MICROBIOTA AND TELOMERE SHORTENING IN GUT-LUNG AXIS OF HUMAN IMMUNODEFICIENCY VIRUS INFECTED DONORS

by

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Microbiota and telomere shortening in gut-lung axis of human immunodeficiency virus infected donors

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Abstract

Previous studies have demonstrated the existence of a gut-lung axis and its proposed role in altering immune response and respiratory disease pathogenesis. The lung and gut microbiomes have been individually studied in the context of Human Immunodeficiency Virus (HIV) infection; however the gut-lung axis in HIV remains relatively unexplored and requires further investigation. This study examined the microbiomes of the gut-lung axis and telomere attrition in HIV subjects using paired human lung and small intestine tissue.

Paired lung and small intestine tissue autopsy specimens were obtained from ART-treated HIV-positive and HIV-negative donors through the National NeuroAIDS Tissue Consortium. Frozen samples were sectioned on dry ice and weighed for DNA extraction using the QIAGEN DNeasy® Blood and Tissue kit. Droplet digital PCR was used to measure bacterial load and quantitative PCR was performed to determine absolute telomere length (aTL). The 16S rRNA V4 region was sequenced using the MiSeq sequencing platform. Extraction negatives were run through the entire workflow alongside samples for quality control. Raw sequence reads were processed using QIIME2 (Quantitative Insights Into Microbial Ecology 2, v2018.2) followed by statistical analysis using R studio. Several t-tests, linear regression, PERMANOVA and ANCOVA were used for statistical analysis.

Microbial composition was not found to differ significantly between ART-treated HIV-positive and HIV-negative donors in both lung and small intestine. However, bacterial load and abundance of individual amplicon sequence variants (ASVs) were found to be correlated in the lung and small intestine. Bacteroides was decreased in ART-treated HIV-positive donors and
identified as one of the bacteria most likely to help explain differences between HIV-negative and ART-treated HIV-positive microbiomes. Telomere length was demonstrated to be shortened in lungs of ART-treated HIV-positive donors in comparison to HIV-negative but no differences were found between the two groups in the small intestine.

ART-treated HIV donors present microbiomes similar to HIV-negative donors, suggesting that ART can provide the microenvironment necessary to maintain a “healthy” microbiome. Furthermore, microbiomes of the gut-lung axis have demonstrated a correlation in bacterial load and abundance of individuals ASVs, suggesting a strong relationship between the two sites in both ART-treated HIV donors and HIV-negative donors.
Lay Summary

Previous studies have demonstrated the existence of a gut-lung axis and its proposed role in altering the immune response and respiratory disease pathogenesis. While the lung and gut bacterial communities, known as microbiomes, in HIV infection have been studied individually, the gut-lung axis in HIV is relatively unknown and still remains to be explored. My project examines the microbiomes of the gut-lung axis in HIV subjects using paired human lung and small intestine tissue. Specifically, the effect of HIV infection on the microbial composition of lung and small intestine while accounting for characteristics such as CD4 count, viral load and telomere length will be investigated.
Preface

This research was approved by the UBC-Providence Health Care Research Ethics Board (certificate number H15-02807).

Identification and design of the research program was done by Dr. Don Sin, Dr. Paul Man, Dr. Janice Leung and I. All droplet digital PCR and quantitative PCR experiments and data analysis were conducted by myself. Library preparation was completed by technician David Ngan and I. Sequencing by MiSeq was performed at the UBC Sequencing and Bioinformatics Consortium by Dr. Sunita Sunha on a fee-per-library service.
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List of Abbreviations

16S: 16 Svedberg Units
AIDS: acquired immunodeficiency syndrome
ANCOVA: analysis of covariance
ANOVA: analysis of variance
AP-1: activator protein 1
ART: antiretroviral therapy
ASV: amplicon sequence variant
aTL: absolute telomere length
AZT: zidovudine
BAL: bronchoalveolar lavage
CCR5: C-C chemokine receptor type 5
Cq: quantitation cycle
COPD: chronic obstructive pulmonary disease
CXCR4: chemokine receptor type 4
ddPCR: droplet digital polymerase chain reaction
DNA: deoxyribonucleic acid
dsDNA: double-stranded deoxyribonucleic acid
EDTA: Ethylenediaminetetraacetic acid
F: Forward
GOLD: Global Initiative for COPD
HAART: highly active antiretroviral therapy
HAND: human immunodeficiency virus-associated neurocognitive disorders
hBD-2: human beta defensin-2
HID: human infectious dose
HIV: human immunodeficiency virus
HIV -: HIV-negative
HIV +: HIV-positive
IBD: inflammatory bowel disease
IBS: irritable bowel syndrome
LEfSe: Linear discriminant analysis effect size
LHMP: Lung HIV Microbiome Project
LPS: lipopolysaccharide
NF-κB: nuclear factor-κB
NMDS: non-metric multidimensional scaling
OD: optical density
OUT: operational taxonomic unit
PCoA: principal coordinate analysis
PCR: polymerase chain reaction
PERMANOVA: permutational multivariate analysis of variance
PLWH: persons living with human immunodeficiency virus
qPCR: quantitative polymerase chain reaction
R: reverse
RNA: ribonucleic acid
rRNA: ribosomal ribonucleic acid
SCFA: short chain fatty acid
Tris-CI: tris(hydroxymethyl)aminomethane hydrochloride
WHO: World Health Organization
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To my dearest family
Chapter 1: Background

1.1 Human Immunodeficiency Virus

1.1.1 Epidemiology and History

According to the UNAIDS 2017 global HIV statistics, an estimated 36.9 million people worldwide are living with HIV\(^1\). Since the start of the HIV pandemic in the early 1980’s, 77.3 million people have been infected with HIV and 35.4 million people have died from AIDS-related illnesses\(^1\). In 2017, 1.8 million people were newly infected, 940,000 died from AIDS-related illnesses and 21.7 million people were accessing antiretroviral therapy\(^1\). The World Health Organization (WHO) African region has the highest HIV population with 4.1% adults living with HIV, accounting for almost two-thirds of all people living with HIV worldwide\(^2\). In Canada, 84,409 cases of HIV have been reported since 1985 with variations in provincial and territorial HIV diagnosis rates\(^3\).

The HIV pandemic originated from zoonotic infections of simian immunodeficiency viruses from African primates with HIV-1 being transmitted by Central African chimpanzees and HIV-2 by West African sooty mangabeys\(^4\). The spread of HIV-1 main (M) subtypes have led to a global pandemic while HIV-2 has been relatively restricted to West Africa and with low prevalence, to Europe, South America and Asia\(^5\). In Canada, the most common subtype is HIV-1 subtype B, making up 79.6% of the 1,811 specimens genotyped in the Canadian HIV Strain and Drug Resistance Surveillance Program (SDR Program) initiated by the Public Health Agency of Canada (PHAC)\(^6\).

HIV was first officially reported in 1981 when cases of Pneumocystis carinii pneumonia, a rare lung infection, were described in five previously healthy, young, gay males by the U.S.
Centers for Disease Control and Prevention (CDC). The disease was named Acquired Immune Deficiency Syndrome (AIDS) in 1982 and the first case in Canada was reported in March of that year. Reports of HIV were first described in homosexual men then intravenous drug users, blood transfusion recipients and heterosexual individuals. HIV was first isolated by Dr. Françoise Barré-Sinoussi and Dr. Luc A. Montagnier at the Pasteur Institute (Paris, France) in 1983 and then implicated to be the cause of AIDS by Dr. Robert Gallo and colleagues at the National Cancer Institute (Maryland, US) in 1984. The US Food and Drug Administration (FDA) approved the first antiretroviral drug, zidovudine (AZT) in 1987, leading the way to the introduction of antiretroviral therapy (ART) in 1995.

ART has become the standard treatment for HIV infection since 1996, achieving this through the use of different combinations of drugs. Standard ART consists of at least three medications (known as highly active antiretroviral therapy or HAART). Potential medications include reverse transcriptase inhibitors, fusion inhibitors, protease inhibitors, chemokine receptor 5 antagonists and integrase strand transfer inhibitors. Effective ART controls HIV multiplication in infected patients, and increases or maintains CD4 count, allowing a prolonged asymptomatic phase and decelerating disease progression. Furthermore, ART reduces the risk of transmission when viral load is kept at undetectable levels. In Canada, a 20-year-old HIV patient on ART can be expected to live into their early 70s, approaching the life expectancy of the general population. However, differences to life expectancy vary based on race, sex, HIV transmission risk group and CD4 count. Based on results from the Strategic Timing of Antiretroviral Therapy (START) trial, the WHO has recommended for all people living with HIV to initiate ART as soon after diagnosis as possible. The START trial presented conclusive evidence that
initiation of ART soon after HIV diagnosis reduces risk of serious illnesses such as Kaposi’s sarcoma and tuberculosis\textsuperscript{14}.

1.1.2 Overview of HIV

HIV is an enveloped ribonucleic acid (RNA) virus classified as part of the Lentivirus genus in the Retroviridae family and Orthoretrovirinae subfamily. The virus consists of two identical single-stranded RNA molecules enclosed within a conical capsid assembled from inner capsid protein p24, followed by a symmetrical outer capsid membrane formed by matrix protein, then viral lipid bilayer envelope and outer lipid membrane envelope\textsuperscript{4}. HIV is present in blood, semen, vaginal secretions and breast milk and can enter the body via eczematous or damaged skin or mucosa, mucous membranes, transplanted organs and parenteral inoculation\textsuperscript{15}. High risk populations in Canada include men who have sex with men (MSM), injection drug users, people from countries where HIV is endemic and indigenous people\textsuperscript{6}.

