Clinical Research Department, London School of Hygiene and Tropical Medicine, London, United Kingdom, [10]Department of Microbiology and Infection Control, University Hospital of North Norway, Tromsø, Norway, [11]Division of Infection and Immunity, School of Biomedical Sciences, University of Western Australia, Perth, Australia

Background. Human immunodeficiency virus (HIV) infection causes impairment of the gastrointestinal barrier, with substantial depletion of $CD4^+$ T cells in the gut. Antiretroviral therapy (ART) restores $CD4^+$ counts and may have beneficial effects on gut microbiota in adults. Little is known about effect of long-term ART on gut microbiome in HIV-infected children. We investigated composition of gut microbiota in HIV-infected and -uninfected children and assessed associations between gut microbiota and patient characteristics.

Methods. In a cross-sectional study, rectal swabs were collected from 177 HIV-infected and 103 HIV-uninfected controls. Gut microbial composition was explored using 16S ribosomal ribonucleic acid sequencing.

Results. Human immunodeficiency virus-infected children had significantly lower alpha-diversity and higher beta-diversity compared to HIV-uninfected. No association was observed between microbiome diversity and $CD4^+$ T-cell count, HIV viral load, or HIV-associated chronic lung disease. We found enriched levels of *Corynebacterium* ($P < .01$), *Finegoldia* ($P < .01$), and *Anaerococcus* ($P < .01$) in HIV-infected participants and enrichment of *Enterobacteriaceae* ($P = .02$) in participants with low $CD4^+$ counts (<400 cells/mm^3). Prolonged ART-treatment (≥10 years) was significantly associated with a richer gut microbiota by alpha diversity.

Conclusions. Human immunodeficiency virus-infected children have altered gut microbiota. Prolonged ART may restore the richness of the microbiota closer to that of HIV-uninfected children.

Keywords. Africa; antiretroviral therapy; children; gut microbiota; HIV infection.

The gastrointestinal (GI) tract plays an important role in the pathogenesis of human immunodeficiency virus (HIV) infection, with the majority of $CD4^+$ T cells residing in the GI tract and associated lymphatic tissue [1]. Human immunodeficiency virus-induced depletion of $CD4^+$ T cells causes structural impairment of the GI epithelial barrier, systemic microbial translocation, and ultimately alteration of the gut microbial community composition [2].

Recent evidence indicates that HIV-associated gut dysbiosis is characterized by decreased abundance of commensal (protective) bacteria and enrichment of potentially pathogenic taxa [3]. For example, the genera *Pseudomonas*, *Enterobacteriaceae*, *Acinetobacter*, and *Campylobacter* are thought to have infectious and inflammatory properties and are enriched in adults with HIV [3, 4].

Studies show that altered gut microbiota is associated with elevated circulating inflammatory markers such as C-reactive protein and interleukin-6 [5–8] as well as markers of microbial translocation such as lipopolysaccharide and lipopolysaccharide binding protein [9, 10]. Furthermore, studies suggest that antiretroviral therapy (ART) may only partially restore the gut microbiota towards levels observed in HIV-uninfected populations, and patients continue to suffer from dysbiosis even when HIV infection is controlled [1, 11, 12].

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Correspondence: Trym Thune Flygel, MD, Paediatric Research Group, Department of Clinical Medicine, Faculty of Health Sciences, UiT -The Arctic University of Norway, Tromsø, Norway, Hansine Hansens veg 18, 9019 Tromsø, Norway (trymtflygel@gmail.com).

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Moreover, gut dysbiosis, and associated microbial translocation may drive systemic chronic inflammation, which increases the risk of chronic noninfectious HIV complications, such as cardiovascular disease and lung complications [13–16].

Few studies have investigated the gut microbiome in sub-Saharan African children and its relation to the development of HIV-associated chronic complications. Most studies to date have been performed in adult populations and potentially confounded by sexual preference and are therefore not directly comparable to our study. The overall aim of our study was to investigate the gut microbiota in HIV-infected and HIV-uninfected children in Harare, Zimbabwe and to evaluate the association between gut microbial composition and clinical and laboratory parameters (chronic lung disease, $CD4^+$ T-cell count, viral load [VL]).

MATERIALS AND METHODS

Study Population

This study investigated bacterial profiles of rectal swabs collected from participants enrolled to the Bronchopulmonary Function in Response to Azithromycin Treatment for Chronic Lung Disease in HIV-infected Children (BREATHE) trial [17] (clinicaltrials.gov identifier NCT02426112). Chronic lung disease was defined as forced expiratory volume in 1 second (FEV_1) z-score less than −1.0 with no reversibility (<12% improvement in FEV_1 after 200 µg of salbutamol inhaled using a spacer). The detailed study protocol has been described previously [17]. For the present substudy, only participants enrolled in Harare, Zimbabwe were included. HIV-infected children aged 6–16 years without chronic lung disease, defined as no prior history of heart/lung diseases, tuberculosis (TB), no chronic cough, reported chest pain or shortness of breath during exercise, and HIV-uninfected participants were recruited at the same outpatient clinic. These were recruited as comparison groups and not randomized into the trial. The route of HIV transmission was likely perinatal for most of the HIV-infected participants. Human immunodeficiency virus-infected participants had to be stable on ART for at least 6 months, to meet eligibility criteria. All study participants completed a detailed questionnaire regarding demographic, socioeconomic characteristics and clinical history.

The study was approved by the following: London School of Hygiene and Tropical Medicine Ethics Committee; Harare Central Hospital Ethics Committee; Medical Research Council of Zimbabwe; The Regional Committee for Medical and Health Research Ethics REC North 2015/1650; and University of Cape Town Human Research Ethics Committee. All participants and/or legal guardians gave written informed consent to participate in the study.

Sample Collection

Rectal swabs were collected from all participants at enrollment into the trial by study nurses. Swabs were immediately preserved in 1.5 mL of transport medium PrimeStore MTM (Longhorn Diagnostics, Bethesda, MD), directly stored on ice for maximum 1 hour, and then frozen at −80°C before shipment on dry ice to the laboratory at the University of Cape Town.

Deoxyribonucleic Acid Extraction

The Zymo Research Quick-DNA Fecal/Soil Microbe Microprep kit (Zymo Research, Irvine, CA) was used for deoxyribonucleic acid (DNA) extractions. Deoxyribonucleic acid was extracted according to the manufacturer's description, with modifications. In brief, a 400-µL aliquot of each sample was mixed with 400 µL BashingBead Buffer in a ZR BashingBead Lysis Tube. Mechanical lysis (bead beating) was performed using the TissueLyser LT (QIAGEN, Hilden, Germany) set to 50 Hz for 5 minutes. Supernatant (500 µL) was transferred to a Zymo-Spin III-F Filter (Zymo Research, Irvine, CA) and centrifuged at 8000 ×*g* for 1 minute. Chemical lysis was done by adding Genomic Lysis Buffer. All other procedures were done according to manufacturer's protocol.

16S Library Preparation and Gene Sequencing

To assess DNA quality and total bacterial load, a real-time quantitative polymerase chain reaction (PCR) was performed as previously described [18]. Subsequently, 2 PCR sets targeting the V4 hypervariable region of the 16S ribosomal ribonucleic acid (rRNA) gene were performed according to previously described protocols [19, 20] (Supplementary Data).

Samples were sequenced on an Illumina Miseq instrument using the Miseq Reagent v3 kit (600 cycles) (Illumina, San Diego, CA). The final library was diluted to a 6-pM concentration, and a 25% PhiX library spike-in was added as internal control [21]. The preprocessing of sequence reads was done using the H3ABioNet 16S rDNA diversity analysis package (https://github.com/h3abionet/h3abionet16S) [20], with the exception that taxonomy of representative reads was assigned using SILVA version 132. Raw sequence files have been submitted to the European Nucleotide Archive, accession number PRJEB32077.

Data Analysis

Statistical analyses were performed in STATA 14 (StataCorp LLC, College Station, TX) and R Statistical software (http://www.r-project.org/). Characteristics between study groups were compared using Fisher's exact test (for categorical parameters) and Kruskal-Wallis or Wilcoxon rank-sum test (for continuous parameters).

Richness of bacterial taxa within a single sample was represented by the number of operational taxonomic units (OTUs) and Chao1 index [22]. Chao1 index uses mark-release-recapture-like ratio to estimate richness by adding a correction factor to the observed number of species. Richness and evenness (relative abundances of the different species) were characterized by Shannon's index [23]. Alpha diversity measures

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were calculated at sampling depth 4000 reads to include 95% of samples.

Interindividual differences, beta diversity, were determined using Bray-Curtis dissimilarity index [24] with sampling depth set at 2000 reads to include 99% of samples. Beta diversity comparisons were explored using Principal Coordinate plots generated by the *stats* package in R (version 3.4.4). Comparisons were made using Wilcoxon rank-sum test where not specified otherwise. We also used Kruskal-Wallis test in cases with more than 2 groups. The same groups were compared using permutational multivariate analysis of variance in QIIME2 (version 2018.4) [25], with number of permutations set to 999. *P* values were adjusted for multiple testing using the Benjamini-Hochberg method [26].

Relative Abundance

To assess relative abundance, a linear discriminant analysis was performed using linear discriminant analysis effect size [27] with default settings (alpha values for the statistical test 0.05). To reduce the number of markers, the effect size threshold was set to 1.0 for the plots. Relative abundance comparison plots were generated using the MicrobiomeAnalyst web-based software tool with standard feature filtering [28]. Heatmaps for comparing relative abundance of specific taxa between groups were generated using only the taxa found to be significantly different by linear discriminant analysis effect size comparison (Supplementary Figures 1–3). The average fraction of each taxa was calculated from all samples within each group. The data were transformed to fractional abundance (Phyloseq) before performing the linear discriminant analysis effect size analysis. All *P* values reported are corrected for multiple testing using false discovery rate (FDR).

Alpha diversity indices between study groups were compared using Wilcoxon rank-sum test. *P* values were corrected for multiple testing using FDR. Spearman's rank correlation with Bonferroni correction was used to assess the association between alpha diversity indices and continuous parameters. We fitted a linear regression model to estimate the association between HIV status and alpha diversity indices. Body mass index (BMI), age, and sex were adjusted for a priori. An interaction term between HIV status and antibiotics the 3 previous months (co-trimoxazole prophylaxis for HIV-infected participants) was included into the regression model to determine whether antibiotics modify the effect of HIV status on alpha diversity estimates. The association between other participant characteristics and alpha diversity indices was further evaluated in regression analysis stratified by HIV status and adjusted for BMI, age, and sex. A 2-tailed significance level of 0.05 was used.

RESULTS

Study Population

In total, 149 HIV-infected participants with chronic lung disease, 28 HIV-infected participants without chronic lung disease, and 103 HIV-uninfected participants were enrolled. All HIV-infected participants were on ART, for a median of 6.6 years for those with chronic lung disease and 8.0 for those without. Eighty-nine percent of HIV-infected participants were taking co-trimoxazole prophylaxis as per World Health Organization guidelines [29]. No HIV-uninfected participants were taking co-trimoxazole. The study group characteristics are presented in Table 1.

Human immunodeficiency virus-infected participants were older compared to the HIV-uninfected participants (15.6 years, interquartile range [IQR] = 12.8–17.7 vs 9.9 years, IQR = 7.4–12.7; $P < .001$) and were more likely to be stunted and underweight compared to HIV-uninfected participants (stunted 41% vs 5%, $P < .001$; underweight 45% vs 5%, $P < .001$). The proportion of participants who experienced diarrhoeal episodes during the last 3 months before enrollment was also higher in the HIV-infected group than in the HIV-uninfected group (11% vs 3%, $P = .03$).

Alpha Diversity

Species richness (OTUs, Chao1) was significantly higher in HIV-uninfected participants compared to HIV-infected participants. There was no difference in Shannon index between these 2 study groups (Figure 1, Supplementary Table 1). After adjustment for BMI, age, and sex using linear regression analysis, the negative association between richness indices and HIV status remained significant ($P = .02$ for OTUs, $P = .001$ for Chao1 index). The use of antibiotics during the 3 previous months did not change the significant effect of positive HIV status for the Chao1 index (Supplementary Table 2). Human immunodeficiency virus-infected participants with suppressed VL had borderline higher OTUs (median 192.5 [IQR, 145.5–228.5] vs 176 [IQR, 138–220], $P = .18$) and higher Chao1 index (median 259.3 [IQR, 201.2–302.1] vs 233.2 [IQR, 175–276], $P = .05$) compared to nonsuppressed participants in regression analysis adjusted for BMI, age, and sex (Supplementary Tables 3 and 4).

We stratified HIV-infected participants based on their time spent on ART (ART <5 years [n = 53]; ART 5–10 years [n = 100]; ART ≥10 years [n = 23]). When comparing HIV-infected participants based on these subgroups, we found that participants who had been on ART ≥10 years had an alpha diversity similar to the HIV-uninfected study group (Table 2).

There was no difference in alpha diversity indices between HIV-infected participants with and without chronic lung disease (Supplementary Table 5). The same was observed after adjusting for BMI, age, and sex using regression analysis. The associations between participant characteristics and alpha diversity indices in HIV-infected participants is presented in Supplementary Table 4.

Prolonged ART treatment was the only parameter significantly associated with richer gut microbiota after adjustment for BMI, age and sex, suggesting a positive effect of prolonged ART. No parameters were found to be significantly associated with alpha diversity estimates in the HIV-uninfected group (Supplementary Table 6).

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Table 1. Characteristics of Study Participants

Parameter	HIV⁻ (N = 103)	HIV⁺ Chronic Lung Disease⁺ (N = 149)	HIV⁺ Chronic Lung Disease⁻ (N = 28)
Age, median (IQR)	9.9 (7.4–12.7)	15.5 (12.8–17.7)	16.7 (11.7–18.1)
Male, N (%)	53 (52)	84 (56)	8 (29)
BMI-for-age z score, median (IQR)[a]	−0.24 (−0.69 to 0.35)	−1.19 (−1.80 to −0.62)	−0.11 (−0.73 to 0.61)
Stunted (height-for-age z-score ≤2), N (%)[a]	5 (5)	66 (44)	7 (25)
Underweight (weight-for-age z-score ≤2), N (%)[a]	5 (5)	78 (52)	2 (7)
Took antibiotics the 3 previous months for HIV-uninfected group or co-trimoxazole prophylaxis for HIV-infected group, N (%)	2 (2)	133 (89)	25 (89)
Episodes of diarrhea during the last 3 months, N (%)[b]	3 (3)	11(13)	1 (4)
Residential Area, N (%)[c]			
High density	107 (95)	83 (98)	24 (86)
Medium density	4 (4)	1 (1)	3 (11)
Low density	2 (2)	1 (1)	1 (4)
HIV-Related Parameters			
ART Regimen, N (%)			
NNRTI-based regimen	-	93 (62)	24 (86)
PI-based regimen	-	56 (38)	4 (14)
CD4 count ≤400 cells/mm, N (%)[d]	-	40 (27)	9 (32)
VL suppression (VL <1000 copies/mL), N (%)	-	87 (58)	17 (61)
Age at ART initiation, median (IQR)[d]	-	8.2 (5.2–11.4)	8.6 (5.0–9.9)
Years spent on ART, median (IQR)[d]	-	6.6 (4.4–8.4)	8.0 (5.0–9.1)
ART Duration Categories, N (%)[d]			
<5 years	-	46 (31)	7 (25)
5–10 years	-	83 (56)	17 (61)
≥10 years	-	19 (13)	4 (14)
Previously treated for TB, N (%)	-	54 (36)	2 (7)

Abbreviations: ART, antiretroviral therapy; BMI, body mass index; HIV, human immunodeficiency virus; IQR, interquartile range; NNRTI, nonnucleoside reverse-transcriptase inhibitor; PI, protease inhibitor; TB, tuberculosis; VL, viral load.

[a]Parameters were calculated using British 1990 Growth Reference Curves.

[b]Data on episodes of diarrhea during the last 3 months were missing for 64 participants.

[c]Data on residential area were missing for 64 participants.

[d]Data was missing for 1 participant.

Beta Diversity

We found significantly higher beta diversity amongst HIV-infected participants compared to HIV-uninfected participants ($P < .01$) (Figure 2a). Antiretroviral therapy duration had no impact on beta diversity when stratified by years spent on ART. There was no association between beta diversity and VL suppression, type of ART regimen, time on ART, or prior TB in HIV-infected participants (Supplementary Table 7).

Human immunodeficiency virus-infected participants with chronic lung disease had higher beta diversity compared to

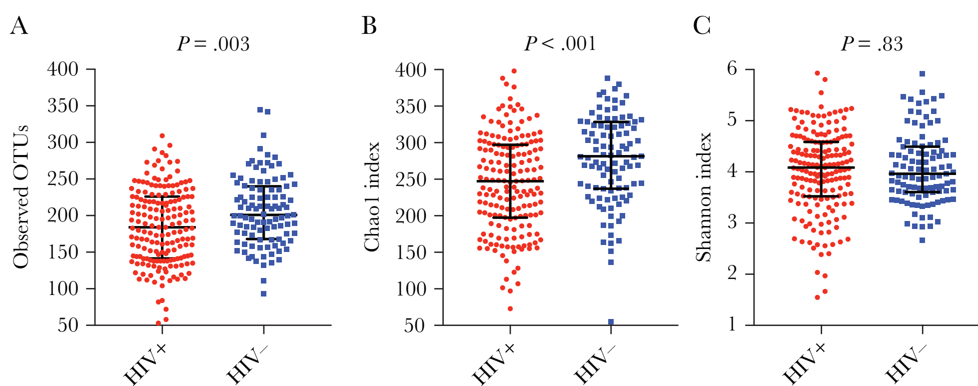

Figure 1. Alpha diversity indices in human immunodeficiency virus (HIV)-infected and there was found a significantly lower richness in HIV infected participants compared to HIV uninfected by (a) Observed OTUs and (b) Chao1 index. Mid line showing median and error bars showing the interquartile range. OTUs, operational taxonomic units.

