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HIV-infection in children and adolescents in Zimbabwe: viral suppression, airway abnormalities and gut microbiota

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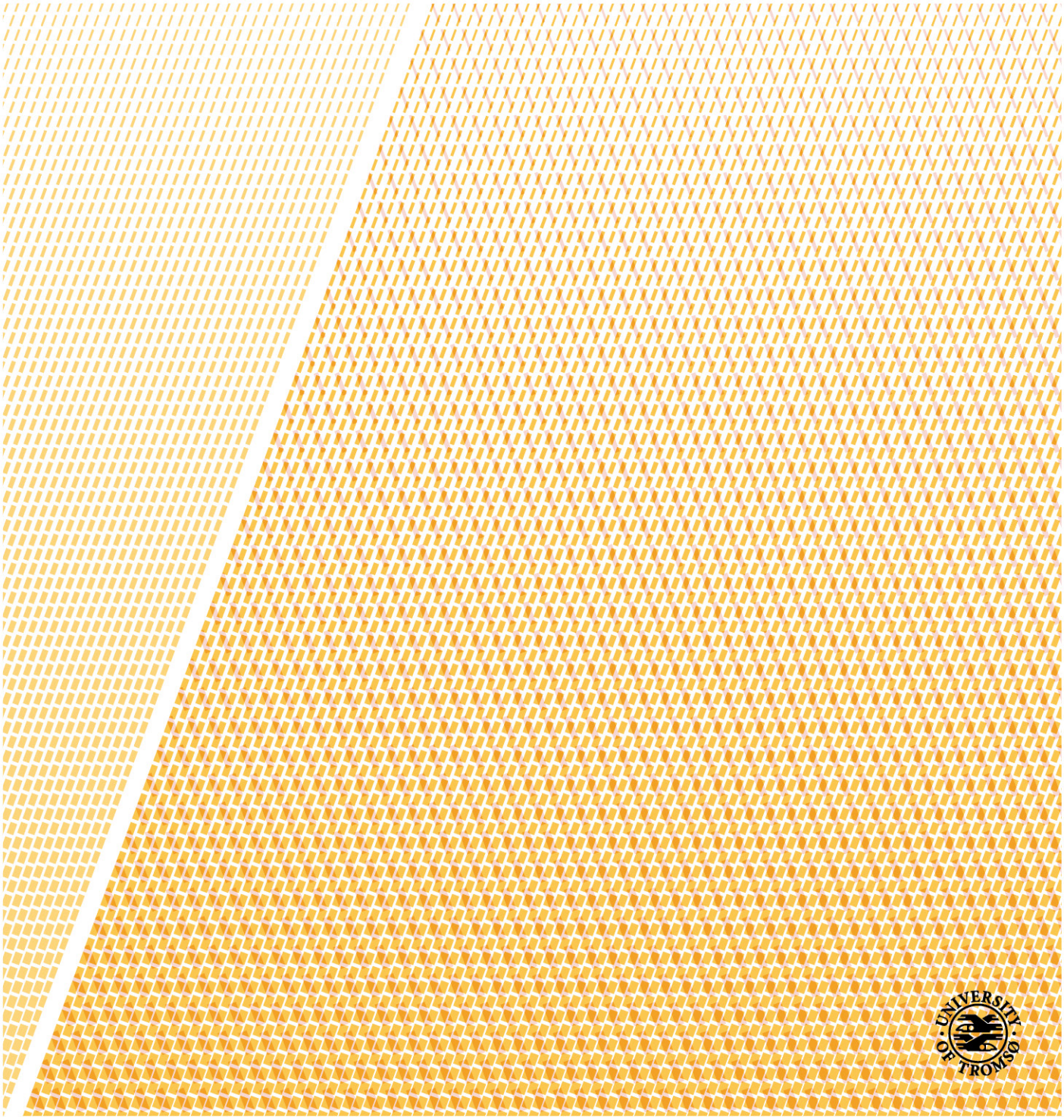

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Summary

Sub-Saharan Africa remains the region with the highest burden of HIV among both children and adults. Access to antiretroviral therapy (ART) has improved survival, yet data on long-term virological outcomes are scarce due to limited access to viral load monitoring. In addition, recent evidence indicates that HIV-infected ART-treated children are at increased risk of lung impairment compared to HIV-uninfected peers. The pathogenesis of lung complications in these children is currently unknown and data on the pathological mechanisms involved are limited. Due to the growing numbers of children with HIV surviving into adulthood, there is an urgent need for studies on the pathogenesis of lung impairment in this population group.

Thus, the overall aim of this work was to study virological outcomes in individuals initiating ART in Harare, Zimbabwe and to investigate two components of host immune defence - nitric oxide (NO) and gut microbiota among HIV-infected ART-treated children and adolescents with and without lung impairment.

In **Paper I** the rates of virological suppression as well as viraemia patterns were estimated in a retrospective cohort study of HIV-infected children and adults. We found that significantly more children than adults did not reach viral load suppression by 12 months ART. Among those who achieved viral suppression during the follow-up period, the incidence of viral failure was higher in children, as was the incidence of low level viraemia and viral blips.

In **Paper II**, we found that HIV status was associated with lower exhaled NO levels. Notably, history of tuberculosis among HIV-infected children was associated with lower exhaled NO levels while airway obstruction alone was not. In addition, higher haemoglobin levels and neutrophil count were associated with higher exhaled NO levels in HIV-infected participants.

Paper III demonstrated that HIV status was associated with lower gut species richness estimates. Prolonged ART treatment was significantly associated with a richer gut microbiota in HIV-infected children. At the same time, no association between airway obstruction or prior tuberculosis and gut biodiversity indices was observed in our study. HIV-infected participants were enriched in *Corynebacterium*, *Finegoldia*, *Anaerococcus* compared to HIV-uninfected participants.

The present work stresses the importance of virological control in the paediatric HIV-infected population. It explores the components potentially involved in the pathogenesis of lung impairment in these individuals. Low levels of exhaled NO may be a marker of HIV- and tuberculosis-induced alterations in pulmonary physiology while low gut biodiversity associated with HIV infection may have further implications in microbiota-targeted interventions.

List of papers

The thesis is based on the following papers:

1. **Sovershaeva E**, Shamu T, Wilsgaard T, Bandason T, Flaegstad T, Katzenstein D, Ferrand RA, Odland JØ. Patterns of detectable viraemia among children and adults with HIV infection taking antiretroviral therapy in Zimbabwe. Int J Infect Dis. 2019;78:65-71.
2. **Sovershaeva E**, Kranzer K, McHugh G, Bandason T, Majonga ED, Usmani OS, Rowland-Jones S, Gutteberg T, Flaegstad T, Ferrand RA, Odland JØ. History of tuberculosis is associated with lower exhaled nitric oxide levels in HIV-infected children. AIDS. 2019;33(11):1711-8.
3. Flygel TT, **Sovershaeva E**, Classen-Weitz S, Hjerde E, Mwaikono KS, Odland JØ, Ferrand RA, McHugh G, Gutteberg T, Nicol MP, Cavanagh JP, Flaegstad T. Composition of gut microbiota of children and adolescents with perinatal HIV infection taking antiretroviral therapy in Zimbabwe. *Submitted*

Abbreviations

AIDS - acquired immunodeficiency syndrome

AM - alveolar macrophages

ART - antiretroviral therapy

ATS - American Thoracic Society

BMI - body mass index

BREATHE - broncho-pulmonary function in response to azithromycin treatment for chronic lung disease in HIV-infected children and adolescents

CD4 cells - CD4 T lymphocytes

CI - confidence interval

CLD - chronic lung disease

COPD - chronic obstructive pulmonary disease

eNO - exhaled nitric oxide

eNOS - endothelial NO synthase

FDR - false discovery rate

FEV1 - forced expiratory volume in one second

FVC - forced vital capacity

GI - gastrointestinal

GOLD - Global Initiative for Chronic Obstructive Lung Disease

HIV - human immunodeficiency virus

iNOS - inducible NO synthase

IQR - interquartile range

LEfSe - linear discriminant analysis effect size

LLV - low level viraemia

Mtb - Mycobacterium tuberculosis

MTCT - mother-to-child transmission

nNOS - neuronal NO synthase

NNRTI - non-nucleoside reverse transcriptase inhibitor

NO - nitric oxide

NOS - NO synthase

NRTI - nucleoside reverse transcriptase inhibitor

OTUs - operational taxonomic units

PCR - polymerase chain reaction

PI - protease inhibitor

ppb - parts per billion

RCT - randomized controlled trial

RLS - resource-limited settings

ROS - reactive oxygen species

rRNA - ribosomal ribonucleic acid

SD - standard deviation

SSA - Sub-Saharan Africa

TAHOD - Treat Asia HIV observational database

TB - tuberculosis

UNAIDS - The Joint United Nations program on HIV and AIDS

VF - viral failure

VL - viral load

WHO - World Health Organization

Introduction

1. HIV infection

1.1. Current status of HIV epidemic

In 2018, it was estimated that 37.9 million people were living with human immunodeficiency virus (HIV) worldwide, with 1.7 million of them younger than 15 years of age. More than half of HIV-infected adults and two-thirds of HIV-infected children reside in Sub-Saharan Africa (SSA) (1).

Increased availability and uptake of antiretroviral therapy (ART) in the last two decades drastically improved survival and led to an increase in the number of people living with HIV worldwide. A large cohort study conducted in the USA reported an increase in life expectancy for HIV-infected persons at age 20 from 19.1 years in 1996-1997 to 53.1 years in 2011 (2). A large population-based cohort study in rural KwaZulu-Natal, South Africa, demonstrated an 11.3 - year gain in adult life expectancy between 2003 (a year before ART became available in the public sector health system) and 2011 (3). However, despite improved survival, there is still at least an 11.8 year gap in life expectancy between HIV-infected and uninfected individuals (2).

The annual number of new HIV infections worldwide has declined from 2.1 million in 2010 to 1.7 million in 2018 (Figure 1). The greatest progress was made in Southern and Eastern Africa with 28% decline in the number of new HIV infections between 2010 and 2018 (1). The number of new HIV infections in children aged below 15 decreased from estimated 280 000 in 2010 to 160 000 in 2018, probably due to widespread introduction of the programs for prevention of mother-to-child transmission (1).

Globally, acquired immunodeficiency syndrome (AIDS)-related deaths decreased from its peak of 1.9 million in 2004 to 770 000 in 2018 (Figure 2) (1). Due to the scaling up of ART, AIDS-related mortality in Southern and Eastern Africa declined from 1 million in 2004 to 310 000 and is now comparable to those reported in high-income settings (4, 5).

In developed countries, the main causes of mortality in HIV-infected individuals have shifted from AIDS-defining to non-AIDS-defining conditions, that is chronic conditions similar to those in the general population. These now account for up to 84% of deaths in HIV infected individuals in the western countries (6-8). A similar trend is also observed in low- and middle-income countries (9, 10). However, in these settings, AIDS-related conditions continue to be the major causes of death, with tuberculosis as the main cause accounting for up to 44% of deaths (10-12).

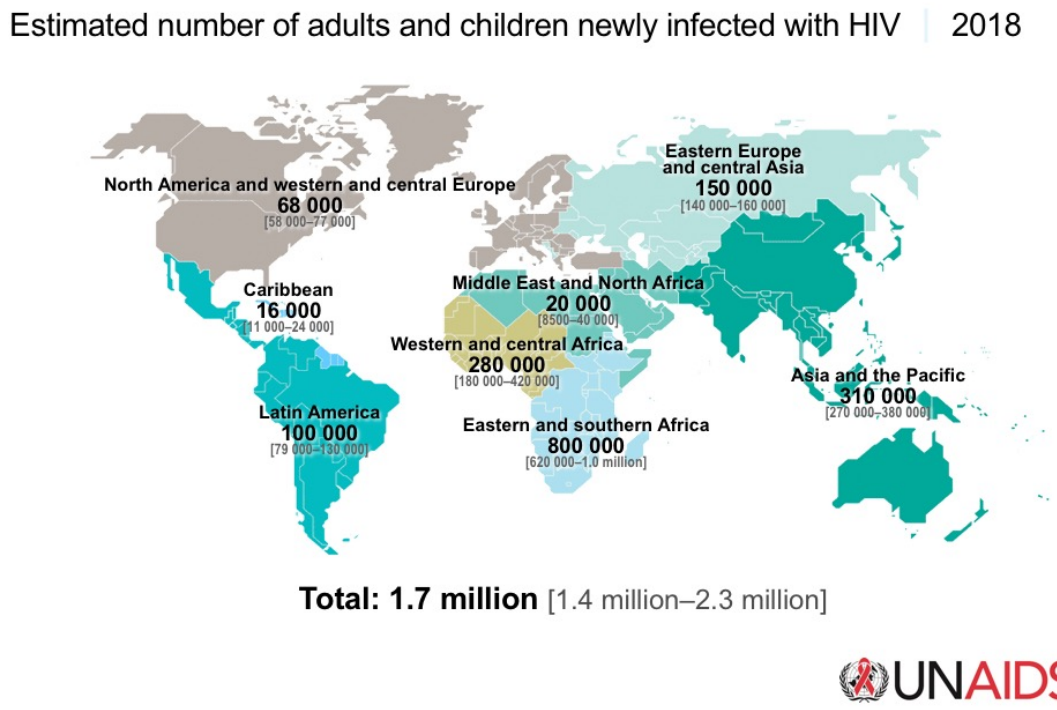

Figure 1. Estimated number of adults and children newly infected with HIV in 2018. Source: UNAIDS 2019 estimates. https://www.unaids.org/sites/default/files/media_asset/2019-UNAIDS-data_en.pdf

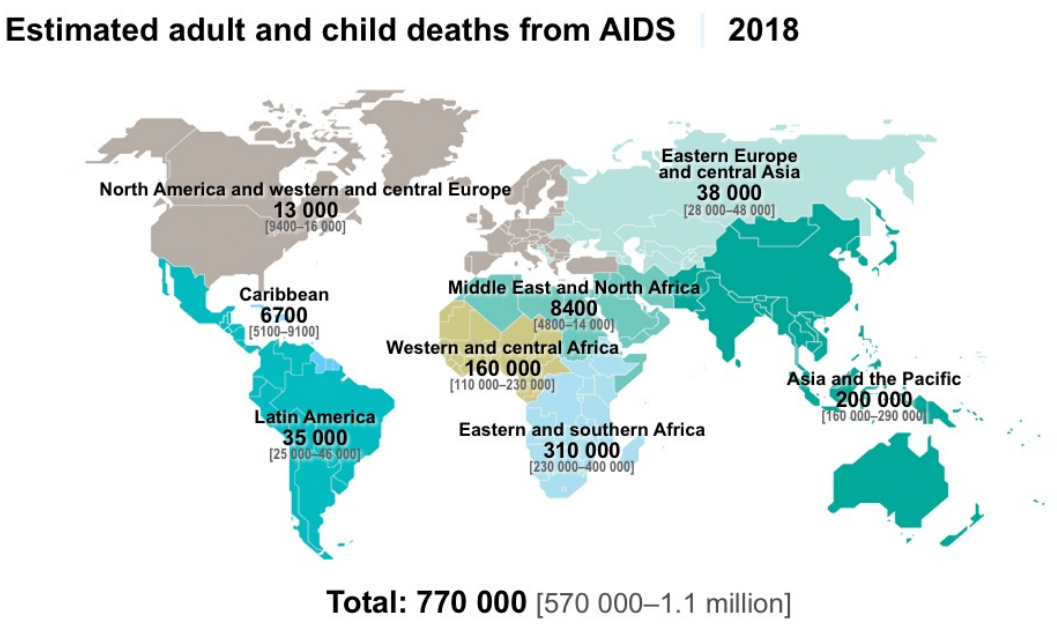

Figure 2. Estimated adult and child deaths from AIDS in 2018. Source: UNAIDS 2019 estimates. https://www.unaids.org/sites/default/files/media_asset/2019-UNAIDS-data_en.pdf

1.2. A historical overview

In 1981 a clinical syndrome characterized by profound immunodeficiency was reported in previously healthy young men who have sex with men in the USA (13). Patients were diagnosed with *Pneumocystis* pneumonia and Kaposi's sarcoma - conditions rarely observed in otherwise healthy individuals. This syndrome was called AIDS and was soon recognized in other groups such as injection drug users and patients with haemophilia (14, 15).

HIV-1 virus - the causative agent for AIDS was isolated for the first time in 1983 from a lymph node biopsy of a patient with generalized lymphadenopathy by Luc Montagnier and Barre-Sinoussi at the Pasteur Institute in Paris (16). Three years later, in 1986, HIV-2 was isolated from two patients from Western Africa (17) at the same institute. Further studies provided evidence that both HIV-1 and HIV-2 are the result of cross-species transmission of simian immunodeficiency virus (18).

The rapid spread of AIDS around the world was accompanied by extensive research of the newly discovered virus and potential treatment options. Already in 1987 zidovudine, a drug which inhibits the HIV enzyme reverse transcriptase, was found to decrease mortality and frequency of opportunistic infections in patients with AIDS (19). However, rapid development of viral resistance to zidovudine forced the scientific community to search for new approaches to treat HIV.

One of the main milestones in HIV research was achieved a decade later (in 1996) with the introduction of highly active ART - a combination of at least three antiretroviral drugs in order to limit the development of resistance. Introduction of combined ART resulted in a dramatic decline in HIV morbidity and mortality and an increase in life expectancy (3, 20, 21).

Due to high treatment costs these achievements were primarily seen in developed countries. However, the Drug Access Initiative launched in 1997 by The Joint United Nations program on HIV and AIDS (UNAIDS) drastically improved ART coverage in resource-limited settings (RLS). The first patients received drugs in Uganda and Cote d'Ivoire soon after in 1998.

1.3. HIV virus, pathogenesis, natural history and treatment

HIV-1 and HIV-2 belong to the Lentiviridae subfamily of retroviruses. Though both viruses cause AIDS, HIV-1 is more aggressive and is responsible for the global HIV pandemic. HIV-2 infection dominates in West African countries, is less virulent, and has lower rates of transmission and a slower progression to AIDS (22). The modes of transmission for HIV-2 are the same as for HIV-1.

The virus is transmitted by sexual intercourse, parenteral inoculation (intravenous injection) and from mother to child. Mother-to-child transmission (MTCT) of HIV occurs *in utero*, during delivery and postnatally through breastfeeding. Late postnatal transmission by breastfeeding represents a considerable further risk, accounting for up to 42% of overall MTCT of HIV (23).

HIV infects CD4 T lymphocytes (CD4 cells), monocytes, macrophages and dendritic cells via binding to CD4 membrane receptors and chemokine coreceptors (C-C chemokine receptor type 5 or C-X-C chemokine receptor type 4) (24). Cells such as astrocytes and renal epithelial cells may also be infected leading to HIV-associated neurocognitive disorders and nephropathy (25, 26). Once the virus enters the cell, HIV RNA and HIV enzymes are released (Figure 3). Reverse transcriptase converts viral HIV RNA into HIV DNA. Viral DNA then migrates into the cell's nucleus and integrates into the host's DNA. This step is mediated by viral integrase. New HIV RNA and HIV proteins are then generated by utilizing the cell's own transcription mechanisms and move further to the cell surface and form immature (noninfectious) HIV particles. Once the virus is released from the cell, HIV protease mediates the formation of the mature infectious virus.

The early phase of HIV infection is characterized by high levels of viral replication and massive depletion of CD4 cells, which are the primary targets of the virus (27-29). Depletion and functional impairment of these cells are crucial points in disease progression. As the result of rapid virus replication, individuals become viraemic shortly after infection. Already this stage of the disease is accompanied by an intense pro-inflammatory response characterized by elevation of a broad range of cytokines and chemokines in the systemic circulation ("intense early cytokine storm") (30). Antibodies against HIV are detectable within 4-6 weeks after contracting HIV. This period is called seroconversion and individuals may develop flu-like symptoms during this period (31, 32). After seroconversion, the plasma viral load (VL) decreases and reaches the steady state level (the so-called "VL set point"). The initial acute decrease in CD4 cells is followed by CD4 cell recovery to almost normal levels which without treatment gradually decline with time. Patients may remain asymptomatic for years until CD4 cell levels fall below 200 cells/mm^3 and the individual becomes susceptible to a wide variety of opportunistic infections (such as *Mycobacterium* tuberculosis, *Pneumocystis* pneumonia, cryptococcal meningitis, candidiasis, etc.) and HIV-related malignancies (Kaposi's sarcoma, non-Hodgkin's lymphoma) (33).

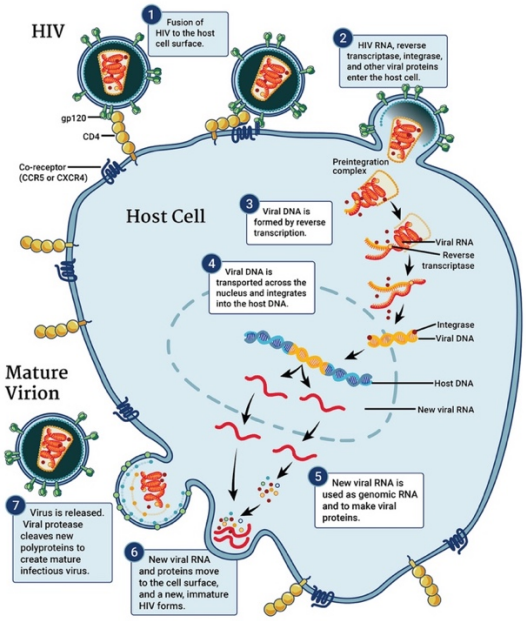

Figure 3. HIV life cycle. Source: National Institute of Allergy and Infectious Diseases. www.niaid.nih.gov/diseases-conditions/hiv-replication-cycle.

A very small proportion of individuals (less than 0.5% of those infected) called long-term nonprogressors may maintain low level viraemia and stable CD4 counts (≥500 copies/ml) for more than 10 years without treatment (34). A subgroup of these individuals called "elite controllers" have no detectable viraemia (35, 36).

ART and current approach to treatment

The HPTN 052 clinical trial, named as the 2011 Breakthrough of the Year by the Journal of Science, demonstrated a 96% reduction of sexual transmission of HIV with early initiation of ART compared with delayed treatment (37). This study became fundamental for our current view on treatment and follow-up of HIV-infected individuals and paved the way for further research. The proposed mechanism of the effect of ART was sustained VL suppression in genital secretions. The findings were later confirmed by a plethora of studies, also in the homosexual population (38-43). A study by Rodger et al. conducted among 1166 serodifferent heterosexual and men who have sex with men (MSM) couples where the HIV-infected partners received ART showed they had undetectable VL and reported condomless sex found no documented cases of within couple HIV transmission during 1.3 years of follow-up (44).

Supported by these and other studies, the World Health Organization (WHO) updated guidelines in 2016, where ART treatment has been recommended to all HIV-infected individuals regardless of WHO clinical stage and CD4 count (45). In addition, these

recommendations highlight HIV VL measurement as the preferred approach to monitor treatment response and detect treatment failure. CD4 count testing (which guided ART initiation and treatment response for more than two decades) is no longer recommended for monitoring of patients who are stable on ART in settings were VL measurement is routinely available (46). However, due to financial constraints (high costs for VL testing) clinicians in RLS still rely on CD4 count monitoring with limited access to routine VL testing.

Currently, treatment of HIV is initiated with an ART regimen consisting of two nucleoside reverse transcriptase inhibitors (NRTIs) plus either a non-nucleoside reverse transcriptase inhibitor (NNRTI), protease inhibitor (PI) or integrase inhibitor (24, 47). Due to similar suppression rates and tolerability of these combinations, clinicians in high-income countries can choose the most appropriate regimen for a patient by taking into account potential adverse effects, ease of administration, drug interactions and costs (48). In case of treatment failure, HIV drug resistance testing is performed with a subsequent switch in ART regimen.

For RLS with limited access to drug resistance testing, the WHO recommends a public health approach with 2 NRTI + NNRTI as a standardized first-line ART for children >3 years and adults and subsequent switch to a second-line ART with 2 NRTI + PI in case of treatment failure. PI-based regimens are recommended for infants and children younger than 3 years (47).

Routine VL monitoring is recommended at 6 and 12 months after ART initiation and then every 12 months in order to monitor treatment response and to detect treatment failure. Detectable VL (thresholds vary) in individuals on ART should prompt enhanced adherence counselling and a repeat VL test within 6 weeks. Adherence counselling is an essential component of ART treatment with data showing that up to 70% of HIV-infected individuals with detectable VL are re-suppressed after adherence counselling (49, 50). Two sequential VL measurements above certain threshold imply viral failure (VF) and require HIV drug resistance testing and ART regimen changes (47).

2. Challenges of HIV in the era of ART

2.1. Lack of universal definitions for virological outcomes

The goal of ART is to achieve sustained VL suppression in order to prevent HIV progression, development of HIV drug resistance mutations and to minimize the risk of HIV transmission. However, thresholds for VL suppression and VF are a matter of debate. Many countries in SSA use WHO cut-offs with VF defined as two sequential VL measurements ≥1000 copies/ml (47). WHO guidelines justify the 1000 copies/ml threshold for VF based on

the data showing low risk of HIV transmission with VL below 1000 copies/ml (47, 51, 52). Another important reason why the WHO recommends this cut-off is that many RLS use dried blood spots for VL measurement which have a substantially higher VL detection threshold compared to plasma samples (550-1000 copies/ml vs 20 copies/ml, respectively), but provide an opportunity for easy transport and storage compared to plasma (53).

However, a number of studies conducted in developed countries demonstrate that patients with VL below 1000 copies/ml are still at increased risk of VF compared to patients with undetectable VL or VL suppression at <50 copies/ml, with some studies advocating for even lower cut-offs (54-56). Therefore, guidelines for high-income settings define VL suppression as VL below the lower limit of detection of commercial assays, which is usually below 50 copies/ml and VF as VL above 200 copies/ml in two or more consecutive VL measurements (57, 58).

HIV-infected individuals may also experience episodes of transient detectable viraemia after initial VL suppression despite being on ART. The spectrum of these episodes includes viral blips, low level viraemia (LLV) and viral rebound. As with VL suppression and VF cut-offs, the definitions used for these viraemia episodesvary across different studies and guidelines (57-59). For example, blips are defined as transiently detectable VL<400 copies/ml in the Department of Health and Human Services guidelines (60), 50-200 copies/ml in Spanish guidelines (61), single VL 50-400 copies/ml preceded and followed by undetectable VL in British guidelines (57) and transient VL 50-1000 copies/ml in WHO guidelines (47).

Different definitions used for VL suppression, VF and detectable viraemia episodes across different settings often complicate the comparison between published data. In addition, due to lack of national VL monitoring programs in many RLS, the majority of data available on VL suppression in SSA comes from cross-sectional studies and clinical trials with a paucity of data from longitudinal cohorts. A recent systematic review and meta-analysis comprising data on 125 distinct populations in SSA found that the proportion of viral suppression was significantly lower in cohort studies compared to trials at 12 months ART and tended to be lower also at 24 months (62). This highlights the fact that clinical trials tend to overestimate VL suppression and may not reflect the “real-world” situation.

2.2. Virological outcomes in individuals on ART

The majority of studies conducted in high-income countries report high rates of VL suppression among adults ranging from 80.9% to up to 95.3% (63-65). Similar suppression rates were reported among perinatally infected children in the UK and Ireland with 92% of

children achieving VL suppression within 12 months since ART initiation (66). An international multicentre trial (PENPACT-1) conducted among HIV-infected children in Europe, North and South America demonstrated that 84% reach VL<400 copies/ml after the median follow-up of 5 years on ART (67).

Due to scaling up of ART in SSA, the proportion of HIV-infected individuals who receive ART treatment in this area has dramatically increased during the last decade. As a result the reported VL suppression rates among the adult HIV-infected population in SSA are now comparable to high-income settings and range from 85.4% to 92.6% (68-71).

However, the results are less encouraging when it comes to VL suppression rates in the paediatric HIV-infected population in RLS. A large meta-analysis of data from 72 studies found that the proportion of children aged <18 years in low- and middle-income countries who achieved VL suppression after 12 months ART ranged from 64.7% to 74.7% during the first two decades of the 21st century (72, 73). This is considerably lower than VL suppression rates previously reported in adults from the same settings and in children from high-income settings (66, 74).

A number of studies conducted both in high- and low-income settings highlight that children and adolescents are less likely to achieve VL suppression and are more prone to VF compared to adults (75-77). Murphy et al. reported that only half (51.4%) of children and adolescents who achieved initial VL suppression maintained undetectable VL for a year (76). The reported rates of VF among children and adolescents in SSA are high, with up to 64% of ART-treated children and adolescents experiencing VF (64% in Senegal; 51.6% in Togo; 30.6% in Zimbabwe) (78-80). A study conducted in Cameroon among HIV-infected adolescents who were on ART for a median of 7 years found that 20.7% of adolescents were in VF (≥1000 copies/ml) (81). It is important to note that, due to lack of resources, regular VL testing is not routinely implemented in many sub-Saharan countries. Therefore, data available on VL levels are often obtained from cross-sectional studies and lack information on initial VL suppression as well as confirmative (second) VL testing in the case of detectable viraemia (79, 80). However, a recent cohort study among children aged<12 years who initiated ART in South Africa found that 20.1% of those who reached initial VL suppression had subsequent VL rebound (defined as at least one VL >1000 copies/m after initial VL suppression) (82).