HIV targets activated CD4 T lymphocytes and enters the cells through interactions with CD4 and chemokine co-receptors C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4)\textsuperscript{9,10,16}. One human infection dose (HID) is approximately 500 to 1,000 HIV particles with a higher amount required for infection through mucous membranes in comparison to infection via bloodstream\textsuperscript{4}. Confirmatory tests in Canada target anti-HIV antibodies, HIV p24 antigen or HIV RNA while CD4 count (cells per mm\textsuperscript{3}) and viral load (copies of virus per mL) help monitor disease progression\textsuperscript{3}.

Clinical progression of HIV occurs in three stages: acute HIV infection, clinical latency and AIDS. After primary infection, people develop acute HIV syndrome in which the virus is
widely disseminated and seeded into lymphoid organs. During this period, flu-like symptoms may occur and high viral loads are found in the blood, making people at this stage very contagious. This is followed by clinical latency in which HIV is inactive or dormant. Patients on ART may experience this stage for decades but patients not on therapy may progress to AIDS in 2 to 25 years or longer. Towards the end of this stage, viral load increases and CD4 cell count decreases. In the final stage, people develop AIDS and display a weakened immune response, opportunistic infections and malignant neoplasms. Opportunistic infections include pneumocystis pneumonia (PCP), toxoplasmosis, cytomegalovirus retinitis and mycobacterium tuberculosis. Common neoplasms observed in HIV infections are human herpes virus type 8 (HHV-8) associated Kaposi’s sarcoma, non-Hodgkin’s lymphomas and human papillomavirus (HPV) induced cervical carcinoma\(^4,10,16,17\).

1.2 Microbiome

1.2.1 Introduction

The human body contains an approximated 38 trillion bacterial cells compared to the 30 trillion human cells estimated based on a 70 kg “reference man”\(^18\). These bacteria are found throughout the human body in variable quantities, inhabiting areas such as the gastrointestinal tract, skin, lungs and oral cavity\(^19\). Microbiota is the term used for a community of microorganisms including bacteria, viruses, archaea and fungi that inhabit a specific location at a specific point in time while the microbiome refers to the genetic material of the microbiota. Both terms have been frequently used in studies to encompass only the bacterial components and are often used interchangeably\(^19\). With the abundance of bacteria and corresponding microbiota in
the human body, it is important to understand the roles of these communities in health and disease processes and interactions within and between communities.

Studies on the microbiome have rapidly increased since 2009 due to the emergence and significantly reduced costs of culture-independent, high-throughput methods such as next-generation sequencing along with the development of molecular phylogenetic approaches to organize taxonomic diversity. With the discovery of 16S ribosomal RNA, researchers are able to classify sequences in the form of amplicon sequence variants (ASVs) or operational taxonomic units (OTUs) using 16S databases such as SILVA, Greengenes and RDP for taxonomic identification\textsuperscript{20-23}.

The interaction between environmental microbiota and various human body sites is highly dynamic, leading to changes in human microbiome due to different birthing methods (vaginal delivery versus caesarean section), dietary changes and disease onset and progression\textsuperscript{19,24,25}. One study demonstrated that infants delivered vaginally had microbiomes resembling their mother’s vaginal microbiomes while microbiomes of caesarean section infants consisted on bacteria found on the skin surface\textsuperscript{24}. Another study showed that it was possible to link a computer mouse to the person’s hand that had used it with up to 95% accuracy after comparing to a database of other hands\textsuperscript{26}.

Previous studies have shown that microbiota are important in metabolism, immunological processes and disease outcome and mechanism. Germ-free mice inoculated with gut microbiota from wild-type mice demonstrated increased energy extraction from diet, leading to increased energy deposition into adipocytes without increases in food intake. E. coli Nissle and lactobacilli
were shown to induced antimicrobial peptide human beta defensin-2 (hBD-2) expression by gut epithelial cells through proinflammatory pathways including nuclear factor-κB (NF-κB) and activator protein 1 (AP-1). Through hBD-2, E. coli Nissle and lactobacilli strengthen the intestinal barrier by counteracting bacterial adherence and invasion. Many studies have presented an inverse relationship between rates of childhood asthma and exposure to microbially rich environments during infancy such as growing up on a farm with livestock\textsuperscript{27–29}, living with two or more dogs\textsuperscript{30} and drinking non-pasteurized milk\textsuperscript{31}.

The Human Microbiome Project was launched in 2008 by the U.S. National Institutes of Health to better understand the impact of microbiomes on human health and disease. The first phase (2008 to 2012) focused on charactering human microbiome and involved 300 healthy individuals, 18 body sites, 5 body regions, resulting in 11,000 primary specimens for sequencing\textsuperscript{32–34}. The focus of the second phase (2014 to 2017), the Integrative Human Microbiome Project, was to develop resources to characterize the microbiome and distinguish its role in health and disease\textsuperscript{35}. Since 2009, publications on the microbiome have rapidly increased to over 8,700 papers published in 2017.

\textbf{1.2.2 Gut Microbiome}

The gut microbiome is one of the earliest and most extensively studied microbiomes in both health and disease due to the abundance of microorganisms in faecal matter which is commonly used in these studies and easily attainable. In the first year of life, microbiome diversity increases until around 2.5 years in which the composition, diversity and functional capabilities resemble that of the adult microbiome\textsuperscript{36,37}. It is not clear whether the gut microbiome shifts later on in life as one study demonstrated an increased abundance of Bacteroidetes in
subjects over 65 years old while another study showed that the microbiome was relatively comparable in subjects over 70 years old to the younger cohort\textsuperscript{38,39}. However, studies have shown that in the elderly, the gut microbiome displays a decreased capacity for metabolic processes like short chain fatty acid (SCFA) production and amylolysis and increased proteolytic activity\textsuperscript{40}. The most common phyla in the gut microbiome are Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes\textsuperscript{36,41,42}.

Many factors are responsible for shaping the gut microbiota including diet, environmental factors, host immune system and physiological properties\textsuperscript{25,36}. Diet is one of the major determinants of gut microbiome composition. Large alterations in the gut microbiome were observed between people on animal-based versus plant-based diets. Animal-based diets had decreased levels of Firmicutes, responsible for metabolising dietary plant polysaccharides and increased abundance of bile-tolerant bacteria such as Bacteroides and Alistipes\textsuperscript{43}. Environmental factors that may affect the composition of the gut microbiome include surgery, geographical location, urban versus rural accommodations and depression\textsuperscript{36,44–46}. Antibiotic usage can cause both short and long-term disruptions to the gut microbiome with studies demonstrating decreased richness and diversity\textsuperscript{47}. The host immune system prevents opportunistic infections of host tissue by stratifying and compartmentalizing bacteria\textsuperscript{48,49}. Secretory IgA helps limit exposure of epithelial cells to bacteria by co-localising with gut bacteria in the mucus layer\textsuperscript{50}. Paneth cells in the epithelial layer of the small intestine produce antimicrobials such as lipopolysaccharide-binding protein, lysozomes and cathelicidins\textsuperscript{51}. Physiological properties such as chemical, nutritional and immunological gradients influence microbial density and composition\textsuperscript{52}. For example, bacterial growth is limited in the small intestine to facultative anaerobes with rapid
growth due to its high acidity, short transit time and presence of oxygen and antimicrobials, while the colon consists mainly of anaerobes that have the ability to break down complex carbohydrates that are not digested in the small intestine\textsuperscript{52}.

In health, the gut microbiota provides nutrients, maintains barrier integrity, protects against pathogens, influences metabolic homeostasis and regulates host immune response\textsuperscript{53}. Nutrients provided by the gut microbiota include vitamin B12 produced by lactic acid bacteria, vitamin K, biotin, riboflavin and folate which is produced by Bifidobacteria and involved in DNA synthesis and repair\textsuperscript{54–57}. Bacteria implicated in maintaining barrier integrity include Lactobacillus plantarum, Akkermansia muciniphila as well as Lactobacilli rhamnosus which promotes cell renewal and wound healing\textsuperscript{58–60}. The healthy gut microbiota protects against colonisation by pathogenic bacteria by competing for attachment sites and nutrient sources and producing antimicrobial substances\textsuperscript{61}. Bacteria in the colon break down complex carbohydrates using carbohydrate-active enzymes to generate short chain fatty acids (SCFA) which are involved in chemotaxis, apoptosis, proliferation and help regulate gene expression and the immune response\textsuperscript{62}. Furthermore, the gut microbiome has been shown to be important in the development of the immune system. Germ-free mice demonstrated an absence of CD4+ T cell population expansion that could be reversed by treatment with polysaccharide A from Bacteroides fragilis\textsuperscript{63}. Perturbations in the gut microbiota have been linked to a wide range of conditions including inflammatory bowel disease, irritable bowel syndrome, Clostridium difficile infection and obesity\textsuperscript{61}. 
1.2.2.1 Gut Microbiome in HIV Infection

Based on current literature, the gut microbiome differs in HIV-negative, HIV-positive and ART-treated individuals although it is unclear the extent to which HIV infection alters the gut microbiome \(^{64-68}\). Various studies have characterized different patterns of composition for each study group. Confounding factors mentioned above such as diet and environmental factors as well as ART, antibiotic use and sexual practices may help explain the differing patterns of gut microbiome composition observed in separate studies. Additionally, level of bacterial classification examined and varying methods of samples collection including stool, tissue biopsy, rectal sponge and stool swabs may also influence the composition of the gut microbiome identified.

Enriched genus Prevotella and decreased Bacteroides in HIV-positive subjects in comparison to HIV-negative subjects have been demonstrated in several studies using both stool and mucosal biopsy samples \(^{69-72}\). Conversely, in a study from Uganda where transmission is predominately heterosexual, Prevotella was not found to be elevated in stool samples of HIV-infected subjects. However, HIV-negative subjects at baseline had a Prevotella-predominant stool microbiome, potentially due to differences in diet, which may have made it difficult to detect small increases in Prevotella \(^{73}\). In a Swedish cohort, Nowak et al did not observe an overabundance of Prevotella at baseline although abundance of Prevotella decreased after ART initiation \(^{74}\). Noguera-Julian et al demonstrated that the increased Prevotella and decreased Bacteroides enterotype was associated with MSM sub-group irrespective of HIV infection \(^{75}\). Increased abundance of Proteobacteria has been observed in HIV-positive subjects in several studies using mucosal samples \(^{70,76}\). Current literature is inconclusive on whether HIV infection
reduces microbial diversity as several studies have demonstrated a decrease in diversity while others have not.