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Table 2. **Alpha Diversity in HIV-Infected Participants Stratified by Years on ART and in HIV-Uninfected Participants**

Alpha diversity indices	HIV⁺, <5 Years on ART (N = 53) [Median (IQR)]	HIV⁺, 5–10 Years on ART (N = 100) [Median (IQR)]	HIV⁺, ≥10 Years on ART (N = 23) [Median (IQR)]	HIV⁻ Group (N = 103) [Median (IQR)]	HIV⁺, <5 Years on ART vs HIV⁻ (*P* Values*)	HIV⁺, 5–10 Years on ART vs HIV⁻ (*P* Values*)	HIV⁺, ≥10 Years on ART vs HIV⁻ (*P* Values*)
Observed OTUs	176 (138–214)	186.5 (143–223.5)	204 (162–242)	201 (168–240)	.001	.10	.28
Chao1	229.4 (175.0–277.9)	249.6 (200.2–299.6)	268.9 (224.4–306)	281.3 (237.2–328.4)	<.001	.02	.08
Shannon index	4.03 (3.48–4.39)	4.12 (3.52–4.58)	4.23 (3.82–4.84)	4.0 (3.6–4.5)	.20	.75	.86

Abbreviations: ART, antiretroviral therapy; BMI, body mass index; HIV, human immunodeficiency virus; IQR, interquartile range; OTUs, operational taxonomic units.

*, Presented *P* values were obtained from regression model adjusted for BMI, age, and sex where HIV status with years of ART was introduced as an independent variable and alpha diversity estimates as a dependent (outcome) variable.

both HIV-uninfected ($P < .01$) and HIV-infected participants without chronic lung disease ($P = .03$). There was no significant difference between HIV-infected participants without chronic lung disease and HIV-uninfected participants ($P = .74$) (Figure 2b). Unweighted UniFrac analysis showed similar results.

Relative Abundance

We identified 26 different phyla in the rectal swabs from all participants. Only 5 phyla contributed more than 1% of the total sequences of the entire dataset. Firmicutes (43.9%), Bacteroidetes (33.9%), Epsilonbacteraeota (9%) (previously within the phylum Proteobacteria), Actinobacteria (5.3%) and Proteobacteria (7.7%) accounted for 99.8% of the bacteria present.

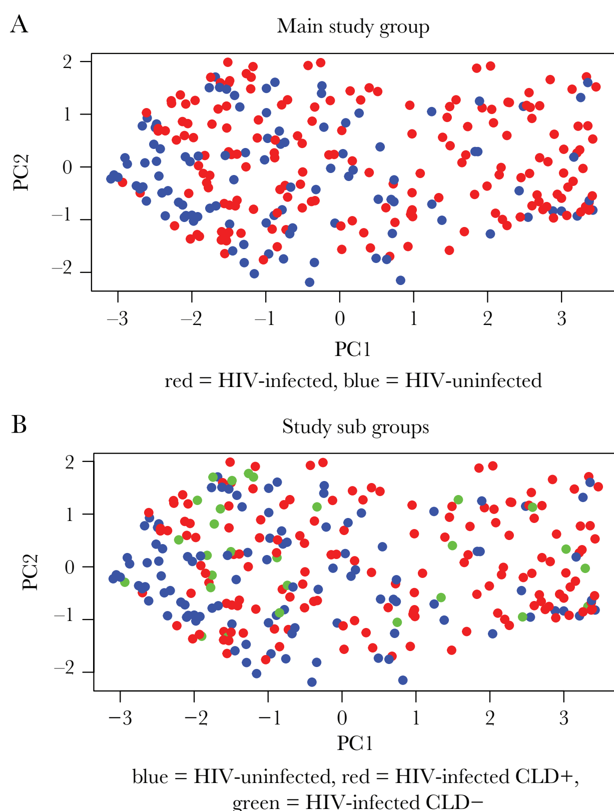

Figure 2. Beta-diversity comparison between study groups. Principal cooridnate (PC) analysis plot showing beta-diversity by Bray-Curtis dissimilarity comparing (a) human immunodeficiency virus (HIV)-infected (red) and HIV-uninfected (blue) participants ($P < .01$) and (b) HIV-infected with chronic lung disease (red), HIV-infected without chronic lung disease (green), and HIV-uninfected (blue) participants. *P* value obtained using Wilcoxon rank-sum test.

Human Immunodeficiency Virus (HIV)-Infected Versus HIV-Uninfected Participants

At phylum level, HIV-infected participants had significantly lower abundance of Epsilonbacteraeota (7%) ($P < .01$) and Bacteroidetes (32%) ($P < .01$) compared to HIV-uninfected participants (with 13% and 38%, respectively) (Supplementary Figure 4).

At genus level, HIV-infected participants had enriched *Corynebacterium* ($P < .01$), *Lawsonella* ($P < .01$), and *Collinsella* ($P = .04$), belonging to the Actinobacteria phylum; whereas in the Firmicutes phylum, *Finegoldia* ($P < .01$), *Anaerococcus* ($P < .01$), *Erysipelotrichaceae* ($P = .02$), and *Lachnoclostridium* ($P = .04$) were enriched when compared to HIV-uninfected participants.

Human immunodeficiency virus-uninfected participants, compared to HIV-infected participants, were enriched in *Campylobacter* ($P < .01$), phylum Epsilonbacteraeota; *Porphyromonas* ($P < .01$) and *Prevotella* ($P = .03$), phylum Bacteroidetes; and *Eubacterium coprostanoligenes_group* ($P < .01$), *Ruminococcaceae* ($P < .01$), *Fastidiosipila* ($P < .01$), *Fournierella* ($P < .01$), *W5053* ($P < .01$), *Coprococcus* ($P = .02$), and *Murdochiella* ($P < .01$), phylum Firmicutes (Figure 3).

Human immunodeficiency virus-infected participants with chronic lung disease had a higher abundance of the genus *Faecalibacterium* ($P = .05$), phylum Firmicutes, compared to participants without chronic lung disease. Participants without chronic lung disease had higher abundance of genus *W5053* ($P < .01$), phylum Firmicutes and *Prevotella* ($P = .05$), phylum Bacteroidetes, compared to participants with chronic lung disease.

Characteristics of Human Immunodeficiency Virus-Infected Participants and Gut Microbiota

When we stratified HIV-infected participants based on CD4 count (CD4 ≤400 cells/mm^3 vs >400 cells/mm^3), we found no statistically significant differences at genus level. However, we found higher proportions at family level of Enterobacteriaceae ($P = .02$) and Burkholderiaceae ($P = .04$) in those with CD4

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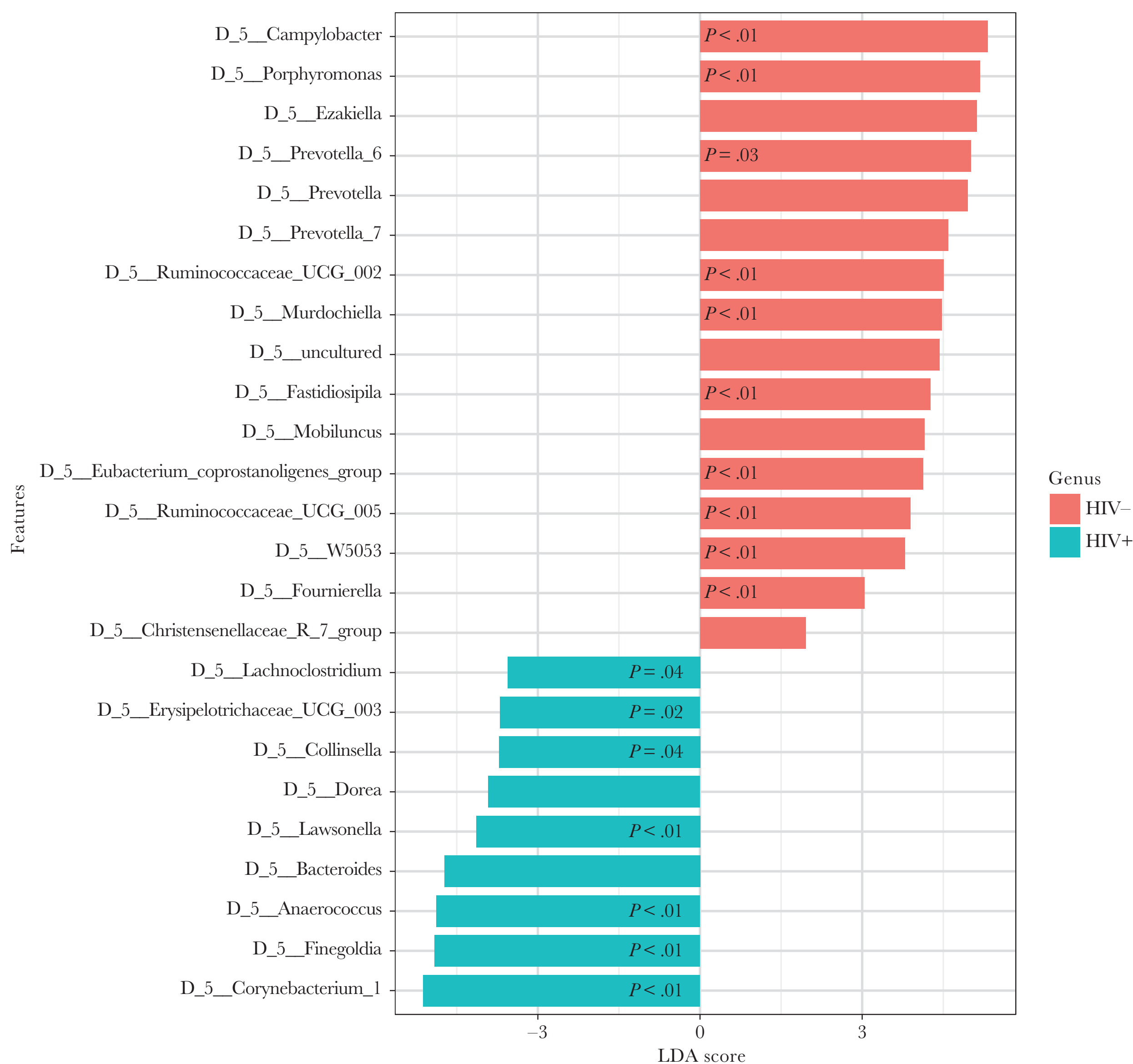

Figure 3. Linear discriminant analysis effect size plot. This plot shows enriched taxa that are significantly different between human immunodeficiency virus (HIV)-infected (blue) and HIV-uninfected (red) participants. Only taxa meeting a significant level of 0.05 and effect size threshold of 1.0 are included. *P* values shown are only those significant after adjustment for false discovery rate.

counts ≤400 cells/mm^3, whereas Succinivibrionaceae (P = .04) was higher in those with CD4 counts >400 cells/mm^3. No differences in relative abundance were found at any taxonomic level between virally suppressed and nonsuppressed participants (<1000 vs ≥1000 copies/mL).

We compared HIV-infected participants based on ART duration subgroups to HIV-uninfected participants using linear discriminant analysis effect size. Genera such as *Bacteroides*, *Prevotella*, *Porphyromonas*, *Blautia*, and *Roseburia* were similarly abundant in HIV-uninfected and HIV-infected participants who have been on ART ≥10 years (Supplementary Figures 1–3 and 5–8). This finding suggests that prolonged ART helps shift microbial composition towards that of HIV uninfected, but this result needs further investigation.We found no differences in relative abundance when comparing HIV-infected participants on ART <5 years to those on ART for 5–10 years or for ≥10 years.

DISCUSSION

Our study showed that gut microbiota in HIV-infected, ART-treated children was less diverse compared to HIV-uninfected children. Children who had been taking ART for 10 years or more had a more diverse microbiota resembling that of HIV-uninfected children. Our results suggest that prolonged ART may minimize differences in gut microbiota between HIV-infected and -uninfected children.

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Impact of Human Immunodeficiency Virus on Gut Microbiota

Several studies in adults demonstrated that untreated HIV infection is associated with intestinal dysbiosis, reduced alpha diversity, and increased beta diversity [9, 30, 31]. These changes may persist despite ART [5, 6, 10, 32, 33]. Our results of overall lower alpha diversity and higher beta diversity in HIV-infected, ART-treated children support these findings.

Published data are less consistent with regards to relative abundance of specific taxa in HIV-infected individuals. Types of specimens used, study populations, geographical area, sequencing method, and false discovery may explain these conflicting results. For example, rectal swab analysis from HIV-infected, ART-treated adults in Nigeria found higher abundance of *Finegoldia* and *Anaerococcus* in HIV-infected individuals [34], which is consistent with our findings. However, in the same study, *Campylobacter* was significantly enriched in HIV-infected participants, whereas we found enriched *Campylobacter* in the HIV-uninfected group.

Several studies showed enriched levels of Proteobacteria in HIV-infected, ART-naive individuals [6, 7, 9], but only 1 study showed similar findings in ART-treated individuals [35]. We found enrichment of Proteobacteria in HIV-infected individuals, but this was not statistically significant.

Impact of Antiretroviral Therapy on Gut Microbiota

At least 2 studies have found a negative impact of ART on gut microbiota diversity [9, 34]. In a longitudinal study, Nowak et al [9] found a significant decrease in the number of observed species and the Shannon index after ART introduction. However, Nowak et al [9] investigated the effect of ART initiation, with a relatively short follow-up of 10 months. In our population, we had no ART-naive participants, and minimum duration of ART was 1 year. We observed lower alpha diversity in those on ART <10 years compared to HIV-uninfected participants.

Previous studies that investigated the gut microbiome in individuals on long-term ART reported similar alpha diversity profiles in HIV-infected, ART-treated and HIV-uninfected individuals [30, 35]. For example, Dinh et al [35] found no significant difference in alpha diversity measures between HIV-infected participants on ART for a median of 13.3 years and HIV-uninfected controls. This is similar to our findings for participants who received ART for 10 or more years. The impact of ART duration on gut microbiota was also noted by Lozupone et al [36] who found that individuals with longer ART duration showed closer resemblance to HIV-uninfected individuals than to subjects with untreated HIV infection. These studies support our findings of the possibility that long-term ART may restore HIV-associated dysbiotic gut microbiota.

We did not observe an association between immunological or virological markers (VL and CD4 count) and gut microbiome diversity measures. In contrast, other studies showed significantly lower microbiome diversity in those with more severe HIV status [9, 37, 38]. Findings of previous studies may have been affected by sample size and ART duration. A longitudinal study with repeated measurements of VL, CD4, and microbiome profiles is needed to uncover the relationship between these parameters.

We found enriched levels of Enterobacteriaceae in HIV-infected participants with low $CD4^+$ T-cell counts (≤400 cells/mm^3). Enterobacteriaceae may cause GI and urinary tract infections in HIV-infected children [38]; however, the clinical significance is unclear, because Enterobacteriaceae are found as part of the normal intestinal flora. Burkholderiaceae, also enriched in those with low $CD4^+$ T-cell counts (≤400 cells/mm^3), includes species known to cause severe lung infections in patients with cystic fibrosis [39].

Gut-Lung Axis

Recent evidence suggests that gut microbiome is involved in maintaining lung health, and an altered gut microbiome composition is often observed in patients with lung diseases [15, 16]. For example, low gut microbiome diversity during infancy has been linked to asthma at school age [32]. In our study, we did not observe any difference in alpha diversity estimates between participants with and without HIV-associated chronic lung disease, but there were some significant differences in relative abundance of specific taxa. For example, the genus *Faecalibacterium* was enriched in HIV-infected individuals with chronic lung disease, whereas *Prevotella* was enriched in HIV-infected individuals without chronic lung disease.

Faecalibacterium have previously been regarded as a protective commensal and is associated with a healthy gut. Depletion of this genus has been linked to the development of inflammatory bowel disease and asthma, and low levels have been shown in patients with cystic fibrosis [16, 40]. Some studies have challenged this, showing increased levels of the species *Faecalibacterium prausnitzii* in gut microbiome of pediatric patients with untreated Crohn's disease at the time of diagnosis [41]. It is interesting to note that a recent study also showed increased levels of *Faecalibacterium* in the gut microbiome of patients with active TB [42].

Co-trimoxazole Prophylaxis

Because the majority (89%) of HIV-infected participants in our study received co-trimoxazole prophylaxis, it is not possible to completely tease apart the effect of HIV from that of cotrimoxazole. Although it is known that antibiotics cause substantial changes in the gut microbiota, data regarding the impact of co-trimoxazole prophylaxis on gut microbiota in HIV-infected, ART-treated individuals are limited. However, recent evidence suggests that co-trimoxazole does not affect global gut microbial composition but rather specific inflammatory pathways in HIV-infected individuals [43]. In our study, the negative impact of positive HIV status on richness estimates remained

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significant after accounting for co-trimoxazole prophylaxis. In addition, no effect of co-trimoxazole administration on alpha diversity in HIV-infected participants was observed. Our results are in line with several other studies where no significant difference in alpha diversity was observed in HIV-infected individuals who took co-trimoxazole and those who did not [44–46].

Study Strengths and Limitations

Our study is one of the few to assess the gut microbiome composition in children and adolescents with perinatally acquired HIV infection. A relatively large sample size and detailed characteristics of study participants allowed us to perform extensive statistical analysis. All participants in our study were from the same region, thus increasing the internal validity of our data.

Our study was cross-sectional and is therefore unable to directly assess relationships over time. The group of HIV-infected participants without chronic lung disease was small and therefore gave limited power to detect differences. Furthermore, we did not assess diet and social factors such as housing or level of education, which may have an impact on gut microbiota. Age imbalance between HIV-infected and -uninfected participants is also a limitation of this study.

CONCLUSIONS

Our study is among the first to assess gut microbial composition of HIV-infected children and adolescents in a very high HIV burden setting. Our results indicate that gut microbiota is altered in HIV-infected children, although diversity improves with increasing duration of ART. Further studies, in which the gut microbiota, markers of microbial translocation, and immunological markers are measured, are warranted to provide better insight to the pathogenesis of HIV and its related complications.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Author contributions. M. P. N., T. F., J. P. C., S. C.-W., E. S., and T. T. F. conceived and designed the study and participated in data analysis and revision of the manuscript. The BREATHE study team was responsible for sample collection and management. T. T. F. performed the laboratory experiments, analyzed the data, and wrote the first draft of the manuscript. S. C.-W. performed the laboratory experiments, participated in data analysis, and reviewed the final manuscript. E. S. analyzed the data and wrote the first draft of the manuscript. E. H. and K. S. M. analyzed the data. J. Ø O., R. A. F., G. M., M. P. N., J. P. C., and T. F. revised the manuscript. All authors approved the final version.