A number of factors may contribute to worse virological outcomes in HIV-infected children and adolescents compared to adults. Lack of paediatric ART formulations, poor tolerability and palatability, and presence of comorbid conditions (low nutritional status, advanced HIV stage) complicate treatment of children with HIV (83). However, lack of

adherence is the biggest challenge in treatment of HIV-infected paediatric population (84, 85). It can be caused by any of the abovementioned factors as well as psychosocial issues and lack of knowledge about why the treatment is needed (83). Adolescence is a period for particularly high risk for poor adherence (86, 87) with data showing that individuals above 15 years of age have a higher risk of non-adherence compared to younger individuals (88). In addition, retention in care is substantially lower among children and adolescents compared to adults with data showing that up to 38% are lost to follow-up after 24 months ART (89, 90).

All these data highlight that a number of challenges exist in treatment, monitoring and retention in care of HIV-infected children and adolescents. Specific models of medical care oriented towards young individuals growing up with HIV should be developed in order to improve treatment outcomes and retention in care.

2.3. Episodes of detectable viraemia

Episodes of detectable viraemia (viral blips, LLV, residual viraemia) after initial VL suppression are common among individuals on ART. Detectable viraemia episodes do not necessarily indicate treatment failure, but may contribute to persistent inflammation and immune activation leading to further HIV progression (91, 92).

Such factors as high baseline (pre-treatment) VL, shorter duration of ART since treatment initiation, specific ART combinations and the presence of transmitted drug resistance mutations have been shown to be independent predictors of detectable viraemia (93-97).

There is also evidence that detectable viraemia may facilitate the selection of drug-resistant HIV variants. This is especially important in SSA, where suppression rates are lower than in developed countries, and diagnostic and treatment options are limited (73). Swenson et al. found that patients with detected drug-resistant mutations during LLV episodes are three times more likely to experience VF in future (98).

HIV viraemia accompanied by chronic inflammation may further contribute to the development of chronic HIV-associated complications which are now increasingly recognized in individuals on ART (99-102).

2.4. Chronic comorbidities in HIV-infected individuals

Due to the introduction of ART, people with HIV live longer and are at risk of developing chronic comorbidities related to ageing. However, recent evidence indicates that chronic comorbidities are more common among HIV-infected individuals compared to the HIV-

uninfected population and that factors other than accelerated ageing may be the main triggers in the development of these complications (7, 103, 104). HIV viraemia, CD4 cell lymphopenia, co-infections, and translocation of microbial products from gastrointestinal (GI) tract may all lead to persistent inflammation and accelerate the development of these complications (99-102, 105).

Among the most commonly observed chronic disorders in HIV-infected adults are cardiovascular disorders (hypertension, myocardial infarction) (106), pulmonary disorders (103, 107), diabetes (108) and non-AIDS defining malignancies (109).

ART has transformed HIV infection into a chronic, manageable disease, with treatment focus shifting towards assessing chronic conditions. With improved access to ART, those complications are more and more common among HIV-infected individuals in RLS.

3. Lung complications associated with HIV infection

3.1. Infectious complications

Ongoing HIV replication accompanied by chronic inflammation impairs the immune response against infectious stimuli thus increasing susceptibility to respiratory infections. This results in the high frequency of respiratory infections observed in HIV-infected individuals, with lung tuberculosis (TB), *Pneumocystis* pneumonia and community-acquired pneumonia being the most commonly observed (110, 111). Although the frequency of these HIV-associated complications has considerably decreased in the ART era, they are still common in HIV-infected individuals. In developed countries bacterial pneumonia (with *Streptococcus pneumoniae* as the causative agent) is the most frequently diagnosed lung infection among people with HIV, while TB remains the major health threat in developing settings (112, 113).

Tuberculosis in HIV-infected individuals

Among the estimated 10 million people who developed TB in 2017, 9% (920 000) of the incident TB cases occurred in people with HIV (113, 114). Although the global incidence of active TB is falling, it remains the leading cause of death in HIV-infected individuals in low- and middle-income countries.

HIV increases the risk of developing active TB in individuals with latent *Mycobacterium tuberculosis (Mtb)* infection and in those who are newly exposed to TB (115, 116). Moreover, HIV-infected children and adults are at higher risk of rapid disease progression and TB

recurrence (117-119). A study conducted in Ethiopia found that HIV-infected children were six times more likely to die during the episode of TB compared to HIV-uninfected children (120).

ART is an effective preventive tool against TB in HIV-infected individuals (121). One of the most impressive effects of ART was shown in Brazil by Miranda et al., with 80% reduction in incident TB in ART-treated HIV-infected individuals compared to ART-naïve (122). Despite this dramatic effect of ART, TB incidence rates in individuals on long-term ART are still higher than in general population (116, 123, 124).

Advanced immunosuppression at treatment initiation (low CD4 count and high VL) and poor nutritional status are associated with increased risk of TB in HIV-infected individuals who receive ART (123, 125, 126). This is especially important for perinatally HIV-infected children who, due to suboptimal early infant diagnosis of HIV, are often diagnosed late in older childhood and adolescence when they have already developed advanced immunosuppression. Moreover, it was recently shown that ongoing HIV replication is an independent risk factor for TB in ART-treated HIV-infected individuals regardless of CD4 count (127). This emphasizes the need to maintain (long-term) suppressed VL and to prioritize routine VL monitoring in individuals on ART.

Given the high risk of progression to active TB, WHO guidelines recommend preventive treatment of TB for adults and adolescents living with HIV who are unlikely to have active TB and have a positive or unknown tuberculin skin test. Treatment with isoniazid should be initiated irrespective of the degree of immunosuppression, history of prior TB and pregnancy (128).

3.2. Burden of chronic lung diseases in HIV-infected individuals

The ART era is characterized by the growing burden of chronic lung complications among HIV-infected individuals, with chronic obstructive pulmonary disease (COPD) being most frequently reported (110, 129-131).

COPD is characterized by reduced lung function assessed by spirometry. In a large prospective study among treated HIV-infected adults in Denmark, HIV was independently associated with 197 ml lower forced expiratory volume in one second (FEV1) and 328 ml lower forced vital capacity (FVC) (132). HIV is currently acknowledged as a risk factor for COPD development in the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines for diagnosis, management and prevention of COPD (133).

According to a recent meta-analysis, the estimated global prevalence of COPD in HIV-infected individuals is 10.5% (134). While the majority of the reports on chronic lung diseases

comes from high-income settings, these complications are now more and more often recognized among HIV-infected individuals in developing settings (135).

Recent data indicate that HIV-infected paediatric populations often present with chronic respiratory symptoms and airway abnormalities (136, 137). The most common patterns of HIV-associated lung impairment in children include lower airway obstruction and reduced diffusion capacity (138). In a study where lung function was assessed by a broad range of tests it was shown that HIV-infected children and adolescents have lower FEV1, FVC, FEV1/FVC, diffusing capacity of carbon monoxide, respiratory system compliance and functional residual capacity and higher airway resistance and lung clearance index compared to age- and ethnicity-matched HIV-uninfected controls (139). Moreover, there is some evidence that HIV-infected children are less likely to have reversibility after bronchodilator probing compared to HIV-exposed uninfected children suggesting that maternal HIV infection may affect fetal lung development (136, 140).

Presence of chronic lung complications may in turn worsen clinical status in individuals with HIV and compromise long-term outcomes. A nationwide population-based COPD diagnoses study found that HIV-infected individuals had higher incidence rates of hospital admissions for COPD and higher in-hospital mortality compared to HIV-uninfected individuals with COPD (141). Another study found that HIV-infected individuals with airway obstruction (FEV1/FVC <0.7) had a 3-fold higher risk of death compared to those without airway obstruction (142).

3.3. Pathogenesis of lung impairment in HIV

Progression of COPD is characterized by accumulation of inflammatory mucous exudate in the lumen of the small airways and infiltration of the airway wall by inflammatory immune cells (143). This is accompanied by airway wall thickening with subsequent lung function decline and development of airway obstruction (144).

Risk behaviours such as cigarette smoking and injection drug use are known risk factors for COPD, and contribute to the development of the disease probably to the same degree as in the general population (103, 137, 145). However, data indicate the presence of pathophysiological factors unique to HIV infection.

HIV in the lung

HIV impairs host defence mechanisms in the lung and persists in alveolar macrophages (AM) and lung CD4 cells even in ART-treated individuals with undetectable plasma VL (146,

147). Ongoing HIV replication in AM leads to progressive depletion of lung CD4 cells and dysregulation of lung immunity (148). In addition, HIV increases H_2O_2 levels in the alveolar space and the expression of oxidative stress markers in AM (149). This in turn stimulates oxidant-mediated lung injury and lung fibrosis.

Systemic inflammation and residual HIV viraemia

Besides the direct effects of the virus on the lung, systemic inflammation and ongoing HIV viraemia may also contribute to lung damage. For example, increased levels of systemic inflammatory biomarkers such as C-reactive protein, interleukin-6, T-cell activation markers (CD25, CD69), endothelin-1, D-dimer, and serum amyloid A were associated with lower lung function parameters in individuals with HIV infection (150-152). Another study found that high plasma VL is associated with increased risk of spirometry-defined obstructive lung disease in HIV-infected individuals (153). This is especially important for perinatally-infected children due to challenges related to long-term virological control, life-long treatment, and longer life expectancy.

Role of respiratory tract infections

High susceptibility to respiratory tract infections in HIV-infected individuals also contributes to the development of chronic lung complications. Impaired immune response to infectious agents may increase the risk of microbial colonization of the respiratory tract or decrease the ability to clear sub-clinical infections (154). Colonization by the microorganisms in turn induces a pro-inflammatory response with increased levels of cytokines, chemokines and proteases. The inability to eliminate pathogens results in tissue re-modelling, airway thickening and lung function decline.

Role of prior TB

History of prior TB infection may also contribute to lung impairment in HIV-infected individuals.

Despite successful treatment, TB leads to incomplete immune restoration. *Mtb* may escape immune mechanisms and persist in AM in a non-replicating state, without symptoms in the host for prolonged periods of time (155, 156). Non-replicating persistence of *Mtb* maintains chronic airway inflammation and facilitates lung impairment (157).

3.4. Role of prior TB in lung impairment in HIV-infected individuals

The ongoing TB epidemic in developing settings and high number of HIV-infected individuals with active TB in anamnesis contribute to the high prevalence of chronic lung disorders in this population. TB-induced scarring results in the loss of parenchymal tissues, which explains restrictive abnormalities observed on spirometry in patients with active TB in anamnesis (158). In addition, a number of studies report that individuals with a history of prior TB are also at increased risk of airway obstruction (158-160). A meta-analysis of eleven studies found a strong association (pooled odds ratio 3.05) between a history of TB and the presence of COPD (161). The mechanisms responsible for obstruction are less clear but may be explained by the development of bronchiectasis and bronchial stenosis as the result of lung tissue remodelling.

Interestingly, in a study conducted among individuals in Tanzania who completed at least 20 weeks of anti-TB treatment, the prevalence of an obstructive spirometry pattern was significantly higher than restrictive (42% vs 13%) and airway obstruction was more common in HIV-infected compared to HIV-uninfected participants (66.7% vs 52.4%) (162). This finding indicates that both TB and HIV may interact and synergistically promote the development of airway obstruction.

Moreover, TB may exacerbate symptoms in individuals with an already established diagnosis of COPD. Jin et al. showed that COPD patients with TB in anamnesis have more severe dyspnoea, a higher prevalence of bronchiectasis and higher percentage of severe bronchiectasis compared to COPD patients without previous TB (163).

Data on post-TB lung impairment in HIV-infected individuals in SSA are scarce. One recent study conducted in Cameroon showed that 45.4% of patients with a history of treated TB have lung function impairment, with restrictive pattern being the most common (164).

Given the high burden of HIV-TB co-infection, it may be difficult to separate the effects of HIV from TB sequelae on lung status. A recent study conducted in South Africa found that the association between HIV status and airway obstruction is mediated by history of TB or pneumonia (135). This may imply the potential underestimation of the role of prior TB in the progression of lung impairment in HIV-infected individuals, especially in high TB-burden settings.

3.5. Methods to assess airway abnormalities

As previously mentioned, the main pattern of lung impairment observed in HIV-infected individuals is chronic airway obstruction that can be detected via lung function tests. A number

of techniques such as spirometry, plethysmography, impulse oscillometry, and exhaled nitric oxide are used to assess lung status.

3.5.1. Spirometry

The most widely used method to assess lung function is spirometry, which measures the volume and flow of the inhaled and exhaled air. It is used for diagnosis, severity grading and monitoring of COPD and asthma patients. Spirometry is the most reproducible and objective measure of airflow limitation and is the gold standard for measurement of lung function according to GOLD guidelines (133). Two of the most widely utilized spirometry parameters are FEV1 (reflects the volume of air that the individual is able to exhale during the first second of forced expiration) and FVC (reflects the total volume of air that the individual is able to forcibly exhale in one breath). An abnormally low ratio of FEV1 and FVC is used to diagnose airflow limitation while FEV1 is used to grade the severity of airway obstruction (165). Since the estimated parameters are age-, height-, sex- and ethnicity-dependent, the estimates obtained from the individual are compared to established reference values. Reference values are estimated from reference equations that contain data from large population surveys.

Standardized criteria and the availability of portable devices makes spirometry the most popular screening tool in individuals with chronic respiratory symptoms and suspected airway abnormalities. The majority of reports on lung abnormalities in HIV-infected individuals utilized spirometry for lung function assessment (135, 139, 166, 167).

3.5.2. Exhaled nitric oxide

Besides lung volumes, a number of techniques exist to measure certain molecules in exhaled air (exhaled breath analysis). These include measurement of nitric oxide (NO), carbon monoxide, volatile organic compounds and biomarkers in exhaled breath condensate. The most extensively studied signalling molecule in exhaled air is NO.

Discovery of nitric oxide

In 1980 Furchgott RF and Zawadzki JV reported that endothelial cells produced a substance that relaxed vascular smooth muscles (168). At that time the molecule was called endothelium-derived relaxing factor. Later, it was concluded that endothelium-derived relaxing factor is a gaseous NO molecule. Since its discovery, the role of NO in various physiological and pathological studies has been widely investigated (169, 170). In 1991 NO was detected for

the first time in the exhaled air in humans and two years later, increased levels of exhaled NO (eNO) were reported in asthmatic patients (171, 172). Due to the discovery of NO as an important signalling molecule in physiology, immunology, cardiology and neuroscience it was named molecule of the year by the Journal of Science in 1992. At present, NO is the most extensively studied exhaled airway biomarker.

Nitric oxide and its role in the lung

NO is generated by the conversion of the amino acid L-arginine to L-citrulline and NO by the enzyme called NO synthase (NOS). At present three isoforms of NOS are known: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). Two isoforms, nNOS and eNOS, are expressed constitutively and are activated by calcium ions to produce NO. This NO plays role in a number of physiological processes such as vasodilation, blood pressure control, inhibition of the haemostatic system and neuronal signalling (173, 174). In the airways NO regulates bronchodilation and vascular basal tone, counteracts hypoxic vasoconstriction and stimulates airway submucosal gland secretion (175). iNOS is not normally expressed, but induced by endogenous (chemokines, cytokines) and exogenous (bacterial toxins, viral infection, allergens, environmental pollutants) factors, leading to the production of large amounts of NO independently of calcium ion influx. There is some evidence that iNOS-derived NO has a role in regulation of airway smooth muscle tone and inflammatory response (175).

Mixing of alveolar NO with NO in the conducting airways during exhalation results in the observed expiratory NO concentration, which can be detected in the exhaled air with special instruments.

Measurement of nitric oxide in exhaled air

A number of techniques are currently available for eNO measurement, including chemiluminescence, electrochemical detection, laser-based technology and extractive electrospray ionization mass spectrometry (176, 177). Though chemiluminescence is the gold standard for NO measurement, portable electrochemical systems for eNO measurements are currently widely used. These machines contain an electrochemical sensor which converts gas concentration into electrical signals. The machine's sensor produces a signal which is directly proportional to the partial NO pressure and thus to NO concentration in the studied sample (176).

Clinical utility of exhaled nitric oxide testing

The clinical utility of eNO testing has been most widely studied in asthma. Increased eNO levels in asthmatic patients are thought to be the result of iNOS overexpression due to eosinophilic airway inflammation. Evidence suggests that eNO may be a useful tool to guide asthma management in both children and adults (178, 179).

Reduced eNO levels are reported in patients with cystic fibrosis (180-182) and active TB (183, 184), though its role in disease management is still unclear. Among mechanisms which may explain reduced eNO levels are lack of the substrate (L-arginine), reduced activity of iNOS in the airways, increased NO catabolism or thick mucus which inhibits the NO diffusion into exhaled air (e.g. in cystic fibrosis) (185-187).

The data regarding the eNO levels in other lung disorders such as bronchiolitis obliterans, bronchiectasis, interstitial lung disease, and COPD are contradictory (188-190). However, one recently published study demonstrated that eNO can predict clinical response to inhaled corticosteroids in adults with nonspecific respiratory symptoms (191).

4. HIV infection and gut microbiota

4.1. Gut microbiota in health and disease

In recent years there has been increased interest in researching the role of gut microbiota both in health and disease. Gut microbiota refers to the trillions of microorganisms which colonize the GI tract. In healthy individuals, over 90% of gut bacterial species are represented by three phyla: Bacteroidetes, Firmicutes and Proteobacteria (192). A balance of commensal microorganisms is needed in order to maintain the integrity of the gut mucosal barrier, the host-microbiota homeostasis and defence against pathogenic organisms (193).

Alteration in the composition of the gut microbiome (gut dysbiosis) has been linked to the pathogenesis of various disorders such as inflammatory bowel syndrome, atopy, type 2 diabetes, obesity, rheumatoid arthritis, multiple sclerosis, and cardiovascular disorders (194-200). Gut dysbiosis is characterized by significantly decreased microbial diversity and a shift towards pathogenic species (201). Moreover, recent evidence indicates that gut microbiota is involved in the maintaining of lung health, with altered gut microbiome composition observed in lung diseases such as asthma and cystic fibrosis (202-204). Low gut microbiome diversity during infancy has been linked to asthma development during school age (205).

4.2. Alterations in gut microbiota associated with HIV infection

The early phase of HIV infection is characterized by massive depletion of CD4 cells, primarily in the intestinal mucosa (29). Studies show that CD4 cells in the GI tract are depleted disproportionately compared to CD4 cells in peripheral blood and lymphoid tissues (27). Massive depletion of mucosal CD4 cells causes structural impairment of the gut epithelial barrier, leading to translocation of microbes and microbial products into systemic circulation (microbial translocation) and alterations in the gut microbiota (206, 207).

Alpha diversity of the gut microbiota is decreased in HIV-infected adults compared to healthy controls (208-210). These findings were reported in both ART-naïve and ART-treated individuals, emphasizing that treatment cannot fully restore the gut microbial composition. In addition, HIV-associated dysbiosis is characterized by decreased abundance of commensal (protective) bacteria and an enrichment in potentially pathogenic taxa such as *Pseudomonas*, *Enterobacteriaceae*, *Acinetobacter* and *Campylobacter* (211-214).

Data also suggest that HIV-induced alterations in gut microbiota are accompanied by immune activation and chronic inflammation (206, 209, 214, 215). For example, a number of studies demonstrated that alpha diversity of the gut microbiota correlates positively with CD4 cell count, and negatively with microbial translocation markers such as plasma lipopolysaccharide and lipopolysaccharide binding protein (208, 216). In addition, associations between specific taxa and markers of microbial translocation and systemic inflammation in HIV-infected individuals were reported in a number of studies (217, 218). Dinh et al. found a significant positive correlation between relative abundance of *Enterobacteriaceae* and interleukin-1b and interferon-γ in patients who received ART and had undetectable plasma VL (218). Increased relative abundance of *Prevotella* in HIV-infected individuals was associated with increased mucosal and systemic T cell activation markers (217). In addition to the microbiota's effect on persistent inflammation, there is some evidence that enrichment in certain species may impact treatment outcomes in individuals initiating ART. For example, Lee et al. showed that a relative abundance of *Fusobacterium* negatively correlated with CD4 count and was independently associated with poorer CD4 cell recovery following ART initiation (219). In addition, a number of studies tried to link gut dysbiosis to the development of chronic complications in HIV-infected individuals, but findings are inconsistent (220-224).

Notably, all these studies were conducted in HIV-infected adults with a paucity of data on gut microbiota in perinatally HIV-infected individuals. So far, only one recent study examined fecal microbiota in 29 HIV-infected children, 14 of whom were treatment naïve and 15 who received ART (225). In this study, decreased alpha diversity and increased relative

abundance of *Prevotella* was observed in HIV-infected children regardless of ART status. Notably, in ART-treated children, relative abundance of *Prevotella* positively correlated with plasma soluble CD14 levels, a marker of microbial translocation and all-cause mortality. These findings emphasize that certain microorganisms that colonize the GI tract may drive chronic inflammation in HIV-infected ART-treated individuals and may potentially contribute to the development of chronic complications.

To the best of our knowledge, no studies on gut microbiota have been conducted in HIV-infected individuals with lung complications. However, recent evidence indicates that the gut microbiome is involved in the maintaining of lung health, and altered gut microbiome composition is often observed in lung diseases in HIV-uninfected populations (202, 203). Low gut microbiome diversity during infancy has been linked to asthma development during school age (205). Antosca et al. found distinct stool microbiota in infants with cystic fibrosis compared to healthy controls and showed a link between gut microbiota and airway exacerbations in infants with cystic fibrosis (226). Given the severe gut dysbiosis observed in HIV, one can hypothesize that it may contribute to the development of lung complications, especially in younger populations.

Aims of the thesis

The overall aim of this thesis was to investigate the role of HIV viraemia and factors that may contribute to the pathogenesis of chronic lung impairment in the HIV-infected population in Zimbabwe.

Specific objectives were:

1. Study the rates of viral suppression among children and adults initiating ART and factors associated with initial viral non-suppression **(Paper I)**.

2. Study the incidence, patterns and risk factors of detectable viraemia in individuals who achieved initial viral suppression **(Paper I)**.

3. Investigate the levels of eNO in HIV-infected children and adolescents and study the association between airway abnormalities (history of prior TB, airway obstruction) in HIV-infected individuals and eNO levels **(Paper II)**.

4. Study the composition of gut microbiota in HIV-infected children and adolescents and investigate the associations between gut microbiota and chronic lung impairment **(Paper III**).

Materials and methods

1. Study setting and study design

The present study was conducted in Harare, Zimbabwe. The country is located in southern Africa with a population of approximately 16 million people (Figure 4). In 2018 there were an estimated 1.3 million HIV-infected people in the country of whom 84 000 were children aged below 15 (1). Unprotected heterosexual sex is the most common HIV transmission route among adults, while MTCT is predominant in children.

ART was introduced into the public health care system in 2004 and is currently provided free of charge by government clinics as well as by a number of non-governmental (not-for-profit) organizations. An estimated 88% of HIV-infected individuals had access to ART in 2018 (1) .

Figure 4. Map of Zimbabwe

For **Paper I** retrospective analysis of data collected at Newlands Clinic (Harare, Zimbabwe) was performed. Newlands Clinic is a not-for-profit HIV clinic established in 2004 by the Swiss not-for-profit organization Ruedi Lüthy Foundation. The clinic provides comprehensive HIV care to over 6000 HIV-infected patients within urban and peri-urban Harare and Chitungwiza. ART as well as routine six-monthly VL monitoring are provided free of charge. Newlands Clinic also offers psychosocial and nutritional support, special services for children and occupational skills training for adolescents and young adults (227).

For **Paper II and Paper III**, a cross-sectional study was conducted in Harare Children's Hospital - a public sector clinic which provides care for more than 4000 HIV-infected children and adolescents.

2. Study population

For **Paper I** the study population was comprised of HIV-infected individuals of all ages who initiated ART at Newlands Clinic during the period August 2013-August 2015 and who had at least two VL tests after treatment initiation. Participants were followed until September 2017.

For **papers II** and **Paper III** individuals found eligible for the BREATHE (Broncho-pulmonary function in response to azithromycin treatment for chronic lung disease in HIV-infected children and adolescents) trial were approached. BREATHE is a randomized controlled trial (RCT) among HIV-infected children and adolescents with chronic lung disease (CLD) attending two hospitals in Harare, Zimbabwe and Blantyre, Malawi (clinicaltrials.gov identifier NCT 02426112). The main objective of the trial is to investigate whether long-term treatment with azithromycin results in improvement of lung function in HIV-infected children with CLD.

For the purpose of the present work only participants enrolled in Harare, Zimbabwe were approached. Participants were eligible for the trial if they were aged 6-19, on ART for at least 6 months, had no evidence of active TB or acute respiratory tract infection and had fixed airway obstruction defined as FEV1 z-score less than -1 with no reversibility (<12% improvement in FEV1 after salbutamol 200 ug inhaled using a spacer). A comparison group of HIV-infected participants with normal lung function, no active TB or acute respiratory tract infection at the time of enrolment, no history of chronic respiratory symptoms in the last 3 months and MRC dyspnoea score <2 was enrolled specifically for the laboratory sub-studies. The detailed BREATHE study protocol has been published elsewhere (228).

In addition, a group of HIV-uninfected participants was recruited from the same catchment area as the HIV-infected group. HIV-uninfected participants aged 6-16 years with no active TB or other respiratory or heart disorders in anamnesis, no reported chest pain after exercise, no shortness of breath during exercise, no chronic cough and normal lung function were enrolled.

HIV-infected participants recruited during the period April 2017-August 2018 and June 2016-January 2018 were included in **Paper II** and **Paper III**, respectively. All HIV-uninfected participants for the present work were recruited during the period April - July 2017.

3. Data collection and study procedures

Paper I

For **Paper I** we obtained anonymized data from medical records on HIV-infected individuals who were in care at Newlands Clinic during the period August 2013-September 2017.

The following variables were extracted from medical records: age, sex, date of ART initiation, ART regimen, adherence, height, weight, clinical history (WHO HIV disease stage, history of TB, opportunistic infections, chronic comorbidities), and laboratory parameters (VL, CD4 count, haemoglobin) at the time of ART initiation and during the follow-up period.

Paper II-III

For **Papers II and III**, questionnaires were used; clinical examination, eNO testing and spirometry were performed; and blood samples and rectal swabs were collected.

Questionnaires

All study participants completed a detailed, standardized questionnaire regarding demographic characteristics, details on HIV diagnosis and treatment, clinical history and presence of respiratory symptoms (chronic cough, wheezing, dyspnoea) at the time of examination and in the past 3 months. Self-reported and physician-diagnosed heart, lung (including history of prior TB and asthma) and other disorders were recorded.

For **Paper II,** an additional questionnaire regarding asthma, eczema (atopic dermatitis), allergic rhinitis, allergic conjunctivitis, food allergy symptoms and diagnosis, the use of medications and exposure to tobacco smoke was administered. Full versions of the questionnaires are presented in Appendices I, II, III (BREATHE forms: B0.03a, B0.03b, B0.15).

Clinical examination included measurement of weight, height, respiratory rate, heart rate and oxygen saturation measured by pulse oximetry (OxyWatch, Beiijing Choice Electronic Technology Co. Ltd)

Exhaled NO measurement

Exhaled NO was measured using the portable electrochemical system NIOX VERO (Circassia, UK). The machine contains an electrochemical sensor which converts gas concentration into electrical signals. The machine's sensor produces a signal which is directly proportional to the partial NO pressure and thus to NO concentration in the studied sample (176). The machine has a measurement range between 5 parts per billion (ppb) and 300 ppb.

eNO measurements were performed according to American Thoracic Society (ATS) guidelines (229). The calibration of the device and quality control were performed according to the manufacturer's instructions. All eNO measurements were performed between 08:00 and 14:00 h prior to spirometry, and the exact time of testing was recorded. The participants were asked to sit and rest for a minimum of 5 minutes before testing. Repeated exhalations with a minimum of 30 seconds rest time in between were performed in order to obtain at least two measurements that agreed within 10%. Up to six measurement attempts were made and the mean eNO value was calculated from two eNO measurements with minimal differences between them.

Spirometry

Spirometry was performed using the EasyOne portable spirometer (ndd Medical Technologies Inc., Andover, MA, USA). Spirometry was performed according to the ATS guidelines (230). Up to eight forced exhalations were recorded. The largest FEV_1 and FVC for each individual were recorded. FEV1 and FVC were expressed as z-scores using Global Lung Function Initiative reference ranges (231). The z-score shows how many standard deviations (SD) the measured value is away from the mean predicted value (for example, a value of 0 is the expected value and the value -1 is one SD below the mean) (165). Participants with FEV1 z-score <-1 repeated spirometry 15 minutes after administration of 200µcg inhaled salbutamol via spacer. The largest values for post-FEV1 and post-FVC were recorded.

Blood tests

Blood samples were collected for full blood count tests, and for participants with HIV, also for HIV VL and CD4 count testing. HIV VL was measured using the Gene Xpert assay (XpertTM HIV-1 Viral Load; Cepheid, Sunnyvale, CA, USA), with a lower limit of detection of 40 copies/ml and CD4 count was measured as a point of care test using a PimaTM Analyser (Alere, Orlando, FL, USA).