With ART, the gut microbiome shifts towards a composition more similar to HIV-negative controls in comparison to untreated HIV-positive subjects. It has been suggested that unmanaged viral replication drives mucosal inflammation leading to changes in microbial composition which may be potentially reversed with ART and the subsequent recovery of the mucosal immune system\textsuperscript{77}.

1.2.3 Lung Microbiome

With the development of culture-independent techniques for microbial identification, the lungs which have previously been considered as sterile in health are now recognized to host diverse and dynamic communities of bacteria. The composition of the lung microbiome is dependent on immigration, elimination and regional growth conditions\textsuperscript{78}. Microbial immigration occurs with microaspiration, direct mucosal dispersion and inhalation of bacteria. Microbial elimination is mediated by cough, mucociliary clearance and innate and adaptive host responses. Regional growth conditions include multiple factors such as pH, temperature, nutrients, competition from local microbes and interaction with host epithelial cells. In health, immigration and elimination are the two main driving forces behind lung microbiota but in disease states such as chronic inflammatory lung diseases and especially during exacerbations, the influence of regional growth conditions increases\textsuperscript{78}.

Within the lung, the composition and abundance of bacterial populations vary based on the anatomical location such as nasal, tracheal, bronchial or alveolar. In the nasal cavity, the
major bacterial phyla are Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria while typical bacterial genera include Moraxella, Streptococcus and Staphylococcus\textsuperscript{79}. The most common phyla of the respiratory tract are Bacteriodes, Firmicutes, Proteobacteria and the most prevalent genera include Fusobacteria, Prevotella, Streptococcus, Pseudomonas and Veillonella\textsuperscript{78}. In a study by Erb-Downward et al, bronchoalveolar lavage (BAL) samples and four to eight distinct tissue samples from each lobe were analyzed and demonstrated regional differences in the lung bacterial microbiome within an individual subject\textsuperscript{80}. Previous studies have shown that bacterial communities in the lung are small in number but diverse\textsuperscript{80–83}.

The microbiome varies in composition between healthy people but also between healthy people and patients with chronic inflammatory lung diseases such as chronic obstructive pulmonary disease (COPD), asthma and cystic fibrosis\textsuperscript{80,84–87}. Studies comparing the lung microbiome in states of health and disease have suggested shifts of microbial composition in the diseased lung. In asthma, the microbial composition in BAL samples shifts away from Bacteroidetes towards Proteobacteria\textsuperscript{88}. In comparing COPD lung tissue samples with control, Sze et al found an increase in the Firmicutes phylum due to an increase in the Lactobacillus genus in patients with very severe COPD (Global Initiative for COPD [GOLD] 4)\textsuperscript{86}.

However, it is still unclear whether chronic diseases are drivers of microbiota imbalance or if microbiota composition contributes to disease pathology. Understanding the relationship between the lung microbiome and the host in health and disease may provide the knowledge required to develop therapeutic targets or supplements to help restore important microbial species and improve lung health.
1.2.3.1 Lung Microbiome in HIV Infection

The NIH Lung HIV Microbiome Project (LHMP, 2009 to 2015) encompassed 6 clinical sites and was created to characterize the lung and respiratory tract microbiomes in order to better understand the changes and effects of the microbiome in health and disease.

Studies under the LHMP have shown the lung microbiome to be similar between HIV-uninfected patients and HIV-infected patients with relatively preserved CD4 cell counts\(^{89-92}\). However, BAL from untreated patients with advanced HIV infection demonstrated a decrease in alpha diversity (richness and diversity) and greater beta diversity in comparison to BAL from HIV-uninfected patients. These differences were mitigated with the use of HAART but were still present up to 3 years after initiation of therapy\(^{89}\). In a study by Twigg III et al, an increased abundance of Streptococcus was found in the HIV population while Flavobacterium was increased in the control population\(^{89}\). When comparing the lung microbiome of BAL from patients with advanced HIV infection and without HIV infection at baseline and after 1 year of HAART, advanced HIV patients demonstrated an increased relative abundance of Prevotella and Veillonella which are associated with lung inflammation\(^{89}\). In particular, the genus Prevotella was associated with Th17 inflammation, development of COPD and pneumonia treatment and outcomes\(^{93}\). Tropheryma whipplei was found to be more frequent in the BAL of HIV-positive patients and ART lead to a reduction in relative abundance of T. whipplei in the lung which was hypothesized to originate from the gut\(^{92}\).

The incidence of bacterial pneumonia is 5% to 30% in HIV infected patients in comparison to less than 1% in immunocompetent individuals\(^{94-97}\). Iwai et al demonstrated that bacterial pneumonia in HIV infected patients were enriched with phylogenetically diverse
bacteria including Firmicutes and Prevotellaceae while HIV-uninfected pneumonia was primarily enriched with Proteobacteria\(^9\). Alterations in the lung microbiome during HIV infection may contribute to lung complications and chronic inflammation.

### 1.2.4 Gut-Lung Axis

Patients with chronic lung diseases such as COPD and asthma have increased prevalence of gastrointestinal diseases including inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS)\(^99,100\). The recently proposed gut-lung axis may help in understanding this phenomenon. Previous studies have shown that disturbance of gut microbiota by infection or antibiotics can change inflammatory signals normally released by the microbiota, contributing to an altered immune response. Exposure to structural ligands such as lipopolysaccharide (LPS) and secreted metabolites from the healthy gut microbiota maintains a homeostatic immune response that is altered when pathogenic bacteria and metabolites stimulate circulating lymphocytes, leading to changes in systemic immune responses\(^101\). Disturbances to gut microbiota during the development of the immune system in the early years can substantially alter the immune response and may potentially lead to chronic inflammatory disorders of the gut and lung later on in life\(^101\). Studies using germ-free mice have demonstrated underdeveloped immune systems and mucosal alterations that can be restored through gut microbiota colonization\(^102,103\).

Proposed mechanisms of bacterial translocation between the gut and lung include ingested bacteria entering the lungs from the gastrointestinal tract through aspiration and direct transfer of bacteria from gut to lungs as observed when intestinal barrier integrity is compromised during sepsis and acute respiratory distress syndrome\(^104\). Entry of bacterial components and metabolites into the circulatory system can cause systemic inflammation\(^101\).
The gut-lung axis has been proposed to play a role in respiratory diseases. Risk of asthma development was shown to be elevated when the gut microbiota contained increased abundance of Bacteroides fragilis and total anaerobes in the early years of life\textsuperscript{104}. Mice with absent or depleted gut microbiota presented an impaired immune response and worsened outcomes after bacterial or viral respiratory infection\textsuperscript{104}. Gut-specific Helicobacter pylori has been linked to decreased incidence of asthma and allergy but positively associated with COPD, suggesting that H. pylori and its subsequent systemic immune response may play different roles in different lung diseases\textsuperscript{105–108}. 

Trompette et al demonstrated that short chain fatty acids (SCFA) originating from gut microbiota can affect the immune response in the lung through reducing dendritic cell promotion of Th2 proliferation by T cells\textsuperscript{109}. Oral administration of SCFA has also been shown to have anti-inflammatory effects that reduced pulmonary pathology post bacterial and viral infection in mice\textsuperscript{109}. Administration of segmented filamentous bacteria (SFB) in the gut has been shown to stimulate T helper 17 response and improve resistance to Staphylococcus aureus pneumonia\textsuperscript{110–112}. In a study by Sze et al, instillation of LPS intratracheally in the lungs led to acute changes in the gut microbiome composition demonstrating that perturbations in the lung could also lead to changes in the gut microbiome\textsuperscript{113}.

Further research on causal links in the gut-lung axis may lead to the development of prophylactic or interventional strategies to modify the microbiota or immune response to improve patient quality of life. While there have been no studies on the gut-lung axis in HIV infection thus far, research on the effects of gut microbiota on the systemic immune response
may help in understanding its potential in mitigating chronic pulmonary diseases of which are more common in HIV patients.

1.3 HIV and Aging

1.3.1 Age-Related Diseases in HIV

With the introduction of ART, HIV patients are living longer and HIV prevalence is increasing worldwide despite a decrease in new infections. Average age for patients living with HIV (PLWH) continues to increase with more than 10% of this population over 50 years of age\textsuperscript{113}. The emergence of an older HIV population at risk of chronic diseases makes it necessary to study the impact of HIV infection on aging and development of chronic diseases.

Even with controlled viral titres and preserved CD4 count, inflammation is elevated in HIV-positive patients and is associated with increased rates of renal, cardiovascular, neurocognitive, oncological and osteoporotic diseases\textsuperscript{114–117}. Mechanisms through which inflammation is increased in HIV-positive patients includes low level viral replication in tissue, chronic reactivation of herpes viruses, microbial translocation and immune dysregulation due to depletion of CD4 T cells and increased senescent CD8 T cells\textsuperscript{76,118–120}.

Whether or not accelerated aging occurs in HIV patients is still a topic of debate. HIV-positive patients experience persistently elevated inflammatory markers, senescent immune changes and increased rates of chronic co-morbidities, geriatric syndromes and frailty\textsuperscript{121}. However, in some cohorts, HIV patients on ART with suppressed viral loads and CD4 counts above 500 cells per mm\textsuperscript{3} experience similar rates of chronic diseases as the general
population\textsuperscript{122,123}. Furthermore, duration of HIV infection does not appear to be associated with an accelerated occurrence of co-morbidities\textsuperscript{124}.