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Paper II

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ORIGINAL ARTICLE

Exhaled nitric oxide is associated with inflammatory biomarkers and risk of acute respiratory exacerbations in children with HIV-associated chronic lung disease

Trym Thune Flygel[1,2] | Dan Hameiri-Bowen[3] | Victoria Simms[4] | Sarah Rowland-Jones[3] | Rashida Abbas Ferrand[5,6] | Tsitsi Bandason[6] | Louis-Marie Yindom[3] | Jon Øyvind Odland[7,8] | Jorunn Pauline Cavanagh[1,2] | Trond Flægstad[1,2] | Evgeniya Sovershaeva[2,9]

[1]Paediatric Research Group, Department of Clinical Medicine, Faculty of Health sciences, UiT – The Arctic University of Norway, Tromsø, Norway

[2]Department of Paediatrics, University Hospital of North Norway, Tromsø, Norway

[3]Nuffield Department of Medicine, University of Oxford, Oxford, UK

[4]Department of Infectious Disease Epidemiology, Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, London, UK

[5]Department of Clinical Research, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK

[6]Biomedical Research and Training Institute, Harare, Zimbabwe

[7]Department of Public Health and Nursing, Faculty of Medicine and Health Sciences, NTNU The Norwegian University of Science and Technology, Trondheim, Norway

[8]School of Health Systems and Public Health, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa

[9]Department of Microbiology and Infection Control, Akershus University Hospital, Nordbyhagen, Norway

Correspondence

Trym Thune Flygel, Paediatric Research Group, Department of Clinical Medicine, Faculty of Health Sciences, UiT – The Arctic University of Norway, Hansine Hansens veg 18, 9019 Tromsø, Norway.

Email: trym.t.flygel@uit.no

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Abstract

Objectives: Chronic lung disease is a recognized complication in children with HIV. Acute respiratory exacerbations (ARE) are common among this group and cause significant morbidity. Exhaled nitric oxide (eNO) is a known marker of local airway inflammation. We investigated the association between eNO and ARE, biomarkers of systemic inflammation, and the effect of azithromycin on eNO levels.

Methods: Individuals aged 6–19 years with HIV-associated chronic lung disease in Harare, Zimbabwe, were enrolled in a placebo-controlled randomized trial investigating the effect of 48-week azithromycin treatment on lung function and ARE. eNO levels and biomarkers were measured at inclusion and after treatment in a consecutively enrolled subset of participants. Linear regression and generalized linear models were used to study associations between eNO and ARE, biomarkers, and the effect of azithromycin on eNO levels.

Results: In total, 172 participants were included in this sub-study, 86 from the placebo group and 86 from the azithromycin group. Participants experiencing

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at least one ARE during follow-up had significantly higher eNO levels at baseline than participants who did not (geometric mean ratio 1.13, 95% confidence interval [CI] 1.03–1.24, $p = 0.015$), adjusted for trial arm, age, sex and history of tuberculosis. Matrix metalloproteinase (MMP)-3, -7, and -10 were significantly associated with higher baseline eNO levels. At 48 weeks, azithromycin treatment did not affect eNO levels (geometric mean ratio 0.86, 95% CI 0.72–1.03, $p = 0.103$).

Conclusion: Higher baseline eNO levels were a risk factor for ARE. eNO was associated with proinflammatory biomarkers previously found to contribute to the development of chronic lung disease. The potential use of eNO as a marker of inflammation and risk factor for ARE in HIV-associated chronic lung disease needs further investigation.

KEYWORDS
Africa, biomarkers, chronic lung disease, exhaled nitric oxide, HIV, inflammation

INTRODUCTION

The scale-up of antiretroviral therapy (ART) has led to increasing numbers of children perinatally infected with HIV surviving into adolescence and adulthood. Data show that ~30% of children and adolescents with HIV in sub-Saharan Africa present with chronic respiratory symptoms and/or airway obstruction [1–3]. Obliterative bronchiolitis and bronchiectasis are the most common forms of HIV-associated chronic lung disease (HCLD) [2, 4]. These children are at high risk of developing acute respiratory exacerbations (ARE); respiratory infections are among the most common causes of hospitalizations and therefore a significant cause of morbidity in this group [5]. The pathogenesis of HCLD is multifactorial and not completely understood. Understanding the aetiology of chronic HIV-associated comorbidities such as HCLD [6, 7] is a growing research priority to establish optimal treatment strategies and tools to monitor disease progression.

Recent studies have suggested that systemic immune activation associated with HIV infection persists despite effective ART and may play a role in in the development of chronic HIV-related comorbidities, including HCLD [8, 9]. Moreover, persistent inflammation due to repeated respiratory infections and a high prevalence of tuberculosis (TB) in this group may also contribute to lung damage [10]. In adults, data show that HCLD is associated with elevated levels of systemic proinflammatory biomarkers such as C-reactive protein, interleukin-6, tumour necrosis factor-α, and T-cell activation [11]. We previously described how matrix metalloproteinases (MMP)-1, -7, and -10, as well as biomarkers of inflammation, interferon (IFN)-γ, C-reactive protein, and interferon gamma-induced protein-10 were associated with HCLD in children and adolescents [12].

Administration of macrolide antibiotics, such as azithromycin, is thought to be beneficial in patients with chronic airway diseases [13, 14]. Azithromycin has bacteriostatic activity and anti-inflammatory properties, reducing the expression of several pro-inflammatory cytokines [13–15]. Macrolide treatment can reduce the number of exacerbations and have immunomodulatory effects in patients with a variety of chronic lung diseases (CLDs) [5, 13, 14, 16, 17]. A recent randomized controlled trial [18] demonstrated that children with HCLD who received once-weekly azithromycin for 48 weeks had a significantly lower risk of ARE than children who received placebo [5]. In the same participants, reduced levels of plasma-soluble biomarkers of inflammation were observed in the azithromycin arm [19].

No studies to date have investigated the role of local airway inflammation in this group of patients and the potential effects of treatment. Exhaled nitric oxide (eNO) is by far the most widely studied biomarker of local inflammation in CLD. Elevated levels of eNO have been used as a marker of eosinophilic airway inflammation in asthma and are suggested to be useful in diagnosing and monitoring of asthmatic disease [20–22]. eNO can easily be measured in exhaled air by non-invasive, standardized methods [21]. These features could make the use of eNO as a clinical marker of inflammation and disease progression relevant in resource-limited settings if prices and availability beyond clinical trials improve.

The aim of this study was to investigate the effect of azithromycin treatment on levels of eNO, a marker of

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local inflammation in the lungs, in children and adolescents with HCLD. We also assessed the association between eNO and the risk of ARE in children with HCLD and described the association between eNO and plasma-soluble biomarkers of systemic inflammation.

MATERIALS AND METHODS

Study participants

This study was nested within the BREATHE trial; a double-blinded, randomized, placebo-controlled trial of weekly weight-adjusted azithromycin for 48 weeks in individuals aged 6–19 years with HCLD in Harare, Zimbabwe, and Blantyre, Malawi (BREATHE trial, clinicaltrials.gov, identifier NCT02426112). The inclusion criteria for the BREATHE trial were age 6–19 years, perinatally acquired HIV, taking ART for at least 6 months, having CLD (defined as forced expiratory volume in 1 second [FEV_1] z-score lower than −1.0), no history or symptoms of active TB or any acute respiratory tract infection. All participants were screened for TB using the Xpert MTB/RIF assay upon enrolment and excluded upon a diagnosis of active TB. Data collection was performed between April 2017 and June 2019. The detailed study protocol and trial main results are published elsewhere [18, 23]. Participants were randomized 1:1 using block randomization stratified by trial site.

In this study, only participants from Harare, Zimbabwe, were included. Measurement of eNO started after the first 68 participants were enrolled to the main study (following an ethics amendment). Thereafter, 173 consecutively enrolled and randomized participants underwent eNO measurements in addition to blood samples and spirometry at baseline and after 48 weeks of treatment.

All participants completed interview-administered questionnaires to ascertain demographic and clinical history. Both self-reported and physician-diagnosed lung diseases and atopic disorders, including rhinitis and atopic dermatitis and asthma, were recorded. Participants were encouraged to attend an unscheduled visit if they developed any acute respiratory symptoms. ARE was defined as new or worsening respiratory symptoms, with or without fever or symptoms of infection as assessed by a clinician. Those presenting with incident acute respiratory symptoms had nasal swabs and sputum samples taken and were treated with co-amoxiclav for 10 days. If they did not show any improvement after treatment, chest X-rays and TB culture on expectorated sputum was performed. A more detailed description is published elsewhere [5].

Ethical approvals

The study was approved by the London School of Hygiene and Tropical Medicine ethics committee, the Harare Central Hospital ethics committee, the Medical Research Council of Zimbabwe, and the Regional Committee for Medical and Health Research Ethics in Norway (2015/1650). Guardians gave written informed consent, and participants aged <18 years provided assent. Those aged >18 years gave independent consent.

Exhaled NO

Levels of eNO were measured using an electrochemical analyser (NIOX VERO, Circassia, UK) according to American Thoracic Society guidelines, recorded in parts per billion [21]. Repeated exhalations of eNO were made to obtain at least two measurements that agreed within 10%. Details of measurements are described elsewhere [24]. The mean eNO value was calculated from the two measurements with the least difference between them. Where the differences between three measurements were the same, we calculated the mean of all three measurements.

Laboratory tests

All participants provided blood samples for full blood count, HIV viral load, and $CD4^+$ T-cell count, as previously described [24]. Soluble biomarkers were measured from cryopreserved plasma, stored at −80°C before use, using a Luminex multiplex bead assay on a MagPix instrument according to manufacturer's protocol (Luminex technology, Hertogenbosch, Netherlands). A detailed description is published elsewhere [12]. Anaemia was defined according to World Health Organization criteria [25].

Data collection/storage

Electronic record forms (for questionnaires) were collected on Google Nexus tablets (Google, Mountain View, CA, USA) with OpenDataKit software. Paper forms were used for data collection of clinical tests. Data from the paper forms were extracted using CARDIFF TELEFORM character optical mark recognition software (version 10.9). Data were managed in a Microsoft Access database (Microsoft, Redmond, WA, USA).

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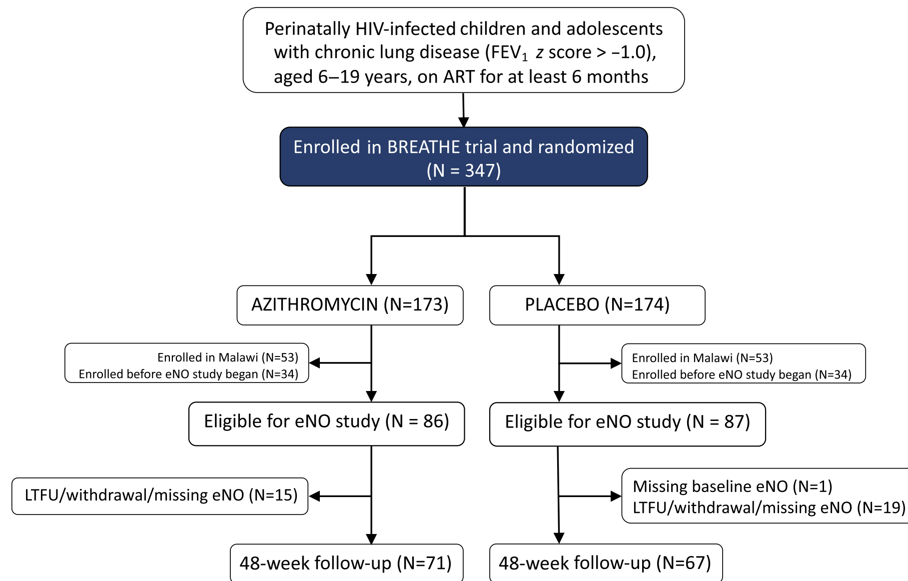

FIGURE 1 Study flow chart. ART, antiretroviral therapy; eNO, exhaled nitric oxide; LTFU, lost to follow-up.

Statistical analysis

Statistical analyses were performed using R (version 4.2.0) and IBM SPSS (version 28.0.0). Characteristics between participants in the azithromycin and placebo groups were compared using the Wilcoxon rank-sum test for continuous parameters and Fisher's exact test for categorical parameters. Data are shown as medians with interquartile ranges or geometric means with 95% confidence intervals (CIs). eNO values were natural log-transformed to approximate normality for statistical analysis and back transformed to geometric means with 95% CIs for presentations. Weight-for-age and height-for-age z-scores were calculated using British 1990 growth reference curves [26]; those with z-scores lower than −2 were characterized as being underweight and stunted, respectively.

T-test and paired *T*-test were used to compare eNO levels between trial groups and time points. Plots were made using the *ggplot2* package in R studio [27]. A generalized linear regression model (GLM) was used to describe the effect of azithromycin on eNO levels. GLM was adjusted for age, sex, history of TB, and baseline eNO levels. The same model was used to determine whether there was an association between baseline levels of eNO and having at least one ARE during the study period. Adjustments were made for trial group, age, sex, and previous history of TB. There were no significant interactions to include in the model. The association between eNO and markers of systemic inflammation was estimated in linear regression analysis.

As history of TB has previously been associated with lower eNO levels [24], we conducted a subgroup analysis comparing eNO levels between participants treated for TB and those without TB. Biomarkers and MMPs included in regression analyses were chosen based on previously confirmed associations with HCLD or response to azithromycin treatment in the same group of participants [19].

RESULTS

Study population

A subgroup of 173 participants of the total 347 recruited in the BREATHE trial was included in this study (Figure 1). One participant missed baseline eNO measurement and was therefore excluded. In total, 86 participants from the azithromycin group and 86 from the placebo group had eNO measured. A total of 15 participants in the azithromycin group and 19 in the placebo group were lost to follow-up, withdrew during the study, or did not provide an eNO test at 48 weeks. This left 71 participants in the azithromycin group and 67 in the placebo group with 48-week follow-up samples (Figure 1).

Baseline characteristics between the groups are presented in Table 1. The groups did not differ regarding age, eNO levels, or prevalence of TB.

Association between eNO and acute respiratory exacerbations

Participants who experienced at least one ARE during the study period had significantly higher levels of eNO at

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TABLE 1 Baseline characteristics of participants by trial group.

Variable	Azithromycin ($N = 86$)	Placebo ($N = 86$)
Age	15.04 (12.65–17.73)	16.45 (13.15–18.45)
Female sex	35 (41)	45 (52)
eNO level (ppb), geometric mean (95% CI)	15.45 (13.69–17.44)	16.72 (14.69–19.02)
Wasted (weight for age z-score lower than −2)	45 (52)	41 (48)
Stunted (height for age z-score lower than −2)	45 (52)	37 (43)
Passive smoking	25 (29)	24 (28)
History of TB	28 (33)	21 (24)
Using cotrimoxazole	80 (93)	76 (88)
Years on ART[a]	5.91 (4.02–8.4)	6.59 (3.93–8.18)
Prescence of atopy (asthma, eczema, or hay fever)	15 (17)	9 (10)
FEV_1 z-score	−1.67 (−2.47 to −1.33)	−1.74 (−2.09 to −1.25)
Obstructive (FEV_1 z-score lower than −1.64)	45 (52)	45 (52)
Anaemia[b]	24 (28)	32 (37)
White blood cell count ($*10^9$/L)[b]	4.08 (3.36–4.83)	4.32 (3.71–5.34)
Eosinophil count ($*10^9$/L)[b]	0.06 (0.03–0.13)	0.08 (0.04–0.13)
Basophil count ($*10^9$/L)[b]	0.02 (0.01–0.03)	0.02 (0.01–0.03)
Neutrophil count ($*10^9$/L)[b,c]	1.5 (1.27–2.02)	1.83 (1.35–2.48)
Lymphocyte count ($*10^9$/L)[b]	1.98 (1.56–2.45)	2.02 (1.6–2.48)
Monocyte count ($*10^9$/L)[b]	0.36 (0.29–0.47)	0.41 (0.3–0.51)
Virally supressed (VL <1000 copies/mL)	56 (65)	52 (60)
CD4 T-cell count (cells/μL)	554 (363–661)	540.5 (323.5–789.5)
CD4 T-cell count <200 cells/μL	11 (13)	11 (13)
Acute respiratory exacerbations during study period	11 (13)	15 (17)
Hospitalization during study period[d]	2 (2)	3 (3)

Note: Azithromycin group: one admitted for pneumonia, one unknown cause of admission. One in the azithromycin group used salbutamol, no-one used corticosteroids.
Note: Data are presented as *N* (%) or median (interquartile range) unless otherwise indicated.
Abbreviations: ART, antiretriviral therapy; CI, confidence interval; eNO, exhaled nitric oxide; FEV_1, forced expiratory volume in 1 s; ppb, parts per billion; TB, tuberculosis; VL, viral load.
[a]One missing data on duration of ART in placebo group.
[b]Two missing data on haemoglobin and white blood cell count in azithromycin group.
[c]One outlier with neutrophil count of 50.80 in placebo group.
[d]Placebo group: one participant admitted for TB treatment, one for ovarian cancer, and one for anaemia.

baseline (geometric mean 20.8, 95% CI 16.5–26.1) than those who did not experience ARE (geometric mean 15.4, 95% CI 14.0–16.9, $p = 0.017$) (Figure 2, Table 2). This difference remained significant in both adjusted linear regression (geometric mean ratio 1.35, 95% CI 1.07–17, $p = 0.013$) and adjusted GLM (geometric mean ratio 1.13, 95% CI 1.03–1.24, $p = 0.015$). We found similar results when adding viral suppression, $CD4^+$ T-cell count, and MMPs associated with HCLD or response to azithromycin treatment to the GLM (Table 2). Baseline characteristics based on having an episode of ARE are presented in Table S1.

Levels of eNO and plasma soluble biomarkers

MMP-3 ($p = 0.024$), MMP-7 ($p = 0.016$), and MMP-10 ($p = 0.005$) were significantly associated with higher levels of eNO at baseline in multivariable linear regression. IFN-γ ($p = 0.039$) was associated with lower baseline eNO levels in multivariable linear regression. MMP-12 was significantly associated with lower eNO levels ($p = 0.034$). However, MMP-12 did have a high proportion of samples (13%) where values were under the limit of detection, so the results are uncertain [12]. None of the

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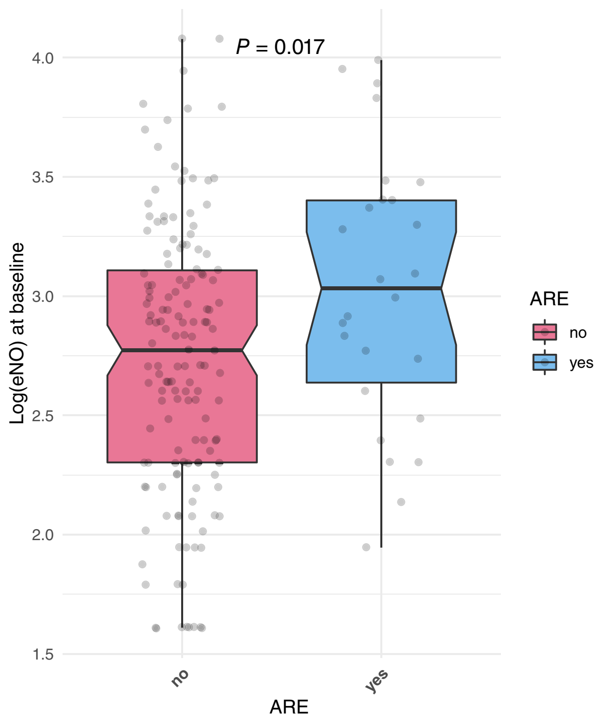

FIGURE 2 Box plot of log-transformed exhaled nitric oxide (eNO) at baseline by experience of at least one acute respiratory exacerbation (ARE) during the study period.