Collection of rectal swabs

Study nurses collected rectal swabs from all participants at study enrolment. Samples were obtained by inserting the swab approximately 2-3 cm inside the rectum, ensuring that swabs were visibly stained with faeces. Swabs were immediately preserved in 1.5 ml of transport medium PrimeStore ® MTM (Longhorn diagnostics, Maryland, USA) and directly stored on ice for a maximum of one hour before being frozen at -80 °C. Frozen samples were shipped on dry ice to the laboratory at Division of Medical Microbiology, University of Cape Town, South Africa. Upon arrival, the samples were stored at -80°C until further processing.

Processing of rectal swabs at the University of Cape Town

DNA extraction

The Zymo Research Quick-DNA™ Fecal/Soil Microbe Microprep kit (Zymo Research, California, USA) was used for DNA extractions. DNA was extracted according to the manufacturer's protocol.

16S library preparation and gene sequencing

In order to assess DNA quality and the total bacterial load extracted, a real-time quantitative polymerase chain reaction (PCR) was performed as previously described (232). Subsequently, two sets of PCRs targeting the V4 hypervariable region of the 16S ribosomal ribonucleic acid (rRNA) gene using the primers 515F and 806R were performed according to previously described protocols (233, 234).

Amplicons were cleaned using the Agencourt® AMPure® XP PCR Purification kit (Beckman Coulter, California, USA) as previously described (234). Each of the purified PCR products was quantified using the QuantiFluor® dsDNA System quantification kit on Promega Glomax®-Multi Detection system (Promega, Wisconsin, USA) (234). Product sizes were confirmed using agarose gel electrophoresis (234). PCR products were pooled to a normalized concentration of 120 ng. Gel excision and purification were performed on the final pool using a 1.6% agarose gel (234). The purified library size was determined using the Agilent 2100 bioanalyzer using hsDNA kit, and quantified using the KAPA PCR quantification kit (KAPA Biosystems, Massachusetts, USA) (234).

Samples were sequenced on an Illumina Miseq® instrument using the Miseq® Reagent v3 kit, (600 cycles) (Illumina, California, USA). The final library was diluted to a 6 pM concentration, and a 25% PhiX library spike-in was added at 6 pM as internal control (235). The pre-processing of sequence reads was done using the H3ABioNet 16S rDNA diversity

analysis package (https://github.com/h3abionet/h3abionet16S), with the exception that the taxonomy of representative reads was assigned using the SILVA version 132 database. The raw sequence files have been submitted to the European Nucleotide Archive under the accession number PRJEB32077.

Sequencing controls, including the ZymoBIOMICS microbial community standards, and biological samples were analysed separately. The average sequence reads of each operational taxonomic unit 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 Storage (Paper II, Paper III)

Electronic clinical record forms (Google Nexus tablets, Google, Mountain View, CA, USA) with OpenDataKit software (opendatakit.org) and paper forms were used for data collection. Data from paper forms were extracted using CARDIFF TELEFORM character optical mark recognition software (Version 10.9, Vista, CA, USA). Data were transferred to Microsoft Access database (Microsoft, Redmond, WA, USA) and then exported for analysis.

4. Definitions

Adherence to ART - number of tablets dispensed at the last visit minus the number of tablets returned at current visit divided by number of tablets that should have been consumed between visits.

Airway obstruction was defined as FEV1 z-score <-1.64 with no reversibility.

Alpha diversity refers to the richness of bacterial taxa and relative abundances of the different species within a single sample.

Anaemia was defined according to WHO criteria (haemoglobin <11 g/dl for children <5 years; <11.5 g/dl for children 5-11.9 years; <12 g/dl for children 12-14.9 years; <12 g/dl for females aged ≥15 years; <13 g/dl for males aged ≥15 years) (236).

Asthma was defined based on clinical history of diagnosed asthma or self-reported symptoms of asthma or treatment with asthma-specific medications.

Atopic disorders were defined based on self-reported episodes of allergic rhinitis, conjunctivitis and eczema (atopic dermatitis) and/or physician diagnosed atopic disorder.

Beta diversity refers to interindividual differences within groups and was determined using Bray-Curtis dissimilarity index (237) with sample depth set at 2000 reads.

Low level viraemia - at least two consecutive viral load measurements 50-1000 copies/ml followed by viral load <50 copies/ml.

Stunting was defined as height-for-age z-score less than -2 for participants aged ≤19. WHO reference standards or British 1990 Growth Reference Curves were used to calculate z-scores (238, 239).

Underweight (wasting) was defined as weight-for-age z-score or body mass index (BMI)-for-age z-score less than -2 for participants aged ≤19 or BMI<18.5 kg/m^2 for participants aged >19. WHO reference standards or British 1990 Growth Reference Curves were used to calculate z-scores (238, 239).

Viral blip – a single episode of viral load ≥50 copies/ml preceded and followed by viral load<50 copies/ml.

Viral failure – viral load ≥1000 copies/ml in two consecutive viral load measurements after initial VL suppression.

Viral load non-suppression – failure to achieve viral load suppression by 12 months on ART.

Viral load refers to the number of HIV RNA copies per millilitre of blood.

Viral load suppression – two consecutive viral load measurements <50 copies/ml after ART initiation.

5. Statistical analyses

Continuous variables were reported as mean and standard deviation (SD) or median and interquartile range (IQR). Continuous parameters between study groups were compared using t-test (in case of normal distribution) and Wilcoxon rank sum or Kruskal-Wallis test (in case of skewed distribution); categorical parameters were compared using chi-squared test or Fisher's exact test. All statistical tests were two-tailed and p-value < 0.05 was considered statistically significant.

Paper I

The proportion of participants who did not achieve VL suppression by 12 months ART (the 12 months cut-off was chosen to allow for a VL test to confirm suppression at month 12)

was estimated and the factors associated with VL non-suppression were studied using logistic regression.

The incidence rates of viral blips, LLV and VF were estimated, and Nelson-Aalen cumulative hazard curves were plotted for those who had at least two VL tests after initial VL suppression. We used survival analysis to study the factors associated with occurrence of viral blips. For the model, participants were included into analysis at time 0 (time of first suppressed VL test after ART initiation) and followed until a viral blip occurred or until the last VL test available. Participants who reported treatment interruption in ART for more than two weeks were excluded from this analysis. Since the estimated cumulative hazard of blips increased exponentially with time, we fitted the parametric survival regression with Weibull distribution stratified by age groups. The value of P (the shape parameter) >1 confirmed that the hazard of failure (viral blip) increased with time.

The following variables were studied as predictors of VL non-suppression and viral blips: age, sex, BMI, stunting (in children only), history of TB before ART initiation, chronic comorbidities (adults only), WHO clinical stage at ART initiation (1-2 vs 3-4), ART regimen at treatment initiation (NNRTI-based vs PI-based), adherence, CD4 count, pre-treatment VL, anaemia. Age, sex and CD4 count at ART initiation were adjusted for *a priori*. All analyses were performed according to age at ART initiation (children aged 0-19 years and adults aged >19 years).

Paper II

The values of eNO were not normally distributed and therefore presented as geometric mean with 95% confidence interval (CI). The analyses were performed with log-transformed eNO data and back transformed to present geometric mean.

The association between eNO levels and explanatory variables was studied using linear regression analysis. Explanatory variables included HIV status, age, sex, anthropometric parameters, haemoglobin level, haematocrit, white blood cell count, neutrophil count, eosinophil count, FEV1 z-score, atopic status, and passive smoking for all participants. The additional variables CD4 count, VL, history of TB and presence of airway obstruction were included for participants with HIV only. Age, sex and exact time of eNO testing were adjusted for *a priori.*

Parameters were included into the multivariable linear regression model if they showed a significant effect on the prediction of eNO level in age-, sex- and time of eNO testing adjusted models at $p<0.05$. Adjustment for unbalanced parameters (for HIV-infected group) was also

performed. Variance inflation factor was used to detect multicollinearity in the multivariable models. The linear association between eNO and continuous variables was estimated graphically. Residual analysis with residual plots and normal probability plots of residuals confirmed no violation of the linear regression assumptions.

Paper III

The following alpha diversity estimates were calculated - the number of operational taxonomic units (OTUs), Chao1 index (240) and Shannon index (241). Alpha diversity indices between study groups were compared using Wilcoxon rank-sum test and p-values were corrected for multiple testing using false discovery rate (FDR) procedure. Spearman´s rank correlation with Bonferroni correction was used to assess the association between alpha diversity indices and continuous parameters. We fitted the linear regression model in order to estimate the association between HIV status and alpha diversity indices. BMI, age, sex were adjusted for *a priori*. An interaction term between HIV status and antibiotics in the three previous months (co-trimoxazole prophylaxis for HIV-infected group) 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. The following participant characteristics were studied: stunted, underweight, FEV1 z-score, episodes of diarrhoea for all participants; VL, CD4 count, years on ART, co-trimoxazole prophylaxis, diagnosis of CLD, ART regimen, history of prior TB - for HIV-infected participants. Age and sex were adjusted for *a priori*.

Beta diversity comparisons were performed using Principal Coordinate plots. Comparisons were made using the Wilcoxon rank sum test. Kruskal-Wallis test was used in cases where more than two groups were compared and the same groups were compared using the permutational multivariate analysis of variance (242), with number of permutations set to 999. P-values were adjusted for multiple testing using the Benjamini-Hochberg method (243).

Relative abundance

Relative abundance was assessed in a linear discriminant analysis using linear discriminant analysis effect size (LEfSe) (244) 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. Heatmaps were generated using only the taxa that were significantly different using the LEfSe comparison. The average fraction of each taxa was calculated from all samples

within each group. The data were transformed to fractional abundance before performing the LEfSe analysis. All p-values reported were corrected for multiple testing using FDR.

Statistical analyses were performed in STATA 14 (StataCorp LLC, College Station, Texas, USA). In addition, for **Paper III,** analyses of beta diversity were performed using *stats* package in R Statistical software (http://www.r-project.org/) and QIIME2 (version 2018.4); relative abundance comparisons plots were generated using the MicrobiomeAnalyst web-based software tool with standard feature filtering (245).

6. Ethical approval

Paper I

Ethical approval was obtained from the Newlands Clinic Research Committee, the Medical Research Council of Zimbabwe, and Regional Committee for Medical and Health Research Ethics (Norway).

Paper II, III

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; Regional Committee for Medical and Health Research Ethics in Norway. Written informed consent by guardian and assent by participants aged <18 years were sought. Consent was obtained from those aged above 18 years.

Results

1. Paper I

In **Paper I**, the rates and risk factors for detectable viraemia were investigated in a cohort of HIV-infected children and adults who initiated ART at Newlands Clinic (Harare, Zimbabwe). Among 725 participants who initiated ART between 2013 and 2015, 127 children (0-19 years) and 503 adults (>19 years) were included in the analysis. The median (IQR) follow-up time was 2.8 (2.3-3.2) and 2.7 (1.8-2.8) years for children and adults, respectively.

Of the 630 participants, significantly more children than adults did not achieve VL suppression by 12 months following ART initiation (19.7% children vs 5.6% adults, p<0.001). In adults, younger age and CD4 count ≤200 cells/mm^3 at ART initiation were significantly associated with not achieving VL suppression in logistic regression analysis.

During the follow-up period, 106 (83.5%) children and 482 (95.8%) adults reached VL suppression. The median (IQR) time to VL suppression was 0.5 (0.4-1.3) and 0.5 (0.2-0.8) years in children and adults, respectively.

Among those who reached VL suppression, 516 participants had sufficient VL data to study the incidence of detectable viraemia episodes. In total, 57 (11%) participants experienced a viral blip. Blips occurred more commonly in children than in adults (22.9% vs 8.3%, p<0.001), with the incidence rate being 10.9 (95% CI 7.2-16.6) and 4.0 (95% CI 2.8-5.5) per 100 person years in children and adults, respectively. In survival analysis no baseline characteristics were found to be significantly associated with increased risk of viral blips in children or adults.

Seven (1.4%) participants (4 children and 3 adults) experienced LLV and 12 (2.3%) participants (8 children and 4 adults) developed VF after initial VL suppression during follow-up. The incidence of LLV was higher in children (1.9 (95% CI 0.7-5.1) vs 0.3 (95% CI 0.1-1.0) per 100 person years, p=0.03), as was the incidence of VF (4.0 (95% CI 2.0-7.9) vs 0.4 (95% CI 0.2-1.2) per 100 person years, p<0.001). All four adults with VF re-suppressed while only three children re-suppressed during the follow-up period.

2. Paper II

In this paper we compared the levels of eNO in children with and without HIV and investigated the association between eNO and history of TB and airway obstruction in HIV-infected children on ART. In total, 222 HIV-infected and 97 HIV-uninfected participants were included in analysis. Among HIV-infected participants 57 (25.7%) had a history of TB in anamnesis and 56 (25.2%) had airway obstruction but no TB in anamnesis. Overall, HIV-

infected children were more likely to be wasted, stunted and to have anaemia compared to HIV-uninfected participants (wasted: 41.9% vs 3.1%, p<0.001; stunted: 42.8% vs 4.1%, p<0.001; anaemia: 31.2% vs 9.3%, p<0.001). HIV status was associated with lower eNO levels after adjusting for age, sex and time of eNO testing (geometric mean ratio 0.79 (95% CI 0.65-0.97), p=0.03).

Among HIV-infected participants, those with a history of TB had significantly lower eNO levels compared to those without TB whether they had airway obstruction (geometric mean 13.7 (95% CI 12.2-15.5) vs 16.9 (95% CI 14.3-19.8), p=0.03) or had no airway abnormalities (geometric mean 17.9 (95% CI 16.0-20.0), p=0.003). History of TB remained significantly associated with lower eNO levels in linear regression analysis after adjustment for age, sex and time of eNO testing. In multivariable analysis history of TB, haemoglobin, neutrophil count, and age were significantly associated with eNO levels in this study group.

Among HIV-uninfected participants, only wasting, haemoglobin and age were significantly associated with eNO levels in multivariable analysis.

3. Paper III

In **Paper III** we investigated the composition of gut microbiota in HIV-infected and HIV-uninfected children and studied the association between participant characteristics and gut microbiota. In total, 149 HIV-infected participants with diagnosis of CLD, 28 HIV-infected participants without diagnosis of CLD, and 103 HIV-uninfected controls were included into the study.

HIV status was associated with lower species richness estimates (OTUs, Chao1 index) in linear regression analysis adjusted for BMI, age and sex (β coefficient (95% CI); OTUs: -18.8 (-35.0 to -2.6) p=0.02; Chao1: -34.7 (-55.7 to -13.7), p=0.001). The association remained borderline significant for OTUs and significant for Chao1 index once antibiotic use in the previous 3 months were introduced into the regression model (OTUs: -26.5 (-54.8 to 1.89), p=0.07; Chao1 index: -42.3 (-79.1 to -5.6), p=0.02). No association between HIV status and Shannon index was observed.

HIV-infected participants with suppressed VL had borderline higher OTUs (Median (IQR): 192.5 (145.5-228.5) vs 176 (138-220), p=0.06) and higher Chao1 index (259.3 (201.2-302.1) vs 233.2 (175-276), p=0.02) compared to participants with VF. The negative association between VF and Chao1 index remained borderline significant in regression analysis adjusted for age and sex (-19.4 (-40.5 to 1.73), p=0.07)).

Once HIV-infected participants were split into groups based on ART treatment duration (<5 y; 5-10 y; ≥10 y), we found that participants who had been on ART ≥10 years had alpha diversity estimates that were similar to the HIV-uninfected participants. Among participant characteristics, prolonged ART treatment was the only parameter significantly associated with a richer gut microbiota in HIV-infected children after adjustment for age and sex. No association between diagnosis of CLD and alpha diversity indices was observed in the study.

Beta diversity

Beta diversity was significantly higher among HIV-infected participants compared to HIV-uninfected group ($p<0.01$).

Relative abundance of specific taxa

At phylum level HIV-infected participants had significantly lower abundance of Epsilonbacteraeota (7% vs 13%, $p<0.01$) and Bacteroidetes (32% vs 38%, $p<0.01$) compared to HIV-uninfected controls. At genus level HIV-infected participants were enriched in *Corynebacterium* ($p<0.01$), *Finegoldia* ($p<0.01$), *Anaerococcus* ($p<0.01$) compared to HIV-uninfected participants.

HIV-infected participants with CLD had a higher abundance of *Faecalibacterium* ($p=0.05$) compared to those without CLD. Enrichment of *Enterobacteriaceae* ($p=0.02$) and *Burkholderiaceae* ($p=0.04$) was observed in HIV-infected children with CD4 counts ≤400 cells/mm^3. Notably, the longer the participants had been on ART, the fewer taxa were found to be significantly different between HIV-infected and HIV-uninfected children.

Discussion

1. Discussion of the main results

1.1. Virological outcomes in individuals initiating ART

In **Paper I** we found that 83.5% of children and 95.8% of adults reached VL suppression after ART initiation. The rates of VL suppression are close to those reported in high-income settings and indicate that Zimbabwe is on its way to achieve the third UNAIDS target of 90% of those on ART have VL suppressed.

Other studies performed in SSA and other RLS report widely varying VF rates, ranging from 4% to 52% depending on study design, population and setting (79, 246-249). In contrast, only a small proportion of HIV-infected individuals developed VF during follow-up in our study. This may be explained by several reasons.

First, VL monitoring is not implemented as a routine test in many RLS and the majority of data on VF come primarily from cross-sectional studies with no information about initial VL suppression or a confirmative VL test in case of detectable viraemia. Moreover, the VF threshold may also vary depending on the guidelines implemented.

If one defines VF based on a single measurement, the results do not discriminate between "true" VF, and transient viraemia. An example of the so-called "VL snapshot" is a study conducted in Togo which demonstrated strikingly high rates of VF among children and adolescents with 52% having VL>1000 copies/ml in a single VL test (79). In contrast to this report, the longitudinal design of our study allowed us to distinguish those who did not reach VL suppression since ART initiation from those who experienced detectable viraemia (either single episodes of raised VL, LLV or VF) after VL suppression.

Another explanation of the relatively low rates of VF in **Paper I** might be that Newlands Clinic aims to provide a standard of care comparable to that of western countries, with routine VL monitoring, adherence counselling and social support. Though data from **Paper I** and **Paper II** are not directly comparable (cross-sectional design, only one VL test was done in **Paper II**), the proportion of "un-suppressed" HIV-infected children and adolescents enrolled in a governmental clinic was substantially higher with 69.2% of children having detectable viraemia (VL≥50 copies/ml) and 36.0% having VL≥1000 copies/ml (potentially implying VF). This underlines the need for follow-up VL tests, which are seldomly performed due to the economical restraints in SSA.

A number of cohort studies conducted in high-income settings found that detectable viraemia is common among individuals on ART and is associated with subsequent VF, yet there

is a lack of longitudinal VL data for HIV-infected individuals in RLS (54, 55, 250). In a study published in 2018, Hermans et al. claimed to be the first to report on LLV episodes among adults on ART in RLS (251), though two prior reports based on data from the Treat Asia HIV observational Database (TAHOD cohort) were published earlier in 2014 (252, 253). TAHOD cohort includes HIV-infected individuals from low- and middle-income settings in Asia and Pacific region (254). In this cohort the incidence rate of LLV in low- and middle-income settings was higher than in our group (0.99 per 100 person-years vs 0.3 per 100 person-years), with LLV being a weak predictor of subsequent VF (252). Another paper, which included 1554 participants from the TAHOD cohort, investigated the predictive value of viral blips. In this study blips occurred in 11% of individuals with an incidence rate 31.7 per 1000 person years in low- and middle-income settings and no association was found between blips and subsequent VF (253). In a large multicenter South African cohort study comprised of data on 70 930 individuals Hermans et al. demonstrated that LLV occurred in 23% of individuals with an incidence rate 11.5 per 100 person-years of follow-up. LLV was also strongly associated with subsequent VF in this study (251). The fact that data were collected from 57 rural-urban centres with differences in treatment practices over the period 2007-2016 and different sensitivity of VL assays used for VL detection may explain the discrepancies between the studies, yet the results are indicative of the real-world situation. The authors concluded that thresholds for VF for RLS should be reconsidered. Due to very low rates of VF over the study follow-up period and relatively short follow-up we could not assess the clinical significance of detectable viraemia episodes.

In the majority of studies (77, 255, 256), children and adolescents have significantly lower suppression rates than adults, with children in our study having a 12% lower suppression rates and 7 % higher VF rate compared to adults over the follow-up period. This is explained by a broad range of challenges during ART treatment in children (83). Poor tolerability to antiretroviral drugs, inadequate ART dosing and dependence on a caregiver while administering the drug contribute to high rates of detectable viraemia among children (84). When reaching adolescence, various psychological, social, environmental issues also come into the picture and compromise adherence to treatment and linkage to care even more. Interestingly, in our study, seven out of eight children who developed VF during follow-up were aged >10 years. Notably, a recent study from Malawi show that women with LLV are at higher risk of MTCT with the majority of women with LLV in this study being adolescents (257).

A number of recently published studies show that one of the instruments to improve suppression rates in both children and adults is adherence counselling (49, 258, 259). In one

study adults who received community-based adherence support had significantly higher suppression rates with OR being 1.33 and 2.66 after 1 and 5 years ART, respectively (260). In another study, children who received community-based adherence support were also more likely to archive VL suppression (OR 1.60) with the most profound effect demonstrated for young children with OR 2.47 (261). In Newlands clinic adherence is checked at every visit when patients pick up their drugs. During the study period average adherence by pill count was 98.4% and 99.6% for children and adults, respectively. Our data showing relatively low rates of detectable viraemia episodes accompanied by good adherence estimates may indicate that more efforts should be moved towards providing adequate routine adherence support for individuals on ART.

To summarize, detectable viraemia among individuals on ART is increasingly recognized as a risk factor for multiple AIDS-related problems (increased risk of VF, MTCT, and development of HIV drug resistance mutations). In order to reach the third 90 UNAIDS goal, more efforts should be put towards increasing adherence and routine VL control.

1.2. Levels of eNO in HIV-infected children

Advancements in ART lead to a shift of focus in management of HIV infection from typical HIV-related opportunistic conditions to less recognized chronic complications. One example of this is a disproportionate burden of chronic lung disorders in HIV-infected children (136, 137, 139, 166). One might argue about the pathogenesis of those conditions, with TB and other chronic infections going hand in hand with HIV, but there is no doubt that the aforementioned conditions are a concern in paediatric HIV-infected population.

Due to the economic restraints of SSA with limited access to modern diagnostic tools, data on pathogenesis of lung impairment in this population group are scarce. In **Paper II** we aimed to evaluate the eNO levels in HIV-infected children and adolescents with and without history of prior TB.

Paper II showed that HIV infection is associated with lower eNO levels in children and adolescents. To our knowledge, only one study investigated this before. In a paper published in 1997, Loveless et al. reported lower levels of eNO in 36 HIV-infected adults compared to relatively healthy hospital staff (262). No studies so far have been conducted in children. The other two studies investigated the levels of eNO in individuals with active TB with or without HIV-infection. While eNO levels were significantly lower in HIV-infected subjects with active TB compared to healthy controls in one study, no association between eNO and HIV status was

observed in another one (183, 184). Taken together, these data indicate an effect of both HIV and TB on eNO levels, though the clinical importance of this warrants further discussion.

There are several pathophysiological mechanisms that can explain the observed low eNO levels. Several studies show that lower levels of L-arginine (a primary substrate for NO in the lung) are observed in individuals with active TB, and may well explain the relative deficiency in eNO in those patients (263, 264). On the other hand, no data indicate a lack of L-arginine in HIV infection. Dirajlal-Fargo et al. did not find decreased arginine availability in HIV-infected ART-treated individuals compared to HIV-uninfected controls (265).

Despite availability of substrate, the HIV virus may prevent cells from producing sufficient amounts of NO. NO is produced by a variety of cells in the respiratory tract, with AM being responsible for primary NO release in response to infection (175, 266). HIV persists in AM even in individuals with undetectable plasma VL (147, 267, 268). Studies show that presence of the virus in AM impairs phagocytic activity of cells and bacterial clearance (146, 149) There is some evidence that NO has a role in intracellular killing and phagocytic activity, though the exact effect of the virus on the NO pathway in AM is unclear (269). Moreover, HIV proteins are known to alter the signalling pathways in monocyte/macrophage cells, changing the normal response to infection (270). Thus, low NO levels may reflect the AM's loss of ability to produce NO as part of host-defence mechanism and compromised lung immune status.

Increased catabolism of NO into NO-metabolites is another plausible reason for low eNO levels observed in our study. There is evidence that the HIV virus increases the levels of H_2O_2 and other reactive oxygen species (ROS) in the alveolar space (149). In the presence of ROS (superoxide or H_2O_2) NO turns rapidly into peroxynitrite and/or stable end products of NO - nitrite and nitrate thus lowering the NO gas concentration. Presence of ROS and highly toxic peroxynitrite in the lung in turn mediates cell dysfunction and damage of the lung extracellular matrix (271, 272). Peroxynitrite is highly unstable and difficult to measure, but may form nitrotyrosine (273). Interestingly, studies found high concentrations of nitrotyrosine, nitrate and nitrite in sputum and exhaled breath condensate alongside low eNO levels in individuals with cystic fibrosis (182, 274-276). As in cystic fibrosis, ongoing oxidative and nitrosative stress in the lung during HIV infection may explain low eNO levels in HIV-infected individuals in **Paper II.**

In addition, low eNO levels could be caused by mechanical factors such as diffusion barriers for NO due to increased mucus production or decrease in airway calibre. However, in line with other studies (277-280), no association was observed between airway obstruction and eNO level in our study population. This indicates that eNO probably has no pathophysiological

role in obstruction, but is rather a marker of the underlying condition, as seen in eosinophilic inflammation in asthma patients (281).

The role of NO during active TB infection has been widely studied. Data show that NO activates certain antimicrobial pathways in AM and also limits the pro-inflammatory response during TB infection (282). At the same time there is evidence that iNOS may be directly inhibited as an immune evasion mechanism by *Mtb (283, 284).* Two studies reported lower levels of eNO in individuals with newly diagnosed untreated lung TB compared to healthy controls (183, 184).

Recent studies showing that *Mtb* may persist in AM after anti-TB treatment have raised interest in post-TB lung complications (155, 285). However, data are scarce regarding NO involvement in post-TB lung impairment. **Paper II** demonstrated that HIV-infected children with prior TB in anamnesis had lower eNO compared to HIV-infected children with airway obstruction or to HIV-infected with no airway abnormalities. Persistence of *Mtb* in AM may maintain ongoing lung inflammation and accelerate the development of lung fibrosis (155-157). Interestingly, *in vitro* studies found that NO has a protective role in the progression of pulmonary fibrosis (286). Thus, low levels of eNO in patients with history of TB may reflect altered lung immune status, which further mediates the development of chronic lung complications.

Age was positively associated with eNO levels in both HIV-infected and HIV-uninfected participants. It is known that values of eNO increase linearly between 6-16 years in parallel with the somatic growth (287). ATS guidelines recommend accounting for age when interpreting the eNO results in children younger than 12 years of age (288). A number of studies also showed a significant association between height and eNO levels (277, 289-291). Total airway mucosal surface area for NO diffusion increases with increasing age and height, thus leading to higher eNO levels (292). In **Paper II,** both age and height were significantly associated with eNO levels in HIV-infected children. As these children are generally smaller than their HIV-uninfected peers, this may partly explain the lower eNO levels among HIV infected children observed in our study.

In **Paper II** we did not observe any association between passive smoking and eNO in study participants. While a number of studies conducted in healthy children and those with asthma showed that passive smoking reduces eNO levels (290, 293, 294), others found no correlation between these two parameters (295, 296). Conflicting results regarding the impact of passive smoking may be explained by genetic factors. For example, Spanier et al. showed that only individuals with specific genetic polymorphisms in the NO synthase gene (namely

GT and TT genotypes) have decreased eNO when exposed to passive smoking (297).

Interestingly, positive associations between several blood parameters and eNO levels were observed in **Paper II**. While a number of studies explored the link between eNO and blood eosinophils in asthmatic individuals, data on associations between eNO and other full blood count parameters in conditions besides asthma are scarce (181, 183, 298). The associations between haemoglobin level, haemotocrit and eNO may reflect physiological processes at the level of endothelium. Elevated haematocrit increases shear stress and production of NO. The positive association between eNO and blood neutrophils observed in HIV-infected participants may be explained by the fact that neutrophils can produce NO in response to foreign stimuli (299). The observed associations emphasize the need to interpret results with caution and thus question the clinical utility of eNO.

eNO measurement is emerging as an easy point-of-care test in monitoring of asthma patients. Although our study does not give evidence for its utility in RLS as a diagnostic test, it provides some important findings. Reduced eNO levels observed in HIV-infected children and in those with prior TB are indicative of impaired lung host defence and may mediate increased susceptibility to respiratory infections in this population group. Further studies are needed to explore pathophysiological pathways that may explain low eNO and what clinical utility it can provide - whether it can serve as a marker of chronic lung non-eosinophilic inflammation or whether approaches towards increasing NO levels could be beneficial.