Multiple studies have observed an increased risk of chronic non-AIDS conditions in HIV-positive patients in comparison to HIV-negative patients as well as high rates of co-morbidities at all ages. HIV patients present geriatric syndromes such as falls, sensory deficits and neurocognitive impairment as well as frailty earlier than uninfected individuals\textsuperscript{124}. Approximately 50\% of all HIV-positive individuals develop HIV-associated neurocognitive disorders (HAND) including myelopathy, peripheral neuropathy and dementia. However, most patients are asymptomatic while up to 12\% have mild and 2\% have severe disorders\textsuperscript{114}. Incidence of chronic renal disease is increased at all ages in comparison to uninfected individuals with risk factors such as low CD4 count, high viral loads and use of certain ART (Tenofovir)\textsuperscript{124}. Risk of chronic cardiovascular diseases including coronary artery disease, congestive heart failure and ischemic stroke with a 1.17 times increased risk of stroke and 1.5 times increased risk of acute myocardial infection at all ages\textsuperscript{125,126}. Prevalence of osteoporosis and fracture is increased in comparison to the general population with a 3-fold increase in risk of fracture\textsuperscript{127}.

An epigenetic clock using CpG DNA methylation signatures has demonstrated accelerated aging in blood of HIV-positive patients being treated with ART and South African adolescents with perinatally acquired HIV (PHIV)\textsuperscript{127}. Extrinsic epigenetic age acceleration was associated with HIV infection in the cohort of PHIV adolescents which is negatively correlated with cognitive functioning measures such as working memory, processing speed and executive functioning\textsuperscript{127}.
1.3.2 Telomere Attrition in HIV

Aging is a time-related deterioration of physiological integrity that eventually leads to impaired function and death. As summarized by López-Otin et al, the nine hallmarks of aging are genomic instability (such as DNA methylation), telomere shortening, mitochondrial dysfunction, cellular senescence, epigenetic alterations, loss of proteostasis, stem cell exhaustion, deregulated nutrient sensing and altered intercellular communication\textsuperscript{128}. In this study, telomere attrition was used as a marker of aging in ART-treated HIV-positive and HIV-negative human lung and gut tissues.

Telomeres are repetitive nucleotide sequences (TTAGGG tandem repeats) located on the ends of each chromosome to preserve chromosome stability and prevent contact with neighbouring chromosomes. At birth, telomeres range from 5,000 to 15,000 base pairs and shorten with each round of cell division by 25 to 200 base pairs\textsuperscript{129,130}. This limits the proliferation of human cells by inducing replicative senescence, differentiation or apoptosis. Telomere integrity depends on length of telomere and catalytic activity of enzyme telomerase and may also be disrupted by a variety of psychosocial, environmental and behavioural factors\textsuperscript{131}.

Multiple studies have examined telomere length in HIV infection. Liu et al demonstrated that mean absolute telomere length (aTL) in peripheral leukocytes of HIV-infected individuals was 27 kilobase pairs per genome shorter than the mean aTL of uninfected individuals. Slopes of aTL versus age were not different between HIV-infected and uninfected individuals. Active hepatitis C virus infection, smoking, worse CT emphysema severity score and reduced FEV1 during HIV infection have all been associated with shorter telomere length\textsuperscript{132}. Pathai et al also
demonstrated significantly shorter telomeres in peripheral blood leukocytes of HIV-infected individuals compared to HIV-seronegative individuals in a South African cohort\textsuperscript{133}. In small airway epithelium, Xu et al showed that telomere length was significantly decreased in patients living with HIV (PLWH) in comparison to HIV-negative patients with adjustments for age, sex and smoking pack-years\textsuperscript{134}. Leung et al observed telomere shortening and methylation changes in peripheral blood during the short term period immediately following HIV seroconversion but not between post-HIV seroconversion and a later follow-up time point\textsuperscript{135}. Shortening of telomere length in peripheral blood after HIV seroconversion was also demonstrated by Gonzalez-Serna et al\textsuperscript{136}. From current literature, accelerated telomere length shortening seems to occur immediately after HIV infection but not during HIV infection.
Chapter 2: Experimental Approach

2.1 Working Hypothesis

The working hypothesis is that ART-treated HIV infection leads to shifts in the compositions of microbiomes in the gut-lung axis and these shifts are associated with changes in host response such as CD4 count, viral load and telomere length.

2.1.1 Specific Aims

To test the hypothesis, experiments were designed to address the following specific aims:

1) Quantify total bacterial population in the lung and small intestine tissue of HIV and non-HIV donors

2) Analyze the effects of HIV infection on the microbiome of lung and small intestine

3) Compare lung and small intestine microbiomes to host characteristics such as telomere length, CD4 count and viral load
Chapter 3: Methods

3.1 Introduction (Cohort)

HIV-positive and HIV-negative human lung and gut tissue samples were obtained from autopsies through the National NeuroAIDS Tissue Consortium (NNTC). Established in 1998, the NNTC collects, stores and distributes samples of central and peripheral nervous system tissue, blood, lung, small intestine and other organs from HIV-positive and negative patients. The NNTC is funded by the National Institute of Mental Health (NIMH) and the National Institute of Neurological Disorders and Stroke (NINDS) to support researchers conducting HIV research. The consortium is composed of four clinical sites: University of Texas (Galveston), University of California San Diego (UCSD), University of California Los Angeles (UCLA) and the Mount Sinai Medical Center (New York). For this study, samples originated from UCLA, Mount Sinai and Texas. Donors were HIV-positive patients previously on ART and HIV-negative patients who had expressed consent for tissue to be used for research purposes. To protect patient privacy, precautions were taken by utilizing unique identifiers for each donor. Procedures detailed by the NNTC were followed for extracting, characterizing, processing and storing tissue post-mortem. Clinical data including HIV status, age at death, gender, organ freeze time, medical history, CD4\(^+\) count, viral load and lung and gut pathology was provided by the NNTC.

<table>
<thead>
<tr>
<th>Clinical Site</th>
<th>Lung</th>
<th>Small Intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIV +</td>
<td>HIV -</td>
</tr>
<tr>
<td>University of California Los Angeles</td>
<td>24</td>
<td>17</td>
</tr>
<tr>
<td>Mount Sinai Medical Center</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>University of Texas</td>
<td>2</td>
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</table>

Table 1. Summary of lung and small intestine samples by clinical site.
Table 2. Summary of study cohort demographics. Statistical analysis performed using student t-test on age at death and organ freeze time and chi-squared test on gender.

<table>
<thead>
<tr>
<th></th>
<th>HIV Positive</th>
<th>HIV Negative</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects</td>
<td>29</td>
<td>19</td>
<td>--</td>
</tr>
<tr>
<td>Age at Death (yrs)</td>
<td>49.6 ± 9.2</td>
<td>61.5 ± 13.5</td>
<td>0.002</td>
</tr>
<tr>
<td>Gender (M:F)</td>
<td>23 : 6</td>
<td>6 : 13</td>
<td>0.012</td>
</tr>
<tr>
<td>Organ Freeze Time (hrs)</td>
<td>8.1 ± 7.4</td>
<td>5.7 ± 3.7</td>
<td>0.124</td>
</tr>
<tr>
<td>CD4$^+$ T cells (cells/mm$^3$)</td>
<td>163.9 ± 213.6</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Detectable Viral Load (%)</td>
<td>73.1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Viral Load (copies/mL)</td>
<td>286473 ± 856709</td>
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</tr>
</tbody>
</table>

Figure 1. Histology of small intestine (left) and lung (right) from cohort.

3.2 Study Design

In this study, DNA was extracted from lung and gut tissue to capture both topical and trans-epithelial microbial composition. Using lung tissue helps reduce the possibility of contamination by fluids from the oral and nasal passages. An advantage in using tissue to
characterize the microbiome is the ability to measure tissue response in the same DNA sample. 54 samples from NNTC were initially used in the study and underwent DNA extraction. NanoDrop and Quant-iT dsDNA high sensitivity assay were then used to determine DNA quality and quantity respectively. After verifying the DNA samples were of satisfactory quality, the samples were diluted separately to the appropriate concentrations or dilution factors for 16S DNA ddPCR, telomere qPCR and 16S DNA MiSeq.

The first experiment of the study involved quantifying the 16S rRNA gene using ddPCR to determine bacterial load. This also gave an idea of what sequence counts could be expected after MiSeq sequencing. Samples were sent to the University of British Columbia Sequencing and Bioinformatics Consortium after library preparation, validation and dilution was completed. From the data generated by MiSeq, alpha, beta diversities and prominence of specific bacteria in experimental groups were investigated using Quantitative Insights Into Microbial Ecology (QIIME2) and various packages from R including phyloseq and vegan. Absolute telomere length was measured using qPCR to investigate changes in cellular senescence in HIV-positive donors in comparison to HIV-negative donors and whether telomere length could be associated with different microbial compositions.

![Experimental workflow of study.](image)
3.2.1 16S rRNA Gene

16S ribosomal RNA is a component of the 30S small subunit of prokaryotic ribosomes and is present in all bacteria. The 16S rRNA gene is widely used in the study of bacteria and microbiomes as it contains nine hypervariable regions (V1 to V9) and conserved regions in between the hypervariable regions. The nine hypervariable regions can help differentiate between bacteria from the phylum to genus levels and in some cases even the species level can be identified. The conserved regions can be used as a target for PCR primers to amplify various combinations of the variable regions for quantitative and qualitative purposes such as investigating bacterial load and microbial community composition. Several databases of 16S rRNA gene sequences exist and this study used the SILVA database to align and classify sequences.

3.3 DNA Extraction and Storage (NNTC Tissue, numbering)

After receiving tissue samples from the NNTC, samples were documented digitally and stored in the -80°C freezer. Lung and gut tissue samples were cut in the biosafety cabinet on a clean plastic surface on dry ice to maintain sample integrity and to ensure samples were kept frozen. Instruments used to excise tissue as well as excision platforms were cleaned with ethanol followed by Cavicide™ (general viral and bacterial decontaminant) after each sample. Approximately 60 mg of sample tissue was cut from each sample, weighed by analytical balance and placed into a previously prepared clean 2 mL screw top micro tube with a single metal bead.