TABLE 2 Association between levels of log eNO at baseline and at least one acute respiratory exacerbation during follow-up, using generalized linear models.

Model	Geometric mean ratio of log eNO at baseline (95% CI)	*p*-value
Unadjusted	1.34 (1.05–1.71)	0.017
Adjusted for trial group, age, sex, and TB history	1.13 (1.03–1.24)	0.015

Abbreviations: CI, confidence interval; eNO, exhaled nitric oxide; TB, tuberculosis; VL, viral load.

other biomarkers investigated were found to be significantly associated with eNO (Table 3).

Effect of azithromycin on levels of eNO

There was no evidence to suggest that azithromycin affected eNO levels after 48 weeks of treatment ($p = 0.189$). Participants in the azithromycin group had a tendency towards lower eNO levels after treatment (geometric mean 14.5, 95% CI 12.6–16.8) compared with participants in the placebo group (geometric mean 17.3,

TABLE 3 Linear regression analyses testing associations of risk factors with eNO at baseline; adjusted for age, sex, and history of TB.

Variable	Geometric mean ratio of log eNO (95% CI)	*p*-value
Age	1.028 (1.003–1.053)	0.028
Sex = male	1.177 (0.992–1.395)	0.061
Study group = azithromycin	0.947 (0.797–1.126)	0.538
ARE during study period = Yes	1.345 (1.065–1.699)	0.013
Atopy = yes	1.148 (0.875–1.507)	0.316
Virally supressed (VL <1000 copies/mL)	1.029 (0.862–1.229)	0.749
CD4 T-cell count	0.998 (0.995–1.001)	0.348
TB treated = yes	0.769 (0.638–0.928)	0.006
FEV_1 z-score	1.021 (0.893–1.168)	0.761
Airway obstruction (FEV_1 z-score lower than −1.64)	1.014 (0.852–1.207)	0.874
Anaemia = yes	0.879 (0.728–1.061)	0.179
White blood cell count (log)	1.288 (0.995–1.666)	0.054
Neutrophils (log)	1.169 (1.006–1.228)	0.041
Eosinophils	1.194 (0.793–1.798)	0.393
Lymphocytes (log)	1.059 (0.884–1.269)	0.532
Monocytes (log)	1.223 (0.985–1.518)	0.068
Wasted (weight-for-age z-score lower than −2)	0.997 (0.828–1.200)	0.974
Stunted (height-for-age z-score lower than −2)	0.924 (0.776–1.100)	0.372
MMP-1 (log)	1.089 (0.974–1.218)	0.132
MMP-3 (log)	1.190 (1.024–1.384)	0.024
MMP-7 (log)	1.186 (1.032–1.363)	0.016
MMP-8 (log)	1.036 (0.963–1.114)	0.337
MMP-10 (log)	1.252 (1.073–1.461)	0.005
MMP-12 (log)	0.919 (0.849–0.993)	0.034
IFN-γ (log)	0.794 (0.638–0.989)	0.039
CRP (log)	1.038 (0.995–1.082)	0.081
sE-selectin (log)	1.146 (0.905–1.450)	0.256
Fas (log)	1.049 (0.795–1.386)	0.732
GCSF (log)	0.922 (0.762–1.115)	0.340

Note: Analyses were done on natural log-transformed values of eNO and back transformed to geometric mean ratio with 95% CI for reporting.
Abbreviations: ARE, acute respiratory exacerbation; CI, confidence interval; CRP, C-reactive protein; eNO, exhaled nitric oxide; FEV_1, forced expiratory volume in 1 s; GCSF, granulocyte colony-stimulating factor; IFN, interferon; MMP, matrix metalloproteinase; sE-Selectin, soluble E-selectin; TB, tuberculosis; VL, viral load.

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95% CI 14.9–20.1) (Table S2, Figure S1). However, this was not statistically significant in adjusted linear regression analysis ($p = 0.538$) (Table 3) or in GLM (geometric mean ratio 0.86, 95% CI 0.72–1.03, $p = 0.103$) (Table S2).

History of TB

As previously described in the same cohort, participants with a history of TB had lower eNO levels at baseline ($p = 0.006$). The only significant change was higher levels of eNO at 48 weeks (geometric mean 18.8, 95% CI 14.6–24.3) than at baseline (geometric mean 12.6, 95% CI 10.2–15.5) in participants with a history of TB within the placebo group ($p = 0.002$).

DISCUSSION

Our study is the first to investigate the effect of long-term azithromycin treatment on eNO levels in children with HCLD. We found that participants who experienced at least one ARE during the study period had significantly higher baseline levels of eNO. eNO levels are known to be higher during asthmatic exacerbations [21]. Studies in other CLDs are few, and the results are conflicting. A meta-analysis found eNO to be mildly elevated in people with chronic obstructive pulmonary disease (COPD) experiencing an exacerbation, although this was not significant [28]. One study showed elevated eNO levels in COPD exacerbations, where participants with viral airway infections had the highest increase in eNO [29]. Another study found that eNO was elevated only in patients with asthmatic exacerbations [30]. Finally, a study of eNO measurements repeated annually in participants with severe asthma showed that higher eNO levels and large variations in eNO were associated with developing ARE [31]. This supports the evidence that increased airway inflammation is a key factor in ARE and emphasizes the potential role of eNO for both monitoring disease and predicting ARE. Although evidence to support the use of eNO as a risk factor for ARE in diseases other than asthma is scarce, our results suggest that its use in children with HCLD could be of interest, but further studies are warranted.

CLD is characterized by both local and systemic inflammation. Hameiri-Bowen et al. previously showed that systemic levels of IFN-γ, a pro-inflammatory cytokine, is associated with having HCLD in children [12]. Our study surprisingly demonstrated a negative association between IFN-γ and eNO in children with HCLD. This is despite previous studies showing that IFN-γ induces NO production through activation of inducible NO synthase (iNOS) in macrophages [32]. Other studies have linked the development of COPD to lung tissue injury promoted by IFN-γ-mediated release of MMPs [33]. Elevated levels of IFN-γ-producing $CD8^+$ T-cells correlating positively with levels of eNO in participants with COPD have also been described [34]. Considering these findings, eNO could potentially be used as a marker of lung injury in COPD. However, the exact signalling pathway and role of IFN-γ in lung tissue injury and development of HCLD need further investigation.

Our study also found a significant positive association between MMP-3, -7, and -10 and eNO levels. Previous studies reported elevated levels of MMPs measured in bronchoalveolar lavage and blood samples in patients with COPD and pulmonary fibrosis, suggesting their role as mediators in the development of CLD [35–37]. One study in adults with COPD found that several MMPs were elevated in bronchoalveolar lavage, including MMP-3, -7, and -10. These three MMPs were also associated with both radiological markers of small airway disease and severity of emphysema [35]. Further, they found that MMP-10 had the strongest association with emphysema. In our study, MMP-10 had the strongest association with elevated levels of eNO, suggesting that these participants may be at higher risk of CLD progression in the future.

It is also known that both MMP-3 and MMP-7 are associated with the development of lung fibrosis by increasing levels of profibrotic mediators [36]. It has further been suggested that MMP-7 measured in blood could be used as a biomarker for progression and disease activity of idiopathic pulmonary fibrosis. MMP-7 has been significantly associated with mortality and disease progression of pulmonary fibrosis [37]. These results highlight that MMPs might play a significant role in airway remodelling, an important part of the pathogenesis of both COPD and lung fibrosis [35, 38]. Our findings of an association between these MMPs and higher levels of eNO can indicate higher levels of local inflammation in participants with a higher degree of airway remodelling.

Published data regarding the effects of macrolides on eNO levels are conflicting. A study in rats found an effect of macrolide antibiotics on iNOS resulting in reduced levels of eNO, suggesting a favourable effect of macrolides on immune-mediated inflammation and lung injury [39]. In our study, we hypothesized that azithromycin would lead to lower eNO levels because of its antimicrobial and immunomodulatory properties. However, we did not find any evidence that azithromycin treatment affects eNO levels after 48 weeks of treatment. Our results are in line with a previously published randomized controlled trial by Diego et al. where azithromycin had no effect on eNO levels in adults with non-cystic

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fibrosis bronchiectasis. However, they did find that azithromycin treatment reduced the frequency of ARE [40].

Two other studies found reduced eNO levels following macrolide treatment. Sadeghdoust et al. found that both azithromycin and prednisolone induced a significant reduction in eNO levels in participants with severe asthma, where those receiving prednisolone had the most reduction in eNO [41]. Another study in children with mild asthma found that a 4-week course of low-dose clarithromycin—another macrolide—in combination with fluticasone both improved lung function as measured by FEV_1 and induced a significant reduction in eNO levels [42]. Of note, these studies were carried out in participants with asthmatic disease, characterized mainly by type 2 inflammation and eosinophilic activity, to which eNO is most strongly linked [22]. In addition, most participants had a chronic and stable disease over time, with few participants experiencing acute inflammation. Hodgson et al. found similar results to ours in adults with treatment-resistant cough after 8 weeks of treatment with azithromycin. They found no significant difference in either symptoms of coughing or levels of eNO, also when controlling for asthma [43]. Our participants had HCLD, and few participants had a history of asthma or atopic disease. The pathogenesis of HCLD is multifactorial and not completely understood. Data suggest that these patients have a form of obliterative bronchiolitis or bronchiectasis. Obliterative bronchiolitis is thought to be a result of repeated injury to the airway epithelium, followed by inflammation and ultimately leading to fibrosis and narrowing of the small airways [2, 17, 44]. Bronchiectasis is characterized by injury and subsequent neutrophilic inflammation [45]. This difference in pathogenesis makes comparisons more difficult. Furthermore, most of the studies included treatment with corticosteroids in addition to macrolides. Corticosteroids are highly potent immunosuppressants, which may influence the results. In summary, corticosteroids together with macrolides are more potent in reducing local inflammation than are macrolides alone. Further investigations regarding the use of corticosteroids for improvement of lung function and reduction of inflammation in HCLD could be considered. However, particular caution is warranted due to the increased risk of infections, especially in a virally non-supressed population.

Few studies on eNO have been conducted in people living with HIV. Previous studies have focused on HIV-TB co-infection or history of prior TB [24, 46, 47]. The results are conflicting, but studies have shown that eNO levels are lower in patients with active or prior TB and cystic fibrosis [48, 49]. This can be explained by the fact that *Mycobacterium tuberculosis* reduces the activity of iNOS, as an immune evasion strategy, thereby decreasing levels of NO in expired air [48]. In cystic fibrosis, diffusion of eNO to the exhaled air and expression of iNOS is reduced by the thick mucus [50]. The local antimicrobial effects of NO can therefore be compromised in these patients, leading to increased lung inflammation, although confirming data are scarce [50].

Two studies found lower eNO levels in adults and children with HIV [24, 46], and prior history of TB was associated with lower eNO levels in one. Another study found eNO to be reduced in participants with TB infection, independent of HIV status [47]. A large study in well-treated adults with HIV in Copenhagen, Denmark, found that eNO levels were higher in adults with HIV than in healthy controls [51]. This study looked at HIV infection alone. Participants had no history of TB or active TB at sample collection and no CLD, defined as no obstruction on spirometry and absence of asthma and airway medications. These results indicate that HIV alone may result in elevated eNO levels and that active lung TB or history of TB may explain the decreased eNO levels seen in other studies in people with HIV. This study was conducted in a western setting in virally supressed adults, whereas our participants were sub-Saharan African children, and almost 40% had HIV viral load >1000 copies/mL, making comparisons complicated.

The strengths of this study are the prospective design and randomization of participants to azithromycin or placebo treatment. The groups were quite similar at enrolment. We have a high rate of follow-up and completeness of data, and no participants used corticosteroids that could influence study results. Limitations include difficulties in adherence to medication for a whole year and the restriction of eNO measurements to enrolment and 48 weeks.

In conclusion, we found that a higher eNO level at baseline was a risk factor for ARE in children with HCLD. eNO was associated with MMPs previously found to be proinflammatory and to play a role in the development of CLD and airway remodelling. We observed no effect of azithromycin on eNO levels. Measurement of eNO at the time of ARE could be beneficial in the quest to further understand the role of eNO in HCLD progression. Further studies on the potential implications of eNO as a tool to monitor local airway inflammation and risk of ARE in patients with HCLD are warranted.

AUTHOR CONTRIBUTIONS

Study conception and design: Trym Thune Flygel, Evgeniya Sovershaeva, Rashida Abbas Ferrand, Trond Flægstad, Jon Øyvind Odland, and Sarah Rowland-Jones. Data collection and management: Dan Hameiri-Bowen, Louis-Marie Yindom, Victoria Simms, Tsitsi Bandason, and BREATHE study team. Data analysis: Trym Thune

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Flygel and Victoria Simms. Manuscript preparations and writing: Trym Thune Flygel and Evgeniya Sovershaeva. Review: All authors. All authors have read and approved the final version.

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CONFLICT OF INTEREST STATEMENT

There were no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Paper III

Appendix I

Supplementary materials Paper I

Supplementary tables, figures and files

Supplementary table 1. Alpha diversity in HIV infected and HIV uninfected participants.

	HIV infected group (N=177)	**HIV uninfected group (N=103)**	
	Median, IQR		p value*
Observed OTUs	184 (142-226)	201 (168-240)	0.003
Chao1	247.5 (197.8-296.8)	281.3 (237.2-328.4)	<0.001
Shannon index	4.1 (3.5-4.6)	4.0 (3.6-4.5)	0.83

* p values were obtained using Wilcoxon rank-sum test and corrected for multiple testing using FDR

Supplementary table 2. Association between HIV status and alpha diversity indices (linear regression analysis where alpha diversity index is the outcome and the HIV status is the dependent variable).

	Univariate analysis		**Adjusted for BMI, age and sex**		**Adjusted for BMI, age, sex, antibiotics in 3 previous months** *	
	β coefficient (95% CI)	p value	β coefficient (95% CI)	p value	β coefficient (95% CI)	p value
Observed OTUs	-18.9 (-31.7 to -6.1)	0.004	-18.8 (-35.0 to -2.6)	0.02	-26.5 (-54.8 to 1.89)	0.07
Chao1	-35.7 (-52.3 to -19.0)	<0.001	-34.7 (-55.7 to -13.7)	0.001	-42.3 (-79.1 to -5.6)	0.02
Shannon index	-0.1 (-0.2 to 0.1)	0.53	-0.1 (-0.3 to 0.1)	0.43	-0.3 (-0.7 to 0.1)	0.16

* interaction term between HIV status and antibiotics in the three previous months status was introduced into the adjusted model.

Supplementary table 3. Alpha diversity in HIV infected-suppressed, HIV infected non-suppressed and HIV uninfected participants.

	HIV+, suppressed (N=104)	**HIV+, non-suppressed (N=73)**	**HIV- group (N=103)**	**HIV+, suppressed vs HIV-**	**HIV+, non-suppressed vs HIV-**
	Median, IQR			p value	
Observed OTUs	192.5 (145.5-228.5)	176 (138-220)	201 (168-240)	0.05*	0.005*
Chao1	259.3 (201.2-302.1)	233.2 (175-276)	281.3 (237.2-328.4)	0.006*	<0.001*
Shannon index	4.10 (3.48-4.61)	4.06 (3.60-4.57)	4.0 (3.6-4.5)	0.51*	0.37*

* presented p values were obtained from regression model adjusted for BMI, age and sex where HIV status was introduced as an independent variable and alpha diversity estimates as a dependent (outcome) variable.

Supplementary table 4. Association between characteristics of HIV infected participants (independent variables) and alpha diversity indices (dependent variables) in linear regression analysis adjusted for BMI, age and sex.

	Richness				**Richness + evenness**	
Covariates	**OTUs**		**Chao1**		**Shannon**	
	b coefficients (95% CI)	p value	b coefficients (95% CI)	p value	b coefficients (95% CI)	p value
Age, years	-0.58 (-3.59 to 2.44)	0.71	-1.19 (-5.01 to 2.62)	0.54	0.01 (-0.03 to 0.06)	0.65
Sex (ref. male)	6.17 (-11.3 to 23.6)	0.49	15.8 (-6.31 to 37.9)	0.16	0.04 (-0.22 to 0.31)	0.74
Stunted (ref. no)	-7.85 (-25.1 to 9.44)	0.37	-11.1 (-33.0 to 10.8)	0.32	0.007 (-0.25 to 0.27)	0.96
Underweight (ref. no)	-8.89 (-30.9 to 13.1)	0.43	-20.8 (-48.6 to 6.87)	0.14	0.05 (-0.28 to 0.39)	0.74
Viral load, log copies/ml	-3.39 (-9.49 to 2.71)	0.27	-6.58 (-14.3 to 1.11)	0.09	0.04 (-0.05 to 0.13)	0.40
VL, categories						
Suppressed	ref.		ref.		ref.	
VL (≥1000 copies/ml)	-11.5 (-28.3 to 5.28)	0.18	-20.6 (-41.8 to 0.47)	0.05	-0.03 (-0.28 to 0.22)	0.82
CD4≤400 cells/mm^3 (ref>400 cells/mm^3)	-4.60 (-23.3 to 14.1)	0.63	-11.6 (-35.2 to 12.1)	0.33	0.16 (-0.12 to 0.44)	0.27

Years on ART	2.85 (0.01 to 5.68)	0.04	3.87 (0.28 to 7.45)	0.03	0.01 (-0.03 to 0.06)	0.55
Years on ART, categories						
<5years	ref.		ref.		ref.	
5-10 years	13.0 (-5.23 to 31.3)	0.16	20.7 (-2.39 to 43.9)	0.08	0.07 (-0.21 to 0.34)	0.63
≥10 years	32.9 (5.65 to 60.1)	0.02	38.3 (3.80 to 72.8)	0.03	0.34 (-0.07 to 0.75)	0.11
Co-trimoxazole prophylaxis (ref.no)	3.27 (-23.0 to 29.5)	0.81	1.89 (-31.4 to 35.1)	0.91	0.18 (-0.22 to 0.57)	0.38
FEV1 z-score	1.32 (-5.12 to 7.76)	0.69	0.08 (-8.07 to 8.24)	0.98	-0.03 (-0.12 to 0.07)	0.58
Diagnosis of chronic lung disease (ref.no)	-0.95 (-24.9 to 23.0)	0.94	3.23 (-27.1 to 33.6)	0.83	-0.04 (-0.40 to 0.32)	0.82
ART regimen (ref. NNRTI)	0.24 (-16.9 to 17.4)	0.98	-5.36 (-27.1 to 16.4)	0.63	0.03 (-0.23 to 0.28)	0.84
Previously treated for TB (ref.no)	-4.10 (-21.5 to 13.3)	0.64	-4.50 (-26.5 to 17.5)	0.69	0.08 (-0.18 to 0.34)	0.55
Episodes of diarrhea (ref.no)	-1.23 (-33.7 to 31.3)	0.94	6.09 (-33.3 to 45.5)	0.76	-0.06 (-0.58 to 0.46)	0.81

Supplementary table 5. HIV infected participants with and without chronic lung disease.