1.3. Composition of gut microbiota in HIV-infected children

In **Paper III** we investigated the differences in the gut microbiota between HIV-infected and HIV-uninfected children, and the association with participant characteristics. Overall, our study demonstrated that the gut microbiota in HIV-infected ART-treated children was less diverse than in HIV-uninfected controls. However, children who received ART for 10 years or more had a more diverse microbiota, resembling that of HIV-uninfected children.

To the best of our knowledge only one study to date has investigated gut microbiota in the paediatric HIV-infected population (225). Kaur et al. demonstrated that alpha diversity was significantly lower in HIV-infected children, mirroring the results of our study. Notably, in Kaur et al. paper there was no difference in alpha diversity estimates between ART-treated and ART-naïve children, though the low sample size (15 participants on ART and 14 ART-naïve) of the study does not allow for conclusion with certainty about the effect of ART.

Studies conducted among adults showed that HIV infection without ART is associated with severe intestinal dysbiosis and reduced alpha diversity (29, 208, 300). These changes may

persist despite ART (5, 6, 10, 38, 39). A recently published individual level meta-analysis comprising data from 17 datasets on 1032 individual samples also demonstrated that HIV infection in adults is associated with decreased alpha diversity of gut microbiota (301). Our results of overall lower alpha diversity in HIV-infected ART-treated children support and extend these findings.

The data are less consistent when it comes to relative abundance of specific taxa in HIV-infected individuals. Such factors as different types of specimens used (stool samples, rectal swabs, etc), study populations, geographical area, sequencing methods and false discovery may explain these discrepancies (302, 303). Kaur et al. studied fecal microbiota and reported significantly higher relative abundance of *Prevotella* in both ART-treated and ART-naïve children compared to HIV-uninfected controls (225). This is in contrast to our findings where HIV-uninfected participants were enriched in *Prevotella*. A few possible explanations exist – the study sample size, different diet and geographical location are certain to influence the microbiome composition (303). Another point is that Kaur et al. used fecal samples, while we used rectal swabs. A study on rectal swabs of HIV-infected ART-treated participants in Nigeria reported higher abundance of *Finegoldia* and *Anaerococcus,* which is consistent with our findings (304). However, in the same study *Campylobacter* was significantly enriched in HIV-infected participants, in contrast to our findings of enriched *Campylobacter* in the HIV-uninfected group.

The effect of ART on gut microbiota composition is contradictory. At least two studies have found a negative impact of ART on gut biodiversity (208, 305). In a longitudinal study, Nowak et al. demonstrated a decrease in gut microbiota diversity after the introduction of ART. Our results are not in direct contradiction to this study, as Nowak et al. investigated the effect of ART initiation, with a relatively short follow-up of 10 months (208). In **Paper III** we had no ART-naïve children, and the minimum duration of ART was 1 year. We observed lower alpha diversity in those who were on ART for less than 10 years compared to HIV-uninfected participants.

A number of studies conducted among individuals on long-term ART reported gut microbiota diversity estimates similar to HIV-uninfected participants (218, 300). For example, Dinh et al. found no significant difference in alpha diversity measures between HIV-infected participants who were on suppressive ART for a median of 13.3 years and HIV-uninfected controls (218). This is similar to our findings for children who received ART for 10 years or more. The impact of ART duration on the gut microbiota was also acknowledged in another study where individuals with longer ART duration showed a closer resemblance to HIV-

uninfected group than to subjects with untreated HIV infection (306). These studies together with our findings indicate the ability of ART to restore the altered gut microbiota in the long run.

We did not find a link between CD4 count and gut microbiome diversity indices, yet there was a weak negative association between VL>1000 copies/ml and Chao1 index. A number of studies showed significantly lower microbiome diversity in those with more severe HIV status (208, 307). However, the findings of previous studies may have been affected by sample size and duration of ART. 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 cell counts, a finding that was also reported in HIV-infected adults with pneumonia in Uganda (308). *Enterobacteriaceae* are part of the normal intestinal flora, though our finding may explain the high incidence of gastrointestinal and urinary tract infections caused by *Enterobacteriaceae* in HIV-infected children (309). *Burkholderiaceae* was also enriched in those with low CD4 cell counts, including the species known to cause severe lung infections in patients with cystic fibrosis (310).

A number of recently conducted studies investigated the gut dysbiosis in relation to chronic HIV-associated complications (222, 224). Hoel et al. found the lowest alpha diversity estimates in HIV-infected participants who also had a diagnosis of type 2 diabetes, while no difference was noted between HIV-infected subjects with no diabetes and healthy controls (222). Another study found no difference in alpha diversity indices between HIV-infected adults with and without metabolic syndrome (224).

Recent years have been characterized by increasing interest in the role of gut microbiome in lung health and lung disease (311). Gut dysbiosis has been reported in HIV-uninfected individuals with lung disorders (202, 203). Notably, a recent study demonstrated a link between the gut and the lung microbiota composition in HIV-infected individuals with bacterial pneumonia. Moreover, this study found an association between lower gut microbiota diversity and mortality (308). The exact mechanism for this is yet to be discovered, though one might speculate that the decrease in normal flora might affect the susceptibility to infections, as well as disturb the normal metabolism in the gut. There is also some evidence from a murine model that gut microbiota enhances alveolar macrophage function and has a protective role during pneumococcal pneumonia (312).

In **Paper III** we did not observe an association between CLD or history of prior TB and alpha diversity estimates, though there were some significant differences in relative abundance

of specific taxa. For example, the genus *Faecalibacterium* was enriched in HIV-infected individuals with CLD while the genus *Prevotella* was enriched in HIV-infected children without CLD. Interestingly, higher proportions of *Prevotella* have previously been shown in lung microbiota of children without asthma compared to asthmatic children and adults with COPD (313). *Faecalibacterium* has previously been regarded as a protective commensal associated with a healthy gut. However, a recent study showed increased levels of *Faecalibacterium* in the gut microbiome of patients with active TB (314). The importance of this finding is unclear, being either a fluke or a protective mechanism, and further studies are warranted if we want to say with certainty whether there is a causal relationship between the gut and the lung.

Our study is one of the largest to assess the gut microbiota of HIV-infected children and adolescents in a high HIV-burden setting. Our findings demonstrate altered gut microbiota in perinatally HIV-infected individuals, which improves with increasing time spent on ART. Further studies where the gut and lung microbiota and markers of microbial translocation and inflammation are measured are necessary in order to provide better insight into the role of gut microbial composition in HIV progression and its related complications.

2. Methodological considerations

The current work is comprised of the findings of three studies, all performed in HIV-infected individuals living in SSA. However, the populations and study designs vary between the studies. In this chapter I have tried to summarize both the positive and the negative aspects of the different study designs used, as well as certain limitations to the methods employed.

2.1. Study designs

The present work is based on observational studies. The choice of study design depends on the research question and available resources. Observational studies allow for the investigation of the "natural course" of risk factors and outcomes (diseases) without any interventions. These studies are practical, relatively inexpensive and quick, allowing researchers to study conditions with a long latent period (315). Observational studies include cohort, cross-sectional and case-control studies. In the present work, cohort and cross-sectional designs were utilized.

Cohort study design

Cohort studies follow individuals over time and allow investigation of the aetiology of the event (study outcome), associations between exposure and outcome as well as estimation of both absolute and relative risks. Absolute risks include incidence rates and cumulative risks which are estimated from the number of new cases in a study population within a given time period (316, 317).

In cohort studies exposures are identified before the outcome (VF, disease, death) happens, thus allowing for the assessment of causality. Another advantage is the opportunity to investigate multiple exposures and multiple outcomes in a single cohort. In addition, compared to RCT, cohort studies generally have broader inclusion criteria and fewer exclusion criteria and therefore may produce more generalizable results (317).

Cohort studies may be prospective or retrospective. Prospective cohort studies allow more accurate data collection but are expensive and time consuming (since long follow-up is usually required). Prospective cohort studies are not appropriate when there is a long latent period between exposure and outcome and for rare events due to the high risk of loss to follow-up. On the other hand, retrospective cohort studies are inexpensive and time-efficient as data are already available (318). Given the relatively low rate of detectable viraemia episodes observed over the follow-up period in **Paper I**, we believe that retrospective observational study design was the most appropriate option.

Cross-sectional study design

Cross-sectional design allows for the estimation of prevalence (number of cases in the population at a given point of time) and the study of multiple outcomes. In cross-sectional studies the data on disease and exposure are collected at one particular time point and therefore temporal relationship between exposure and study outcome cannot be established (317, 319). Even though no causal relationship can be stated, the investigators may formulate questions in a way that the subject is asked about his/her previous exposures (treatment for a disease, lifestyle, etc), thus making room for extrapolation of the previous exposures as a possible cause for the outcome. This allows for categorization of subjects based on previous exposures even if the information is collected at one point in time. Since multiple risk factors and outcomes can be assessed, these studies may be particularly useful for understanding disease aetiology and for hypothesis generation. The associations observed in cross-sectional studies may be investigated in more detail in cohort studies or RCT. Given the exploratory nature of studies in **Paper II** and **III**, we believe that cross-sectional design was the most appropriate.

2.2. Internal validity and technical limitations

Research studies are evaluated in terms of internal and external validity. Internal validity refers to how well the study was conducted and depends on the degree to which bias is minimized (320, 321). Bias is a systematic error which may occur at any stage of conducting a study, including study design and data collection, as well as analysis and publication (322). The presence of selection bias, information bias, or confounding may lead to inaccurate estimates of the association between exposure and outcome and thus affect internal validity of the study (321).

Selection bias

Selection bias occurs when the participants selected for a study are systematically different from eligible participants who are not selected for the study (321). Therefore, the study population does not represent the target population for which a statement (conclusion) is to be made. Selection bias may occur at the study enrolment or retention of participants in the study (for example when those who were exposed to a certain factor are less likely to be followed than those in the unexposed group). The latter is relevant for cohort studies and is usually referred to as "differential loss to follow-up".

Data for **Paper I** was collected in a not-for-profit clinic which provides care to an unselected population of more than 6500 HIV-infected individuals in Harare. The demographical profile of study participants was comparable to the country's HIV profile, suggesting that the study participants are representative of the population at risk. For Paper **II** and **III**, participants were recruited in Harare central hospital, a government funded hospital, which has a catchment area of 1.5 million residents and provides care for around 3500 HIV-infected children.

Information bias

Information bias occurs when data are measured or recorded inaccurately resulting in a misclassification of exposure and/or outcome. Two types of misclassification may occur: differential and nondifferential. Nondifferential misclassification occurs when the degree of misclassification of exposure among those with and without outcome (disease) is the same (321, 323). This will bias results towards the null hypothesis (no association) in the case of binary exposure variables. Differential misclassification occurs when the degree of misclassification is different between the compared groups and may bias estimates in any direction. The latter is

especially likely when the information on exposure is recorded after the outcome occurred. Recall bias, measurement error, and technical limitations usually contribute to information bias.

Recall bias

Differential misclassification may be introduced into the study as a result of recall bias. It occurs when the participants' ability to recall past exposures depends on the outcome status. Recall bias is usually a minor concern in cohort studies due to the temporal sequence of the exposure and outcome. However, it is a common issue in studies that use self-reported information. Self-reporting (use of questionnaires, surveys, interviews) is a common approach to collect information about participants (320, 321). It is generally acknowledged that self-reporting is a less reliable approach for gathering information about participants than medical records.

The risk of recall bias in **Paper I** is low since data were extracted from medical records. In **Paper II** and **III**, clinical data were collected via questionnaires that were administered after the participant's airway obstruction was diagnosed by spirometry. Therefore, differential misclassification of exposure could be the introduced when participants were classified into groups based on history of TB. It is possible that those who were found eligible for the trial could overreport prior TB in anamnesis while those with normal lung function could underreport prior TB. A reasonable approach to minimize the risk of recall bias could be verification of exposure information by review of hospital or pharmacy records, though this was not feasible in the given setting.

Measurement error bias

Lack of accuracy, precision and/or reliability of procedures performed refers to measurement (instrument) error and may also cause misclassification (324). A number of measurement techniques were used in the present work.

<u>HIV VL measurement</u>

In **Paper I** HIV VL was detected using an FDA-approved Roche COBAS AmpliPrep / COBAS TaqMan48 version 2.0 assay which has high sensitivity and specificity (325). In **Paper II** and **III**, point-of-care GeneXpert VL assay was used. It shows good precision on plasma samples and good correlation with reference assays (326, 327). For WHO prequalification Xpert HIV-1 VL assay had 94.1% sensitivity and 98.5% specificity for VF at 1000 copies/ml (328).

Measurement of eNO

In **Paper II** we used NIOX VERO, a portable device to measure eNO levels. It was shown to have better reproducibility and reliability compared to the chemiluminescence technique (the gold standard for eNO measurement) (329, 330). Measurements were performed according to ATS guidelines (229) and the same instrument was used in order to standardize measurement conditions.

Use of FEV1 z-scores instead of predicted values

In **Paper II and III** we used FEV1 z-scores instead of predicted values for interpreting spirometry results. The advantage of using z-scores is that it takes into account the underlying distribution of normal lung function data when classifying the estimated values into the severity categories of lung impairment. This approach eliminates biases due to age, height and sex differences in underlying lung function distribution when using FEV1 %predicted (165).

Use of rectal swabs for gut microbiota analysis

Rectal swabs were used as specimens for sequencing in **Paper III**. Swabs are relatively low biomass samples which may not be optimal with regards to profiling the gut microbiota. However, a number of studies showed good concordance between the use of rectal swabs and solid stool samples for profiling and comparisons of the gut microbiota (331, 332).

Confounding

Confounding is a situation when an apparently causal association observed between the exposure and an outcome is due to the influence of the third variable (or variables). A confounder must fulfil certain criteria: 1) it is related to both exposure and outcome; 2) it is unequally distributed between the study groups; 3) not part of the causal pathway between the exposure and the outcome (321). Confounding can be addressed by statistical approaches such as stratification or multivariable modelling (where confounders are included as covariates in multivariable regression models) (333).

In **Paper I**, age, sex and baseline CD4 count were considered as the most likely confounders. Therefore, the analyses were performed separately for children and adults and age, sex and baseline CD4 count were adjusted for. Age, sex and the time of the day when the measurement was performed may influence eNO level and were the factors we adjusted for in **Paper II.** When investigating gut biodiversity in **Paper III**, age, sex and BMI were adjusted for.

Unmeasured confounding may decrease the validity of the findings. There are a number of parameters that were not assessed in **Paper II** and **III** and which could have affected the study outcomes (eNO or gut microbiota). For example, among parameters which may influence eNO levels and which were not assessed in the **Paper II** are genetic factors (activity of NO synthase) and environmental factors (air pollution). In **Paper III** we did not assess diet and social factors such as housing or level of education of the participants, which could also have an impact on gut microbiota.

2.3. External validity

External validity refers to the generalizability of the results (whether the study conclusions will hold for other subjects, places and times). The closer the recruited subjects are to the target population the better the external validity. Adequate internal validity is a prerequisite for external validity (319, 321). Despite certain limitations mentioned above, we believe that the results of **Paper I** can be generalized to HIV-infected individuals living in HIV high-burden countries. External validity of **Paper II** and **III** findings may be limited to African population since both eNO and gut microbiota may be influenced by ethnicity (334).

Conclusions and future perspectives

More than half of HIV-infected adults and two-thirds of HIV-infected children reside in Eastern and Southern Africa. Despite limited healthcare resources, our study demonstrated that adequate VL suppression is an achievable and realistic goal even for this area. The rates of VL suppression following ART initiation observed in **Paper I** are close to the third "90" UNAIDS goal (90% of those on ART treatment have suppressed VL). At the same time, our study revealed that more children than adults fail to reach VL suppression and are more prone to experience detectable viraemia after initial VL suppression. High rates of detectable viraemia observed in Paper **II-III** also support insufficient VL suppression in paediatric HIV-infected population.

Given that detectable viraemia is the main driver of HIV progression and transmission, our results emphasize the need to prioritize routine VL monitoring in the paediatric population in areas where access to VL testing is limited. Development and implementation of specific programs oriented towards young individuals growing up with HIV is justified in order to improve virological outcomes and reduce the risk of HIV transmission. The significance of detectable viraemia episodes in RLS requires further studies.

As the result of improved survival, a growing number of HIV-infected individuals on ART develop chronic comorbidities. Recent evidence indicates that HIV-infected children and adolescents are at increased risk of lung disorders. However, to date, few studies have focused on pathological pathways that may be involved in lung impairment in this population group. **Paper II** showed that both HIV infection and prior TB are associated with lower eNO levels, an important component of lung host defence.

Whether approaches to increase NO levels may be beneficial in this group or whether measurement of eNO may be useful in management of long-term lung complications in HIV-infected individuals needs further investigation. At the same time, certain recommendations may be given already now. In light of the high burden of lung impairment in HIV-infected ART-treated children in SSA, monitoring of lung health in this group is warranted. There is a need to adapt care delivery services in a way that early detection of these chronic comorbidities becomes feasible. In addition, in light of the persistently high incidence of TB in ART-treated individuals, development of joint longitudinal HIV and TB care programs would be beneficial for management of long-term lung impairment.

The present work demonstrated that perinatally acquired HIV infection is associated with altered gut microbiota, which improves with increasing duration of ART. Further studies assessing microbial translocation markers, immune activation, and microbe-derived

metabolites are needed to find out what implication gut dysbiosis may have on HIV disease progression. Though we did not observe an association between gut biodiversity and presence of lung impairment, studies where both gut and lung microbiota are investigated simultaneously may uncover potential links between these sites. Future studies are warranted to find out whether microbiota-based interventions may be beneficial for individuals growing up with HIV.

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Patterns of detectable viraemia among children and adults with HIV infection taking antiretroviral therapy in Zimbabwe

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ABSTRACT

Objective: To investigate the incidence and predictors of viraemia among individuals on antiretroviral therapy (ART) in Harare, Zimbabwe.
Methods: Children (0–19 years) and adults (>19 years) starting ART between 2013 and 2015 were followed for a median of 2.8 and 2.7 years, respectively. The incidence rates of virological failure (VF), low-level viraemia (LLV), and viral blips were assessed and the predictors of viraemia were determined using logistic and parametric survival regression analyses.
Results: A total of 630 individuals initiated ART, and 19.7% of children and 5.6% of adults did not achieve viral suppression by 12 months. Younger age and CD4 count ≤200 cells/mm^3 at baseline were associated with not being virally suppressed at 12 months in adults. Among those who achieved viral suppression during the follow-up period, the incidence of VF was higher in children (4.0/100 person-years vs. 0.4/100 person-years in adults; $p < 0.001$), as was the incidence of LLV (1.9/100 person-years vs. 0.3/100 person-years in adults; $p = 0.03$). The incidence rate of blips was 10.9 per 100 person-years in children and 4.0 per 100 person-years in adults.
Conclusions: Children are less likely to reach viral suppression and are at higher risk of viraemia while on ART than adults. The significance of LLV and blips needs further study.

Introduction

Access to antiretroviral therapy (ART) has substantially increased survival and improved quality of life for HIV-infected individuals worldwide (Antiretroviral Therapy Cohort C, 2017; De La Mata et al., 2016). Sustained viral suppression achieved on ART reduces the risk of immunodeficiency, clinical progression, and HIV transmission (Cohen et al., 2016). According to World Health Organization (WHO) guidelines, an HIV viral load (VL) of ≥1000 copies/ml should prompt enhanced counselling and a repeat VL test within 6 weeks, and two sequential VL measurements of ≥1000 copies is considered virological failure (VF) and should lead to regimen change (World Health Organization, 2016).

A proportion of individuals on ART experience low-level viraemia (LLV) and/or transient viraemia; for example, viral blips have been reported in up to 40% of HIV-positive individuals on ART (Grennan et al., 2012; Havlir et al., 2001; Sorstedt et al., 2016). Viral blips have been shown to be associated with higher HIV pre-treatment VL and lower CD4 count at the time of ART initiation (Farmer et al., 2016; Havlir et al., 2001; Sorstedt et al., 2016). There is some evidence that they may impair CD4 cell recovery and maintain ongoing low-grade immune activation (Taiwo et al., 2013; Zoufaly et al., 2014).

Data on detectable viraemia after ART initiation are scarce and particularly the incidence and significance of transient and/or LLV in low-income settings, where – unlike high-income settings –

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regular VL monitoring is not the norm (Haas et al., 2015). The few studies that have been conducted in low-income countries have focused mainly on adults (Kanapathipillai et al., 2014). However, children are potentially at a higher risk of developing viraemia due to weight-based dosing, which may lead to variable drug levels, poor tolerability of drugs, and suboptimal adherence (Easterbrook et al., 2002; Young et al., 2015). Moreover, psychosocial factors including dependence on a caretaker and delayed disclosure of HIV status to the child may put children at higher risk of remaining viraemic after ART initiation (Lall et al., 2015). Adolescence is a specific high-risk period for poor adherence in many chronic conditions (Taddeo et al., 2008).

In this study, the incidence of and risk factors for detectable viraemia including VF, LLV, and blips were investigated in a cohort of HIV-infected children and adults initiated on ART in Harare, Zimbabwe.

Methods

A retrospective cohort study was conducted using data collected from patients who attended Newlands Clinic, Harare, Zimbabwe, a not-for-profit HIV clinic that provides care for children and adults. ART is provided free of charge according to national guidelines. Routine 6-monthly VL monitoring is performed (during the study period, this was done using the Roche COBAS AmpliPrep/COBAS TaqMan48 version 2.0 test; Roche Molecular Systems, Pleasanton, CA, USA). Adherence and psychosocial counselling is provided at each clinic visit (at least every 3 months). Adherence is assessed by pill count for each antiretroviral drug at every clinic visit. In the case of suspected VF (a VL $\geq$1000 copies/ml), the patient receives intensified adherence counselling for 6 weeks and repeated VL testing. Those with a VL $\geq$ 1000 copies/ml in two consecutive VL measurements are considered to have VF and are switched to second-line ART (protease inhibitor (PI)-based regimen and change of at least one nucleoside reverse transcriptase inhibitor (NRTI)). Those on second-line ART who have a VL $\geq$1000 copies/ml despite counselling undergo HIV drug resistance testing and are considered for third-line ART.

Individuals who were ART-naïve and initiated ART between August 2013 and August 2015 and who had at least two VL tests after ART initiation were included in the study. The following data were extracted: age, sex, date of ART initiation, ART regimen, adherence, height, weight, clinical history (WHO HIV disease stage, history of tuberculosis, opportunistic infections, chronic comorbidities), and laboratory parameters (VL, CD4 count, haemoglobin) at the time of ART initiation and during the follow-up period until September 28, 2017.

Data analysis

All patient data are stored in a secure electronic database. The data were anonymized prior to analysis. Statistical analyses were performed in Stata 14 (StataCorp LLC, College Station, TX, USA). The outcomes were the incidence of VF, LLV, and viral blips. Viral blip was defined as a VL measurement $\geq$50 copies/ml preceded and followed by a VL below the limit of detection (<50 copies/ml) (Havlir et al., 2001; Kanapathipillai et al., 2014; Martinez et al., 2005). LLV was defined as a VL $\geq$50 to <1000 copies/ml in at least two consecutive VL tests. The WHO definition for VF was used (VL $\geq$1000 copies/ml in two consecutive VL measurements) (World Health Organization, 2016).

The proportion of participants who did not achieve viral suppression by 12 months of ART (the 12-month cut-off was chosen to allow for a VL test to confirm suppression at month 12) was estimated and the factors associated with virological non-suppression were studied using logistic regression.

The incidence rates of viral blips, LLV, and VF among those who had achieved viral suppression during the follow-up period and had at least two VL tests after initial VL suppression were estimated. Nelson–Aalen cumulative hazard curves were plotted to evaluate the incidence of VF, LLV, and viral blips after VL suppression by age at ART initiation (children aged 0–19 years and adults aged >19 years).

The factors associated with the occurrence of viral blips were investigated using survival analysis. For the model, participants were included in the analysis at time 0 (time of first suppressed VL test after ART initiation) and followed until a viral blip occurred, or for those patients who remained suppressed until the last VL test available, by the end of the follow-up. Participants who reported treatment interruption in ART for more than 2 weeks were excluded from this analysis. Since the estimated cumulative hazard of blips increased exponentially with time, we fitted the parametric survival regression with Weibull distribution stratified by age group. A value of P (the shape parameter) >1 confirmed that the hazard of failure (viral blip) increased with time.

Age, sex, body mass index (BMI), stunting (in children only), pre-treatment VL, CD4 count, anaemia, WHO clinical stage at ART initiation, history of tuberculosis before ART initiation, chronic comorbidities (adults only), ART regimen, and average adherence were investigated as predictors of detectable viraemia. Stunting was defined as a height-for-age *Z*-score of <-2 (children only). Height-for-age *Z*-scores and BMI-for-age *Z*-scores in children were calculated using WHO reference standards. Anaemia was defined according to WHO criteria (haemoglobin <11 g/dl for children <5 years; haemoglobin <11.5 g/dl for children 5–11.99 years; haemoglobin <12 g/dl for children 12–14.99 years; haemoglobin <12 g/dl for females aged $\geq$15 years; haemoglobin <13 g/dl for males aged $\geq$15 years). Adherence was calculated as a percentage of the number of tablets dispensed at the last visit minus the number of tablets returned at the current visit divided by number of tablets that should have been consumed between visits. The average adherence over the study period was calculated for each participant. Age, sex, and CD4 count were adjusted for a priori. All statistical tests were two-tailed and *p*-values of <0.05 were considered statistically significant.

Ethical approval for the study was obtained from the Newlands Clinic Research Committee, the Medical Research Council of Zimbabwe, and Regional Committee for Medical and Health Research Ethics (Norway).

Results

Of the 725 participants who initiated ART during the study period, 17 were excluded as they were suppressed at baseline (and thus may have received ART previously) and 78 were excluded for having <2 VL measurements available following ART initiation (Figure 1). The baseline demographic, clinical, and laboratory characteristics of the remaining 630 participants (127 children and 503 adults) are shown in Table 1. The median follow-up time was 2.8 (interquartile range (IQR) 2.3–3.2) years for children and 2.7 (IQR 1.8–2.8) years for adults. The sex distribution was equal in the paediatric group (49.6% female vs. 50.4% male), while there were more adult female than male participants (62.2% female vs. 37.8% male), consistent with studies in Sub-Saharan Africa, which have shown higher HIV treatment coverage among women (UNAIDS, 2013). Twenty-one children, all aged below 3 years at ART initiation, were commenced on PI-based regimens, as per the national guidelines, which recommend PIs as part of the first-line regimen in children below 3 years. Ten adults initiated treatment with a PI-based regimen based on clinician judgement, with

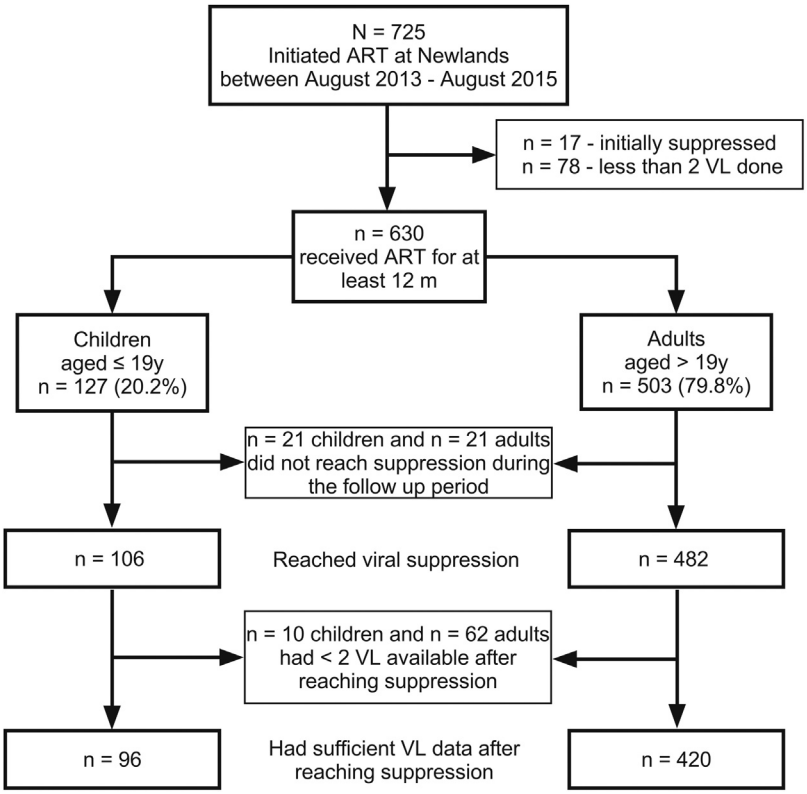

Figure 1. Flow chart of participant recruitment.

Table 1
Characteristics of study participants. Results are presented as the number (percentage), or as the median (interquartile range), unless indicated otherwise.