DNA extraction was performed following the user-developed protocol “Purification of total DNA from soft tissues using the TissueLyser and the DNeasy® Blood & Tissue Kit found through Qiagen® (Maryland, USA) with the following exceptions. Tissue homogenization (step
4) was performed using the “Purification of DNA from Animal and Human Tissues” protocol (pages 23 to 24) from the Qiagen® TissueLyser LT Handbook (May 2009). Incubation at 56°C in a shaker incubator was performed overnight, 4 μL of RNase (100 mg/mL) was added after 56°C overnight incubation and 100 μL of AE buffer (elution buffer comprised of 10 mM Tris-Cl and 0.5 mM EDTA; pH 9.0 from Qiagen®) was used to elute all samples. Extraction negative controls were included in the tissue extraction process to account for potential contamination in disposables and reagents.

After DNA extraction, NanoDrop (Delaware, USA) was utilized to determine quality and estimate quantity through the use of optical density (OD) 260/280, OD 260/230 and OD 260 measurements. The 260/280 ratio is used to assess purity of nucleic acids with a ratio of approximately 1.8 being accepted as “pure” DNA. The 260/230 ratio is a secondary measure of nucleic acid purity with values typically in the 2.0 to 2.2 range. OD 260 is used to determine the quantity of nucleic acid in a sample. DNA concentration were determined using Quant-iT™ dsDNA High-Sensitivity Assay Kit (Thermo Fisher Scientific, Massachusetts, USA) and comparing samples to a standard curve after measuring fluorescence at 485/530 nm.

All samples were placed into 4°C after DNA extraction to avoid repeated freeze and thaw cycles. After completion of MiSeq library preparation, 16S droplet digital polymerase chain reaction (ddPCR) and telomere quantitative polymerase chain reaction (qPCR) samples were organized and placed into -80°C freezer for long-term storage.
3.4 Experiment 1: Bacterial Load

3.4.1 ddPCR Theory and Conditions

Droplet digital PCR was used to examine bacterial load in samples. Small intestine DNA samples were sequentially diluted to 1:1000 and lung DNA samples were diluted 1:50. Prior to droplet formation, the PCR reaction volume was 20 µL with the following components: 10 µL of BioRad 2x QX200™ ddPCR™ EvaGreen® Supermix, 0.2 µL of 10 µM 16S forward primer (63F), 0.2 µL of 10 µM 16S reverse primer (355R), 7.6 µL DNase/RNase free water and 2 µL DNA template. Using the QX200™ Droplet Generator, 70 µL of droplet generation oil was dispensed into the DG8 droplet generator cartridges along with 20 µL of PCR reaction in separate wells. The sample is then partitioned into approximately 20,000 nano-liter sized droplets using water-oil emulsion with a final volume of 40 µL. All samples including plate controls, water negative and extraction negatives were run in duplicate. The plate is then sealed with PCR foil at 180 °C and then placed into the Bio-Rad MyCycler Thermal Cycler to amplify the 16S region according to the following conditions:

1 x (5 minutes at 95°C)

40 x (30 seconds at 95°C, 1 minute at 60°C)

Ramp rate 2°C/second between temperature changes

1 x (5 minutes at 4°C)

1 x (5 minutes at 90°C)
The 16S rRNA gene V1-V2 region was amplified using universal 16S primers 63F and 355R.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5’ – 3’)</th>
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<tbody>
<tr>
<td>63F</td>
<td>GCAGGCCTAACACATGCAAGTC</td>
</tr>
<tr>
<td>355R</td>
<td>CTGCTGCCTCCCGTAGGAGT</td>
</tr>
</tbody>
</table>

Table 3. Forward and reverse primer sequences used in 16S rRNA ddPCR.

During PCR, the inactive form of EvaGreen was heat-activated into the active variation which forms a complex with hybridized DNA. After thermal cycling, samples were analyzed using the QX200™ Droplet Reader which analyzes each droplet individually using a binary detection system to differentiate between PCR-positive and PCR-negative droplets based on fluorescence. Determining the amount of PCR-positive droplets then allows the absolute quantification of 16S DNA template concentration in the original sample.

3.4.2 ddPCR Data Analysis

The QX200™ Droplet Reader reads approximately 12,000 to 16,000 droplets and connects to the QuantaSoft™ Software which was used for assembling plate layout and data acquisition. Quality control was performed by ensuring that the droplet count was above 12,000 for each sample and observing a clear separation in fluorescence amplitude between PCR-positive and PCR-negative droplets. Using a threshold of 10,000 fluorescent units to differentiate between PCR-positive and PCR-negative droplets, copies per microliter (µL) was generated through the software and further adjusted for dilution factor, plate differences and extraction negative. Previously determined DNA concentrations were then used to normalize the results to copies per nanogram of DNA to account for variation in initial DNA concentrations.
3.5 Experiment 2: Telomere qPCR

3.5.1 qPCR Theory and Conditions

Quantitative PCR was used to determine absolute telomere length in lung and small intestine DNA samples using the protocol described by O’Callaghan and Fenech (2011)\textsuperscript{137}. Samples were diluted to 5 ng/µL excluding those that had initial concentrations below 5 ng/µL. The final qPCR reaction volume was 20 µL and included the following: 10 µL 2 x Bio-Rad iTaq\textsuperscript{™} Universal SYBR\textsuperscript{®} Green Supermix, 1µL of 2 µM forward primer, 1 µL of 2 µM reverse primer, 4 µL RNase free water and 4 µL DNA template at 5 ng/µL. Samples with concentrations below 5 ng/µL were added individually with adjustment of water volume so that total DNA amount was 20 ng in each reaction.
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Species</th>
<th>Primer Sequence (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>teloF</td>
<td>Human/rodent</td>
<td>CGTTTGGTTTTGGGTTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT</td>
</tr>
<tr>
<td>teloR</td>
<td>Human/rodent</td>
<td>GGCTTGCCTACCCCTTACCCCTTACCCCTTACCCCTTACCCCTTACCCCT</td>
</tr>
<tr>
<td>36B4F</td>
<td>Human</td>
<td>CAGCAAGTGGAAGGTGTAATCC</td>
</tr>
<tr>
<td>36B4R</td>
<td>Human</td>
<td>CCCATTCTATCATCAACGGGTACAA</td>
</tr>
</tbody>
</table>

Table 4. Telomere and 36B4 primers.

For the telomere standard, a 10-fold serial dilution of 1 ng/µL stock telomere concentration was performed with 8 standard points and the first point starting at the 1:10 dilution. The single copy gene standard used was 36B4 which encodes the acidic ribosomal phosphoprotein P0 (RPLP0) and was used to determine genome copies per sample. For the 36B4 standard, a 4-fold serial dilution of 40 pg/µL stock 36B4 concentration was performed with 8 standard points and the first point starting at the 1:16 dilution.

Standard sequences and absolute telomere qPCR standards kB calculations shown below:

Telomere standard 84 bp: (TTAGGG)\(^{14}\)

36B4 standard 75 bp: CAGCAAGTGGAAGGTGTAATCCGTCTCCACAGACAAAGGCGG GACTCGTTTGTACCGTTGATGATAGAATGGG

Telomere standard starting concentration: 1 ng/µL (use 4 ng/reaction)

\[
\frac{4 \times 10^{-9} g}{0.44 \times 10^{-19} g \text{ (telomere oligomer weight)}} = 9.09 \times 10^{10} \text{(molecules of telomere oligomer) \times 84 (oligomer length)}
\]

= 7.64 \times 10^{9}kB

36B4 standard starting concentration: 4 pg/µL (use 16 pg/reaction)

\[
\frac{16 \times 10^{-12} g}{0.38 \times 10^{-19} g \text{ (36B4 oligomer weight)}} = \frac{4.211 \times 10^{8} \text{(molecules of 36B4 oligomer)}}{2 \text{ (diploid copies)}}
\]

= 2.11 \times 10^{8} \text{diploid copies}
<table>
<thead>
<tr>
<th>Telomere Standard</th>
<th>36B4 Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution</td>
<td>Concentration</td>
</tr>
<tr>
<td>Stock</td>
<td>1 ng/uL</td>
</tr>
<tr>
<td>1:10</td>
<td>0.1 ng/uL</td>
</tr>
<tr>
<td>1:100</td>
<td>10 pg/uL</td>
</tr>
<tr>
<td>1:1000</td>
<td>1 pg/uL</td>
</tr>
<tr>
<td>1:10000</td>
<td>0.1 pg/uL</td>
</tr>
<tr>
<td>1:100,000</td>
<td>10 fg/uL</td>
</tr>
<tr>
<td>1:1,000,000</td>
<td>1 fg/uL</td>
</tr>
<tr>
<td>1:10,000,000</td>
<td>0.1 fg/uL</td>
</tr>
<tr>
<td>1:100,000,000</td>
<td>10 ag/uL</td>
</tr>
<tr>
<td>1:1,000,000,000</td>
<td>100 ag/uL</td>
</tr>
</tbody>
</table>

**Table 5.** Telomere and 36B4 standard curve concentrations. Eight standard points were used in both telomere (1:10 dilution to 1:100,000,000 dilution) and single copy gene 36B4 (1:16 dilution to 1:262,144 dilution) standards.

Samples were run in triplicate using telomere and 36B4 primers separately on the same 384-well plate. Positive controls included K562 (human chronic myelogenous leukemia cell line) DNA, HEK293 (human embryonic kidney cell line) DNA and two human controls extracted from buffy coat. RNase free water was used as no template control. qPCR was run on Bio-Rad CFX96 Touch™ Real-Time PCR Detection System according to the following conditions:

1 x (10 minutes at 95°C)

40 x (15 seconds at 95°C, 1 minute at 60°C)

Melt curve

Quality control was performed by ensuring that standard curves had at least 5 points, an efficiency value between the range of 90% to 110%, an R-squared value of above 0.98 and the
no template control had a quantitation cycle (Cq) value above 30. All measurements had to be within range of the standard curve and Cq standard deviations less than 1.000 between triplicates.