	HIV infected with chronic lung disease (N=149)	**HIV infected without chronic lung disease (N=28)**	
	Median, IQR		p value*
Observed OTUs	183 (144-228)	191 (140-216)	0.64
Chao1	245.9 (199.3-297.6)	248.7 (191.9-276.8)	0.68
Shannon index	4.1 (3.5-4.6)	4.0 (3.5-4.7)	0.80

* p values were obtained using Wilcoxon rank-sum test and corrected for multiple testing using FDR

Supplementary table 6. Association between characteristics of HIV uninfected participants (independent variables) and alpha diversity indices (dependent variables) in linear regression analysis adjusted for BMI, age and sex.

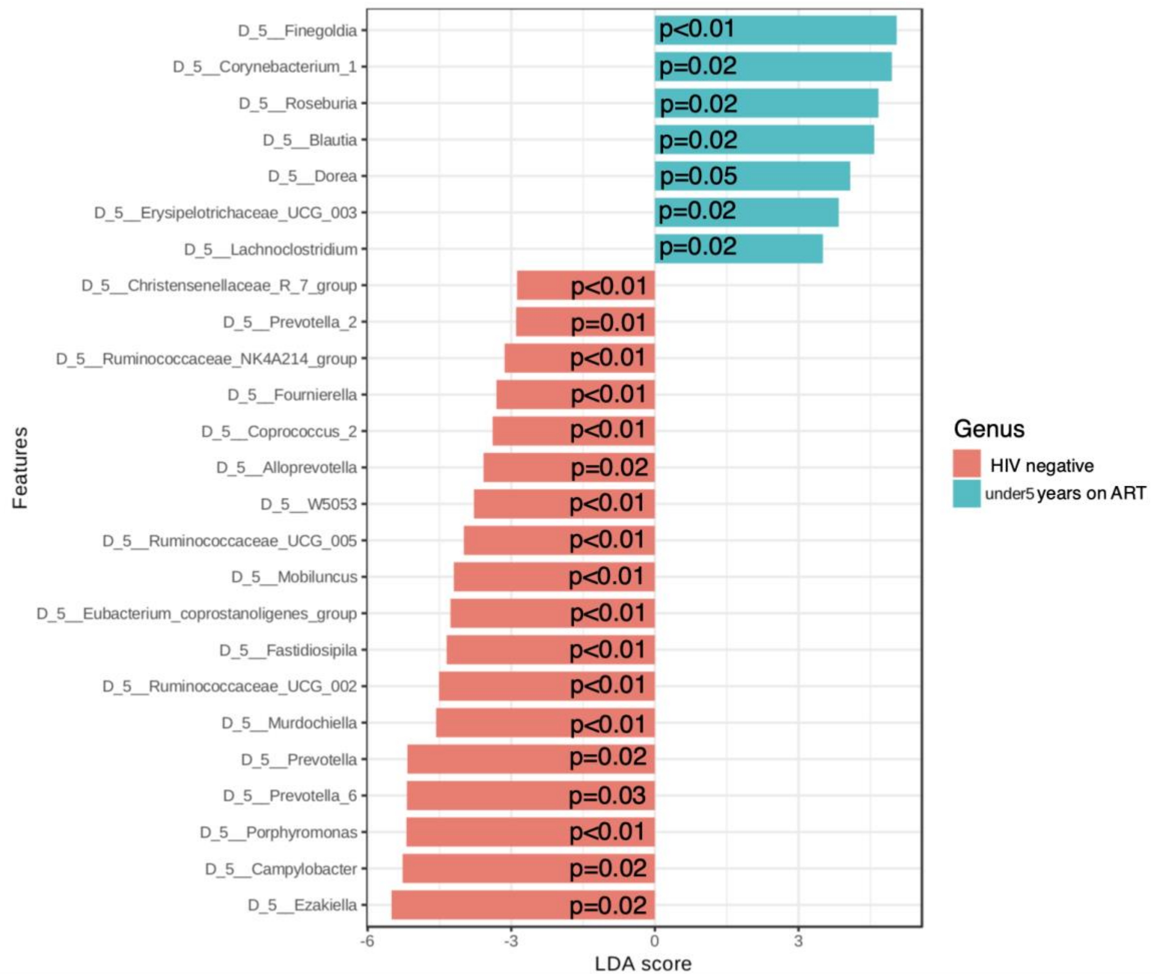
	Richness				**Richness + evenness**	
Covariates	**OTUs**		**Chao1**		**Shannon**	
	b coefficients (95% CI)	p value	b coefficients (95% CI)	p value	b coefficients (95% CI)	p value
Age, years	1.96 (-2.10 to 6.03)	0.34	2.92 (-2.62 to 8.45)	0.30	0.004 (-0.05 to 0.06)	0.89
Sex (ref. male)	-2.97 (-23.0 to 17.1)	0.77	3.92 (-23.4 to 31.2)	0.78	0.22 (-0.05 to 0.50)	0.11
Stunted (ref. no)	-9.94 (-56.1 to 36.2)	0.67	-31.9 (-94.5 to 30.8)	0.31	-0.11 (-0.75 to 0.53)	0.74
Underweight (ref. no)	-3.52 (-51.4 to 44.4)	0.88	-27.6 (-92.6 to 37.5)	0.40	0.27 (-0.39 to 0.93)	0.42
FEV1 z-score	4.83 (-8.48 to 18.1)	0.47	4.62 (-13.8 to 23.1)	0.62	0.06 (-0.11 to 0.24)	0.48
Episodes of diarrhea (ref.no)	19.9 (-38.2 to 78.1)	0.50	40.4 (-38.6 to 119.4)	0.31	0.06 (-0.75 to 0.87)	0.88

Supplementary table 7. Comparison of beta diversity in HIV infected participants based on different participant characteristics.

	Wilcoxon rank sum test	**permutational multivariate analysis of variance (PERMANOVA)**
TB-treated (n=56) vs. no TB-treatment (n=121)	p=0.40	p=0.86
Viral load suppressed (n=104) vs. non-supressed (n=73)	p=0.43	p=0.69
CD4 counts >400 cells/mm^3 (n=128) vs. CD4 <400 cells/mm^3 (n=49)	p=0.34	p=0.19
Duration of ART <5 years (n=53) vs. 5-10 years (n=100) vs. ≥10 years (n=23)	–	p=0.32

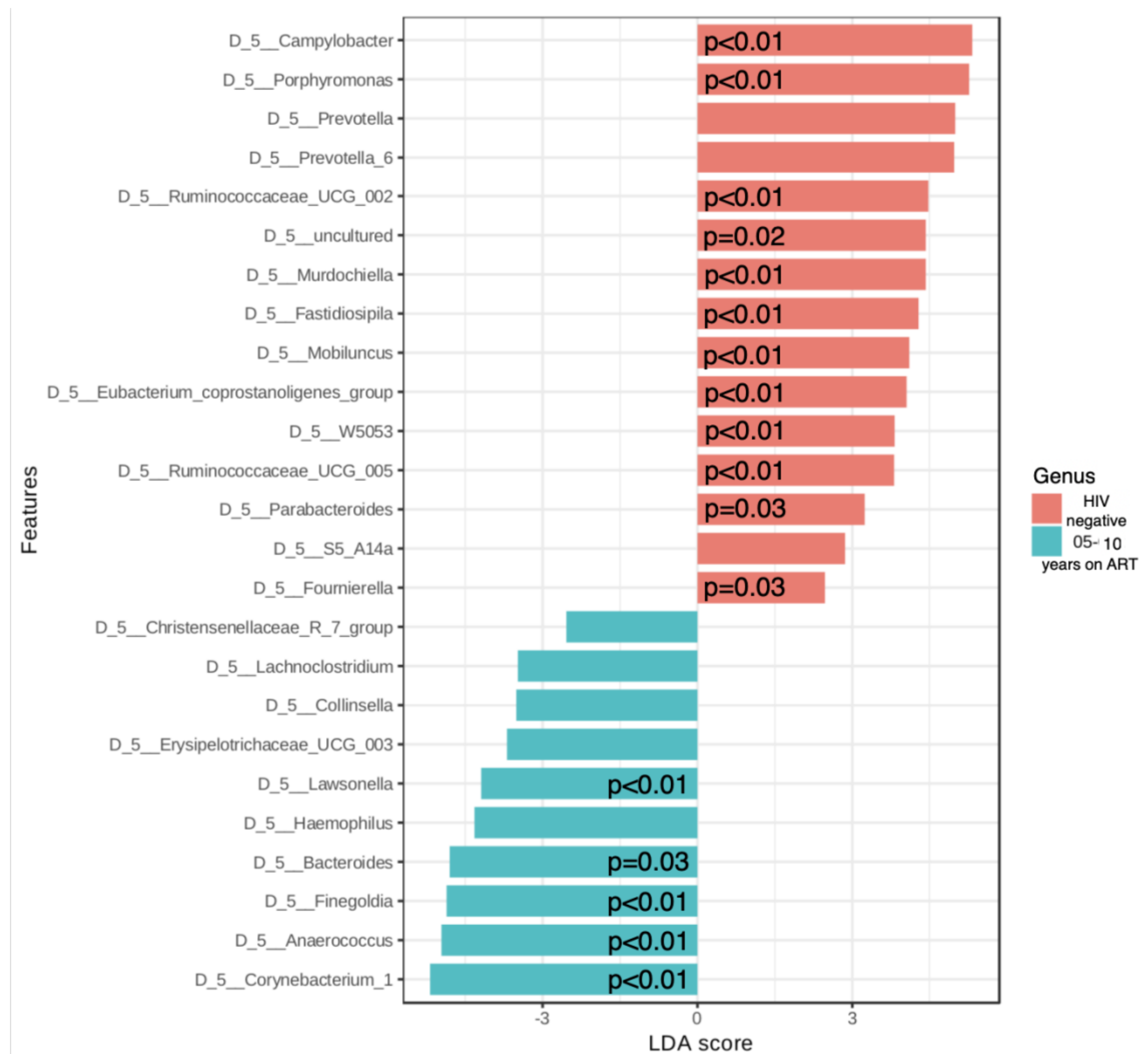
Table showing no significant differences in beta diversicy in HIV infected group based on patient charachteristics. For PERMANOVA-analysis umber of permutations was set to 999. P-values were adjusted for multiple testing using the Benjamini-Hochberg method.

Supplementary figure 1. LEfSe (linear discriminant analysis effect size)-plot.



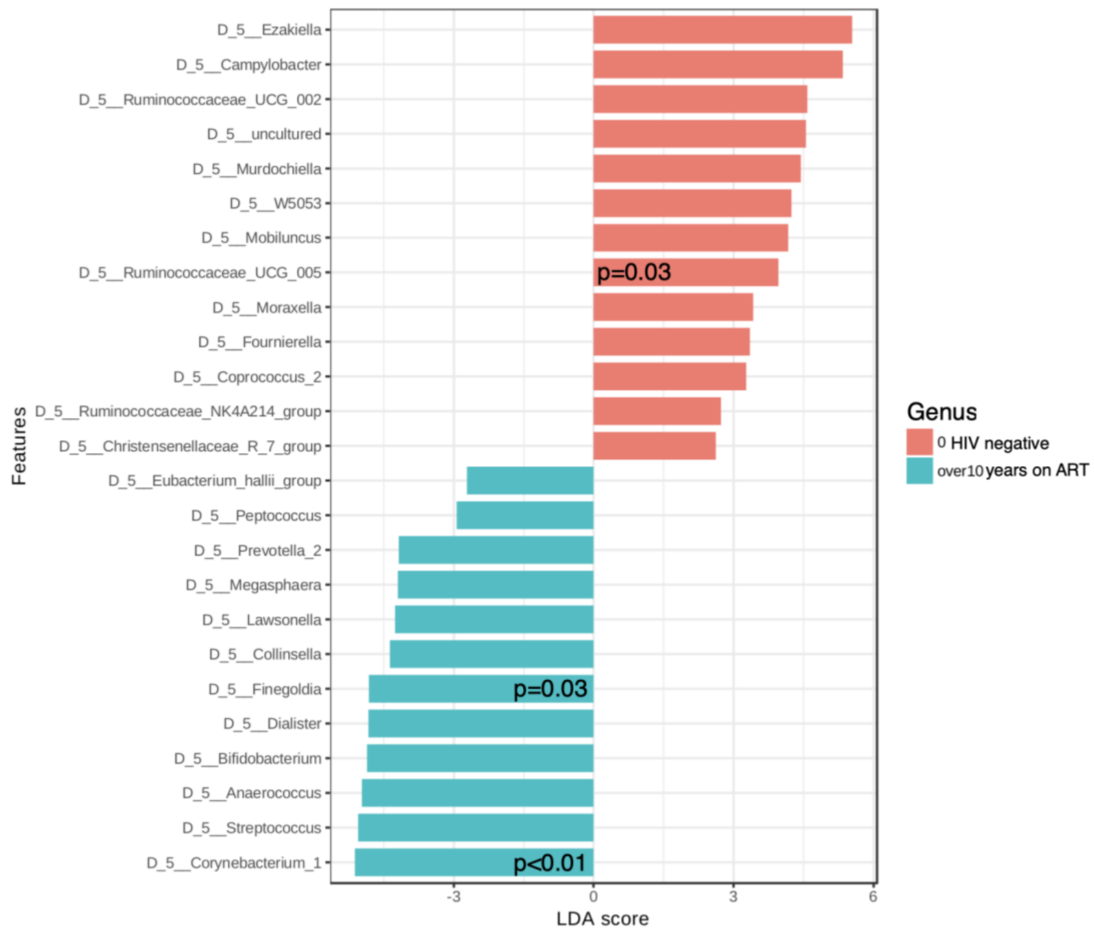
Showing enriched taxa that are significantly different between HIV infected participants on ART < 5 years (blue) and HIV uninfected participants (red). Only taxa meeting a significant level of 0.05 and effect size threshold of 1.0 are included. P-values shown are only those significant after adjustment for FDR. The longer participants had taken ART, the fewer taxa was significantly different between HIV infected and HIV uninfected

Supplementary figure 2. LEfSe (linear discriminant analysis effect size)-plot.



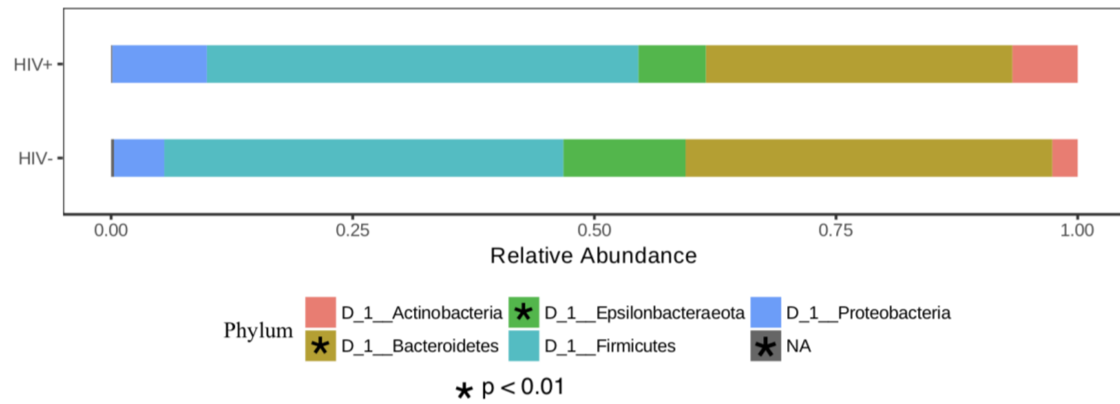
Showing enriched taxa that are significantly different between HIV infected participants on ART for 5-10 years (blue) and HIV uninfected participants in Zimbabwe (red). Only taxa meeting a significant level of 0.05 and effect size threshold of 1.0 are included. P-values shown are only those significant after adjustment for FDR. The longer participants had taken ART, the fewer taxa was significantly different between HIV infected and HIV uninfected

Supplementary figure 3. LEfSe (linear discriminant analysis effect size)-plot.