Characteristics	Children ($n = 127$)	Adults ($n = 503$)
Demographic		
Male	64 (50.4%)	190 (37.8%)
Age at ART initiation, years	10 (3–15)	37 (31–44)
Stunted at ART initiation (height-for-age *Z*-score <−2)	24 (18.9%)	
Underweight at ART initiation (BMI *Z*-score <−2 for children or BMI <18.5 kg/m^2 for adults)	13 (10.2%)	40 (8.0%)
Clinical		
History of tuberculosis	10 (7.9%)	59 (11.7%)
Prevalence of chronic comorbidities[a]	2 (1.6%)	90 (17.9%)
Opportunistic infections[b]	13 (10.2%)	75 (14.9%)
WHO clinical stage 3 or 4 at ART initiation[c]	31 (24.8%)	144 (28.9%)
ART regimen at treatment initiation		
2NRTI + NNRTI	106 (83.5%)	493 (98.0%)
PI-based	21 (16.5%)	10 (2.0%)
Average adherence to ART by pill-count	98.4%	99.6%
Years on ART	2.8 (2.3–3.2)	2.8 (1.8–2.8)
Laboratory		
CD4 count at ART initiation, cells/mm^3	341 (137–733)	220 (104–334)
Viral load at ART initiation, $\log_{10}$ copies/ml[d]	4.8 (4.4–5.3)	4.8 (4.3–5.2)
Anaemia at ART initiation	82 (64.6%)	248 (49.4%)

ART, antiretroviral therapy; BMI, body mass index; WHO, World Health Organization; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.
[a] Chronic comorbidities included hypertension, diabetes, or renal failure diagnosed before inclusion in the study.
[b] After ART initiation, had at least one episode of one of the following: oral candidiasis, necrotizing gingivitis, herpes zoster, histoplasmosis, cryptococcal meningitis, molluscum contagiosum, genital warts, tonsillitis.
[c] Data missing for two children and five adults.
[d] Data missing for 42 children and 159 adults.

reasons including severe anaemia, Kaposi sarcoma, WHO stage 4, third trimester of pregnancy, and breastfeeding.

Of the 630 participants, significantly more children than adults did not achieve viral suppression by 12 months post ART initiation (19.7% children vs. 5.6% adults; $p < 0.001$). Younger age and CD4 count ≤200 cells/mm^3 at baseline were associated with not achieving viral suppression in adults (Table 2). Over the follow-up period, 106 (83.5%) children and 482 (95.8%) adults reached VL suppression, with the median time to VL suppression being 0.5 (IQR 0.4–1.3) years for children and 0.5 (IQR 0.2–0.8) years for adults.

Table 2
Logistic regression analysis of the risk factors for not achieving viral suppression by 12 months of ART.

Characteristic	Children (≤19 years of age) (*n* = 127)				Adults (>19 years of age) (*n* = 503)			
	Unadjusted analysis		Adjusted analysis[a]		Unadjusted analysis		Adjusted analysis[a]	
	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value
Female	1.69 (0.69–4.11)	0.25	1.71 (0.69–4.19)	0.24	0.59 (0.27–1.26)	0.17	0.72 (0.33–1.60)	0.43
Age at ART initiation (per 1 year older)	1.05 (0.98–1.13)	0.18	1.06 (0.96–1.18)	0.23	0.97 (0.93–1.01)	0.15	0.95 (0.91–1.00)	0.04
Stunted at ART initiation (height-for-age *Z*-score <−2), yes vs. no	0.53 (0.14–1.93)	0.33	–	–	–	–	–	–
BMI *Z*-score (children) or BMI kg/m^2 (adults) at ART initiation	0.78 (0.57–1.07)	0.12	0.79 (0.56–1.13)	0.21	0.91 (0.84–0.98)	0.01	0.94 (0.87–1.03)	0.22
History of TB, yes vs. no	–	–	–	–	1.70 (0.62–4.64)	0.30	0.92 (0.31–2.74)	0.89
Chronic comorbidities[b], yes vs. no	–	–	–	–	0.53 (0.16–1.81)	0.32	0.86 (0.24–3.16)	0.83
WHO clinical stage 3 or 4 at ART initiation, yes vs. no	1.23 (0.46–3.30)	0.68	1.44 (0.51–4.06)	0.49	2.24 (1.04–4.84)	0.04	1.18 (0.50–2.83)	0.70
ART regimen at treatment initiation (Ref. 2NRTI + NNRTI)[c]	0.64 (0.17–2.36)	0.50	1.06 (0.17–6.72)	0.95	–	–	–	–
Average adherence to ART by pill-count, %	0.88 (0.72–1.07)	0.21	0.85 (0.69–1.05)	0.14	0.97 (0.89–1.06)	0.54	1.00 (0.91–1.09)	0.92
CD4 count at ART initiation, cells/mm^3								
≤200 cells/mm^3	2.55 (1.02–6.36)	0.04	2.21 (0.78–6.29)	0.14	5.66 (2.11–15.1)	0.001	5.92 (2.18–16.1)	<0.001
>200 cells/mm^3	Ref.		Ref.		Ref.		Ref.	
Viral load at ART initiation, $\log_{10}$ copies/ml	1.15 (0.63–2.11)	0.65	1.48 (0.73–3.00)	0.27	1.78 (1.00–3.18)	0.05	1.26 (0.68–2.33)	0.46
Anaemia at ART initiation, yes vs. no	1.53 (0.58–4.00)	0.38	1.46 (0.55–3.88)	0.45	2.26 (1.00–5.10)	0.05	1.73 (0.72–4.19)	0.22

ART, antiretroviral therapy; OR, odds ratio; CI, confidence interval; BMI, body mass index; TB, tuberculosis; WHO, World Health Organization; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

[a] Adjusted for CD4 count at ART initiation, age, and sex.

[b] Chronic comorbidities included hypertension, diabetes, or renal failure diagnosed before inclusion in the study.

[c] PI-based regimen as the exposure.

Of the 588 participants who achieved viral suppression after ART initiation, 516 had sufficient VL data to study the incidence of detectable viraemia. Over the follow-up period, 57 (11%) participants experienced a viral blip. Blips occurred more commonly in children than in adults (22.9% vs. 8.3%; $p < 0.001$), with the incidence rate being 10.9 (95% confidence interval (CI) 7.2–16.6) per 100 person-years in children and 4.0 (95% CI 2.8–5.5) per 100 person-years in adults (Figure 2A). Fifty percent of blips in children and 71.4% of blips in adults were of low magnitude (50–199 copies/ml). The median time from viral suppression to a blip was 1.9 (IQR 1.4–2.3) years in children and 1.8 (IQR 1.0–2.1) years in adults.

Seven (1.4%) participants (four children and three adults) experienced LLV and 12 (2.3%) participants (eight children and four adults) developed VF after initial suppression during follow-up. The incidence of LLV was 1.9 (95% CI 0.7–5.1) per 100 person-years in children and 0.3 (95% CI 0.1–1.0) per 100 person-years in adults

Figure 2. Nelson–Aalen cumulative hazard curves for the development of (A) viral blips, (B) low-level viraemia (LLV), and (C) virological failure (VF) after viral load suppression. Curves for children are presented in red and curves for adults in blue.

(Figure 2B). VF was more common in children compared to adults (8.3% vs. 0.9%; $p < 0.001$). The incidence of VF was 4.0 (95% CI 2.0–7.9) per 100 person-years in children and 0.4 (95% CI 0.2–1.2) per 100 person-years in adults (Figure 2C). Notably, 2.2% of children <10 years and 13.7% of those ≥10 years developed VF ($p = 0.05$). All four adults with VF had re-suppression (three following a switch from a non-nucleoside reverse transcriptase inhibitor (NNRTI)-based to a PI-based regimen, one remaining on the same NNRTI-based regimen), while only three children had re-suppression (two following a switch from an NNRTI-based to a PI-based regimen, one remaining on the same NNRTI-based regimen) during the follow-up period.

Four out of 22 children (18.2%) experienced both viral blips and VF during the follow-up. Blips were followed by VF in two participants, while blips occurred after VF and re-suppression in the other two cases. In those participants with blips followed by VF, the magnitude of blips was low, while in those who had blips after VF, blips were of medium (200–499 copies/ml) and high (>500 copies/ml) magnitude. Among two children with blips after VF, one had re-suppression on the same ART regimen with adherence counselling, while another had re-suppression after a switch to a PI-based regimen. Two children with blips followed by VF remained unsuppressed by the end of follow-up.

Due to low rates of LLV in the study participants after initial VL suppression, risk factors for only viral blips were investigated in the survival analysis. The survival analysis included 507 participants (94 in the paediatric group and 413 in the adult group). No baseline characteristics were found to be associated with an increased risk of viral blips in children or adults (Table 3).

Discussion

Among individuals starting ART, a significantly higher proportion of children compared to adults did not achieve viral suppression by 12 months. Likely reasons include poor tolerability to antiretroviral drugs, inadequate ART dosing, or suboptimal adherence (Boerma et al., 2016). This is in line with the findings of Jobanputra et al., who showed that being a child or adolescent is associated with detectable viraemia (Jobanputra et al., 2015). Some individuals may require longer periods to achieve suppression, as shown in the present study, where over the follow-up period, 83.5% of children and 95.8% of adults did achieve viral suppression. One possibility is that perinatally HIV-infected young children tend to have sustained high VL in the first years of life with slow reduction of peak VL with increasing age (McIntosh et al., 1996). This potentially could contribute to incomplete initial VL suppression and a higher risk of viraemia. Yet the present study results do not support this, as the exclusion of children aged ≤3 years did not change the rates of viral non-suppression in the paediatric study group. Lower CD4 count at baseline was associated with not being suppressed at 12 months in adults, a finding consistent with other studies (Anude et al., 2013; Collaboration of Observational et al., 2008; Mujugira et al., 2016; Samuel et al., 2014).

This study found a significantly higher incidence of viral blips among children compared to adults. The reported incidence of blips varies in different populations (Farmer et al., 2016; Grennan et al., 2012; Havlir et al., 2001; Kanapathipillai et al., 2014; Sorstedt et al., 2016), largely due to different definitions and variability of the assays for VL. We used a common definition of viral blip, as a single VL measurement ≥50 copies/ml preceded and followed by VL <50 copies/ml (Fung et al., 2012; Ryscavage et al., 2014). Compared to studies that have used a similar definition, it was found that a lower proportion of adults in the present study experienced blips: 8.3% during 2.1 years of follow-up compared to 18.6–40% followed up from 1.08 to 1.58 years (Havlir et al., 2001;

Table 3
Risk factors for the development of viral blips by age.

Characteristic	Children (≤19 years)				Adults (>19 years)			
	Unadjusted analysis		Adjusted analysis[a]		Unadjusted analysis		Adjusted analysis[a]	
	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
Female	1.41 (0.60–3.32)	0.43	1.08 (0.42–2.76)	0.87	0.79 (0.39–1.58)	0.50	0.76 (0.37–1.56)	0.45
Age at ART initiation, years	0.99 (0.91–1.07)	0.74	1.06 (0.92–1.22)	0.43	1.00 (0.96–1.04)	0.87	1.00 (0.96–1.04)	0.91
Stunted at ART initiation (height-for-age *Z*-score <−2)	0.46 (0.13–1.67)	0.24	0.39 (0.08–2.01)	0.26	–	–	–	–
BMI *Z*-score (children) or BMI kg/m^2 (adults) at ART initiation	1.12 (0.84–1.49)	0.44	0.87 (0.55–1.37)	0.54	0.97 (0.92–1.03)	0.31	0.97 (0.91–1.03)	0.33
History of TB	2.17 (0.60–7.94)	0.24	2.99 (0.87–10.3)	0.08	0.87 (0.27–2.81)	0.82	0.85 (0.24–3.00)	0.80
Chronic comorbidities[b]	–	–	–	–	1.17 (0.50–2.76)	0.71	1.19 (0.50–2.86)	0.69
WHO clinical stage 3 or 4 at ART initiation	0.62 (0.19–1.98)	0.42	0.75 (0.21–2.74)	0.66	0.78 (0.33–1.83)	0.57	0.78 (0.31–1.94)	0.60
ART regimen at treatment initiation (Ref. 2NRTI + NNRTI)[c]	1.92 (0.72–5.11)	0.19	1.65 (0.44–6.26)	0.46	0.94 (0.13–6.97)	0.95	0.99 (0.13–7.42)	0.99
Average adherence to ART by pill-count, %	0.91 (0.67–1.24)	0.56	0.90 (0.66–1.22)	0.50	1.16 (0.86–1.56)	0.34	1.17 (0.86–1.58)	0.31
Months to viral load suppression	1.06 (0.99–1.14)	0.09	1.06 (0.98–1.16)	0.14	1.03 (0.96–1.12)	0.38	1.03 (0.96–1.11)	0.35
CD4 count at ART initiation, $\log_{10}$ cells/mm^3	2.21 (0.84–5.82)	0.11	3.83 (0.53–27.5)	0.18	1.19 (0.57–2.50)	0.64	1.29 (0.61–2.73)	0.50
Viral load at ART initiation, $\log_{10}$ copies/ml	1.01 (0.43–2.34)	0.99	1.06 (0.41–2.73)	0.91	1.58 (0.95–2.62)	0.08	1.56 (0.98–2.50)	0.06
Anaemia at ART initiation	2.33 (0.78–6.97)	0.13	1.99 (0.65–6.10)	0.23	0.91 (0.46–1.81)	0.79	0.97 (0.44–2.13)	0.94

HR, hazard ratio; CI, confidence interval; ART, antiretroviral therapy; BMI, body mass index; TB, tuberculosis; WHO, World Health Organization; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

[a] Adjusted for CD4 count at ART initiation, age, and sex.

[b] Chronic comorbidities included hypertension, diabetes, or renal failure diagnosed before inclusion in the study.

[c] PI-based regimen as the exposure.

Martinez et al., 2005; Sklar et al., 2002). Several studies have shown that patients on PI regimens are more prone to developing viral blips (Grennan et al., 2012; Sorstedt et al., 2016). Havlir et al. (2001) reported a high incidence of blips (40%) in a cohort of patients who received an unboosted PI-based treatment regimen in a high-income country. In addition, the median number of VL measurements was 17 per participant, compared to an average of six measurements in the present study in which only 2.1% of adults were receiving a ritonavir-boosted PI-based regimen.

It is likely that more frequent VL testing will increase the probability of detecting blips, which may explain the discrepancies between our findings and those of studies conducted in high-income settings. However, in a study comparing the incidence of blips in high-, middle-, and low-income countries, 21% of the individuals in the first category experienced blips, while only 11% of participants did so in the middle/low income group, despite a similar ratio of blips to the number of VL measurements across the settings (Kanapathipillai et al., 2014). HIV plasma RNA measurements are subject to pre-analytic and laboratory errors, variations in cut-off point, and variations in lower limit of detection among the different VL assays, which may have an impact on the incidence of blips (Grennan et al., 2012; Young et al., 2015). Even though good agreement between the different assays is observed at high VL levels, there is substantial variability at low VL levels (Ruelle et al., 2012; Swenson et al., 2014a).

There appear to be limited data on the incidence of viral blips in children in the low-income setting (Jobanputra et al., 2015; Szubert et al., 2017). In a retrospective analysis of VL tests among children who initiated ART in Uganda and Zimbabwe, 46% of children experienced viral blips (Szubert et al., 2017). This is considerably higher than the results of the present study, but may be explained by the use of WHO 2006 criteria for ART initiation, which relied on clinical and/or immunological assessment and not just the diagnosis of HIV infection. The majority of studies in low-income countries have investigated the incidence of any detectable viraemia in one or two VL measurements. For example, a cross-sectional study among adolescents with HIV conducted in an urban setting in Cameroon utilized two consecutive VL measurements to detect sustained VL suppression (VL < 50 copies/ml in two VL tests) (Fokam et al., 2017). In that study, 18.6% of participants had a VL measurement above 50 copies/ml in a single test. Another study conducted in Ethiopia found detectable viraemia (defined as an HIV-1 RNA of 41–1000 copies/ml) in a single VL measurement in 13% of HIV-positive children who had received first-line ART for a median of 24 months (Mulu et al., 2014). In a study by Jobanputra et al., 38% of children aged <10 years and 34% of children aged 10–19 years had single detectable VL (>100 copies/ml) followed by VL re-suppression (Jobanputra et al., 2015). Although the results from these studies are not directly comparable to the present study findings, they show that detectable viraemia is common in paediatric patients. A multicentre cohort study of data on children with HIV in the UK and Ireland showed that 22% of participants with sustained viral suppression experienced transient viraemia (defined as a single VL >50 copies/ml between two VL tests below 50 copies/ml), with an incidence of 12 per 100 person-years, similar to the findings in our study (Lee et al., 2007).

The incidence of LLV and VF after reaching VL suppression was also significantly higher in children than in adults. Furthermore, in the present study, adolescents (those aged ≥10 years) were more likely to experience VF compared to younger children, a finding that has also been reported by other studies conducted in Africa (Makadzange et al., 2015). Adolescence is a period of particularly high risk for poor adherence (Mukui et al., 2016; Nachega et al., 2009). Taken together, these emphasize the need for investigating and addressing factors that impede viral suppression among adolescents.

The rate of VF in children who reached VL suppression is lower than those reported by other studies (Makadzange et al., 2015; Salou et al., 2016). A possible reason for the higher VF rates reported in other studies is the cross-sectional design of studies and lack of information regarding initial VL suppression prior to inclusion in the study (Fokam et al., 2017; Salou et al., 2016). Due to regular VL monitoring and the longitudinal design of our study, it was possible to distinguish between those who developed VF and those who never reached suppression. Another explanation for low VF in our cohort is the high standard of care provided at Newlands Clinic, with routine VL testing, continuous adherence monitoring support, and a rapid response with intensified adherence counselling and/or ART switch among those who have a non-suppressed VL (Haas et al., 2015). Routine VL testing, which is not available as standard of care in many low-income settings, can detect VF earlier than targeted VL monitoring, i.e., VL testing prompted by clinical or immunological deterioration.

A number of studies have shown that episodes of LLV and viral blips are associated with an increased risk of subsequent VF in adults (Antiretroviral Therapy Cohort C et al., 2015; Grennan et al., 2012; Laprise et al., 2013; Leierer et al., 2016). While some studies have suggested that episodes of transient viraemia may result in the selection of drug-resistant HIV strains, leading to an increased risk of VF (Clutter et al., 2016; Gonzalez-Serna et al., 2014; Swenson et al., 2014b), others have found no link between blips and HIV progression (Havlir et al., 2001; Kanapathipillai et al., 2014; Nettles et al., 2005). Young et al. found a gradual increase in the risk of VF with increasing blip magnitude (Young et al., 2015). In another study, only blips of 500–999 copies/ml magnitude were associated with the increased risk of viral rebound (Grennan et al., 2012). In contrast, other studies have shown no evidence of an association between blips and VF in adults (Kanapathipillai et al., 2014; Martinez et al., 2005; Sklar et al., 2002). However, these studies have been conducted largely in adults. In the present study, not only did children have a higher risk of VF, but they also had a higher incidence of viral blips as well as LLV, and the significance of these in predicting VF needs further study. Furthermore, careful virological monitoring is warranted in children.

The strengths of this study are the use of a standardized definition for viral blips, regular VL monitoring, availability of detailed clinical data including adherence data, and good follow-up rates. The limitations of the study include the relatively small sample size and low number of blips in each age group, and there may therefore be inadequate power to detect an association between the covariates and the incidence of viral blips. Furthermore, the follow-up period was not long enough to investigate whether blips or LLV increase the risk of subsequent VF. Adherence may have been over-estimated, as the pill count is not the optimal measure of adherence.

In conclusion, this study demonstrated that detectable viraemia is common among children, although its role with regard to long-term outcomes necessitates further study in low-income settings, especially where HIV prevalence is high and the availability of VL monitoring remains limited.

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Ethical approval

Ethical approval was given by Newlands Clinic Research Committee, the Medical Research Council of Zimbabwe (MRCZ/E/188), and the Regional Committee for Medical and Health Research Ethics, Norway (REK 2015/1650).

Conflict of interest

None declared.

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PAPER II

OPEN

History of tuberculosis is associated with lower exhaled nitric oxide levels in HIV-infected children

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Objective: HIV disrupts host defense mechanisms and maintains chronic inflammation in the lung. Nitric oxide is a marker of lung inflammation and can be measured in the exhaled air. We investigated the relationship between exhaled nitric oxide (eNO), HIV status and airway abnormalities in perinatally HIV-infected children aged 6–19 years.

Design: A cross-sectional study.

Methods: HIV-infected individuals on antiretroviral therapy and HIV-uninfected children with no active tuberculosis (TB) or acute respiratory tract infection were recruited from a public hospital in Harare, Zimbabwe. Clinical history was collected and eNO testing and spirometry was performed. The association between eNO and explanatory variables (HIV, FEV1 *z*-score, $CD4^+$ cell count, viral load, history of TB) was investigated using linear regression analysis adjusted for age, sex and time of eNO testing.

Results: In total, 222 HIV-infected and 97 HIV-uninfected participants were included. Among HIV-infected participants, 57 (25.7%) had a history of past TB; 56 (25.2%) had airway obstruction, but no prior TB. HIV status was associated with lower eNO level [mean ratio 0.79 (95% confidence interval, 95% CI 0.65–0.97), $P = 0.03$]. Within the HIV-infected group, history of past TB was associated with lower eNO levels after controlling for age, sex and time of eNO testing [0.79 (95% CI 0.67–0.94), $P = 0.007$].

Conclusion: HIV infection and history of TB were associated with lower eNO levels. eNO levels may be a marker of HIV and TB-induced alteration in pulmonary physiology; further studies focused on potential causes for lower eNO levels in HIV and TB are warranted.

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Keywords: airway obstruction, exhaled nitric oxide, HIV infection, sub-Saharan Africa, tuberculosis

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Background

Access to antiretroviral therapy (ART) has substantially increased survival of children with HIV, with increasing numbers now reaching adolescence and adulthood. However, it is becoming increasingly apparent that HIV infection is associated with chronic comorbidities in the ART era [1,2].

In recent years, several studies from sub-Saharan Africa have shown that chronic respiratory symptoms and airway obstruction are common among children taking ART [3–6], with obliterative bronchiolitis and bronchiectasis being common noncommunicable causes of chronic respiratory symptoms [7–12]. Furthermore, ART is not fully protective against tuberculosis (TB) and individuals with HIV on ART have an estimated 10% four-year risk of developing TB compared with a 10% lifetime risk in the general population of South Africa [13,14]. Moreover, children with HIV experience more rapid TB progression, poorer response to treatment and are at a higher risk of TB recurrence [15,16]. The long-term consequences of TB often persist despite successful treatment, adding to the burden of chronic lung disease [17].

Even though the pathogenesis of chronic lung disease in HIV-infected individuals is not completely understood, chronic airway inflammation and oxidative stress are thought to play a distinct role [12,18]. One of the markers of airway inflammation is nitric oxide that is produced by various cells in the body, including the respiratory tract. Under normal physiological conditions, there is constant production of NO in the lung that regulates bronchodilation, neurotransmission, mucous secretion and the inflammatory response [19,20]. The presence of NO can be easily measured in exhaled air using a noninvasive, quantitative, standardized method [21]. Increased levels of exhaled nitric oxide (eNO) are a well known marker of eosinophilic airway inflammation in asthma, while reduced eNO levels have been reported in patients with active lung TB, cystic fibrosis and in smokers [22–24]. Increased eNO levels are explained by overexpression of the inducible NO synthase (iNOS), which often correlates with systemic and lung inflammation, while decreased eNO levels may be due to lack of the substrate (L-arginine); reduced activity of iNOS in the airways (which may be directly inhibited as an immune evasion mechanism by *Mycobacterium tuberculosis*) or thick mucus that may inhibit the NO diffusion into exhaled air (e.g. in cystic fibrosis) [25–27].

Current data suggest that eNO may be a useful tool to guide asthma management in both children and adults [28,29], though its clinical application for other lung disorders is unclear. A recent study showed that increased eNO levels may predict clinical response to inhaled corticosteroids in adults with nonspecific respiratory symptoms [30].

Data investigating the association between HIV infection and eNO levels are scarce [31]. Although lower levels of eNO were found in one study among 36 HIV-infected patients [31] compared with HIV-uninfected healthcare workers, no significant difference in eNO levels were reported in patients with HIV-TB coinfection compared with HIV-uninfected patients with TB [22,32]. No studies so far have investigated the level of eNO in children with HIV-associated chronic lung disease.

In this study, we aimed to compare the levels of eNO in children with and without HIV and investigated the association between eNO and history of TB and airway obstruction in HIV-infected children on ART.

Materials and methods

Study population

This cross-sectional study was conducted between April 2017 and August 2018 as a substudy of a randomized controlled clinical trial investigating the effect of azithromycin in children with HIV-associated chronic lung disease (BREATHE trial, clinicaltrials.gov identifier NCT02426112). The detailed study protocol has been published elsewhere [33]. Participants were eligible for the trial if they were aged 6–19 years, had perinatally acquired HIV and had been taking ART for at least 6 months, and had no evidence of active TB or acute respiratory tract infection. The trial is being conducted in Malawi and Zimbabwe, but the substudy was conducted in Zimbabwe only at the Harare Central hospital paediatric HIV clinic, the largest public sector HIV clinic in Harare.

TB may cause lung tissue changes (bronchovascular distortion, fibrotic lesions and cavitation) persisting beyond the acute episode [17]; these morphological changes may influence eNO levels. We expected to find a high prevalence of past TB because of the high incidence of TB in people living with HIV. Thus, we divided HIV-infected participants into three groups on the basis of clinical history and spirometry results: normal lung function and no TB history; history of TB irrespective of airway obstruction; and airway obstruction and no TB history. Airway obstruction was defined as forced expiratory volume in 1 s (FEV_1) *z*-score less than -1.64 with no reversibility (<12% improvement in FEV1 after salbutamol 200 μg inhaled using a spacer).

In addition, a group of HIV-uninfected participants was recruited from the same catchment area as the HIV-infected group. HIV-uninfected children (were tested for HIV at enrolment) aged 6–16 years with no prior history of heart/lung diseases (including history of TB), no reported chest pain after exercise, shortness of breath

during exercise or chronic cough and normal lung function were eligible for the study as a control group.

Ethical approval

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; Regional Committee for Medical and Health Research Ethics in Norway (2015/1650). Written informed consent by guardian and assent by participants aged less than 18 years were obtained. Those aged 18 years and older gave independent consent.

Study procedures

A detailed questionnaire regarding demographic and clinical history was administered to all participants. Participants were explicitly asked about current and previous respiratory symptoms (chronic cough, wheezing and dyspnoea) in the past 3 months. Self-reported and/or physician-diagnosed lung diseases and/or atopic disorders including rhinitis and atopic dermatitis were recorded. Participants were classified as having asthma based on clinical history of diagnosed asthma or self-reported symptoms of asthma (episodes of wheezing, nocturnal tightness in the chest, attacks of shortness of breath following strenuous activity, at rest or at night-time) or treatment with asthma-specific medications (short-term/long-term β2 agonists; inhaled corticosteroids; leukotriene receptor antagonists; methylxanthines).

Measurement of exhaled nitric oxide

The level of eNO was measured by the electrochemical analyser (NIOX VERO, Circassia, UK) according to American Thoracic Society (ATS) guidelines [21] and expressed in parts per billion (ppb). The calibration of the machine and quality control were performed according to the manufacturer's instructions. The participants were asked to sit and rest for a minimum of 5 min before the measurement. Repeated exhalations were performed in order to obtain at least two measurements that agreed within 10%. Measurements were taken with a minimum of 30 s rest time in between. Up to six eNO measurement attempts were made and the mean eNO value was calculated from two eNO measurements with minimal difference between them. All eNO measurements were performed between 0800 and 1400 h and exact time of the testing was recorded.

Spirometry

Spirometry was performed after eNO measurement using EasyOne spirometer (ndd Medical Technologies Inc., Andover, Massachusetts, USA) according to the ATS guidelines. FEV1 *z*-scores were calculated using prediction equations from the Global Lung Function Initiative [34].

Laboratory tests

All participants provided blood samples for full blood count, and for participants with HIV, also for HIV viral load and $CD4^+$ cell count testing. HIV viral load was measured using the Gene Xpert assay, with a limit of detection of 40 copies/ml (XpertTM HIV-1 Viral Load; Cepheid, Sunnyvale, California, USA) and $CD4^+$ cell count was measured as a point of care test using a PimaTM Analyser (Alere, Orlando, Florida, USA). Anaemia was defined according to WHO criteria (haemoglobin <11.5 g/dl for children 6–11.9 years; haemoglobin <12 g/dl for children 12–14.9 years; haemoglobin <12 g/dl for girls aged ≥ 15 years; haemoglobin <13 g/dl for boys aged ≥ 15 years) [35].

Data collection

Electronic record forms (for questionnaires) collected on Google Nexus tablets (Google, Mountain View, California, USA) with OpenDataKit software and paper forms (for clinical tests) were used for data collection. Data from 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).

Statistical analysis

All statistical analyses were performed using STATA Version 14 (StataCorp LLC, College Station, Texas, USA). Values for eNO were presented as geometric mean with 95% confidence interval. Weight-for age and height-for-age *z*-scores were calculated using British 1990 Growth Reference Curves [36], with *z*-scores less than -2 representing wasting and stunting, respectively.