![Standard Curve](image.png)

**Figure 4.** Example of experimental run to measure absolute telomere length using qPCR.

### 3.5.2 qPCR Telomere Data Analysis

Starting quantity (SQ) of both telomere and 36B4 were calculated from the standard curve and Cq values. Absolute telomere length (aTL) was then calculated using the following method:

\[
\frac{SQ_{Telomere}}{SQ_{36B4}} = \frac{kB}{SCG}
\]

\[
\frac{Total \ length \ (kB/SCG)}{92 \ (number \ of \ telomeres \ in \ 23 \ pairs \ of \ chromosomes)} = length \ per \ telomere \ (kB)
\]

To correct for plate-to-plate variability, positive controls were run on each plate and the ratios between plates were used to normalize aTL results.
3.6  Experiment 3: Illumina Miseq Sequencing

3.6.1  MiSeq Sequencing Platform

The Illumina MiSeq next generation sequencer was used to characterize bacterial community composition in the lung and small intestine tissue of HIV-positive and HIV-negative donors. The Illumina NGS workflow consisted of four basic steps: library preparation, cluster generation, sequencing and data analysis. Library preparation, which will be detailed in the next section, consisted of amplifying the target of interest and labeling the individual samples with unique barcode combinations and adapter fragments to allow for high-throughput. Cluster generation involved loading the library into a flow cell, ligation of library adaptors to complementary surface-bound oligos and bridge amplification of bound fragments into distinct, clonal clusters.

Illumina uses sequencing by synthesis technology in which four fluorescently-labeled nucleotides are used to sequence the clonal clusters on the flow cell surface. In a single sequencing cycle, a labeled deoxyribonucleotide triphosphate (dNTP) with a reversible terminator was added onto the end of the nucleic acid chain. The reversible terminator inhibited polymerization which allowed the fluorescent dye to be imaged after each cycle to identify the base by different colours of fluorescence. The terminator was then enzymatically cleaved to permit incorporation of the subsequent dNTP. This cycle was then repeated for 250 cycles to fully sequence the forward and reverse reads amplified during library preparation. QIIME2, a next-generation microbiome bioinformatics platform and SILVA, an rRNA database were then used for processing, alignment and sequence quality control. Further data analysis was performed using packages phyloseq and vegan in R studio.
3.6.2 Library Preparation

MiSeq library preparation was performed by adapting the Schloss laboratory dual-index sequencing protocol published by Kozich et al\textsuperscript{138}. The V4 region of the 16S rRNA gene was used as the target for MiSeq sequencing of the microbiome. After DNA extraction of samples, touchdown PCR was used to amplify the target gene and attach sequence identifiers and adaptors. The touchdown technique was chosen to increase specificity by starting at an annealing temperature higher than the optimal and gradually decreasing the temperature in subsequent cycles. At temperatures higher than the calculated optimal temperature, the primer and template bind more specifically. As the temperature gradually lowers in subsequent cycles, annealing of primer to template becomes less specific but more frequent allowing increased amplification of amplicons created in the earlier high-temperature cycles.

The final touchdown PCR reaction volume was 20 µL and included the following: 2 µL of 10x Buffer II, 0.15 µL of AccuPrime Taq Polymerase, 2 µL of 10µM reverse and forward primer combination, and a combined 15.85 µL of extracted DNA sample and RNase free water adjusted for an input of 20 ng of DNA due to varying concentrations of extracted DNA. Touchdown PCR was run on the Bio-Rad Mycycler Thermal Cycler using the following conditions adapted from a protocol by Korbie & Mattick:

1 x (2 minutes at 95°C)

20 x (20 seconds at 95°C, 15 seconds at 60°C/54°C, 90 seconds at 72 °C)

Temperature decrease of 0.3°C at each cycle from 60°C to 54°C

20 x (20 seconds at 95°C, 15 seconds at 55°C, 90 seconds at 72 °C)

1 x (5 minutes at 72°C)
The samples are run along with a negative control (water) and positive control (mock community). Final product including adaptor, barcode, pad, link, primer and target sequence was approximately 400 base pairs with the target sequence being 250 base pairs long.

<table>
<thead>
<tr>
<th>Forward Primers (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA501 AATGATACGCGACCACCAGATCTACACATCGTAGTTATGGTAATTGTGTGCAGCMGCCGCCGCTAA</td>
</tr>
<tr>
<td>SA502 AATGATACGCGACCACCAGATCTACACAATCTATCTGTATGGTAATTGTGTGCAGCMGCCGCCGCTAA</td>
</tr>
<tr>
<td>SA503 AATGATACGCGACCACCAGATCTACACTACATCGAGTTATGGTAATTGTGTGCAGCMGCCGCCGCTAA</td>
</tr>
<tr>
<td>SA504 AATGATACGCGACCACCAGATCTACACTAGCGTTATGGTAATTGTGTGCAGCMGCCGCCGCTAA</td>
</tr>
<tr>
<td>SA505 AATGATACGCGACCACCAGATCTACACTCTGCTAATGGTAATTGTGTGCAGCMGCCGCCGCTAA</td>
</tr>
<tr>
<td>SA506 AATGATACGCGACCACCAGATCTACACTAGCGTTATGGTAATTGTGTGCAGCMGCCGCCGCTAA</td>
</tr>
<tr>
<td>SA507 AATGATACGCGACCACCAGATCTACACTAGCGTTATGGTAATTGTGTGCAGCMGCCGCCGCTAA</td>
</tr>
<tr>
<td>SA508 AATGATACGCGACCACCAGATCTACACTAGCGTTATGGTAATTGTGTGCAGCMGCCGCCGCTAA</td>
</tr>
</tbody>
</table>

**Table 6.** List of forward sequencing primers adapted from the Schloss Lab (Adaptor, Barcode, Pad, Link and Primer).

<table>
<thead>
<tr>
<th>Reverse Primers (3’ to 5’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB701 CAAGCAGAAGACGGCATACGAGATAGTCGAGATTCGTACGAGCCGAATTACHVGGGTWCTTAAT</td>
</tr>
<tr>
<td>SB702 CAAGCAGAAGACGGCATACGAGATATATCTCGAGTCGTACGAGCCGAATTACHVGGGTWCTTAAT</td>
</tr>
<tr>
<td>SB703 CAAGCAGAAGACGGCATACGAGATAGCGTCTAGTCGTACGAGCCGAATTACHVGGGTWCTTAAT</td>
</tr>
<tr>
<td>SB704 CAAGCAGAAGACGGCATACGAGATATAGTCGAGTCGTACGAGCCGAATTACHVGGGTWCTTAAT</td>
</tr>
<tr>
<td>SB705 CAAGCAGAAGACGGCATACGAGATAGTCGAGTCGTACGAGCCGAATTACHVGGGTWCTTAAT</td>
</tr>
<tr>
<td>SB706 CAAGCAGAAGACGGCATACGAGATCTCGTATAGTCGTACGAGCCGAATTACHVGGGTWCTTAAT</td>
</tr>
<tr>
<td>SB707 CAAGCAGAAGACGGCATACGAGATGCGACCTGTGCTACGAGCCGAATTACHVGGGTWCTTAAT</td>
</tr>
<tr>
<td>SB708 CAAGCAGAAGACGGCATACGAGATGGTACTATAGTCGTACGAGCCGAATTACHVGGGTWCTTAAT</td>
</tr>
<tr>
<td>SB709 CAAGCAGAAGACGGCATACGAGATTATACGAGTCGTACGAGCCGAATTACHVGGGTWCTTAAT</td>
</tr>
<tr>
<td>SB710 CAAGCAGAAGACGGCATACGAGATACGAGTCGTACGAGCCGAATTACHVGGGTWCTTAAT</td>
</tr>
<tr>
<td>SB711 CAAGCAGAAGACGGCATACGAGATACGAGTCGTACGAGCCGAATTACHVGGGTWCTTAAT</td>
</tr>
<tr>
<td>SB712 CAAGCAGAAGACGGCATACGAGATCGCTACGAGTCGTACGAGCCGAATTACHVGGGTWCTTAAT</td>
</tr>
</tbody>
</table>

**Table 7.** List of reverse sequencing primers adapted from the Schloss Lab (Adaptor, Barcode, Pad, Link and Primer).
All samples then underwent 1.5% agarose gel electrophoresis to check that the target amplicon was successfully amplified and no additional bands were present.

![Image of agarose gel electrophoresis](image)

**Figure 5.** Example of 1.5% agarose gel ran to visual PCR product with 100 base pair (bp) DNA ladder.

The Invitrogen SequalPrep Plate Normalization Kit was then used for library clean up and normalization of all samples to 5 ng. AMPure XP system magnetic beads (Beckman Coulter, Brea, California) were used to clean up the library sample by removing nucleotides, primers, and components that were not the target amplicon. Quality control was performed using the DNA 1000 assay and Agilent Bioanalyzer 2100 (Agilent Technologies Inc, Santa Clara, CA, USA) to determine whether the amplicon was the correct size, sample purity and approximate concentration.
Figure 6. DNA 1000 Assay on Agilent Bioanalyzer 2100 to check for amplicon size, sample purity and concentration.

Actual concentration was measured by Quant-iT dsDNA high sensitivity assay and then diluted to 4 µM with 9.0 pH TE buffer. The prepared sample was then sent for Illumina MiSeq sequencing (Illumina, Redwood City, California) with 2 x 250 paired end read chemistry at the UBC Sequencing and Bioinformatics Consortium by Dr. Sunita Sunha.