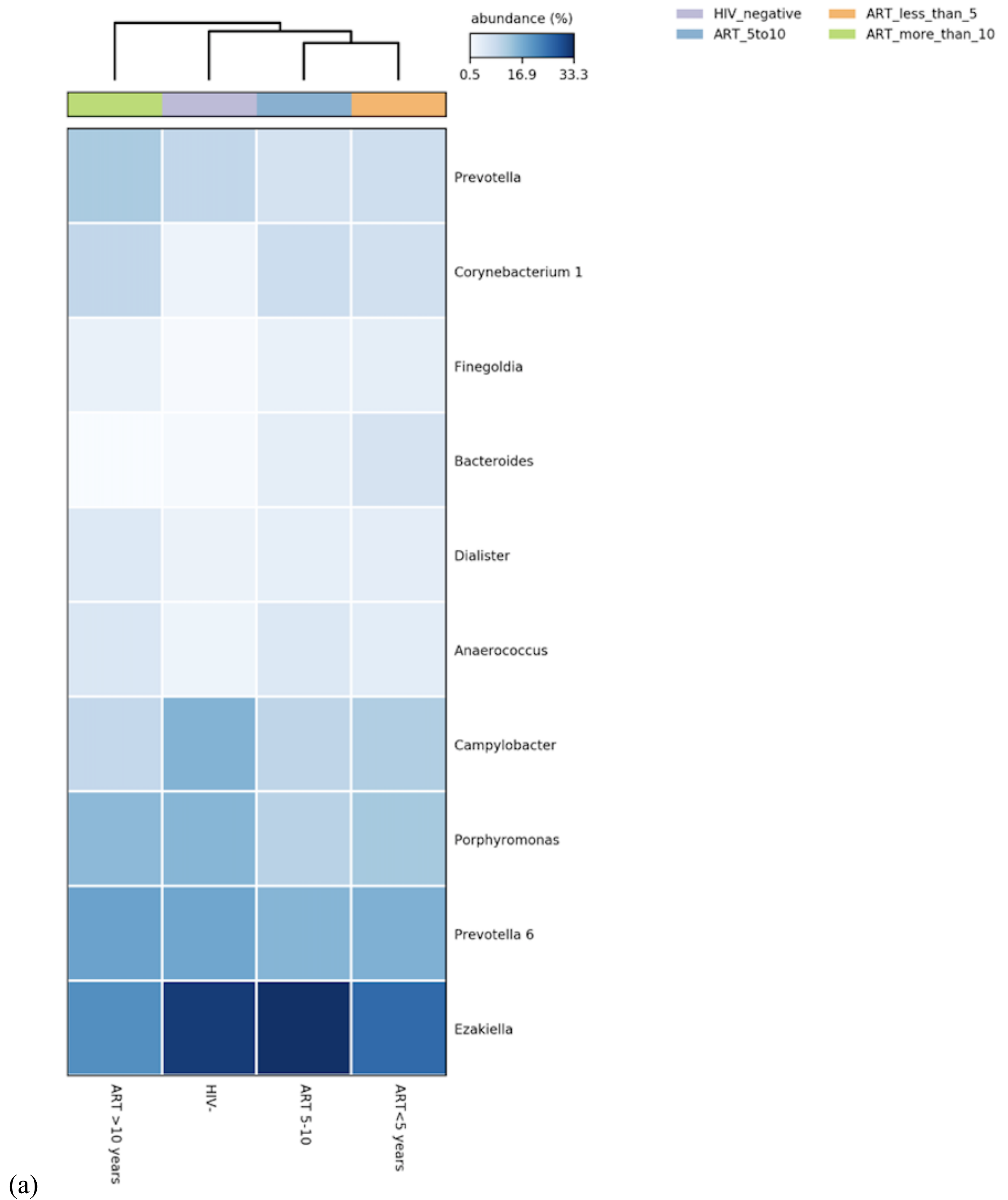


Showing enriched taxa that are significantly different between HIV infected participants on ART > 10 years (blue) and HIV uninfected participants (red). Only taxa meeting a significant level of 0.05 and effect size threshold of 1.0 are included. P-values shown are only those significant after adjustment for FDR. The longer participants had taken ART, the fewer taxa was significantly different between HIV infected and HIV uninfected

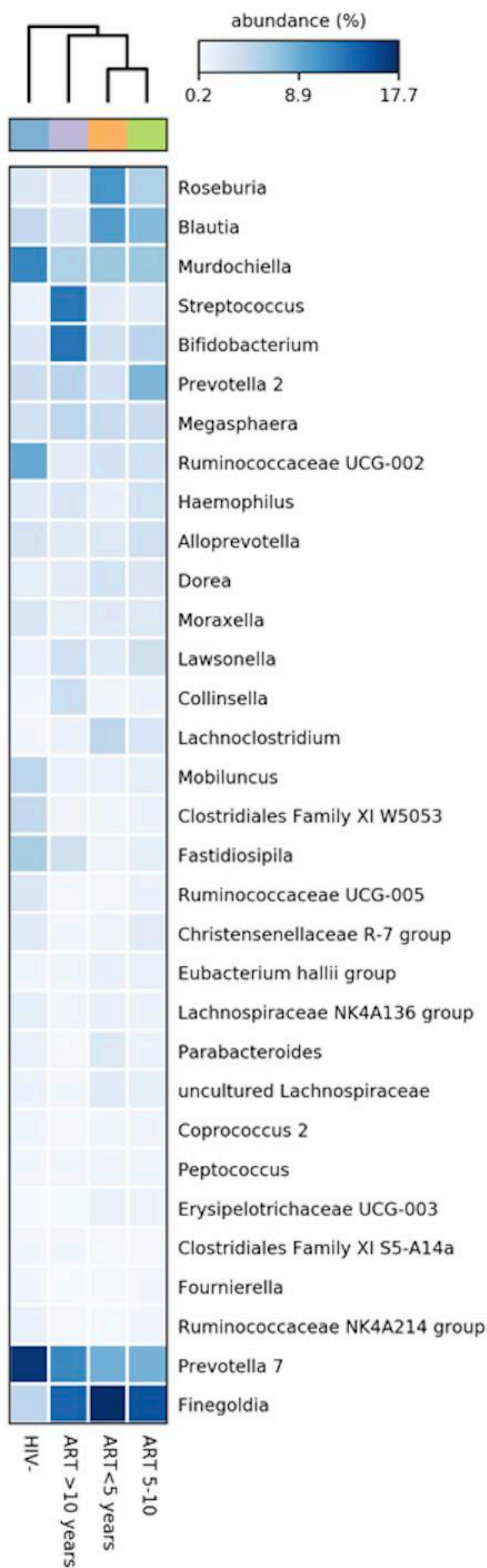
Supplementary figure 4. Relative abundance at phylum level for HIV infected and HIV uninfected participants. P-value is adjusted for FDR.



Supplementary figure 5. Heatmap showing differences of the (a) more abundant genera and (b) less abundant genera, between HIV uninfected participants and HIV infected participants grouped by time spent on ART (>5 years, 5-10 years and ≥10 years).

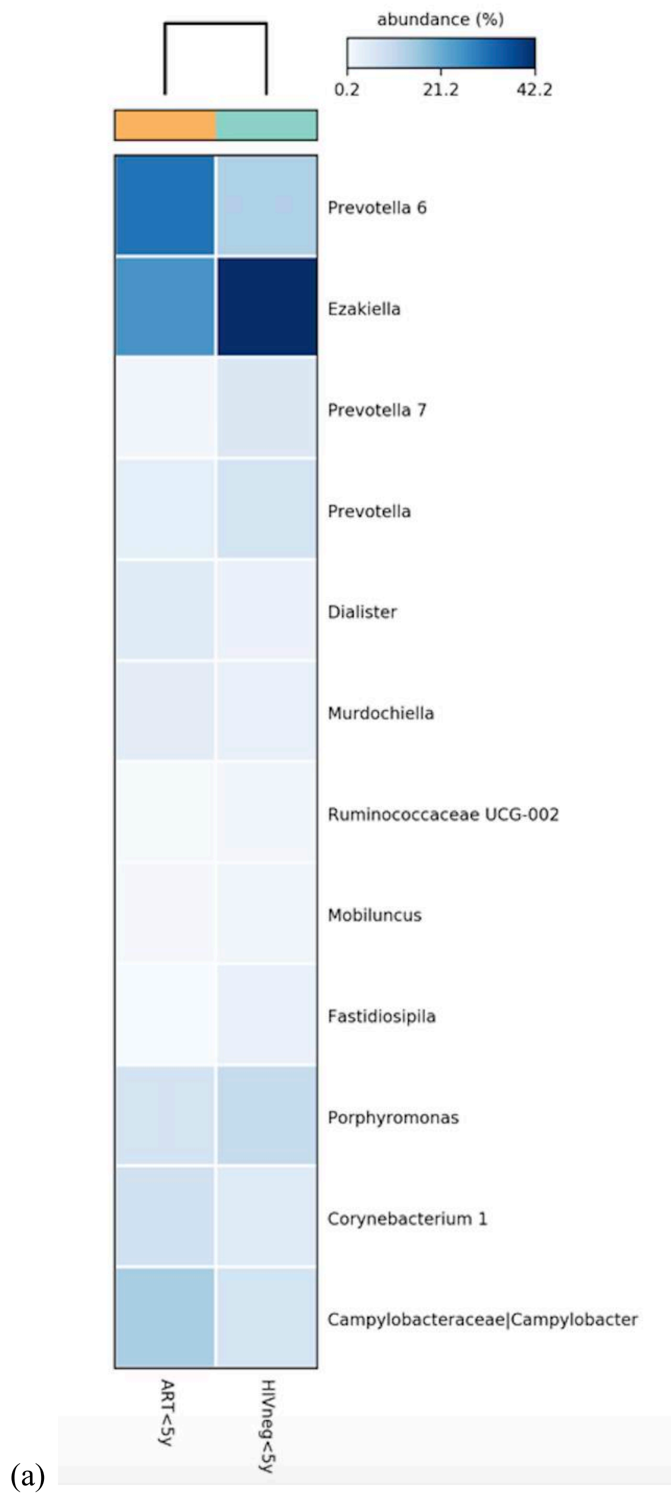


(a)

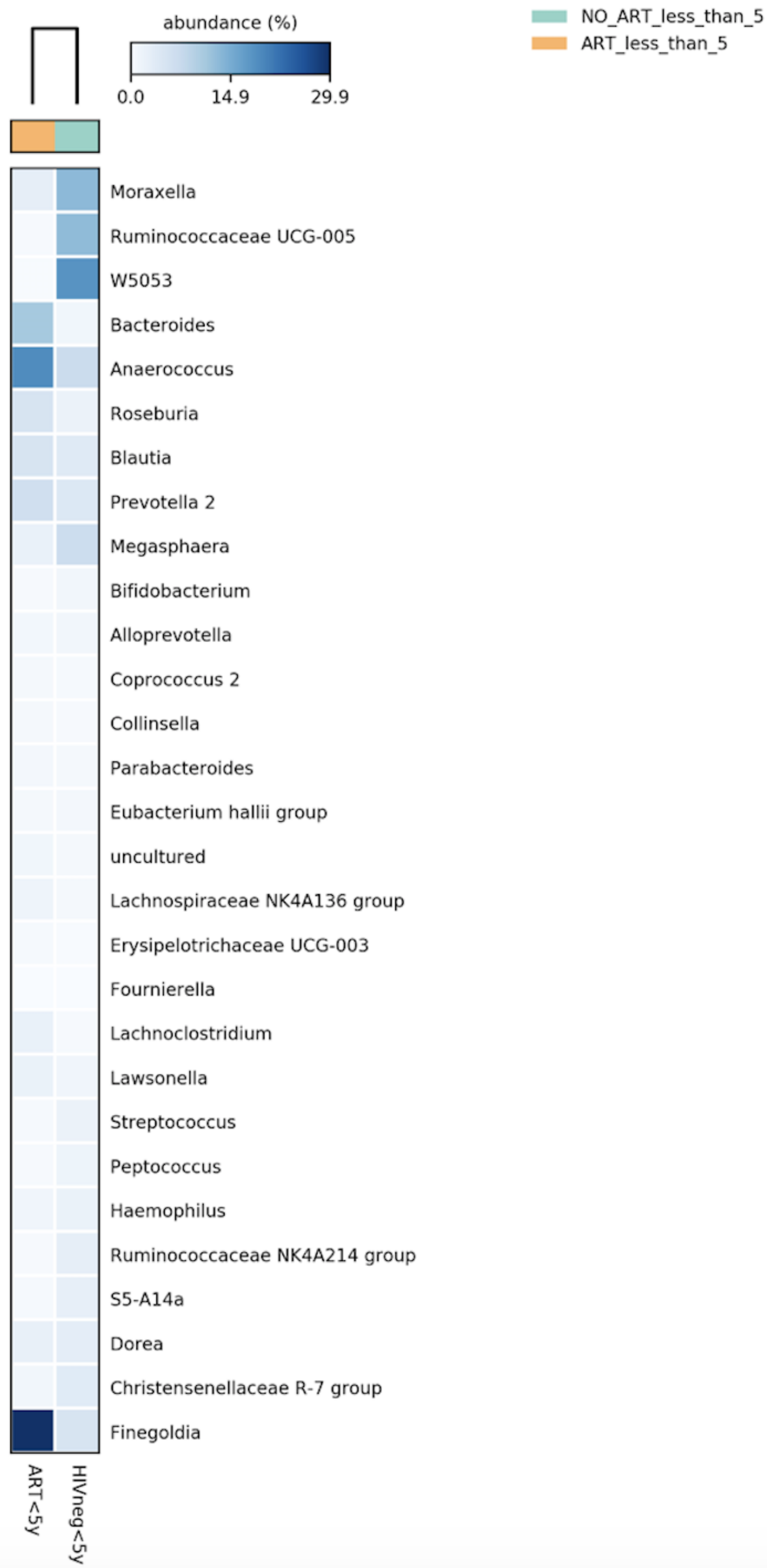


(b)

Supplementary figure 6. Heatmap showing differences of the (a) more abundant genera and (b) less abundant genera, between age matched HIV uninfected participants and HIV infected participants on ART<5 years.

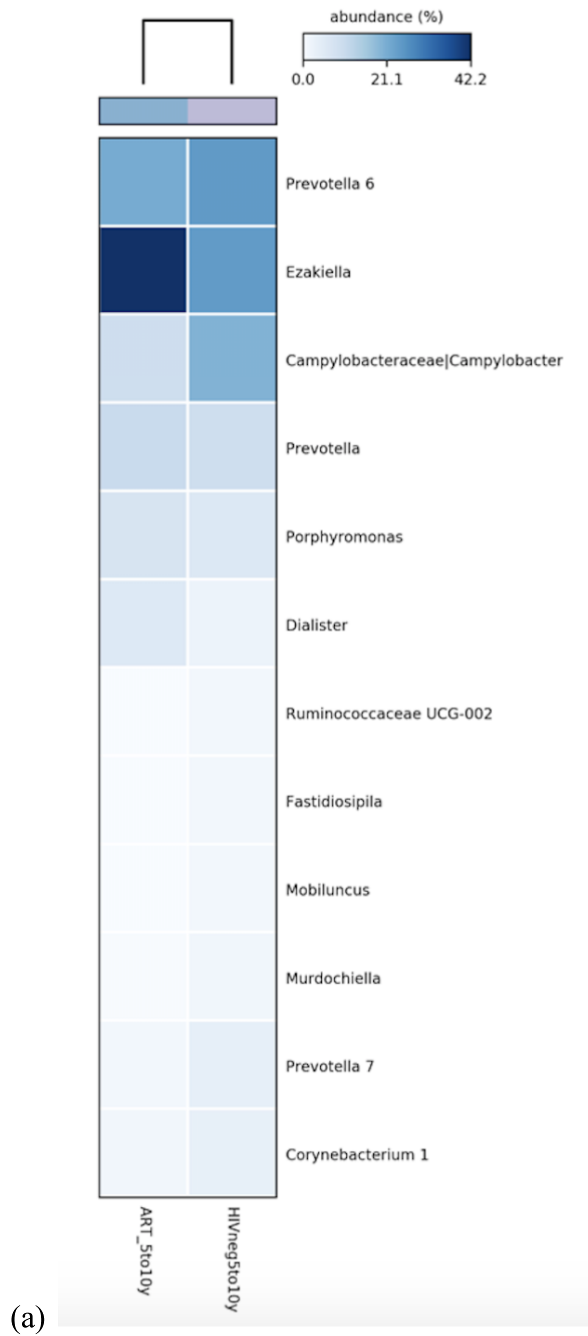


(a)

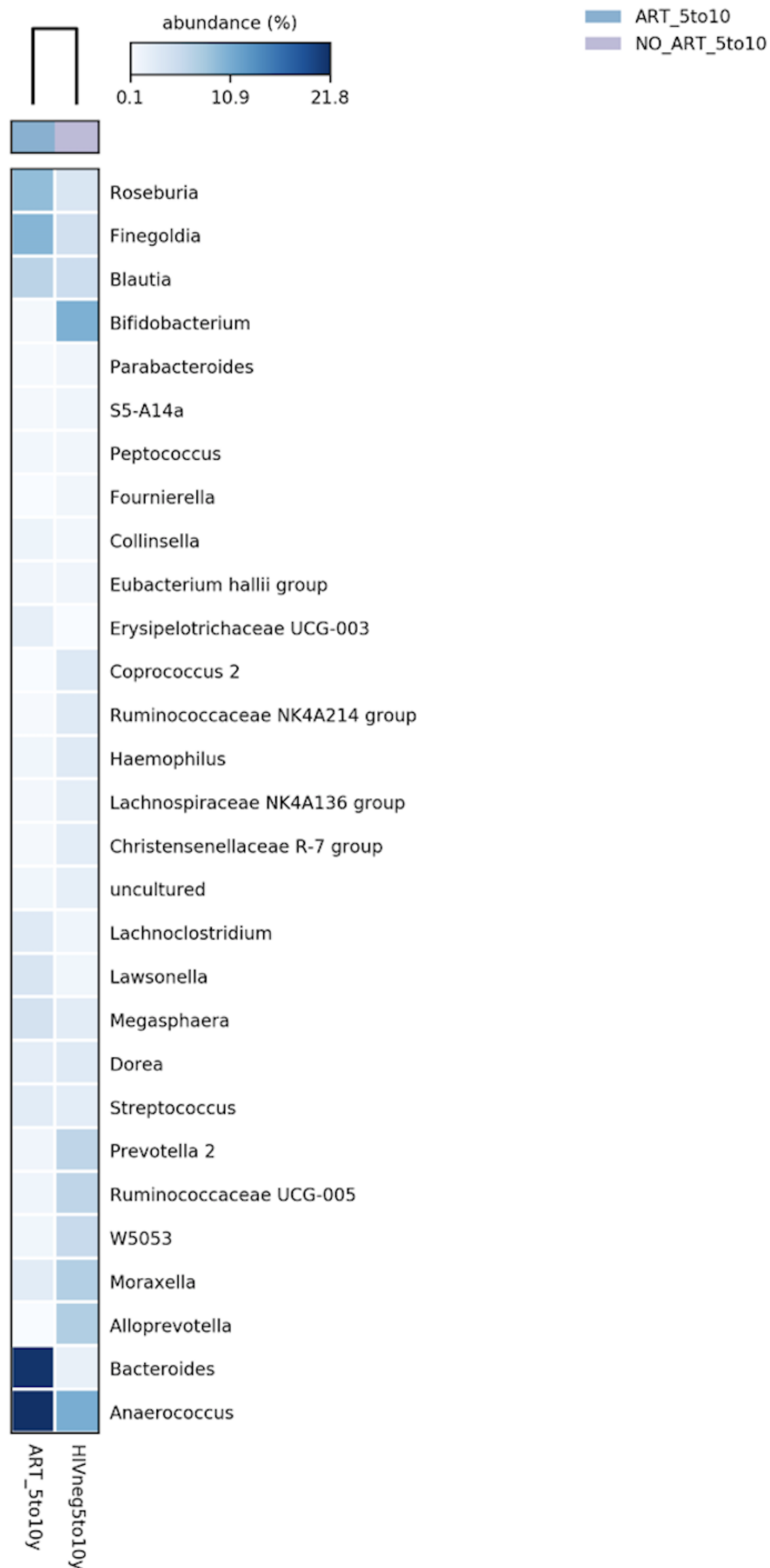


(b)

Supplementary figure 7. Heatmap showing differences of the (a) more abundant genera and (b) less abundant genera, between age matched HIV uninfected participants and HIV infected participants on ART 5-10 years.