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). The values of eNO were not normally distributed and therefore were log transformed to approximate normality. The analyses were performed with log-transformed eNO data and back transformed to present geometric mean. The association between eNO levels and *a priori* defined explanatory variables was studied using linear regression analysis. Explanatory variables included HIV status, age, sex, anthropometric parameters, haemoglobin level, haematocrit, white blood cell count, neutrophil count, eosinophil count, FEV1 *z* score, atopic status, passive smoking, for all participants; $CD4^+$ cell count, viral load, history of TB and presence of airway obstruction, for participants with HIV only. Age, sex and exact time of eNO testing were adjusted for *a priori*. Parameters were included into multivariable linear regression model if they showed a significant effect on the prediction of eNO level in age, sex and time of eNO testing adjusted models at P value less than 0.05. Adjustment for unbalanced parameters (for HIV-infected group) was also performed. Variance inflation factor was used to detect multicollinearity in the multivariable models. Height was not included into multivariable regression models due to high collinearity with age. The linear association between eNO and continuous variables

was estimated graphically. Residual analysis with residual plots and normal probability plots of residuals confirmed no violation of the linear regression assumptions.

Results

In total, 227 HIV-infected and 104 HIV-uninfected children were enrolled. Five participants ($n = 1$ HIV-infected, $n = 4$ HIV-uninfected) aged 6–7 years did not understand the procedure and were excluded from the study. Of the remaining participants, acceptable eNO measurements were obtained from 225 HIV-infected and 98 HIV-uninfected children. A total of four participants were excluded from the study for the following reasons: abnormal spirometry ($n = 1$, HIV-uninfected) and missing spirometry data ($n = 3$, HIV-infected).

Baseline characteristics of study participants and their distribution across the four groups are presented in Table 1. The median time on ART among HIV-infected children was 6.6 years (IQR 4.0–8.4) and comparable across the three different groups. HIV-infected children overall were more likely to be wasted and stunted compared with HIV-uninfected children (wasted: 41.9 vs. 3.1%, $P < 0.001$; stunted: 42.8 vs. 4.1%, $P < 0.001$). Anaemia was more common in HIV-infected than HIV-uninfected children (31.2 vs. 9.3%, $P < 0.001$). Exposure to passive smoking was more common in HIV-infected than HIV-uninfected participants.

Among HIV-infected participants, those with a history of prior TB had significantly higher prevalence of wasting than participants with no airway abnormalities (52.6 vs. 28.4%, $P = 0.001$, respectively). In addition, participants with a history of prior TB received a protease inhibitor based regimen more often than other two groups. The prevalence of both stunting and wasting was higher in participants with airway obstruction when compared with those with no airway abnormalities (stunted: 58.9 vs. 33.0%, $P < 0.001$; wasted: 57.1 vs. 28.4%, $P < 0.001$).

HIV status was associated with lower eNO levels after adjusting for age, sex and time of eNO testing [geometric mean ratio 0.79 (95% CI 0.65–0.97), $P = 0.03$]. Among HIV-infected participants, those with a history of TB had significantly lower eNO levels than those without TB whether they had airway obstruction [geometric mean 13.7 (95% CI 12.2–15.5) vs. 16.9 (95% CI 14.3–19.8), $P = 0.03$] or had no airway abnormalities [geometric mean 17.9 (95% CI 16.0–20.0), $P = 0.003$] (Fig. 1). Linear regression analysis controlled for age, sex and time of eNO testing confirmed the association between past TB and lower eNO levels (Table 2).

The association between participant characteristics and eNO levels in HIV-infected and HIV-uninfected participants are presented in Tables 2 and 3, respectively. The level of eNO increased with increasing age and height in HIV-infected children. Higher haemoglobin level and neutrophil count were associated with higher eNO level in adjusted analysis. Female sex was associated

Table 1. Characteristics of study participants.

Variables	HIV-infected			HIV-uninfected (*N* = 97)
	History of TB (*N* = 57)	Airway obstruction, but no history of TB (*N* = 56)	No TB, no obstruction (*N* = 109)	
Age (years), Median (IQR)	15 (12–18)	15 (13–18)	16 (12–18)	10 (7–12)
Female sex, *N* (%)	26 (45.6)	18 (32.1)	71 (65.1)	47 (48.4)
Wasting (weight for age *z*-score < −2), *N* (%)	30 (52.6)	32 (57.1)	31 (28.4)	3 (3.1)
Stunted (height-for-age *z*-score < −2), *N* (%)	26 (45.6)	33 (58.9)	36 (33.0)	4 (4.1)
Passive smoking, *N* (%)	21 (36.8)	15 (26.8)	25 (22.9)	3 (3.1)
Living in high-density area, *N* (%)	53 (94.6)	49 (96.1)	100 (93.5)	95 (97.9)
eNO level (ppb), geometric mean (95% CI)	13.7 (12.2–15.5)	16.9 (14.3–19.8)	17.9 (16.0–20.0)	16.5 (14.8–18.5)
FEV1 *z*-score, Median (IQR)	−1.8 (−2.3 to −1.3)	−2.1 (−2.7 to −1.9)	−1.1 (−1.3 to 0.4)	−0.2 (−0.6 to 0.3)
Presence of atopy (asthma, eczema, allergic rhinitis), *N* (%)	12 (21.0)	12 (21.4)	13 (11.9)	10 (10.3)
Haemoglobin (g/dl), Median (IQR)	13 (11.9–13.7)	12.7 (11.6–13.6)	12.8 (11.7–14)	13.2 (12.4–13.9)
Haematocrit, %, Median (IQR)	36.7 (34.0–38.4)	35.2 (33.3–37.9)	36 (33.4–38.6)	36.3 (34.8–39)
White blood cell count ($*10^9$/l), Median (IQR)	4.3 (3.8–5.4)	4.6 (3.3–5.3)	4.2 (3.6–5)	5.3 (4.5–6.3)
Eosinophil count ($*10^9$/l), Median (IQR)	0.075 (0.04–0.12)	0.065 (0.02–0.13)	0.06 (0.03–0.16)	0.1 (0.06–0.2)
Neutrophil count ($*10^9$/l), Median (IQR)	1.7 (1.3–2.2)	1.7 (1.1–2.5)	1.8 (1.4–2.4)	2.2 (1.8–2.9)
Lymphocyte count ($*10^9$/l), Median (IQR)	2.0 (1.6–2.6)	2.1 (1.6–2.7)	2.0 (1.6–2.3)	2.3 (2.0–2.8)
Monocyte count ($*10^9$/l), Median (IQR)	0.39 (0.32–0.48)	0.39 (0.30–0.5)	0.35 (0.27–0.46)	0.47 (0.36–0.63)
Anaemia, *N* (%)	15 (26.8)	17 (32.7)	35 (32.7)	9 (9.3)
Viral load ($\log_{10}$ copies/ml), Median (IQR)	2.7 (1.6–4.0)	2.5 (1.6–3.7)	2.1 (1.6–3.4)	–
$CD4^+$ cell count (cells/μl), Median (IQR)	509 (339–702)	559 (326–728)	624 (355–779)	–
Years on ART, Median (IQR)	6.8 (4.6–9.1)	5.9 (3.6–7.8)	6.5 (4.0–8.4)	–
PI-based regimen, *N* (%)	25 (43.9)	12 (21.4)	12 (11.0)	–

ART, antiretroviral therapy; IQR, interquartile range; eNO, exhaled nitric oxide; FEV1, forced expiratory volume in 1 s; PI, protease inhibitor.

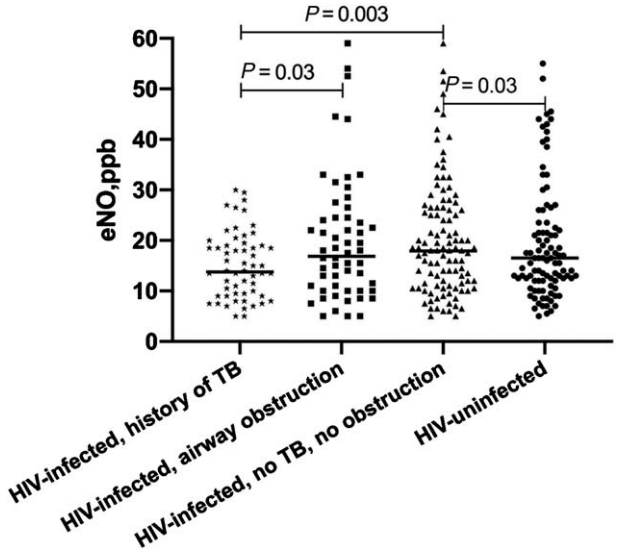

Fig. 1. Level of exhaled nitric oxide in HIV-infected and HIV-uninfected participants. Horizontal lines indicate geometric mean values.

with lower eNO level in adjusted analysis. Due to imbalances in wasting, stunting and use of protease inhibitor based regimen by HIV-infected groups, these variables were adjusted for in multivariable analysis. On multivariable linear regression, haemoglobin, neutrophil count, history of TB and age remained significantly associated with eNO levels in HIV-infected participants.

In HIV-uninfected individuals, anthropometric parameters (height and weight) were significantly positively associated with eNO level in univariate analysis, though the association became nonsignificant after accounting for age, sex and time of eNO testing. Higher haemoglobin level and haematocrit were associated with higher eNO level, while wasting was associated with lower eNO level in univariate and adjusted analysis. On multivariable analysis, wasting, haemoglobin and age remained significantly associated with eNO levels in HIV-uninfected participants.

Discussion

Our study showed that children with HIV infection have lower eNO levels than HIV-uninfected children. HIV-infected children with a prior history of TB had significantly lower eNO levels than those without prior TB.

There are few data on the levels of eNO in HIV-infected adults [31], and none among children. Two studies reported lower eNO levels associated with HIV infection among adults with [32] and without TB [31], mirroring the results of our study. However, a study including 19 HIV-infected and 126 HIV-uninfected adults with TB did not find any difference in eNO, but was likely underpowered [22]. The observed low eNO levels in

Table 2. Analysis of factors associated with exhaled nitric oxide level in HIV-infected participants.

	Unadjusted analysis		Adjusted for age, sex and time of eNO testing		Multivariable model	
Variables	GMR[a] (95% CI)	*P*	GMR[a] (95% CI)	*P*	GMR[a] (95% CI)	*P*
Age (years)	1.03 (1.00–1.05)	0.004	1.03 (1.01–1.05)	0.003	1.03 (1.01–1.05)	0.005
Female sex	0.89 (0.77–1.03)	0.13	0.85 (0.74–0.99)	0.04	0.86 (0.74–1.01)	0.07
Weight (kg)	1.01 (1.00–1.01)	0.002	1.01 (1.00–1.02)	0.16		
Height (cm)	1.01 (1.00–1.02)	<0.001	1.02 (1.01–1.03)	<0.001		
Weight-for-age *z*-score	1.01 (0.96–1–06)	0.72	1.00 (0.94–1.05)	0.94		
Height-for-age *z*-score	1.06 (1.00–1.13)	0.07	1.05 (0.99–1.13)	0.12		
Wasting (weight for age *z*-score < −2)	0.97 (0.83–1.13)	0.68	0.99 (0.85–1.16)	0.95	1.01 (0.84–1.22)	0.89
Stunted (height-for-age *z*-score < −2)	0.93 (0.80–1.08)	0.33	0.94 (0.81–1.09)	0.41	0.94 (0.80–1.12)	0.51
Passive smoking (ref. no)	1.19 (1.00–1.40)	0.04	1.17 (0.99–1.38)	0.06		
History of tuberculosis	0.78 (0.67–0.90)	0.004	0.79 (0.67–0.94)	0.007	0.80 (0.68–0.95)	0.013
FEV1 *z*-score	1.01 (0.95–1.07)	0.73	0.98 (0.92–1.05)	0.59		
FEV1 *z*-score < −1.64 (airway obstruction)	0.92 (0.79–1.08)	0.33	0.92 (0.78–1.09)	0.33		
Presence of atopy	0.94 (0.77–1.15)	0.54	0.94 (0.77–1.15)	0.55		
Haemoglobin (g/dl^2)	1.07 (1.03–1.12)	0.002	1.06 (1.01–1.11)	0.009	1.07 (1.02–1.12)	0.004
Haematocrit, %	1.02 (1.00–1.03)	0.023	1.01 (1.00–1.02)	0.12		
White blood cell count (log transformed)	1.17 (0.92–1.49)	0.20	1.57 (0.90–2.71)	0.11		
Eosinophil count (log transformed)	1.03 (0.97–1.10)	0.33	1.04 (0.97–1.11)	0.26		
Neutrophil count (log transformed)	1.19 (1.05–1.35)	0.012	1.46 (1.07–1.99)	0.016	1.19 (1.05–1.36)	0.008
Lymphocyte count (log transformed)	0.91 (0.78–1.08)	0.28	0.96 (0.66–1.41)	0.84		
Monocyte count (log transformed)	1.16 (0.96–1.40)	0.12	1.36 (0.89–2.08)	0.15		
Anaemia (ref. no)	0.97 (0.82–1.14)	0.71	0.97 (0.83–1.15)	0.75		
HIV viral load ($\log_{10}$ copies/ml)	1.00 (0.95–1.05)	0.95	0.99 (0.95–1.05)	0.86		
CD4$^+$ cell count (cells/μl)	1.00 (0.99–1.00)	0.07	1.00 (0.99–1.01)	0.34		
Years on ART	0.99 (0.97–1.02)	0.70	0.98 (0.95–1.00)	0.13		
PI-based regimen (NNRTI-based as ref.)	0.85 (0.71–1.02)	0.09	0.86 (0.72–1.03)	0.11	0.90 (0.75–1.08)	0.27

ART, antiretroviral therapy; CI, confidence interval; eNO, exhaled nitric oxide; FEV1, forced expiratory volume in 1 s; PI, protease inhibitor.
[a]Geometric mean ratio.

Table 3. Analysis of factors associated with exhaled nitric oxide level in HIV-uninfected participants.

	]Unadjusted analysis		Adjusted for age, sex and time of eNO testing		Multivariable model	
Variables	GMR[a] (95% CI)	*P*	GMR[a] (95% CI)	*P*	GMR[a] (95% CI)	*P*
Age	1.09 (1.05–1.13)	<0.001	1.08 (1.05–1.12)	<0.001	1.07 (1.03–1.11)	0.001
Female sex	1.25 (1.01–1.56)	0.04	1.14 (0.93–1.40)	0.21	1.14 (0.92–1.41)	0.22
Weight (kg)	1.02 (1.01–1.03)	<0.001	1.01 (0.99–1.03)	0.42		
Height (cm)	1.02 (1.00–1.02)	<0.001	1.01 (0.99–1.03)	0.15		
Weight-for-age *z*-score	1.00 (0.88–1.13)	0.99	1.00 (0.88–1.13)	0.99		
Height-for-age *z*-score	1.04 (0.93–1.16)	0.50	1.04 (0.93–1.17)	0.50		
Wasting (weight for age *z*-score < −2)	0.45 (0.24–0.85)	0.014	0.45 (0.24–0.85)	0.015	0.52 (0.29–0.92)	0.03
Stunted (height-for-age *z*-score < −2)	0.70 (0.40–1.22)	0.21	0.70 (0.40–1.23)	0.21		
Passive smoking (ref. no)	1.52 (0.80–2.88)	0.20	1.12 (0.61–2.04)	0.71		
FEV1 *z*-score	1.01 (0.87–1.18)	0.87	1.01 (0.86–1.18)	0.90		
Presence of atopy	1.08 (0.75–1.56)	0.67	1.05 (0.75–1.47)	0.75		
Haemoglobin (g/dl^2)	1.13 (1.05–1.23)	0.003	1.09 (1.01–1.19)	0.031	1.09 (1.00–1.18)	0.04
Haematocrit, %	1.07 (1.04–1.11)	<0.001	1.05 (1.01–1.08)	0.009		
White blood cell count (log transformed)	0.89 (0.66–1.20)	0.45	0.94 (0.49–1.81)	0.86		
Eosinophil count (log transformed)	1.03 (0.92–1.14)	0.62	1.05 (0.95–1.16)	0.32		
Neutrophil count (log transformed)	0.97 (0.78–1.20)	0.76	1.06 (0.66–1.71)	0.79		
Lymphocyte count (log transformed)	0.94 (0.64–1.37)	0.74	1.04 (0.45–2.40)	0.92		
Monocyte count (log transformed)	0.81 (0.62–1.05)	0.12	0.78 (0.44–1.40)	0.41		
Anaemia (ref. no)	1.13 (0.77–1.66)	0.52	1.13 (0.77–1.67)	0.53		

CI, confidence interval; eNO, exhaled nitric oxide; FEV1, forced expiratory volume in 1 s.
[a]Geometric mean ratio.

HIV-infected children may be explained by immunological and pathophysiological mechanisms. Alveolar macrophages play a central role in the defense against bacterial and mycobacterial infection; they may become infected with HIV and constitute the primary reservoir of the virus in the lung [37,38]. During HIV infection, alveolar macrophage function is compromised [37], with evidence of oxidative stress [39] and reduced ability to phagocytose and kill bacteria [40,41]. HIV-associated chronic activation of inflammatory cells in the alveolar space may further compromise the host response against infectious stimuli [42,43]. Thus, low levels of eNO detected in patients with HIV may reflect the inability of alveolar macrophages to produce eNO as part of the lung innate immune system. This may contribute to persistent lung immune dysfunction.

eNO levels are known to increase with age. This needs to be taken into consideration when interpreting eNO levels measured in children. Values of eNO increase linearly between 6 and 16 years in parallel with the somatic growth [44]. The ATS guidelines recommend adjustment for age when interpreting the eNO levels in children younger than 12 years of age [45]. In addition, the majority of published studies have reported an association between height and eNO levels [46–49]. Total airway mucosal surface area for NO diffusion increases with increasing age and height, thus leading to higher eNO levels [50]. In our study, both age and height were significantly associated with eNO levels in HIV-infected children. Given that HIV-infected children are generally smaller than their HIV-uninfected peers, this might partly explain the lower eNO levels among HIV-infected children observed in our study.

An interesting finding of our study was that history of TB in HIV-infected participants was associated with lower eNO levels. No studies have investigated the level of eNO in individuals with a history of TB, though a number of studies were conducted in adult patients with active lung TB [22,32,51]. At least two studies found that eNO levels were lower in patients with newly diagnosed untreated lung TB than healthy controls [22,32]. Moreover, one of these studies reported an association between low eNO levels at the time of TB diagnosis and severity of disease [22].

M. tuberculosis (*Mtb*) may persist in alveolar macrophages for a prolonged period of time [52,53]. Nonreplicating persistence of *Mtb* may maintain chronic airway inflammation and may lead to lung fibrosis [17]. There is some evidence from in-vitro studies that nitric oxide has a protective role in the progression of lung fibrosis [54]. Thus, lower levels of eNO in patients treated for TB in our study may reflect altered lung immune response mediating the development of chronic lung complications.

No association was found between airway obstruction measured as FEV1 *z* score less than -1.64 and eNO levels in HIV-infected children. This is in line with several other studies reporting no association between FEV1 and eNO in adults with chronic obstructive lung disease [55,56], implying that eNO has probably no pathophysiological role in the obstruction, but rather a marker of the underlying condition, as seen in eosinophilic inflammation in asthma patients [57].

In our study, we found significant positive associations between several blood parameters and eNO levels. The

associations between haemoglobin level, haemotocrit and eNO may reflect physiological processes at the level of endothelium. Increased haematocrit results in increased shear stress and production of NO. The positive association between eNO and blood neutrophils observed in HIV-infected participants may be explained by the fact that neutrophils can produce nitric oxide in response to foreign stimuli [58]. However, in cystic fibrosis patients, an inverse correlation between blood neutrophils and eNO levels was reported, which was thought to be due to the ability of neutrophils to produce superoxide that downregulates nitric oxide production [24]. The observed associations emphasize the need to interpret eNO results with caution. eNO levels are influenced by a multitude of factors, including demographic, anthropometric and biological factors, thus questioning the utility of eNO as a clinical tool in conditions beyond asthma. Although noninvasive nature and measurement simplicity make eNO testing an attractive tool for resource-limited settings, it is too early to forecast its applicability and clinical usefulness among HIV-infected children with airway abnormalities in a given setting.

The strengths of the study are its prospective design, relatively large sample size, use of standardized protocol for eNO testing and spirometry performed by well trained nurses. Study limitations include self-report of TB with no information regarding TB localization (pulmonary or extrapulmonary), raising concerns of recall bias. Among other determinants that may have an impact on eNO levels and that were not assessed in the present study are genetic factors (affecting the activity of nitric oxide synthase), environmental factors (air pollution) and socioeconomic factors.

In summary, our study shows that both HIV infection and history of TB are associated with lower eNO levels, with no association found between eNO and airway obstruction. The low eNO levels may be a marker of abnormal pulmonary physiology due to perinatally acquired HIV infection and post-TB lung changes. The role of nitric oxide in the pathogenesis of HIV infection needs further investigation.

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Conflicts of interest

There are no conflicts of interest.

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PAPER III

Composition of gut microbiota of children and adolescents with perinatal HIV infection taking antiretroviral therapy in Zimbabwe

Running head: Gut microbiota of HIV infected children on ART

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Abstract

Background: HIV infection causes impairment of the gastrointestinal barrier, with substantial depletion of $CD4^+$ T-cells in the gut. Antiretroviral therapy (ART) restores the $CD4^+$ 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: A cross-sectional study. Rectal swabs were collected from 177 HIV infected and 103 HIV uninfected controls. Gut microbial composition was explored using 16S rRNA sequencing (Illumina Miseq).

Results: HIV 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 counts, HIV viral load or HIV-associated CLD. We found enriched levels of *Corynebacterium* ($p<0.01$), *Finegoldia* ($p<0.01$) and *Anaerococcus* ($p<0,01$) in HIV infected, and enrichment of *Enterobacteriaceae* ($p=0.02$) 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.

Conclusion: HIV infected children have altered gut microbiota**.** Our results suggest that prolonged ART minimize the differences in gut microbiota between HIV infected and uninfected participants.

Keywords: HIV infection; antiretroviral therapy; gut microbiota; children; adolescents; Africa

Introduction

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]. HIV-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 an 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 significantly enriched in adults with HIV [3, 4].

Studies show that altered gut microbiota is associated with elevated inflammatory markers such as C-reactive protein and interleukin-6 [5-8] as well as with markers of microbial translocation such as lipopolysaccharide and lipopolysaccharide binding protein [9, 10]. Dinh et al. found a positive correlation between relative abundance of *Enterobacteriaceae* and markers of chronic inflammation in HIV infected, antiretroviral (ART)-treated individuals with undetectable plasma HIV RNA [11]. Further, studies suggest that ART may only partially restore the gut microbiota towards levels observed in HIV uninfected population, and patients continue to suffer from dysbiosis even when HIV infection is controlled [1, 12, 13].

Moreover, gut dysbiosis may be associated with the development of chronic non-infectious HIV complications, such as cardiovascular disease and lung complications [14, 15]. Systemic chronic inflammation due to gut microbial translocation and gut dysbiosis may have some relation to the development of chronic complications [16, 17].

So far, no studies have investigated the gut microbiome in children in sub-Saharan Africa and its relation to the development of HIV-associated chronic complications. 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 ($CD4^+$ T-cell count, viral load).

Materials and methods

Study population

This study investigated bacterial profiles of rectal swabs collected from participants of the Bronchopulmonary Function in Response to Azithromycin Treatment for Chronic Lung Disease in HIV-infected Children (BREATHE) trial [18] (clinicaltrials.gov identifier NCT02426112). CLD was defined as forced expiratory volume in one second (FEV_1) z-score less than -1.0 with no reversibility (<12% improvement in FEV1 after 200 ug of salbutamol inhaled using a spacer). The detailed study protocol has been described previously [18]. For the present sub study only participants enrolled in Harare (Zimbabwe) were included. In addition, HIV uninfected children aged 6-16 years from the same area with no prior history of heart/lung diseases, tuberculosis (TB), no chronic cough, reported chest pain or shortness of breath during exercise were recruited for the control group. All study participants completed a detailed questionnaire regarding demographic, socio-economic characteristics and clinical history.

The study was approved by the 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 enrolment into the trial by study nurses. Swabs were immediately preserved in 1,5 ml of transport medium PrimeStore ® MTM (Longhorn diagnostics, Maryland, USA) and directly stored on ice for a maximum of one hour, and then frozen at -80 °C before shipment on dry ice to the laboratory at Division of Medical Microbiology, University of Cape Town (UCT).

Laboratory procedures

DNA extraction

The Zymo Research Quick-DNA™ Fecal/Soil Microbe Microprep kit (Zymo Research, California, USA) was used for DNA extractions. DNA was extracted according to the manufacturer's description, with modifications. Briefly, an aliquot 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, Hilden, Germany) set to 50 Hz for 5 minutes. Then 500 µl of supernatant was transferred to a Zymo-Spin™ III-F Filter (Zymo Research, 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 library preparation and gene sequencing

In order to assess DNA quality and the total bacterial load extracted, a real-time quantitative polymerase chain reaction (qPCR) was performed as previously described [19]. Subsequently, two sets of PCRs targeting the V4 hypervariable region of the 16S ribosomal ribonucleic acid (rRNA) gene using the primers 515F and 806R were performed according to previously described protocols [20, 21]. For details on laboratory procedure see Supplementary file 1.

Samples were sequenced on an Illumina Miseq® instrument using the Miseq® Reagent v3 kit, (600 cycles) (Illumina, California, USA). The final library was diluted to a 6 pM concentration, and a 25% PhiX library spike-in was added at 6 pM as internal control [22]. The pre-processing of sequence reads was done using the H3ABioNet 16S rDNA diversity analysis package (https://github.com/h3abionet/h3abionet16S) [21], with the exception that the taxonomy of representative reads was assigned using the SILVA version 132 database. The raw sequence files have been submitted to the European Nucleotide Archive (ENA) under the accession number PRJEB32077.

Data analysis

Statistical analyses were performed in STATA 14 (StataCorp LLC, Texas USA) 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).

The richness of bacterial taxa within a single sample was represented by the number of operational taxonomic units (OTUs) and Chao1 index. Both richness and evenness (relative abundances of the different species) was characterized by Shannon's index.

Interindividual differences, beta diversity, was determined using Bray-Curtis dissimilarity index [23] with sample depth set at 2000 reads. Beta diversity comparisons were explored using Principal Coordinate plots generated by the *stats* package in R Statistical software (http://www.r-project.org/). Comparisons were made using the Wilcoxon rank sum test where not specified otherwise. We also used Kruskal-Wallis test in cases where more than two groups were compared and the same groups were compared using the permutational multivariate analysis of variance (PERMANOVA) in QIIME2 (version 2018.4) [24], with number of permutations set to 999. P-values were adjusted for multiple testing using the Benjamini-Hochberg method [25].

Relative abundance

To assess relative abundance a linear discriminant analysis (LDA) was performed using LEfSe (linear discriminant analysis (LDA) effect size) [26] 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 comparisons plots were generated using the MicrobiomeAnalyst web-based software tool with standard feature filtering [27]. Heatmaps were generated using only the taxa that were significantly different using the LEfSe comparison (Supplementary figure 1-3). The average fraction of each taxa was calculated from all samples within each group. The data was transformed to fractional abundance (Phyloseq) before performing the LEfSe 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 and p-values were corrected for multiple testing using false discovery rate (FDR) procedure. Spearman´s rank correlation with Bonferroni correction was used to assess the association between alpha diversity indices and continuous parameters. We fitted linear regression model in order to estimate the association between HIV status, participant characteristics, presence of HIV-associated CLD and alpha diversity indices. Age and sex were adjusted for a priori. A two-tailed significance level of 0.05 was used.

Results

Study population

In total 149 HIV infected participants with CLD, 28 HIV infected participants without CLD, and 103 HIV uninfected participants were enrolled. The characteristics of study groups are presented in Table 1.

HIV infected participants were older compared to the HIV uninfected participants ((15.6 years (IQR 12.8-17.7) vs 9.9 (IQR 7.4-12.7), $p<0.001$)) and were more likely to be stunted and underweight compared to HIV uninfected participants (stunted: 41.2% vs 4.8%, $p<0.001$; underweight: 45.2% vs 4.8%, $p<0.001$). The proportion of participants who

experienced episodes of diarrhoea during the last three months prior to enrolment was also higher in the HIV infected than in HIV uninfected group (10.6% vs 2.9%, p=0.03).

Alpha diversity

Species richness estimates (OTUs, Chao1) were significantly higher in HIV uninfected compared to HIV infected participants. However, there was no difference in the Shannon index between these two study groups (Figure 1, Supplementary table 1). After adjustment for age and sex using linear regression analysis the negative association between richness indices and HIV status remained significant (p =0.03 for OTUs and p=0.002 for Chao1 index). The use of antibiotics during the three previous months did not change the significant effect of positive HIV status for the Chao1 index (Supplementary table 2). A sub-group analysis of virally suppressed/non-suppressed participants revealed no association between viral suppression and estimates of alpha diversity in our study population (Supplementary table 3). We stratified the 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 that was similar to the HIV uninfected study group (Table 2).

There was no difference in alpha diversity indices between HIV infected participants with and without CLD (Supplementary table 4**).** The same was observed after adjusting for age and sex using regression analysis (Supplementary table 5).