3.6.3 QIIME2 16S Sequencing Data Analysis

16S sequencing data was analysed using next-generation microbiome bioinformatics platform QIIME 2™ (2018.4) for clean-up of raw sequencing data. Raw sequencing data was imported as fastq.gz files and sequence quality control and feature table construction were
completed using the Divisive Amplicon Denoising Algorithm 2 (DADA2) QIIME 2 plugin. The DADA2 pipeline detects and corrects sequence data and filters out PhiX and chimeric sequences. PhiX was used as a control library to provide quality control for cluster generation and sequencing for the Illumina MiSeq run. Chimeric sequences are artifacts created during PCR and contain DNA from two or more parent sequences. When an amplicon terminates prematurely, it may be extended again in a subsequent cycle if another DNA strand with a similar starting region attaches onto the existing amplicon. Chimeric sequences can lead to the false “discovery” of novel organisms and are removed during sequence quality control.

Sequences were aligned to the SILVA database (version) for identification of bacteria based on 16S. All samples were subsampled to 2486 sequence reads for further analysis in R studio (version 1.1.453, R version 3.5.0) using packages phyloseq (version 1.25.2) and vegan (version 2.5-2) to determine alpha and beta diversities. Alpha diversity was measured by richness (total number of features), evenness (similarity in numbers of individual features) and Shannon diversity index which accounts for both richness and evenness. Beta diversity was measured using a principal coordinates analysis of weighted Unifrac distances. Distribution of features at the phylum and genus taxonomic levels and comparison of relative abundance in specific phyla and genera was investigated.

3.7 Troubleshooting

As previously mentioned, this study originally consisted of 54 samples from the NNTC cohort. After completion of 16S ddPCR, qPCR telomere and 16S MiSeq, a request for additional samples was made to the NNTC and 32 more samples were processed for analysis. The initial 54
samples were re-extracted to reduce batch effect and all samples went through the same pipeline of experiments as previously mentioned.

One limitation of the study was that the NNTC performed the autopsies and therefore there was no method to define where specifically in the lung and small intestine the tissues are from. The lung tissue may have originated from different areas and may therefore have differences in microbial composition as shown through previous studies on locations differing in microbial composition in the lung. Another limitation was the inability to control organ freeze time and potential contaminants from autopsy using negative controls such as instrument washes.

3.7.1 Experiment 1: Bacterial Load

Experimental conditions for ddPCR were optimized through testing one sample each of high, medium and low DNA concentrations at different dilutions for both lung and small intestine samples. 1:1000 dilution (through 1:10 serial dilutions) for small intestine samples and 1:50 dilution for lung samples were determined to be the optimal settings for sufficient separation of PCR-positive and PCR-negative droplets.

3.7.2 Experiment 2: qPCR Telomere

Telomere and single copy gene 36B4 standard curve optimization tests were run prior to sample measurement to determine the optimal serial dilution and starting concentration. However, after comparing Cq values to earlier experiments, it was determined that the previous stock for telomere and 36B4 standards were underperforming which may have been due to DNA degradation from storage and freeze-thaw cycles. New stocks of telomere and 36B4 standards
(Integrated DNA Technologies, Coralville, Iowa) were ordered, reconstituted and aliquoted into working concentrations.

**Figure 7.** Telomere standard optimization

**Figure 8.** 36B4 standard optimization
3.7.3 Experiment 3: 16S MiSeq

Prior to the MiSeq library preparation method mentioned above, the “16S Metagenomic Sequencing Library Preparation” protocol from Illumina was used. This protocol amplified the V3V4 region of 16S and used 2 x 300 paired end read chemistry to sequence the prepared library. However, this resulted in a lower overlap of the paired end reads and increased error rates compared to previous studies (based on sequenced Mock community). Therefore, the Schloss laboratory MiSeq protocol amplifying the V4 regions was utilized for the final results. Experimental optimizations for library preparation included testing for optimal input DNA concentration for touchdown PCR and re-extracting samples with very low DNA concentrations in initial extractions.
Chapter 4: Results

4.1 Experiment 1: 16S Bacterial Load

Bacterial load was expressed as 16S copies amplified normalized to total nanograms of DNA measured on the ddPCR platform. For lung samples, there were 29 HIV-positive samples and 21 HIV-negative samples. For small intestine samples, there were 22 HIV-positive samples and 12 HIV-negative samples. Out of 14 HIV-negative small intestine samples, two samples were deemed to be outliers and removed from the analysis. A two-way analysis of variance (ANOVA) was applied in the figure below, demonstrating that HIV status did not have a significant effect on 16S bacterial load while location of sample (lung or small intestine) had a statistically significant effect.

![16S Quantification](image)

**Figure 9.** Bacterial load in lung and small intestine of HIV-positive and HIV-negative donors by 16S rRNA. Error bars represent standard error of mean. Statistical analysis completed using two-way ANOVA.
To determine whether 16S bacterial load in lung and small intestine were correlated, sample values measured by ddPCR were graphed on the x- and y-axis respectively. Two samples were omitted as outliers as previously mentioned. The figure below shows evidence of correlation between lung and small intestine 16S quantification (p=0.039) using a linear regression model.

![Figure 10. 16s rRNA bacterial load correlation between lung and small intestine samples. Statistical analysis completed using linear regression.](image)

### 4.2 Experiment 2: Absolute Telomere Length

Absolute telomere length (aTL) was measured in kilobases per genome using qPCR targeting the telomere gene and normalizing to single copy gene (SCG) 36B4. In the lung, aTL was found to be significantly shorter in HIV-positive donors in comparison to HIV-negative donors (p=0.039) while aTL did not differ between HIV-positive and HIV-negative donors in the small intestine.
Figure 11. Comparison of absolute telomere length (kilobases per SCG 36B4) between HIV-positive and HIV-negative donors in lung and small intestine. Box extends from 25th to 75th percentiles and whiskers represent minimum to maximum values. Statistical analysis completed using Mann-Whitney test.

The effect of HIV status on the relationship between aTL and age was determined to be not significant in both the lung (p=0.449) and small intestine (p=0.066) using linear regression and analysis of covariance.
Figure 12. Effect of HIV status on relationship between aTL and age in lung and small intestine. Statistical analysis completed using linear regression on aTL and age and ANCOVA on difference between slopes generated from HIV-positive and HIV-negative donors.

There was no correlation between bacterial load and aTL in both lung and small intestine of HIV-positive and HIV-negative donors.
4.3 Experiment 3: 16S MiSeq Sequencing

4.3.1 Lung

4.3.1.1 Lung Alpha and Beta Diversity

Alpha diversity measures of observed ASVs (number present), Shannon and Simpson indices were used to quantify diversity within individual lung samples. As previously mentioned, ASVs are categorizations of sequences in QIIME2 and used to identify sequences through alignment with 16S databases. Shannon and Simpson indices are methods of calculating in-sample diversity by accounting for both number of species (richness) and distribution of species.
evenness). Microbiomes of HIV-positive and HIV-negative donors did not differ in terms of observed ASVs, Shannon index and Simpson index with p-values of 0.760, 0.401 and 0.401 respectively.

**Figure 14.** Alpha diversity measures comparing microbiota in lungs of HIV-positive to HIV-negative donors. Comparisons of observed ASVs, Shannon index and Simpson index yielded p-values of 0.760, 0.401 and 0.401 respectively. Statistical analysis completed using Wilcoxon rank sum test with continuity correction.

Beta diversity determines dissimilarities between individual samples using ordination methods to demonstrate differences in microbial composition visually. In this study, a principle coordinate analysis (PCoA) and weighted UniFrac methods were used to visualize the microbiome data. Principle coordinate analysis is a method to visualize dissimilarities using a distance matrix and assigns each sample a location in low-dimensional space. UniFrac is a distance matrix used to compare communities and incorporates information on phylogenetic distances between observed organisms. Weighted UniFrac incorporates abundances of ASVs,
diminishing the impact of low abundance features. In the lung, HIV-positive and HIV-negative microbial compositions were not different with a p-value of 0.799 using permutational multivariate analysis of variance (PERMANOVA).

**Figure 15.** PCoA plot using weighted unifrac of microbial compositions in lung of HIV-positive and HIV-negative donors. Statistical analysis completed using PERMANOVA (p=0.799).

Further beta diversity analysis of microbial compositions based on telomere length, viral load, CD4 count and clinical site were performed. Telomere length was separated into two groups with samples over and less than 250 kilobases per genome which was chosen as it gave the most even separation of samples. Viral load was separated by samples with less than or more than 75 HIV copies per milliliter as this was the lowest detectable amount in this database. CD4 count was grouped by samples under and over 200 cells per mm$^3$ as patients with a CD4 count of under 200 cells per mm$^3$ are diagnosed with AIDS.
Microbial compositions did not differ based as telomere length, viral load, CD4 count and clinical site with PERMANOVA generated p-values of 0.987, 0.191, 0.903 and 0.228 respectively.

Figure 16. PCoA plot using weighted unifrac of microbial compositions in lungs categorized by telomere length (aTL using kB/SCG 36B4), viral load (copies/mL), CD4 count (cells/mm$^3$) and clinical site (p-values 0.987, 0.191, 0.903 and 0.228 respectively). Statistical analysis completed using PERMANOVA.
4.3.1.2 Lung Taxonomic Analysis

The figure below shows the relative abundance of major phyla in the lungs of HIV-positive and HIV-negative donors. In both groups Firmicutes and Proteobacteria made up the largest proportion of the microbial community with the rest being mostly Bacteroides, Actinobacteria, Fusobacteria and Verrucomicrobia. Phyla with relative abundance lower than 1% were grouped into the category “Others”.