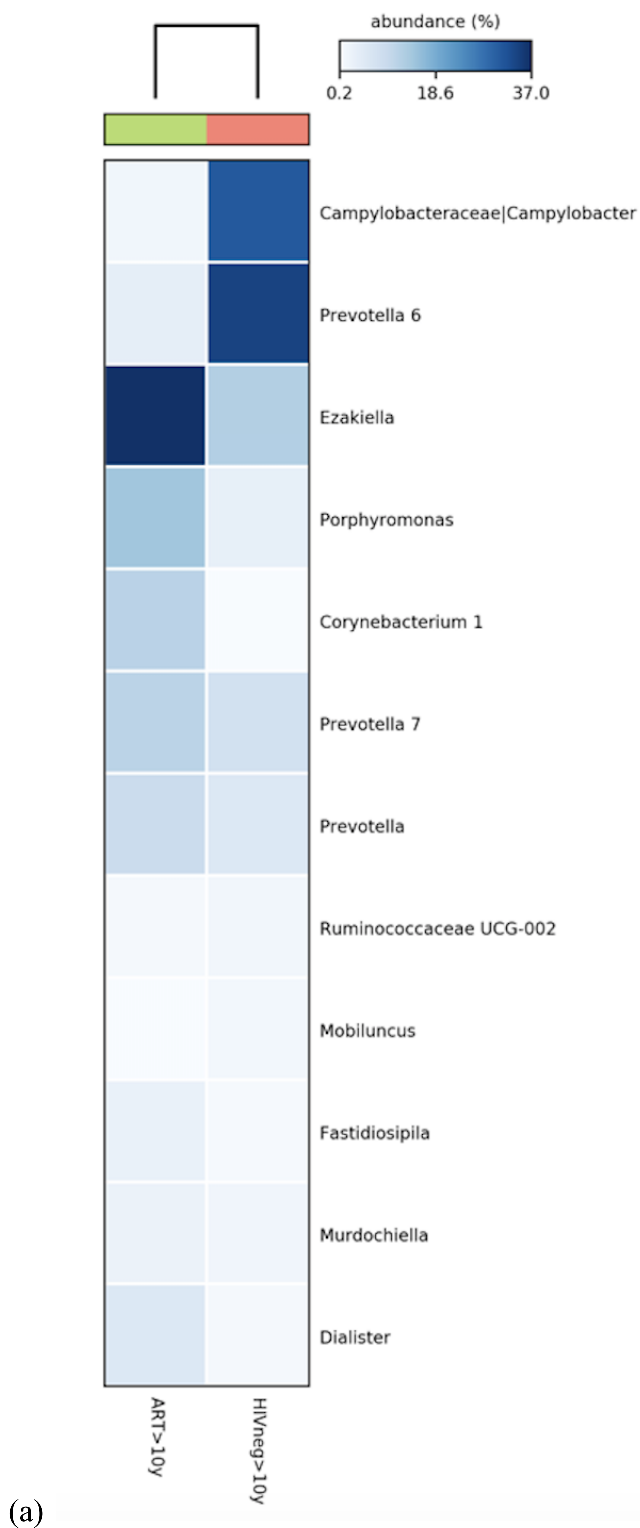


(a)

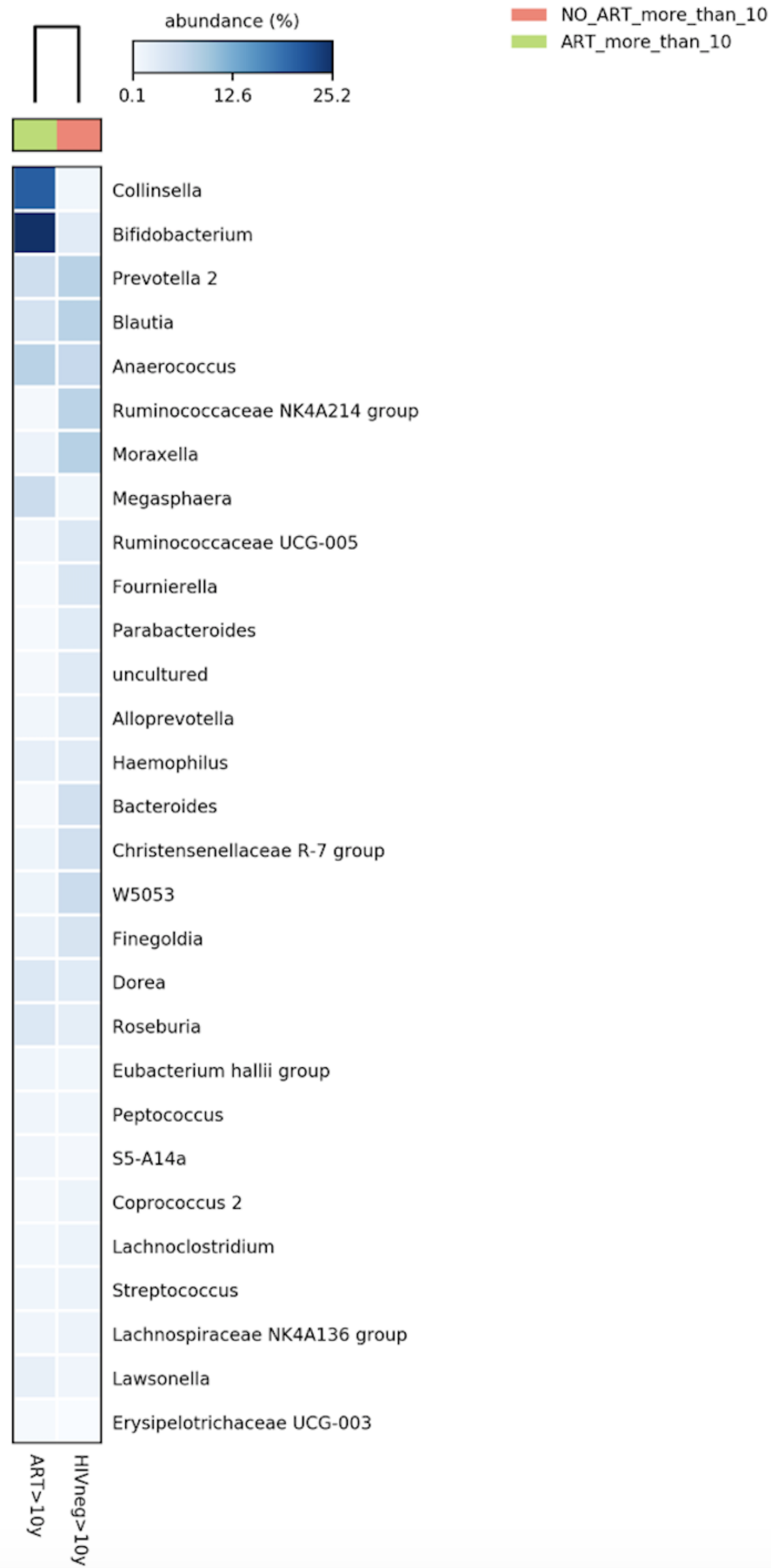


(b)

Supplementary figure 8. Heatmap showing differences of the (a) more abundant genera and (b) less abundant genera, between age matched HIV uninfected participants and HIV infected participants on ART>10 years.



(a)



(b)

Supplementary file 1. Laboratory procedures

Sample collection

Rectal swabs were collected from all participants at enrolment into the trial. Rectal swabs were obtained by study nurses, inserting the swab approximately 2-3 cm inside the rectum. All samples were visibly stained with faeces before immediately being preserved in 1,5 ml of transport medium PrimeStore ® MTM (Langhorn, Bethesda, Maryland, USA). Upon sampling, specimens were directly stored on ice for a maximum one hour, and then frozen at minus 80 °C before shipment on dry ice to the laboratory at Division of Medical Microbiology, University of Cape Town (UCT).

DNA extraction

We used tthe Zymo Research Quick-DNA™ Fecal/Soil Microbe Microprep kit (Irvine, California, USA) for DNA extractions, as preliminary experiments performed at UCT showed that this kit yielded better quality of DNA from low biomass samples, compared to other kits and automated methods. DNA was extracted according to the manufacturer's description, with a few modifications. Briefly, aliquots of 400 µl of each sample was mixed with 400 µl of BashingBead™ Buffer in a ZR BashingBead™ Lysis Tube. Mechanical lysis (bead beating) was performed using the TissueLyser LT™ (QIAGEN) set to 50 Hz for 5 minutes. Then 500 µl of supernatant was transferred to Zymo-Spin™ III-F Filter (Irvine, California, USA) and centrifuged at 8000 x g for 1 minute. Further chemical lysis was done by adding Genomic Lysis Buffer. All other procedures were done according to the manufacturers protocol.

16S real time quantitative PCR

After extractions of nucleic acids, we performed a real-time quantitative polymerase chain reaction (qPCR) on the extracted product to see if the DNA was giving amplicons of good

quality for further analysis, and to establish the total bacterial load of the samples as previously described [1]. The PCR reaction consisted of 15 µl SensiFAST™ Probe No-ROX (catalogue no. BIO-86020, Bioline, London, UK), 1 µl of forward primer 16S-F1 (5′-CGA AAG CGT GGG GAG CAA A-3′) at 10µM , 1 µl of reverse primer 16S-R1 (5′-GTT CGT ACT CCC CAG GCG G-3′) at 10 µM, 1 µl of the probe 16S-P1 (FAM-ATT AGA TAC CCT GGT AGT CCA-MGB) at 5 µM, 2,5 µl of DNA template and 9,5 µl MilliQ water, giving a total volume of 30 µl per sample [1]. We used a set of 7 bacterial DNA standards by Zymo Research (Irvine, California, USA) with 10-fold dilutions, and 1 non-template control (Femto Bacterial DNA quantification, catalogue no. ZR E2006-2) as a standard curve. The amplifications were done using a BioRad C1000™ thermal cycler with CFX96™ Real-Time system (Hercules, California, USA), using the previously described protocol: 50 °C for 2 min, 95 °C for 5 min, and 45 repetitive cycles denaturation at 95 °C for 15 s and annealing at 60 °C for 60 s.

Nanodrop

All sample concentrations were also measured by spectrophotometry, using the NanoDrop™ ND100 (Thermo Fisher Scientific, Massachusetts, USA), for quantification and purity assessment, with varying results. Using 1,5 µl of each sample, most of the samples gave low NanoDrop-concentrations (< 5ng/ml), but all samples gave good amplifications by real time qPCR, with most samples having a CQ-value of < 28. In comparison MilliQ water gave an average CQ-value of 34 within the 5 runs.

16S short and long PCR

We performed two sets of PCRs targeting the hypervariable V4 region of the 16S ribosomal ribonucleic acid (rRNA) gene using the primers 515F (5’ GTGCCAGCHGCYGCGGT 3’) and 806R (3’ TAATCTWTGGGNNCATCAGG 5’), according to previously described protocols

[2, 3]. The first PCR run, aiming to amplify the V4 region using target-only primers, consisted of 12.5 µl of 2X MyTaq™ HS Mix (Bioline, London, UK), 2 µl of the forward and reverse primers at concentrations of 10 µM each, 0,75 µl dimethyl sulfoxide (catalogue no D2650, Sigma-Aldrich®, Missouri, USA) and 4 µl template, made to a final volume of 25.25 µl using PCR-grade water (Thermo Fisher Scientific Inc., Massachusetts, USA). Amplifications were done under the following conditions: Denaturation at 95 °C for 3 min, 10 repetitive cycles of amplification at 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 s, with a final extension at 72 °C for 5 min.

The second run of PCR used 4 µl of the amplified V4 product from the first run as template and the primers 515Fmod4_SM_12N(-15N) (5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC GATCTNNNNNNNNNNGTGCCAGCHGCYGCGGT 3') and 806Rmod1_SM_12N(-15N) (5' CAAGCAGAAGACGGCATACGAGATACGAGACTGATTGTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTNNNNNNNNNNNNGGACTACNNGGGTWTCTAAT 3') to further amplify the V4 product from run one. The long, modified primers also include the needed sequencing adapters, primer region and 12-15 staggered random nucleotides (NNNNNNNNNNNN) serving as a spacer. To reduce non-specific binding risks and introduction of additional PCR bias when adding extra components to the primers, the PCR were performed using this two-step approach [4]. Golay barcodes were incorporated in the reverse primer 860R_SMod_long (12 underlined bases) to identify each sample individually [2], with the modifications of adding 20 cycles to the amplification step [3].

Agencourt® AMPure® XP PCR Purification kit (Beckman Coulter, California, USA) was used to clean the amplicons as previously described [3]. Modified from manufacturers protocol with a 0.65:1 ratio of Agencourt AMPure XP solution to PCR products in step 2. Using a 2% agarose gel electrophoresis at 110V for 90 min, we verified the PCR products, and amplicons were quantified using QuantiFluor® dsDNA System quantification kit on Promega Glomax®-Multi Detection system (Promega, Wisconsin, USA). Determined by the calculations from the quantification, we pooled the samples at 120 ng, followed by purification using a 1:1 ratio of Agencourt AMPure XP solution. The pooled and purified samples were again quantified using the Qubit™ dsDNA BR Assay Kit (Thermo Fischer Scientific Inc., Massachusetts, USA), and run on a 1,6% agarose gel electrophoresis for 30 min at 35V, 45 min at 40V, 3 hours at 70V, and 60 min at 50V. The products were excised from the gel and purified using QIAquick Gel Extraction Kit (50) (QIAGEN, Hilden, Germany) with slight modifications as describes by Claassen et al. [3]. Modifications included incubation of sample for 5 min at 37 ˚C at step 10, and heating of elution buffer, Tris-EDTA (pH 8.0) at 60-70 ˚C at step 13.

16S rRNA gene sequencing (done at CPGR)

The final 16S library from rectal swabs at 50 µl was measured to a concentration of 49,2 ng/ µl using the Qubit™ dsDNA BR Assay Kit (Thermo Fischer Scientific Inc., Massachusetts, USA). KAPA qPCR quantification kit (KAPA Biosystems, Massachusetts, USA) and Agilend DNA 1000 kit (Agilent Technologies, California, USA) were used to quantify and size the library. The library was then diluted to 4 nM using Buffer EB (QIAGEN) and denatured using 0,2 N NaOH and finally diluted to a concentration of 6 pM using HT1 buffer, before being sequenced on the Illumina Miseq® using the Miseq® Reagent v3 kit, 600 cycles (Illumina, California, USA), adding a 25% PhiX library at 6 pM as internal control, as per manufacturer's instructions [5].

Sequencing controls

Sequencing controls used in the run included two no-template water controls and two PrimeStore controls, one of each spiked with mycobacterium smegmatis. ZymoBIOMICS microbial community standard and ZymoBIOMICS microbial community DNA standards (Zymo Research, Irvine, California, USA) were used, as well as four randomly selected biological samples that were run in duplicates to check reproducibility and control for technical variations within the single sequencing run. Both PrimeStore and ZymoBIOMICS microbial community standard went through the entire extraction protocol, to control for contamination in all steps of the library preparations.

Controls, including the ZymoBIOMICS standards, and biological samples were analysed separately. The average sequence reads of each OTU detected in the spiked controls (except from the DNA these controls were spiked with) were calculated and subtracted from the biological samples. In the biological replicates the number of reads between the two replicates varied (+/- 20%), but the taxonomic profiles were completely overlapping. In addition, the replicated samples separated into four clusters both after alpha and beta diversity analysis, confirming technical reproducibility of the run (data not shown).

Bioinformatics

Pre-processing of sample reads were done according to previously described protocols using the H3ABioNet 16S rDNA diversity analysis package (https://github.com/h3abionet/h3abionet16S) [3]. Sequencing quality was assessed by FASTQ files using Fastqc and SolexaQA. The forward and reverse sequences were merged using USEARCH7 fastq_mergepairs (fastq_maxdiffs set to 3) and then quality filtered using

USEARCH7 fastq_filter. To remove potential contaminants, no-template water controls were aligned to biological samples using USEARCH7 usearch-global. The average number of reads from each unique sequence from the no-template controls were removed from biological samples where reads matched at 100% similarity. For dereplication and selection of sequences occurring more than twice, the USEARCH sortbysize was used. Sequences were clustered to operational taxonomic units (OTUs) using USEARCH7 cluster_otus, and ChimeraSlayer reference database and USEARCH7 uchime_ref tool were used to remove chimeras [3]. The only difference from the protocol described by Claassen-Weitz et. al. was that further processing of data was performed using Quantitative Insights Into Microbial Ecology 2 (QIIME2, version 2018.4) [6]. And finally, taxonomy of representative reads was assigned using the SILVA version 132 database [7] with 97% sequence similarity. The raw sequence files have been submitted to the European Nucleotide Archive (ENA) under the accession number PRJEB32077.

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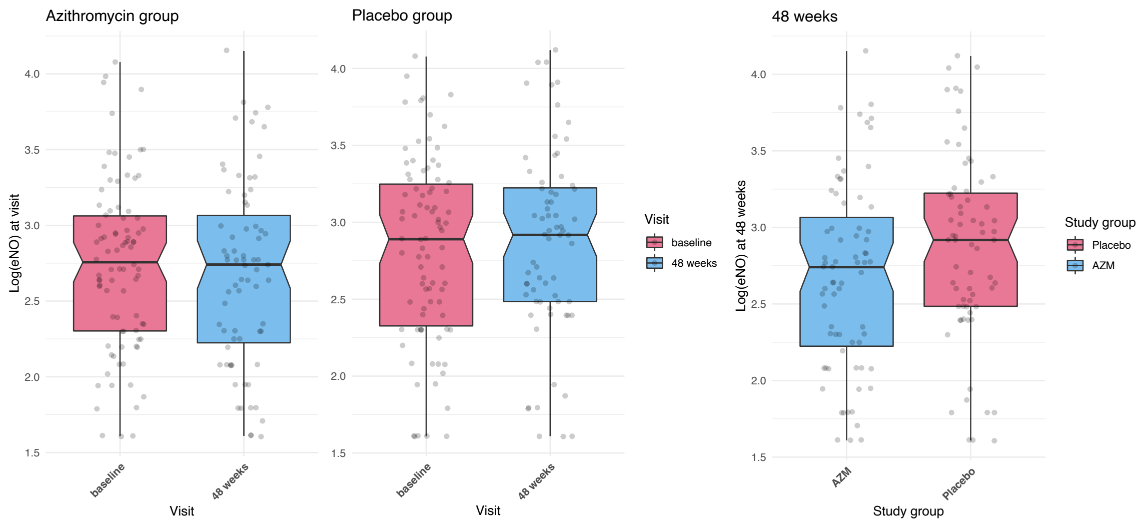
Appendix II

Supplementary materials Paper II



Supplementary figure 1. Box plot of eNO at baseline and 48 weeks by trial group and overall.

eNO, exhaled nitric oxide; AZM, azithromycin.