The association between participant characteristics and alpha diversity indices in HIV infected participants is presented in Supplementary table 5. Prolonged ART treatment was the only parameter significantly associated with a richer gut microbiota after adjustment for age and sex, suggesting a positive effect of prolonged ART. No parameters were found to be significantly associated with alpha diversity estimates in HIV uninfected group.

Beta diversity

We found a significantly higher beta diversity amongst HIV infected participants, compared to HIV uninfected (p<0.01) (Figure 2a). Duration of ART had no impact on beta diversity when stratified by years spent on ART. There was no association between beta diversity and viral load suppression, type of ART regimen, time on ART and prior TB in HIV infected participants.

HIV infected participants with CLD had higher beta diversity compared to both HIV uninfected (p<0.01) and HIV infected participants without CLD (p=0.03). There was no significant difference between HIV infected participants without CLD and HIV uninfected (p=0.74) (Figure 2b). Unweighted UniFrac analysis show similar results.

Relative abundance of specific taxa

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

HIV infected participants versus HIV uninfected participants

At phylum level HIV infected participants had significantly lower abundance of Epsilonbacteraeota (7%) ($p<0.01$) and Bacteroidetes (32%) ($p<0.01$) compared to HIV uninfected participants (with 13% and 38% respectively) (Supplementary figure 4).

At genus level HIV infected participants had enriched *Corynebacterium* ($p<0.01$), *Lawsonella* ($p<0.01$) and *Collinsella* ($p=0.04$) belonging to the Actinobacteria phylum; while in the Firmicutes phylum *Finegoldia* ($p<0.01$), *Anaerococcus* ($p<0,01$), *Erysipelotrichaceae* ($p=0.02$) and *Lachnoclostridium* ($p=0.04$) were enriched when compared to HIV uninfected.

HIV uninfected participants, when compared to HIV infected, were enriched in *Campylobacter* ($p < 0.01$) belonging to the Epsilonbacteraeota phylum*; Porphyromonas* ($p<0.01$) and *Prevotella* ($p=0.03$) belonging to Bacteroidetes phylum; *Eubacterium coprostanoligenes_group* ($p<0.01$), *Ruminococcaceae* ($p<0.01$), *Fastidiosipila* ($p<0.01$), *Fournierella* ($p<0.01$), *W5053* ($p<0.01$), *Coprococcus* ($p=0.02$) and *Murdochiella* ($p<0.01$) all belonging to the Firmicutes phylum (Figure 3).

HIV infected participants with CLD had a higher abundance of the genus *Faecalibacterium* ($p=0.05$) belonging to the phylum Firmicutes*,* compared to participants without CLD. Participants without CLD had higher abundance of genus *W5053* ($p<0.01$), phylum Firmicutes and *Prevotella* ($p=0.05$), phylum Bacteroidetes, compared to participants with CLD.

Associations between characteristics of HIV infected participants and gut microbiota

When we stratified HIV infected participants based on CD4 count (CD4≤400 cells/mm vs >400 cells/mm), we found no statistically significant differences at the genus level. However, we found higher proportions at the family level of Enterobacteriaceae ($p=0.02$) and Burkholderiaceae ($p=0.04$) in those with CD4 counts ≤ 400 cells/mm, whereas Succinivibrionaceae ($p=0.04$) was higher in those with CD4 counts > 400 cells/mm. No differences in relative abundance were found at any taxonomic level between virally suppressed and non-suppressed participants (<1000 copies/ml vs ≥1000 copies/ml).

We compared the HIV infected participants based on ART duration sub groups to HIV uninfected using the LEfSe analysis. The longer the participants had been on ART, the fewer taxa was found to be significantly different between HIV infected and HIV uninfected (Supplementary figure 1-3), illustrated in heatmaps (Supplementary figure 5-8). HIV infected participants who had been on ART≥10 years are more similar to HIV uninfected than those on ART for less than 10 years. Genera such as *Bacteroides*, *Prevotella*, *Porphyromonas*, *Blautia* and *Roseburia* are similarly abundant in HIV uninfected and HIV infected participants who have been on ART≥10 years. However, the genera *Finegoldia* and *Corynebacterium* remained significantly enriched in HIV infected participants on ART≥10 years compared to HIV uninfected, whereas *Ruminococcaceae* remained significantly enriched in HIV uninfected compared to HIV infected participants on ART≥10 years.

We found no differences in relative abundance when comparing HIV infected participants on ART for <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 than in HIV uninfected children, however 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 minimize the differences in gut microbiota between HIV infected and uninfected participants.

Impact of HIV on gut microbiota

A number of studies in adults demonstrated that HIV infection without treatment is associated with severe intestinal dysbiosis, reduced alpha diversity and increased beta diversity [9, 28, 29]. These changes may persist despite ART (5, 6, 10, 38, 39). Our results of overall lower alpha diversity and higher beta diversity in HIV infected ART-treated children support these findings.

The published data are less consistent when it comes 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, analysis of rectal swabs of HIV infected, ART treated participants in Nigeria found higher abundance of *Finegoldia* and *Anaerococcus* in the HIV infected population [30], which is consistent with our findings. However, in the same study *Campylobacter* was significantly enriched in HIV infected participants in contrast to our findings of enriched *Campylobacter* in the HIV uninfected group.

Several studies showed enriched levels of Proteobacteria in HIV infected, ART naive individuals [6, 7, 9], with only one study showing similar findings in ART treated individuals [11]. We found an enrichment of Proteobacteria in HIV infected individuals, yet not statistically significant. However, we found a trend towards lower abundance of Proteobacteria with increasing time on ART.

Impact of ART on gut microbiota

At least two studies have found a negative impact of ART on gut microbiota diversity [9, 30]. In a longitudinal study, Nowak P. *et al.* found significant decrease in the number of observed species and Shannon index after the introduction of ART. Our results does not contradict this, as they investigated the effect of ART initiation, with a relatively short follow up of 10 months [9]. In our population, we had no ART-naive participants, and minimum duration of ART was 1 year. We observed lower alpha diversity in those who were on ART for less than 10 years compared to HIV uninfected participants.

Previous studies that have investigated the gut microbiome in individuals on long-term ART reported similar alpha diversity profiles in HIV infected, ART-treated individuals and HIV uninfected participants [11, 28]. For example, Dinh *et al.* found no significant difference in alpha diversity measures between HIV infected participants who were on suppressive ART for a median of 13.3 years and HIV uninfected controls [11]. This is similar to our findings for those participants who received ART for 10 or more years. The impact of ART duration on the gut microbiota was also noted by Lozupone *et al.* who

found that individuals with longer ART duration showed a closer resemblance to HIV uninfected individuals than to subjects with untreated chronic HIV infection [31]. These studies together with our findings support the ability of long-term ART to restore the 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, 32, 33]. The findings of previous studies may have been affected by sample size and duration of ART. 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 gastrointestinal and urinary tract infections in HIV infected children [33], however the clinical significance of this finding is unclear, as 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 [34].

Gut-lung axis

Recent evidence suggests that the gut microbiome is involved in maintaining lung health and an altered gut microbiome composition is often observed in patients with lung diseases [16, 17]. For example, low gut microbiome diversity during infancy has been linked to asthma at school age [35]. In our study, however, we did not observe any difference in alpha diversity estimates between participants with and without HIV-associated CLD, though there were some significant differences in relative abundance of specific taxa. For example, the genus *Faecalibacterium* was enriched in HIV infected individuals with CLD while the genus *Prevotella* was enriched in HIV infected individuals without CLD. Interestingly, higher proportions of *Prevotella* have previously been shown in lung microbiota of children without asthma compared to asthmatic children and adults with chronic obstructive pulmonary disease (COPD) [36].

Faecalibacterium have previously been regarded as a protective commensal, and is associated with a healthy gut. Depletion of this bacteria have been linked to the development of inflammatory bowel disease and asthma, and low levels have been shown in patients with cystic fibrosis [17, 37]. However, some studies have challenged this, by showing increased levels of the species *Faecalibacterium prausnitzii* in the gut microbiome of paediatric patients with untreated Crohn's disease at the time of diagnosis [38]. Interestingly, a recent study also showed increased levels of *Faecalibacterium* in the gut microbiome of patients with active TB, suggesting that its role in disease is not entirely beneficial [39].

Co-trimoxazole prophylaxis

Since the majority (90.8%) 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. Though 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. In our study, the negative

impact of positive HIV status on richness estimates remained significant after accounting for co-trimoxazole prophylaxis. We also did not observe any effect of co-trimoxazole administration on alpha diversity in HIV infected participants. Our results are in line with several other studies where no significant difference in alpha diversity indices (OTUs, Shannon index) was observed in HIV infected individuals who took co-trimoxazole and those who did not [40-42].

Study strengths and limitations

Our study is one of the first to assess the gut microbiome composition in children and adolescents with perinatally acquired HIV infection. 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. Further, we did not assess diet, social factors such as housing or level of education of the participants which may also have impact on gut microbiota.

Conclusion

To our knowledge, this study is the first to assess gut microbial composition of HIV infected children and adolescents in a setting with a very high HIV burden. Our results indicate that gut microbiota is altered in HIV infected children, though it improves with increasing duration of ART. Further studies, where 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.

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Tables and figures

Table 1. Characteristics of study participants.

Parameter	**HIV- (N=103)**	**HIV+CLD+ (N = 149)**	**HIV+CLD- (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 (51.5)	84 (56.4)	8 (28.6)
Stunted (height-for-age z-score <-2), N (%)	5 (4.9)	66 (44.3)	7 (25.0)
Underweight (weight-for-age z-score <-2), N (%)	5 (4.9)	78 (52.3)	2 (7.1)
Took antibiotics the three previous months for HIV uninfected group or Co-trimoxazole prophylaxis for HIV infected group, N (%)	2 (1.9)	133 (89.3)	25 (89.3)
Episodes of diarrhea during the last three months, N (%) [1]	3 (2.9)	11(12.9)	1 (3.6)
Residential area, N (%) [2]			
High density	107 (94.7)	83 (97.7)	24 (85.7)
Medium density	4 (3.5)	1 (1.2)	3 (10.7)
Low density	2 (1.8)	1 (1.2)	1 (3.6)
HIV-related parameters			
ART regimen, N (%)			
NNRTI-based regimen	-	93 (62.4)	24 (85.7)
PI-based regimen	-	56 (37.6)	4 (14.3)
CD4 count ≤400 cells/mm, N (%)	-	40 (26.9)	9 (32.1)
VL suppression (VL<1000 copies/ml), N (%)	-	87 (58.4)	17 (60.7)
Years spent on ART [3]	-	6.6 (4.4-8.4)	8.0 (5.0-9.1)
Previously treated for TB, N (%)	-	54 (36.2)	2 (7.1)

[1] Data on episodes of diarrhea during the last three months was missing for 64 participants
[2] Data on residential area was missing for 64 participants
[3] Data on years spent on ART was missing for one participant

Figure 1. Alpha diversity indices in HIV infected and HIV uninfected participants.

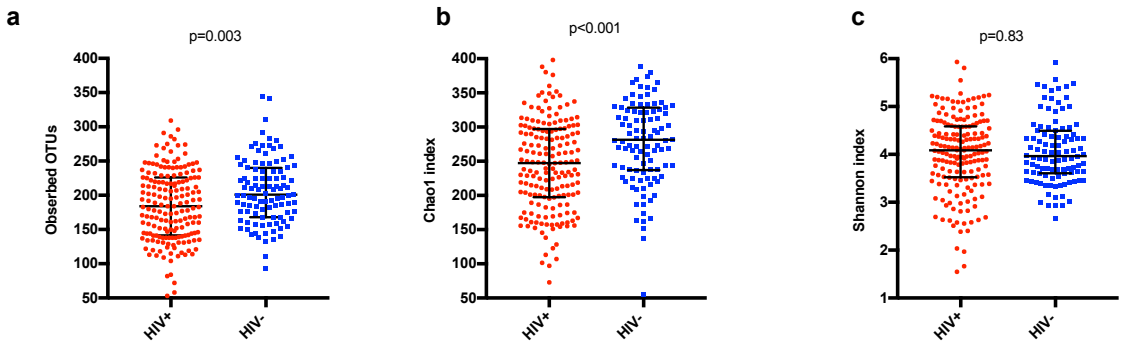
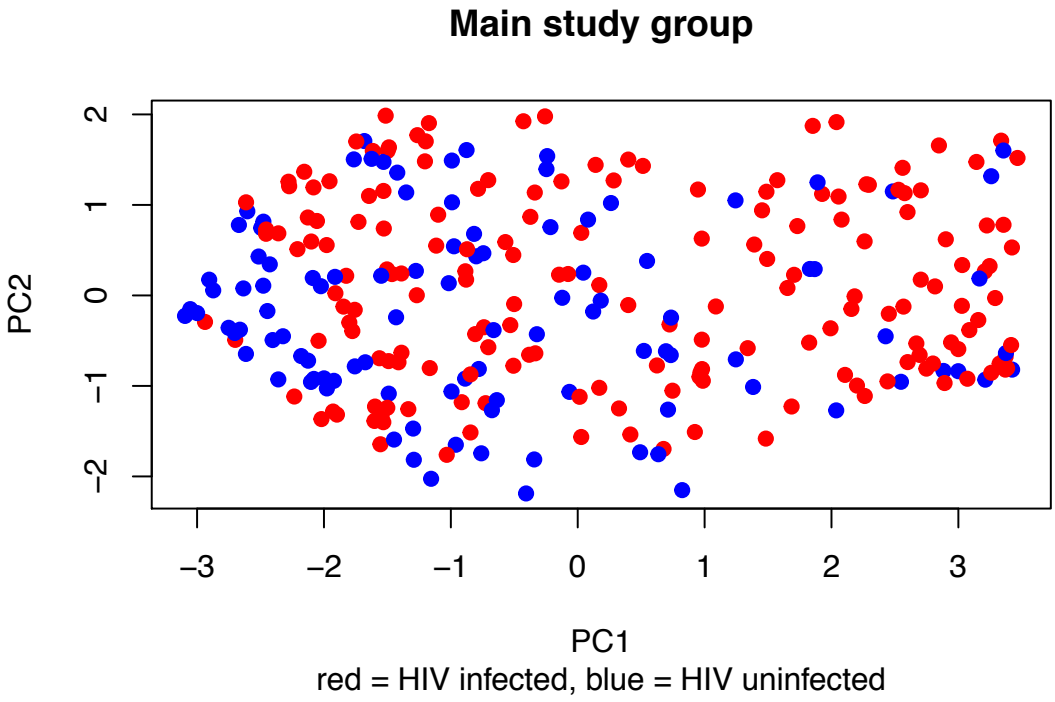


Table 2. Alpha diversity in HIV infected participants stratified by years on ART and in HIV uninfected participants.

	HIV+, <5 years on ART (N=53)	**HIV+, 5-10 years on ART (N=100)**	**HIV+, ≥10 years on ART (N=23)**	**HIV- group, (N=103)**	**HIV+, <5 years on ART vs HIV-**	**HIV+, 5-10 years on ART vs HIV-**	**HIV+, ≥10 years on ART vs HIV-**
	Median, IQR				p values *		
Observed OTUs	176 (138-214)	186.5 (143-223.5)	204 (162-242)	201 (168-240)	0.001	0.10	0.25
Chao1	229.4 (175.0-277.9)	249.6 (200.2-299.6)	268.9 (224.4-306)	281.3 (237.2-328.4)	<0.001	0.01	0.07
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)	0.28	0.82	0.90

* presented p values were obtained from regression model adjusted for 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.



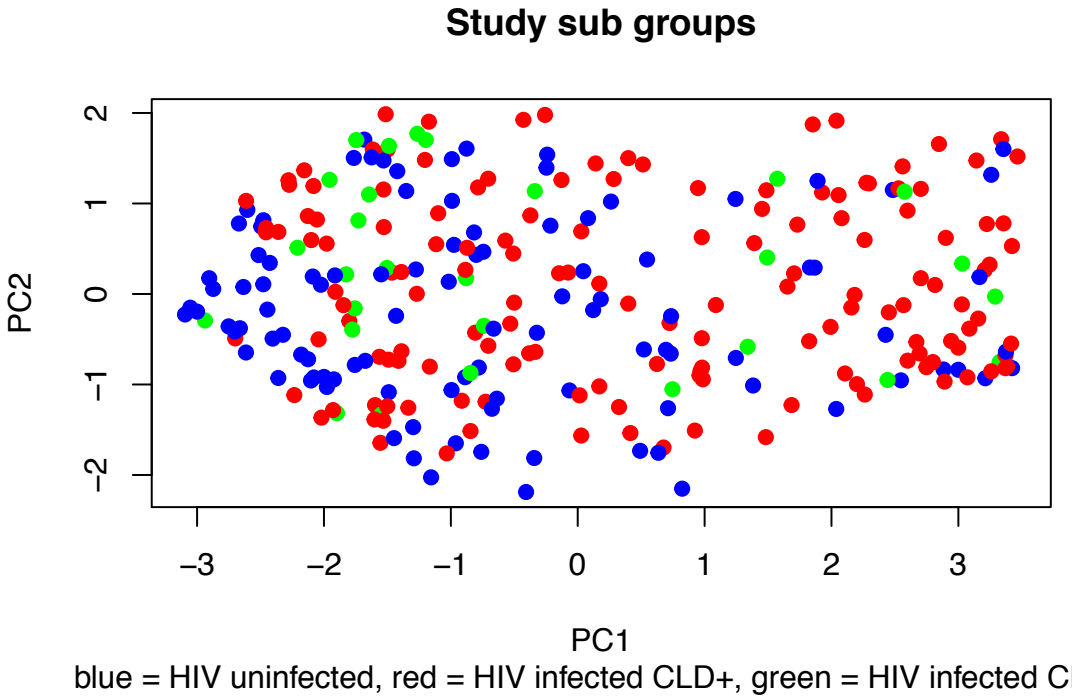


Figure 2. Beta-diversity comparison between study groups
Primcipal cooridnate analysis-plot showing beta-diversity by Bray-Curtis dissimilarity comparing (a) HIV infected (red) and HIV uninfected (blue) participants ($p<0.01$) and (b) HIV infected with CLD (red), HIV infected without CLD (green) and HIV uninfected (blue) participants. P-value obtained using wilcoxon rank sum test.

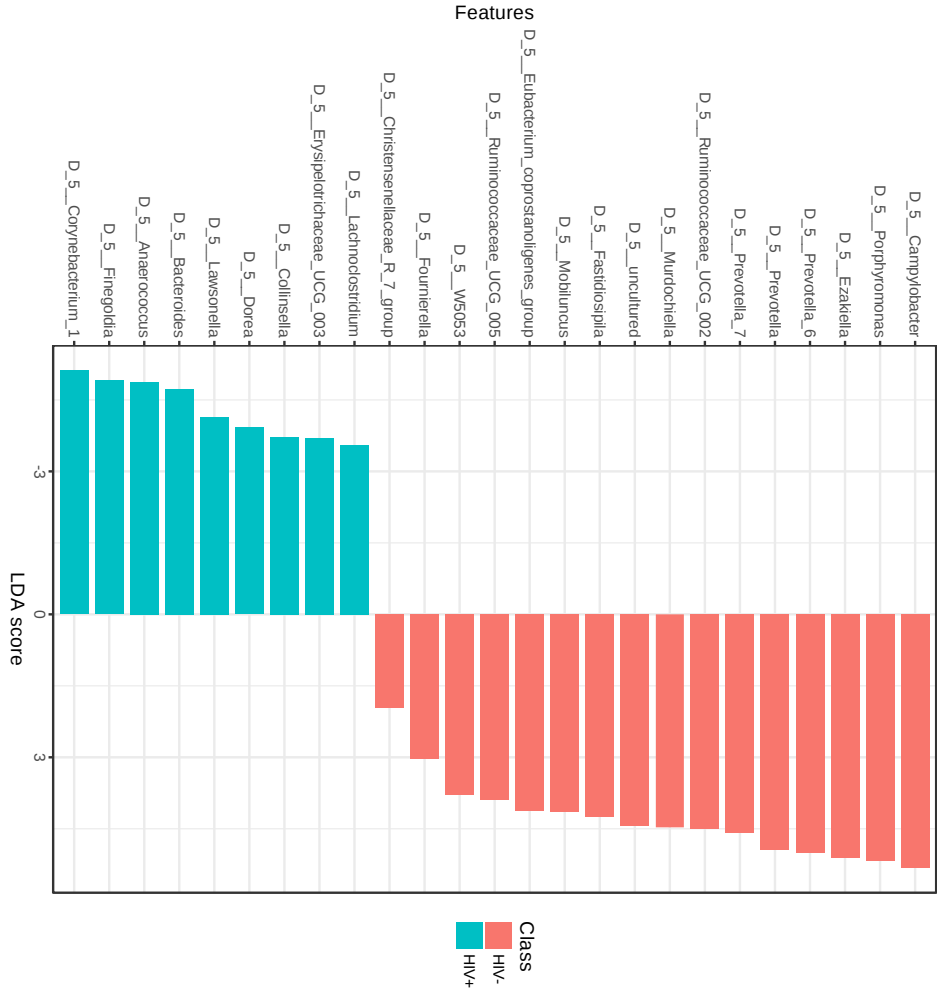


Figure 3. LEfSe plot (linear discriminant analysis (LDA) effect size) showing enriched taxa that are significantly different between HIV infected (blue) and HIV uninfected (red) participants. Only taxa meeting a significant level of 0.05 and effect size threshold of 1.0 are shown.

Supplementary files, tables and figures

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-**	**HIV+, suppressed vs HIV+, non-suppressed**
	Median, IQR			p value		
Observed OTUs	192.5 (145.5-228.5)	176 (138-220)	201 (168-240)	0.05*	0.005*	0.06**
Chao1	259.3 (201.2-302.1)	233.2 (175-276)	281.3 (237.2-328.4)	0.006*	<0.001*	0.02**
Shannon index	4.10 (3.48-4.61)	4.06 (3.60-4.57)	4.0 (3.6-4.5)	0.51*	0.37*	0.47**

* 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.

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

Supplementary table 4. 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 5. Association between characteristics of HIV infected participants (independent variables) and alpha diversity indices (dependent variables) in linear regression analysis adjusted for 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	-1.94 (-4.33 to 0.46)	0.11	-2.63 (-5.66 to 0.40)	0.09	-0.007 (-0.04 to 0.03)	0.68
Sex (ref. male)	1.07 (-15.0 to 17.1)	0.90	10.4 (-9.92 to 30.7)	0.31	-0.02 (-0.26 to 0.22)	0.85
Stunted (ref. no)	-4.23 (-20.6 to 12.1)	0.61	-9.01 (-29.7 to 11.7)	0.39	0.05 (-0.19 to 0.30)	0.67
Underweight (ref. no)	-0.35 (-16.5 to 15.8)	0.97	-10.3 (-30.8 to 10.1)	0.32	0.12 (-0.12 to 0.36)	0.33
Viral load, log copies/ml	-2.91 (-9.01 to 3.19)	0.35	-6.05 (-13.7 to 1.62)	0.12	0.04 (-0.05 to 0.14)	0.34
VL, categories						
Suppressed	ref.		ref.		ref.	
VL (≥1000 copies/ml)	-10.3 (-27.1 to 6.44)	0.23	-19.4 (-40.5 to 1.73)	0.07	-0.01 (-0.27 to 0.24)	0.90
CD4≤400 cells/mm^3 (ref>400 cells/mm^3)	-5.16 (-23.9 to 13.6)	0.59	-12.1 (-35.8 to 11.5)	0.31	0.15 (-0.13 to 0.43)	0.29

Years on ART	2.98 (0.14 to 5.81)	0.04	4.00 (0.43 to 7.58)	0.03	0.01 (-0.03 to 0.06)	0.49
Years on ART, categories						
<5years	ref.		ref.		ref.	
5-10 years	14.5 (-3.57 to 32.7)	0.11	22.2 (-0.67 to 45.1)	0.06	0.09 (-0.18 to 0.36)	0.52
≥10 years	34.5 (7.37 to 61.6)	0.01	39.9 (5.58 to 74.2)	0.02	0.36 (-0.05 to 0.77)	0.08
Co-trimoxazole prophylaxis (ref.no)	4.24 (-22.1 to 30.6)	0.75	2.92 (-30.3 to 36.2)	0.86	0.19 (-0.21 to 0.58)	0.35
FEV1 z-score	-0.38 (-6.15 to 5.38)	0.89	-0.95 (-8.26 to 6.35)	0.80	-0.04 (-0.13 to 0.04)	0.30
Diagnosis of chronic lung diaseade (ref.no)	4.71 (-17.4 to 26.8)	0.67	6.00 (-22.0 to 34.0)	0.67	0.05 (-0.28 to 0.37)	0.78
ART regimen (ref. NNRTI)	0.25 (-17.0 to 17.5)	0.98	-5.34 (-27.1 to 16.4)	0.63	0.03 (-0.23 to 0.28)	0.84
Previously treated for TB (ref.no)	-3.09 (-20.5 to 14.3)	0.73	-3.42 (-25.4 to 18.5)	0.76	0.09 (-0.17 to 0.35)	0.48
Episodes of diarrhea (ref.no)	-0.53 (-33.4 to 32.3)	0.97	6.84 (-32.9 to 46.5)	0.73	-0.06 (-0.57 to 0.46)	0.83

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

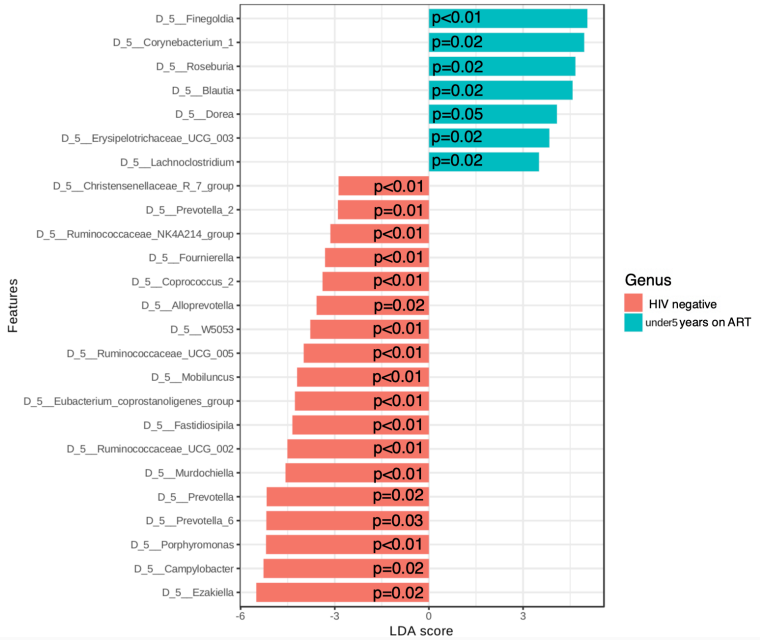
	Richness				**Richness + evenness**	
Covariates	**OTUs**		**Chao1**		**Shannon**	
	b coefficients (95% CI)	p value	b coefficients (95% CI)	p value	b coefficients (95% CI)	p value
Age, years	3.14 (-0.14 to 6.43)	0.06	4.24 (-0.23 to 8.70)	0.06	0.02 (-0.02 to 0.07)	0.27
Sex (ref. male)	-1.11(-20.9 to 18.6)	0.91	5.63 (-21.2 to 32.4)	0.68	0.24 (-0.03 to 0.52)	0.08
Stunted (ref. no)	-18.6 (-63.9 to 26.8)	0.42	-40.8 (-102.1 to 20.5)	0.19	-0.13 (-0.76 to 0.51)	0.69
Underweight (ref. no)	-15.4 (-60.8 to 30.0)	0.50	-38.1 (-99.5 to 23.3)	0.22	0.10 (-0.53 to 0.74)	0.74
FEV1 z-score	4.10 (-9.39 to 17.6)	0.55	3.94 (-14.7 to 22.5)	0.67	0.07 (-0.11 to 0.25)	0.43
Episodes of diarrhea (ref.no)	19.6 (-38.6 to 77.7)	0.51	40.1 (-38.7 to 118.9)	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

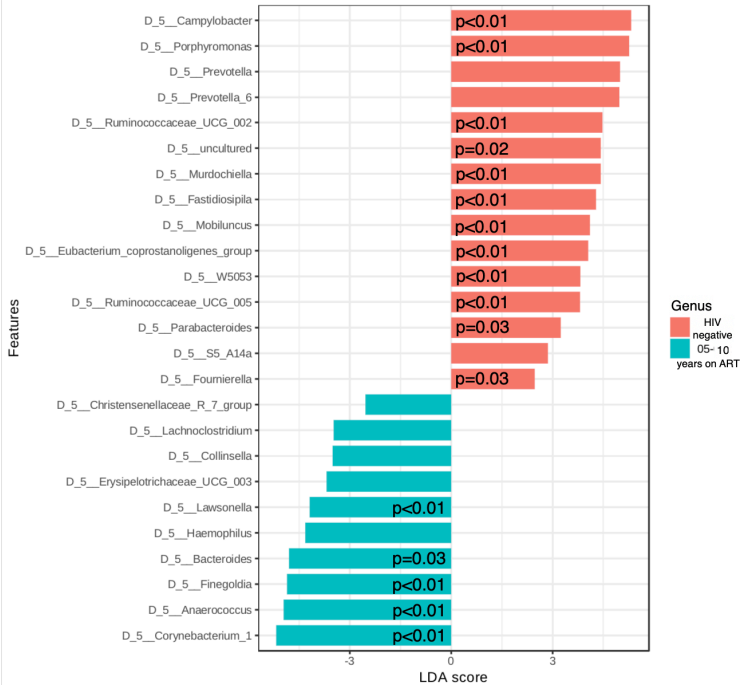
Table showing no significant differences in beta diversicy in HIV infected group based on patient charachteristics. For PERMANOVA-analysis umber of permutations was set to 999. P-values were adjusted for multiple testing using the Benjamini-Hochberg method.

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



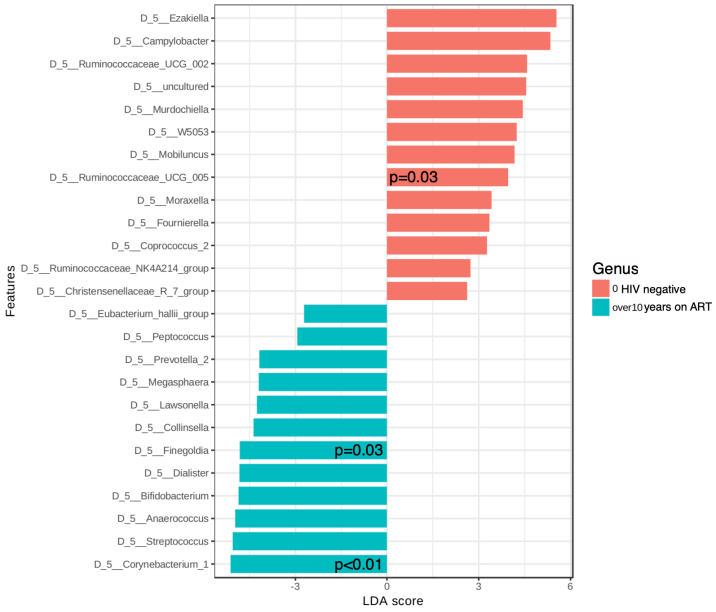
Showing enriched taxa that are significantly different between HIV infected participants on ART < 5 years (blue) and HIV uninfected participants (red). Only taxa meeting a significant level of 0.05 and effect size threshold of 1.0 are included. P-values shown are only those significant after adjustment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



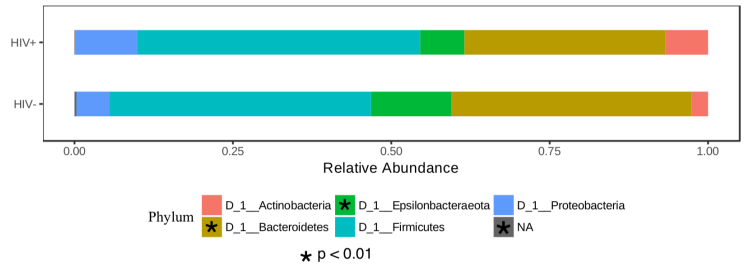
Showing enriched taxa that are significantly different between HIV infected participants on ART for 5-10 years (blue) and HIV uninfected participants in Zimbabwe (red). Only taxa meeting a significant level of 0.05 and effect size threshold of 1.0 are included. P-values shown are only those significant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

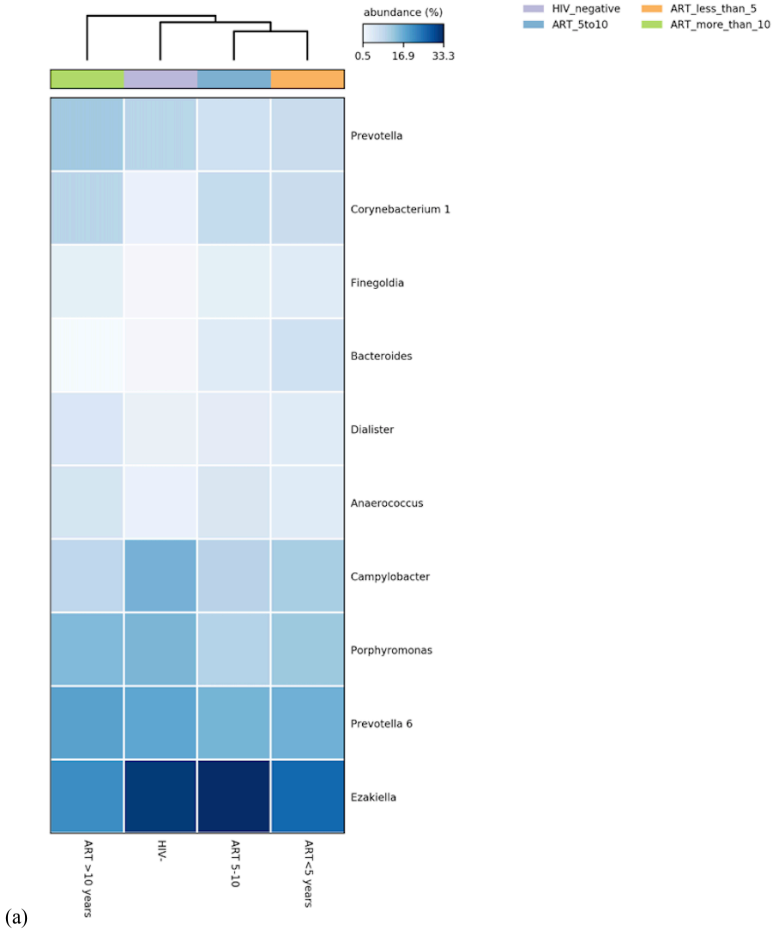


Showing enriched taxa that are significantly different between HIV infected participants on ART > 10 years (blue) and HIV uninfected participants (red). Only taxa meeting a significant level of 0.05 and effect size threshold of 1.0 are included. P-values shown are only those significant after adjustment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

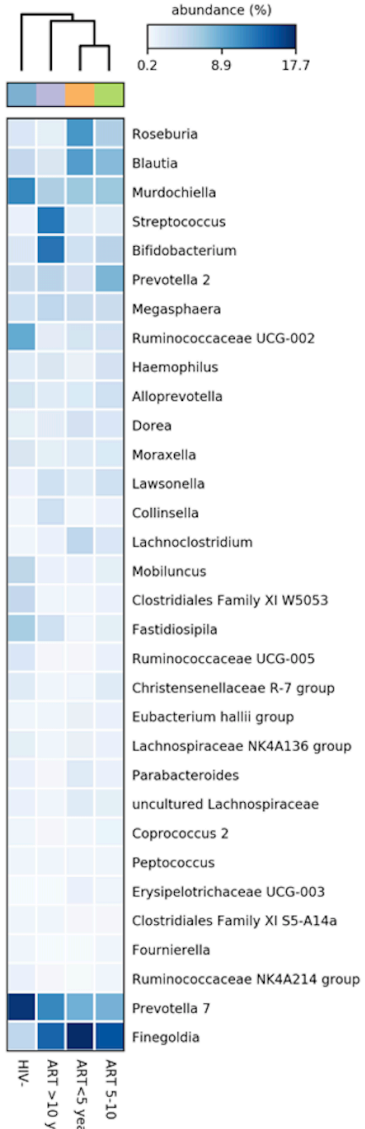
Supplementary figure 4. Relative abundance at phylum level for HIV infected and HIV uninfected participants. P-value is adjusted for FDR.



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)



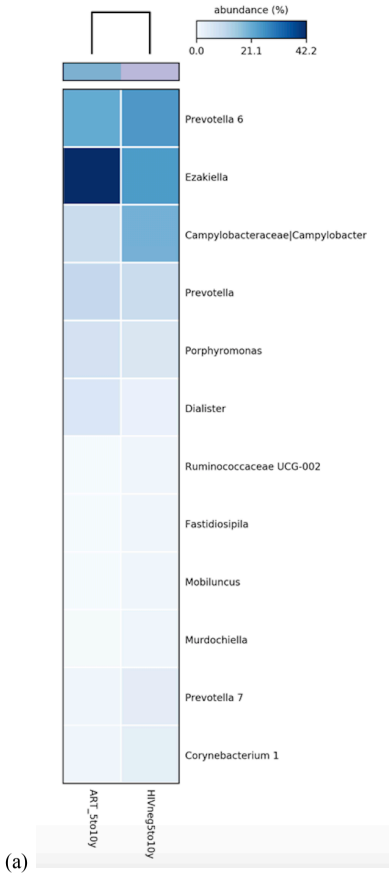
(b)

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)

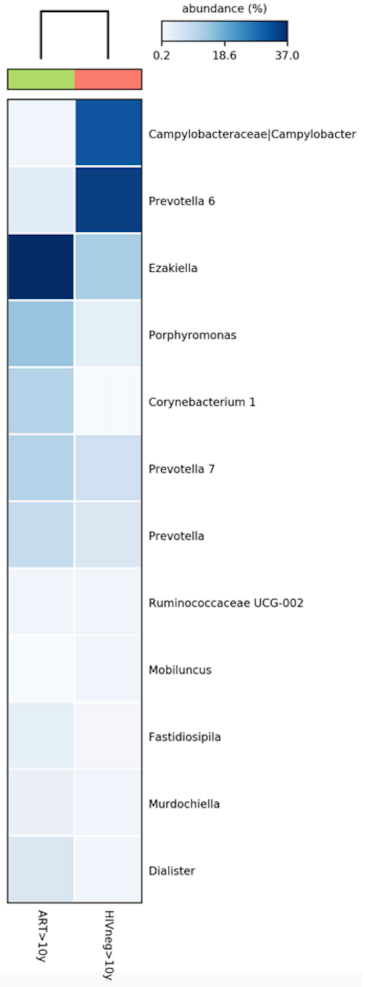
Supplementary figure 7. Heatmap showing differences of the (a) more abundant genera and (b) less abundant genera, between age matched HIV uninfected participants and HIV infected participants on ART 5-10 years.



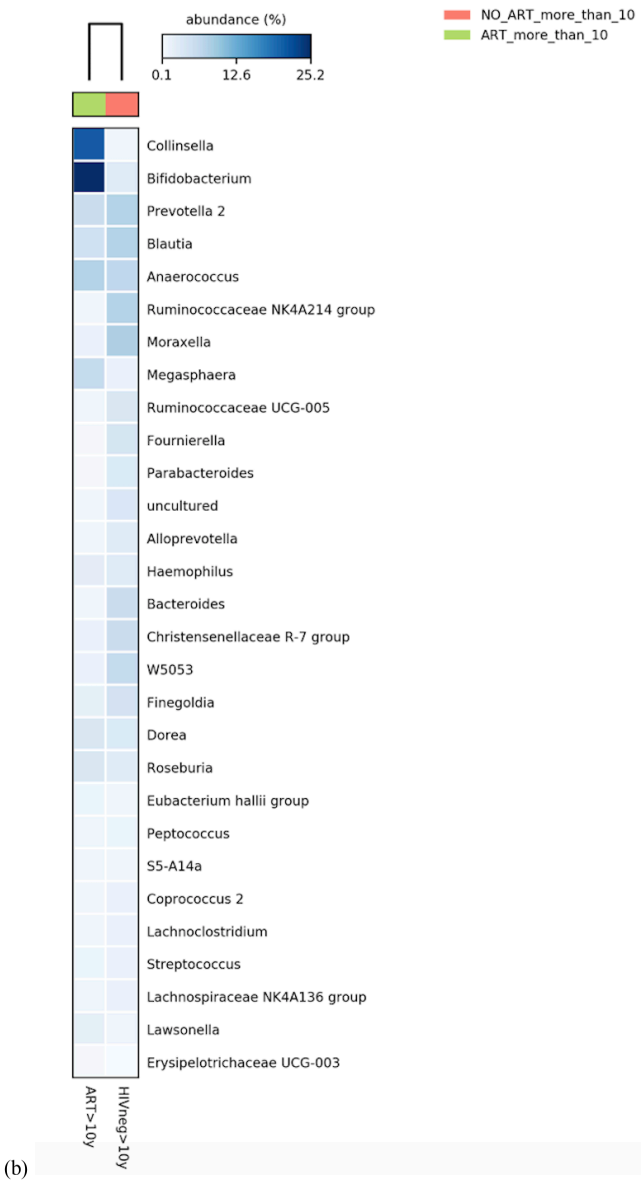
(a)

(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 (NNNNNNNNNNN) 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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7. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res **2012**; 41:D590-D6.

APPENDIX I

BO.03a BREATHE Trial

Bronchopulmonary response to azithromycin treatment for chronic lung disease in HIV-infected children

BASELINE CLINICAL FORM - MAIN STUDY

F01	*STUDN*	Study number	☐☐☐☐☐
F02	*DATE*	Date of interview *dd/MMM/yyyy*	☐☐/☐☐☐/20☐☐
HIV HISTORY			
F03	*HIVNO*	HIV clinic number	☐☐☐☐☐☐☐☐☐☐☐☐
F05	*STATUS*	Is the participant aware of his/her HIV status?	Yes☐ No☐
F06	*DATEHIV*	Date of HIV diagnosis ***(if day unknown assign 15, if month unknown assign JUN)***	☐☐/☐☐☐/20☐☐ Unknown ☐
F07	*AGEHIV*	Age at HIV diagnosis **(Years completed, 0 if under one year)**	☐☐ years
F08	*REASONHIV*	Was HIV diagnosis done because: ***(check ONE box only)***	Child was sick☐ Routine screening☐ Other☐ Specify other…… Not known☐
F09	*DATEART*	Date of ART initiation ***(if day unknown assign 15, if month unknown assign JUN)***	☐☐/☐☐☐/☐☐☐☐ Unknown ☐
F10	*AGEART*	Age at ART initiation **(Years completed, 0 if under one year)**	☐☐
F11	*COTRI*	Is the participant taking cotrimoxazole?	Yes☐ No☐ Don't Know☐

BASELINE CLINICAL FORM - MAIN STUDY

F13	*DRUGS*	Please tick current ART drugs the child is taking: ***(tick all that apply)***	
		AZT (zidovudine)	Yes☐ No☐
		D4T (stavudine)	Yes☐ No☐
		TNF (tenofovir)	Yes☐ No☐
		ABC(Abacavir)	Yes☐ No☐
		3TC (lamuvidine)	Yes☐ No☐
		DDI (didanosine)	Yes☐ No☐
		NVP(Nevirapine)	Yes☐ No☐
		EFV(Efavirenz)	Yes☐ No☐
		ATV (atazanavir)/R (ritonavir)	Yes☐ No☐
		LPV (kaletra, alluvia)/R(ritonavir)	Yes☐ No☐
		Other ART Drug	Yes☐ No☐
		Specify Other ART Drug ……..	
CLINICAL HISTORY			
F14	*ADM*	Has the participant been admitted to hospital for chest problems in the last 12 months?	Yes☐ No☐ Don't Know☐
F15	*NADM*	If yes, how many times has the participant been admitted to hospital for chest problems in the last 12 months	☐☐ NA ☐
F16	*TBTREAT*	Has the participant ever been treated for TB?	Yes☐ No☐ Don't Know☐
F17	*NOTBTREAT*	If yes, how many times has the participant been treated for TB?	☐☐ NA ☐
F18	*BREATH*	Is the participant currently breathless?	Yes☐ No☐
F19	*MRC5*	Does the participant get breathless when dressing or too breathless to leave the house?	Yes☐ No☐
F20	*MRC4*	Does the participant have to stop for breath after walking 100 m?	Yes☐ No☐
F21	*MRC3*	Does the participant walk slower than most people or has to stop after 15 minutes walking?	Yes☐ No☐

BASELINE CLINICAL FORM - MAIN STUDY

F22	*MRC2*	Is the participant short of breath when hurrying on the level or walking uphill?	Yes☐ No☐
F23	*MRC1*	Does the participant get breathless on moderate exercise?	Yes☐ No☐
F24	*MRCSCORE*	What is the MRC Dyspnoea Scale score? ***(please check consistency with answers on questions F18 to F23)***	☐
F25	*COUGH*	Does the participant have a cough now?	Yes☐ No☐
F26	*COUGHT*	Has the cough changed over time? ***(tick what applies)***	Same ☐ Improving ☐ Getting worse ☐ Do not know ☐ Not coughing ☐
F27	*SPUTUM*	Does the participant cough up sputum?	Yes☐ No☐ N/A☐
F28	*SPUTUMQ*	How much sputum does the participant cough up each day? ***(tick what applies)***	No Sputum☐ Less than a table spoon ☐ A few table spoons ☐ A cup ☐ Do not know ☐ Not coughing ☐
F29	*LYWH*	Does the participant get wheezing or whistling in the chest?	Yes☐ No☐ Don't Know☐
F30	*INH*	Does the participant use an inhaler?	Yes☐ No☐ Don't Know☐
F31	*FINH*	How often does the participant use an inhaler? ***(tick what applies)***	Once a week ☐ Daily ☐ More than once a week ☐ Don´t know ☐ Not applicable ☐

BASELINE CLINICAL FORM - MAIN STUDY

F32	*SALBT*	Does the participant use salbutamol tablets?	Yes☐ No☐ Don't Know☐
F33	*ASTHMA*	Has the participant ever been told by a doctor or nurse that he or she has asthma?	Yes☐ No☐ Don't Know☐
EXAMINATION			
F34	*WEIGHT*	Weight	☐☐.☐ Kg
F35	*HEIGHT*	Height	☐☐☐.☐ cm
F36	*RR*	Respiratory rate	☐☐ breaths per minute
F37	*HR*	Heart rate	☐☐☐ per minute
F38	*SAT*	Oxygen saturation	☐☐☐ %
ELIGIBILITY FOR SHUTTLE WALK TEST			
F41	*SHUTY*	Is the participant capable of doing the shuttle walk test	Yes☐ No☐
F42	*SHUTN*	If not capable of doing a shuttle walk test, give reason why not (check all reasons that apply):	SpO_2 <88% ☐ RR>30/min☐ Resting heart rate >120/min☐ Feels too ill to exercise ☐ Unable to stand/ walk☐ Other☐ If other, specify _____ _____
FOR THOSE ELIGIBLE FOR SHUTTLE WALK TEST			
F43	*SHUTM*	Time participant walked	☐☐minutes ☐☐seconds
F44	*SHUTO2N*	O2 saturation **immediately after SWT**	☐☐☐%
F45	*SHUTHRN*	Heart rate **immediately after SWT**	☐☐☐/min
F46	*SHUTRRN*	Respiratory rate **immediately after SWT**	☐☐/min
F48	*SHUTHR1*	Heart rate **1 minute after SWT**	☐☐☐/min
F50	*SHUTDNC*	When the participant had to stop, what was the reason?	Chest pain☐ Breathlessness☐ Leg tiredness☐

BASELINE CLINICAL FORM - MAIN STUDY

			Staggering ☐ Excessive sweating (diaphoresis) ☐ Did not reach next point(beep) ☐ Other ☐ If other, Specify _____
FORMS AND TESTS			
F51	*TESTS*	Which tests were collected? ***(tick all that applies)***	Blood sample immunology Yes ☐ No ☐ N/A ☐ (BO.25) Sputum storage Yes ☐ No ☐ N/A ☐ (BO.26) NPA Yes ☐ No ☐ N/A ☐ (BO.28) Rectal Swab storage Yes ☐ No ☐ N/A ☐ (BO.29) Stool storage Yes ☐ No ☐ N/A ☐ (BO.27) Viral Load Yes ☐ No ☐ N/A ☐ (BO.19) CD4 Yes ☐ No ☐ N/A ☐ (BO.18) Cardiac Echo Yes ☐ No ☐ N/A ☐ (BO.14)
F52	*LOGD*	Drug recording diary given to designated caregiver?	Yes ☐ No ☐
F53	*PHARM*	Has the pharmacy form been completed (BO.13)?	Yes ☐ No ☐
F54	*FORM*	Form BO.04 (SCHOOLING FORM) Completed	Yes ☐ No ☐
NEXT VISIT			
F55	*ECHOD*	Cardiac echo appointment date ***(if not possible to be done on the baseline visit date)***	☐☐/☐☐☐/20☐☐ NA ☐
F56	*NVISIT*	Follow up appointment date	☐☐/☐☐☐/20☐☐

APPENDIX II

BO.03b BREATHE Trial

Bronchopulmonary response to azithromycin treatment for chronic lung disease in HIV-infected children

BASELINE CLINICAL FORM COMPARISON GROUP

G01	*STUDN*	Study number	☐☐☐☐☐
G02	*DATE*	Date of interview *dd/MMM/yyyy*	☐☐/☐☐☐/20☐☐
HIV HISTORY			
G03	*HIVNO*	HIV clinic number	☐☐☐☐☐☐☐☐☐☐☐☐
G05	*STATUS*	Is the participant aware of his/her HIV status?	Yes☐ No☐
G06	*DATEHIV*	Date of HIV diagnosis ***(if day unknown assign 15, if month unknown assign JUN))***	☐☐/☐☐☐/20☐☐ Unknown ☐
G07	*AGEHIV*	Age at HIV diagnosis **(Years completed, 0 if under one year)**	☐☐ years
G08	*REASONHIV*	Was diagnosis done because: ***(check ONE box only)***	Child was sick☐ Routine screening☐ Other☐ Specify other…… Not known☐
G09	*DATEART*	Date of ART initiation ***(if day unknown assign 15, if month unknown assign JUN))***	☐☐/☐☐☐/☐☐☐☐ Unknown ☐
G10	*AGEART*	Age at ART initiation **(Years completed, 0 if under one year)**	☐☐
G11	*COTRI*	Is the participant taking cotrimoxazole?	Yes☐ No☐ Don't Know☐

BO.03b BREATHE Trial

Bronchopulmonary response to azithromycin treatment for chronic lung disease in HIV-infected children

BASELINE CLINICAL FORM COMPARISON GROUP

G13	*DRUGS*	Please tick current ART drugs the child is taking: ***(tick all that apply)***	
		AZT (zidovudine)	Yes☐ No☐
		D4T (stavudine)	Yes☐ No☐
		TNF (tenofovir)	Yes☐ No☐
		ABC(Abacavir)	Yes☐ No☐
		3TC (lamuvidine)	Yes☐ No☐
		DDI (didanosine)	Yes☐ No☐
		NVP(Nevirapine)	Yes☐ No☐
		EFV(Efavirenz)	Yes☐ No☐
		ATV (atazanavir)/R (ritonavir)	Yes☐ No☐
		LPV (kaletra, alluvia)/R(ritonavir)	Yes☐ No☐
		Other ART Drug	Yes☐ No☐
		Specify Other ART Drug ……..	
CLINICAL HISTORY			
G14	*ADM*	Has the participant been admitted to hospital for chest problems in the last 12 months?	Yes☐ No☐ Don't Know☐
G15	*NADM*	If yes, how many times has the participant been admitted to hospital for chest problems in the last 12 months	☐☐ NA ☐
G16	*TBTREAT*	Has the participant ever been treated for TB?	Yes☐ No☐ Don't Know☐
G17	*NOTBTREAT*	If yes, how many times has the participant been treated for TB?	☐☐ NA ☐
LABORATORY EVALUATIONS			
G18	*TESTS*	Which tests were collected? ***(tick all that applies)?***	CD4 Yes☐ No☐ N/A☐ (BO.18) Viral Load Yes☐ No☐ N/A☐ (BO.18 Blood sample immunology Yes☐ No☐ N/A☐ (BO.25) Sputum storage Yes☐ No☐ N/A☐ (BO.26) NPA Yes☐ No☐ N/A☐ (BO.28) Rectal Swab storage Yes☐ No☐ N/A☐ (BO.29) Stool storage Yes☐ No☐ N/A☐ (BO.27)
G19	*FORM*	FORM B04 (SCHOOLING CRF) Completed	Yes☐ No☐
THIS IS THE END OF THE STUDY FOR THE COMPARISON GROUP PARTICIPANTS			

APPENDIX III

BO.15 BREATHE Trial

Bronchopulmonary response to azithromycin treatment for chronic lung disease in HIV-infected children

Exhaled Nitric Oxide FORM

Measurement of fractional exhaled nitric oxide in children and adolescents			
N00	STUDTYPE	What kind of study is it?	Breathe Main☐ Breathe Control☐
N01	STUDN	Study number	☐☐☐☐☐
N02	DATE	Date of interview and eNO test (dd/mm/yyyy)	☐☐/☐☐/☐☐☐☐
N03	TIME	Time of eNO test	
N04	VISIT	Type of visit	Enrol☐12months☐
N04b	MEASURE	Acceptable measurement done (If Yes Complete the rest of the questionnaire, If No Stop here, end of test))	YES☐NO☐
Fractional exhaled nitric oxide (eNO) measurement			
N35	TIMEXHALE	The exhalation time of the measurement	10 s☐, 6 s☐
N35	NO1	First measurement *ppb (5-300)*	☐☐
N36	NO2	Second measurement *ppb (5-300)*	☐☐
N37	DIFF	Percent difference between two measurements ≤10%	YES☐NO☐
N38	NO3	If >10%, the third measurement *ppb (5-300)*	☐☐
COM		Any Comment (Patient could not do second test	
N05	BRTH	Does the participant currently have any breathing problems?	YES☐NO☐
N06	PAIN	Does the participant have any pain or physical problem that may prevent him/her from taking a deep breath and exhaling forcefully?	YES☐NO☐
N07	SMKH	**Within the last hour,** has the participant smoked a cigarette, cigar,pipe or used any other tobacco product?	YES☐NO☐
N08	EXCH	**Within the last hour,** has the participant exercised strenuously?	YES☐NO☐
N09	DRKH	**Within the last hour,** has the participant had anything to eat or drink ?	YES☐NO☐
N10	VEGH	W**ithin the last 3 hours** has the participant eaten any vegetables?	YES☐NO☐
N11	VGN	If (N10) **YES,** please name: *(e.g: beets, covo/rape, broccolo, cabbage, celery, lettuce, spinach, radishes)*	----------

BO.15 BREATHE Trial

Bronchopulmonary response to azithromycin treatment for chronic lung disease in HIV-infected children

Exhaled Nitric Oxide FORM

N12	MEATH	Within the last three hours, has the participant eaten any meat?	YES☐NO☐
N13	MEATN	If (N12) **YES,** please name *(e.g: bacon,beef,pork, ham, hot dogs)*	---------- --
N14	CORTD	**Within the last two days,** has the participant used any oral or inhaled corticosteroids or β2 agonists?	YES☐NO☐
N15	RESPD	Within the last 7 days, has the participant had a cough, cold, phlegm, runny nose or other respiratory illness? Do not count allergies or hay fever.	YES☐NO☐

CLINICAL HISTORY			
N16	SMKE	Does the participant smoke?	YES☐NO☐quit smoking☐
N17	SMKF	Are the members of participant's family/people the participant living with smoking?	YES☐NO☐Don't know☐
N18	MEDS	Does the participant take any medications/drugs besides ART?	YES☐NO☐Don't know☐
N19	MEDSN	If yes, please name what medications/drugs.	----------
N20	SULB	Does the participant use salbutamol tablets/inhaler?	YES☐NO☐Don't know☐
N21	CORTE	Has the participant ever taken corticosteroids, long-term β2 agonists, leukotriene receptor antagonists(LRA), methylxanthines?	YES☐NO☐Don't know☐
N22	CORT	If the answer (N21) **YES**, please name (select all that apply): corticosteroids (budesonide/fluticasone) -brown/orange inhaler☐ long-term b2 agonists (formoterol/salmeterol)-purple inhaler☐ LRA (montelukast (egsingulair) ☐ methylxanthines(theophylline) ☐	
N23	ASTH	Has the participant ever had asthma?	YES☐NO☐Don't know☐
N24	WHZ	Has the participant everexperienced wheezing, nocturnal tightness in the chest, attacks of shortness of breath following strenuous activity, at rest or at night time?	YES☐NO☐Don't know☐
N25	HAY	Has the participant ever had hay fever?	YES☐NO☐Don't know☐

BO.15 BREATHE Trial

Bronchopulmonary response to azithromycin treatment for chronic lung disease in HIV-infected children

Exhaled Nitric Oxide FORM

N26	SNZ	Has the participant) ever had a problem with sneezing, or a runny, or a blocked nose, or nasal itching when the participant **DID NOT hav**e a cold or the flu?	YES☐NO☐Don't know☐
N27	WATE	If (N26) **YES,** has this nose problems been accompanied by itchy-watery eyes?	YES☐NO☐Don't know☐
N28	CONJ	Has the participant ever had allergic conjunctivitis?	YES☐NO☐Don't know☐
N29	DERM	Has the participant ever had atopic dermatitis (eczema)?	YES☐NO☐Don't know☐
N30	RASH	Has the participant ever had a rash which was coming and going?	YES☐NO☐Don't know☐
N31	ITCH	If (N30) **YES**, had this caused itching or scratching?	YES☐NO☐Don't know☐
N32	ALLGF	Does the participant have any known food allergies?	YES☐NO☐Don't know☐
N33	ALLEGN	If (N32) **YES**, please name: (e.g: *cow's milk, egg whites, poultry, seafood, nuts)*	----------
N34	REACTF	Has the participant ever experienced bloating, itchy skin rash, tingling in the tongue/lips/throat, swelling or wheezing after eating some types of food? Do not count food poisoning.	YES☐NO☐Don't know☐