Supplementary table 1: Baseline characteristics of participants by experiencing ARE

Variable	ARE = yes (N = 26)	ARE = no (N = 146)	P-value
Age, median (IQR)	15.3 (12.6-17.9)	15.7 (12.9-18.2)	0.842
Female sex, N (%)	14 (54%)	66 (45%)	0.523
eNO level (ppb), geometric mean (95% CI)	15.4 (14.0-16.9)	20.8 (16.5-26.2)	0.019
Azithromycin group, N (%)	11 (42%)	75 (51%)	0.524
Wasted (weight for age Z-score <-2), N (%)	10 (39%)	76 (52%)	0.287
Stunted (height for age z-score <-2), N (%)	14 (54%)	76 (52%)	1.0
Passive smoking, N (%)	7 (27%)	42 (29%)	-
History of TB, N (%)	6 (23%)	43 (29%)	0.639
Using cotrimoxazole, N (%)	23 (88%)	133 (91%)	0.713
Years on ART, median (IQR)[1]	6.4 (4.6-9.1)	6.4 (3.9-8.3)	0.374
Prescence of atopy (asthma, eczema, or hay fever), N (%)	4 (15%)	20 (14%)	0.764
FEV_1 Z-score, median (IQR)	-1.62 (-2.4 to -1.2)	-1.72 (-2.2 to -1.3)	0.876
Obstructive (FEV_1 Z-score <-1.64), N (%)	13 (50%)	77 (53%)	0.834
Anaemia, N (%)[2]	8 (31%)	48 (33%)	1.0
White blood cell count ($*10^9$/l), median (IQR)[2]	4.1 (3.5-5.3)	4.2 (3.6-5.1)	0.989
Eosinophil count ($*10^9$/l), median (IQR)[2]	0.06 (0.02-0.11)	0.07 (0.04-0.13)	0.679

Basophil count (*10^9/l), median (IQR)[2]	0.02 (0.01-0.03)	0.02 (0.01-0.03)	0.271
Neutrophil count (*10^9/l), median (IQR)[2,3]	1.91 (1.4-2.82)	1.65 (1.27-2.17)	0.661
Lymphocyte count (*10^9/l), median (IQR)[2]	1.74 (1.41-2.17)	2.01 (1.59-2.47)	0.222
Monocyte count (*10^9/l), median (IQR)[2]	0.39 (0.3-0.56)	0.39 (0.3-0.49)	0.969
Virally supressed (VL <1000 copies/ml), N (%)	16 (62%)	92 (63%)	1.0
CD4 T cell count (cells/µl), median (IQR)	579 (357-746)	552 (338-716)	0.863
CD4 T-cell count <200 cells/µl, N (%)	4 (15%)	18 (12%)	0.749
Hospitalization during study period, N (%)[4]	2 (8%)	3 (2%)	0.165

[1] 1 missing data on duration of ART in Placebo group

[2] 2 missing data on Hb and white blood cell count in AZM group.

[3] 1 outlier with neutrophil count of 50.80 in Placebo group.

[4] Placebo group: 1 participant admitted for TB treatment, 1 for ovarian cancer and 1 for anemia. AZM group: 1 admitted for pneumonia and 1 unknown cause of admission.

1 in AZM group used salbutamol, no one used corticosteroids.

ARE; acute respiratory exacerbation, IQR, interquartile range; eNO, exhaled nitric oxide; ppb, parts per billion; CI, confidence interval, TB, tuberculosis; ART, Antiretroviral therapy; FEV_1, forced expiratory volume in 1 s; VL – viral load.

Supplementary Table 2. Effect of azithromycin on levels of eNO after 48 weeks of treatment using a generalized linear model.

Study group	N	Mean eNO at 48 weeks (95% CI) (log transformed)	Geometric mean ratio of log eNO at 48 weeks (95% CI)	p-value
Azithromycin	71	14.5 (12.6-16.8)	0.86 (0.72-1.03)	0.103*
Placebo	67	17.3 (15.0-20.1)		

eNO, exhaled nitric oxide; CI, confidence interval

Appendix III

Supplementary materials Paper III

Effect of azithromycin on Gut Microbial Composition in Children and Adolescents with HIV-associated Chronic Lung Disease

SUPPLEMENTARY TABLES AND FIGURES

Supplementary table 1. *Alpha diversity indices compared between study sites at baseline using generalized linear model (GLM).*

Alpha diversity	**Baseline (N = 346)**		
	Zimbabwe (N = 241)	**Malawi (N = 105)**	**P-value**
	Coefficient (95% CI)*		
Observed ASVs	4.04 (-12.24 – 20.32)	Ref.	0.626
Chao1	4.85 (-11.72 – 21.42)	Ref.	0.565
Shannon	-0.09 (-0.25 – 0.07)	Ref.	0.285
Total bacterial load (16S copy number, log transformed)	1.86 (1.34 – 2.39)	Ref.	<0.001

**Estimate of coefficient with 95% confidence interval and P values calculated using a linear regression model by the lm-function from the lme4 package in R version 4.2.2 (R studio version 2022.12.0+353).*

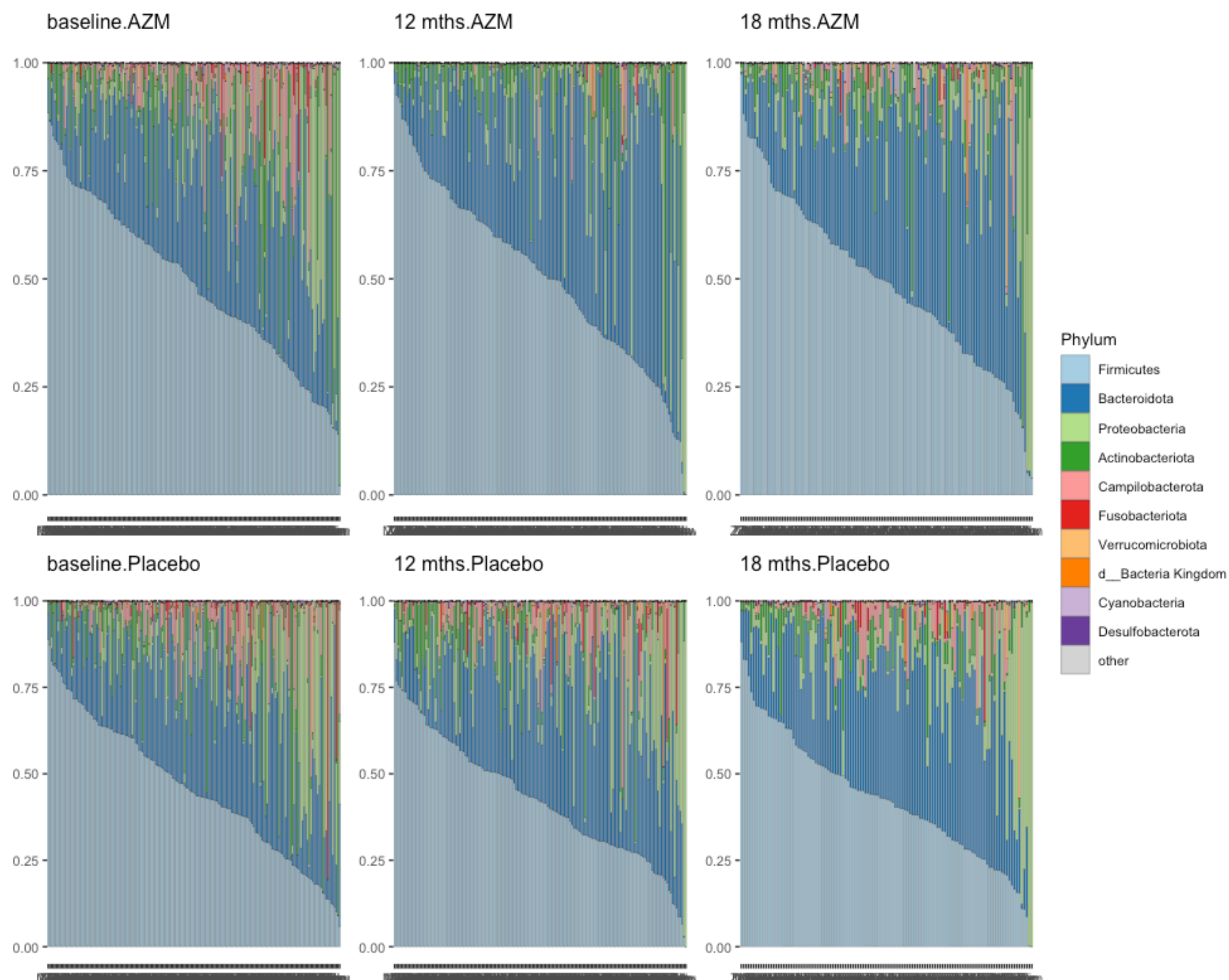
The model was adjusted for age, sex, history of TB, reported diarrhea, being stunted (HAZ < -2), season of sampling, being on 1st line ART regimen, CD4 T-cell count <200 and age at ART initiation.

Supplementary table 2. *Differentially abundant taxa between Zimbabwe and Malawi at baseline.*

Phylum	Genus	Effect size*	Difference (Between)	Difference (Within)	P-value**
Actinobacteriota	Corynebacterium	-0.34	-3.04	8.04	<0.001
Actinobacteriota	Brachybacterium	-0.36	-2.56	6.44	<0.001
Actinobacteriota	Brevibacterium	-0.31	-2.35	7.03	0.003
Firmicutes	Gallicola	-0.28	-2.07	6.97	0.002
Firmicutes	Anaerococcus	-0.31	-1.86	5.27	<0.001
Actinobacteriota	Actinomyces	-0.26	-1.74	6.28	0.018
Firmicutes	Fastidiosipila	-0.24	-1.67	6.79	0.013
Actinobacteriota	Kocuria	-0.25	-1.66	5.95	0.012
Firmicutes	Clostridium sensu stricto 1	-0.26	-1.65	5.61	0.005
Proteobacteria	Acinetobacter	-0.23	-1.61	5.93	0.026
Firmicutes	Ezakiella	-0.22	-1.57	6.39	0.009
Firmicutes	Peptococcus	-0.25	-1.53	5.58	0.005
Firmicutes	Helcococcus	-0.21	-1.48	6.20	0.036
Firmicutes	Finegoldia	-0.20	-1.37	6.13	0.042
Firmicutes	Murdochiella	-0.20	-1.35	6.22	0.020
Firmicutes	Holdemanella	-0.21	-1.34	5.96	0.018
Firmicutes	Lachnospiraceae (Family)	0.22	0.86	3.38	0.003
Firmicutes	Lachnospiraceae UCG-004	0.17	0.90	4.69	0.03
Firmicutes	Dialister	0.21	1.26	5.15	0.006
Proteobacteria	Sutterella	0.27	1.53	5.37	<0.001
Bacteroidota	Prevotella	0.34	1.57	4.18	<0.001
Firmicutes	Lachnoclostridium	0.33	1.66	4.33	<0.001
Bacteroidota	Parabacteroides	0.41	2.31	4.93	<0.001
Bacteroidota	Bacteroides	0.39	2.50	5.72	<0.001
Campylobacterota	Campylobacter	0.36	2.66	6.76	<0.001

* Negative effect size indicates higher abundance in Malawi and positive effect size indicates higher abundance in Zimbabwe.

** Wilcoxon test with FDR set to 0.05 using the Benjamini-Hochberg method



Supplementary figure 1. *Bar plot of relative abundance at phylum level compared between study groups at all three time points sorted by relative abundance of Firmicutes.*

Supplementary table 3. *Differentially abundant taxa between azithromycin and placebo group at 48 weeks in Zimbabwe (N = 25)*

Phylum	Genus	Effect size*	Difference (Between)	Difference (Within)	P-value**
Proteobacteria	Parasutterella	-0.61	-4.40	6.28	<0.001
Firmicutes	Blautia	-0.34	-1.12	2.72	<0.001
Firmicutes	Flavonifractor	-0.29	-1.70	5.53	0.029
Actinobacteriota	Eggerthella	-0.28	-1.64	5.17	0.022
Firmicutes	Dorea	-0.28	-1.04	3.01	<0.001
Firmicutes	Lachnoclostridium	-0.28	-1.18	3.71	0.002
Firmicutes	Fusicatenibacter	-0.27	-1.41	4.45	0.004
Firmicutes	Anaerostipes	-0.25	-1.59	5.70	0.023
Firmicutes	[Ruminococcus] torques group	-0.20	-0.95	4.19	0.040
Firmicutes	Lachnospiraceae UCG-004	-0.19	-0.91	3.95	0.029
Firmicutes	Butyricicoccus	-0.19	-0.99	4.40	0.040
Firmicutes	Fenollaria	0.23	2.03	8.19	0.039
Bacteroidota	Porphyromonas	0.25	1.84	6.68	0.023
Firmicutes	Clostridium sensu stricto 1	0.26	1.61	5.77	0.037
Cyanobacteria	Gastranaerophilales (Order)	0.26	1.48	5.38	0.044
Firmicutes	Fastidiosipila	0.27	1.90	6.34	0.026
Firmicutes	Mitsuokella	0.27	1.72	5.76	0.040
Fusobacteriota	Fusobacterium	0.31	2.44	7.52	0.013
Firmicutes	Bacilli_RF39 (Order)	0.35	2.07	5.38	0.004
Desulfobacterota	Desulfovibrio	0.40	2.56	5.61	0.001
Firmicutes	Negativicoccus	0.43	3.34	6.78	<0.001
Actinobacteriota	Bifidobacterium	0.46	3.34	6.63	<0.001
Firmicutes	Clostridia UCG-014 (Order)	0.59	3.52	5.34	<0.001
Campylobacterota	Campylobacter	0.88	6.57	6.72	<0.001
Proteobacteria	Sutterella	1.06	6.23	5.24	<0.001

Supplementary table 4. *Differentially abundant taxa between baseline and 48 weeks in the azithromycin group in Zimbabwe (N = 34)*

Phylum	Genus	Effect size*	Difference (Between)	Difference (Within)	P-value**
Proteobacteria	Parasutterella	-0.48	-3.41	6.41	<0.001
Bacteroidota	Bacteroides	-0.44	-2.27	4.72	<0.001
Firmicutes	Blautia	-0.33	-1.13	2.87	<0.001
Firmicutes	Lachnoclostridium	-0.32	-1.47	4.12	<0.001
Firmicutes	Butyricicoccus	-0.31	-1.46	4.29	<0.001
Firmicutes	Fusicatenibacter	-0.28	-1.49	4.78	0.003
Firmicutes	Anaerostipes	-0.25	-1.56	5.49	0.012
Firmicutes	Dorea	-0.25	-0.99	3.19	0.002
Bacteroidota	Alistipes	-0.25	-1.42	5.16	0.029
Firmicutes	Lachnospiraceae UCG-004	-0.25	-1.19	4.11	0.002
Bacteroidota	Parabacteroides	-0.20	-1.09	4.58	0.025
Firmicutes	[Ruminococcus] torques group	-0.19	-0.99	4.60	0.025
Firmicutes	Murdochiella	0.21	1.62	7.14	0.048
Bacteroidota	Alloprevotella	0.24	1.74	6.77	0.034
Firmicutes	Christensenellaceae R-7 group	0.24	1.45	5.39	0.025
Actinobacteriota	Actinobacteriota (Phylum)	0.24	1.55	5.76	0.031
Firmicutes	Romboutsia	0.25	1.51	5.36	0.028
Spirochaetota	Treponema	0.26	1.62	5.56	0.024
Fusobacteriota	Fusobacterium	0.26	1.95	7.19	0.018
Firmicutes	Clostridia (Class)	0.26	1.64	6.03	0.031
Firmicutes	Intestinimonas	0.27	1.53	4.97	0.013
Firmicutes	Ezakiella	0.27	2.18	7.62	0.006
Actinobacteriota	Bifidobacterium	0.28	2.13	6.74	0.012
Firmicutes	Bacilli_RF39 (Order)	0.29	1.69	5.48	0.011
Bacteroidota	Porphyromonas	0.34	2.78	7.53	<0.001
Firmicutes	Fastidiosipila	0.35	2.65	7.05	0.001
Cyanobacteria	Gastranaerophilales (Order)	0.36	2.10	5.41	0.002
Firmicutes	Fenollaria	0.37	3.44	8.42	<0.001
Firmicutes	Mitsuokella	0.45	2.90	5.74	<0.001
Firmicutes	Clostridia UCG-014 (Order)_	0.52	3.08	5.32	<0.001
Desulfobacterota	Desulfovibrio	0.54	3.27	5.31	<0.001
Firmicutes	Negativicoccus	0.65	5.23	7.12	<0.001
Proteobacteria	Sutterella	0.87	5.71	5.68	<0.001
Campylobacterota	Campylobacter	1.00	7.75	7.00	<0.001

Supplementary table 5. *Differentially abundant taxa between azithromycin and placebo group at 72 weeks*

Phylum	Genus	Effect size*	Difference (Between)	Difference (Within)	P-value**
Proteobacteria	Parasutterella	-0.47	-3.02	6.08	<0.001
Firmicutes	Lachnospiraceae UCG-004	-0.21	-1.07	4.40	0.031
Firmicutes	Fastidiosipila	0.30	2.27	6.84	0.033
Campylobacterota	Campylobacter	0.35	2.66	6.90	<0.001
Proteobacteria	Sutterella	0.51	3.35	5.85	<0.001

* Negative effect size indicates higher abundance in azithromycin group and positive effect size indicates higher abundance in placebo group.

** Wilcoxon test with FDR set to 0.05 using the Benjamini-Hochberg method

Supplementary table 6. *Differentially abundant taxa between 48 and 72 weeks in the azithromycin group*

Phylum	Genus	Effect size*	Difference (Between)	Difference (Within)	P-value*
Proteobacteria	Escherichia-Shigella	0.26	1.99	7.00	0.028
Firmicutes	Negativicoccus	0.28	2.16	6.94	0.018
Firmicutes	Clostridia UCG-014 (Order)	0.34	2.17	5.75	0.006
Campylobacterota	Campylobacter	0.45	3.57	7.14	<0.001

* Negative effect size indicates higher abundance at 48 weeks and positive effect size indicates higher abundance at 72 weeks.

** Wilcoxon test with FDR set to 0.05 using the Benjamini-Hochberg method