![Lung-Phyla Composition](image)

**Figure 17.** Phyla composition of microbiota in lungs of HIV-positive and HIV-negative donors.

However, statistical analysis using the Wilcoxon rank sum test with continuity correction demonstrated that relative abundance of all major phyla did not differ between HIV-positive and HIV-negative donors in the lung.
<table>
<thead>
<tr>
<th>Phylum</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Firmicutes</td>
<td>0.504</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>1.000</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>0.656</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>0.768</td>
</tr>
<tr>
<td>Fusobacteria</td>
<td>0.172</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>0.428</td>
</tr>
</tbody>
</table>

**Table 8.** Comparison of major phyla of microbiota in lungs. Comparison between HIV-positive and HIV-negative donors completed using Wilcoxon rank sum test with continuity correction.

Comparison of top 15 genera between HIV-positive and HIV-negative donors demonstrated no significant differences in relative abundance as shown in the table below.

<table>
<thead>
<tr>
<th>Genus</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia-Shigella</td>
<td>0.869</td>
</tr>
<tr>
<td>Streptococcus</td>
<td>0.533</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>0.834</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td>0.475</td>
</tr>
<tr>
<td>Veillonella</td>
<td>0.407</td>
</tr>
<tr>
<td>Enterococcus</td>
<td>0.599</td>
</tr>
<tr>
<td>Paenibacillus</td>
<td>0.357</td>
</tr>
<tr>
<td>Prevotella</td>
<td>0.557</td>
</tr>
<tr>
<td>Prevotella 7</td>
<td>0.983</td>
</tr>
<tr>
<td>Corynebacterium 1</td>
<td>0.524</td>
</tr>
<tr>
<td>Proteus</td>
<td>0.332</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>0.468</td>
</tr>
<tr>
<td>Fusobacterium</td>
<td>0.184</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>0.503</td>
</tr>
<tr>
<td>Pelomonas</td>
<td>0.950</td>
</tr>
</tbody>
</table>

**Table 9.** Comparison of top 15 genera of microbiota in lungs. Comparison between HIV-positive and HIV-negative donors completed using Wilcoxon rank sum test with continuity correction.

Linear discriminant analysis effect size (LEfSe) is a tool used to determine features that would most likely explain differences between groups by combining standard statistical tests with tests for biological consistency and effect relevance. Significant differences were defined as an linear-discriminant analysis (LDA) above 2. In the figure below, in which LDA represents the effect size, four features (Pasteurellales, Pastuerellaceae, Burkholderiaceae and Lactobacillus)
were identified for HIV-positive and three features (Ruminococcus torques, Eisenbergiella and Carnobacteriaceae) for HIV-negative as most likely to explain differences between the two groups.

![LEfSe analysis on lung microbiota of HIV-positive and HIV-negative donors.](image)

**Figure 18.** LEfSe analysis on lung microbiota of HIV-positive and HIV-negative donors.

### 4.3.2 Small Intestine

#### 4.3.2.1 Small Intestine Alpha and Beta Diversity

Alpha diversity measures of observed ASVs (number present), Shannon and Simpson indices were used to quantify diversity within individual small intestine samples. Microbiomes of HIV-positive and HIV-negative donors did not differ in terms of observed ASVs, Shannon index and Simpson index with p-values of 0.242, 0.713 and 0.860 respectively.
Figure 19. Alpha diversity measures comparing microbiota in small intestine of HIV-positive to HIV-negative donors. Comparisons of observed ASVs, Shannon index and Simpson index yielded p-values of 0.242, 0.713 and 0.860 respectively. Statistical analysis completed using Wilcoxon rank sum test with continuity correction.

Beta diversity analysis was performed for HIV-positive and HIV-negative microbial compositions in the small intestine which were shown to not be statistically different with a p-value of 0.799 using PERMANOVA.
Figure 20. PCoA plot using weighted unifrac of microbial compositions in small intestine of HIV-positive and HIV-negative donors. Statistical analysis completed using PERMANOVA (p=0.799).

Further beta diversity analysis of microbial compositions based on telomere length, viral load, CD4 count and clinical site were performed. Microbial compositions did not differ based as telomere length, viral load and CD4 count with PERMANOVA generated p-values of 0.641, 0.330 and 0.115 respectively. PERMANOVA on clinical site generated a p-value of 0.054, nearing significance.
**Figure 21.** PCoA plot using weighted unifrac of microbial compositions in small intestine categorized by telomere length (aTL using kB/SCG 36B4), viral load (copies/mL), CD4 count (cells/mm³) and clinical site (p-values 0.641, 0.330, 0.115 and 0.054 respectively). Statistical analysis completed using PERMANOVA.
4.3.2.2 Small Intestine Taxonomic Analysis

The figure below shows the relative abundance of major phyla in small intestine of HIV-positive and HIV-negative donors. Similar to the lung, Firmicutes and Proteobacteria made up the largest proportion of the microbial community with the rest being mostly Bacteroides, Actinobacteria, Fusobacteria and Verrucomicrobia.

![Phylum Composition Graph]

**Figure 22.** Phyla composition of microbiota in small intestine of HIV-positive and HIV-negative donors.

Statistical analysis using the Wilcoxon rank sum test with continuity correction demonstrated that relative abundance differ significantly only in the Verrucomicrobia phylum when comparing between HIV-positive and HIV-negative donors in small intestine.
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<tr>
<td>Verrucomicrobia</td>
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**Table 10.** Comparison of major phyla of microbiota in small intestine. Comparison between HIV-positive and HIV-negative donors using Wilcoxon rank sum test with continuity correction.

However, the difference in Verrucomicrobia appears to be driven by high relative abundance in a small proportion of samples as shown in the figure below.

**Figure 23.** Comparison of relative abundance of Verrucomicrobia between HIV-positive and HIV-negative donors in the small intestine.

Comparison of top 15 genera between HIV-positive and HIV-negative donors demonstrated significant differences in relative abundance of Bacteroides (p=0.021) and Akkermansia (p=0.022) and near significant (p=0.068) in Prevotella.
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<td>Phyllobacterium</td>
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<td>Faecalibacterium</td>
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</tr>
<tr>
<td>Prevotella</td>
<td>0.068</td>
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</table>

Table 11. Comparison of top 15 genera of microbiota in small intestine. Comparison between HIV-positive and HIV-negative donors using Wilcoxon rank sum test with continuity correction.

However, differences in relative abundance of both Akkermansia and Prevotella appeared to be driven by high relative abundance in a few samples as shown in the figures below.

Figure 24. Comparison of relative abundances of Akkermansia and Prevotella between HIV-positive and HIV-negative donors in the small intestine.
Differences in relative abundance of Bacteroides may be a true signal as demonstrated in the figure below.

**Figure 25.** Comparison of relative abundance of Bacteroides between HIV-positive and HIV-negative donors in the small intestine.

LEfSe analysis identified one feature (Ruminococcaceae) for HIV-positive and ten features for HIV-negative that were most likely to explain differences between the two groups.

**Figure 26.** LEfSe analysis on small intestine microbiota of HIV-positive and HIV-negative donors.
4.3.3 Gut-Lung Axis Comparison

Comparison of average ASV abundance in lung and small intestine yielded a significant relationship in both HIV-positive and HIV-negative cohorts (p<0.0001 for both). Changes in ASV abundance in the small intestine are related to changes in the same ASV in the lung.

**Figure 27.** Comparison of average ASV abundance (log transformed) in lungs and small intestine of HIV-positive and HIV-negative donors (both p<0.0001). Statistical analysis completed using linear regression. Graph shows best fit line with 95% confidence band.
Chapter 5: Discussion

5.1 Experiment 1: 16S Bacterial Load

In this study, 16S bacterial load was investigated to determine whether bacterial load changes with HIV infection. The results of this cohort demonstrated that HIV infection does not significantly alter bacterial load. Differences in bacterial load between locations (lung and small intestine) were significant as expected. Studies on the lung and gut microbiomes in HIV typically include sequencing data and relative abundances and do not compare bacterial loads between HIV-positive and HIV-negative. However, with multiple studies demonstrating the “normalizing” effect of ART on the microbiome, it is not surprising that the bacterial load does not differ in this cohort of donors who were on ART\textsuperscript{89–92}.

Evidence of correlation between lung and small intestine 16S bacterial load was shown with a p-value of 0.039 and an R-squared value of 0.126 using linear regression. Although the p-value was below 0.05, the R-squared value was low which indicates that there is some variability of the data from the linear regression model. As samples in this cohort are from autopsies, some donors had pulmonary edema, lung carcinoma and 77% and 73% of HIV-positive and HIV-negative patients had pneumonia respectively. Structural changes as well as bacterial pneumonia may have affected the bacterial load in the lung. As previously mentioned in the introduction, metabolites produced by microbiota of a specific site may affect the immune response in distal sites due to entry into systemic circulation. This could be one factor explaining the correlation between bacterial load in the small intestine and lung in this project.

One of the limitations of this experiment is the inability to dilute lung and small intestine samples to the same dilution which may create bias when comparing the calculated bacterial
load. Small intestine samples without dilution typically exceed the maximum detection limit and therefore a 1:1000 dilution was tested to be the optimal dilution for clear separation. Lung tissue samples are inherently difficult to procure microbial DNA and were diluted to a 1:50 dilution which may cause potential bias when compared to the 1:1000 diluted small intestine samples.

5.2 Experiment 2: Absolute Telomere Length

Comparison of absolute telomere length in the lung and small intestine of HIV-positive and HIV-negative donors demonstrated shortened telomere length in HIV-positive lung in comparison to HIV-negative but no differences in small intestine. This was quite surprising as increased small intestine epithelial turnover has been demonstrated in HIV infection and therefore we hypothesized that aTL would be decreased in HIV-positive donors in comparison to HIV-negative donors. However, sampling location may have an effect on measured aTL as the small intestine samples from this cohort may not all contain tissue from the same cross-section or location. This is one of the limitations in this study as tissue collection was performed by clinical sites in the NNCT consortium and was therefore not controlled specifically for this project. Further comparison of bacterial load and aTL demonstrated no correlation in both lung and small intestine of HIV-positive and HIV-negative donors.

Previous studies have shown aTL to be shorter in peripheral leukocytes and small airway epithelium of HIV-infected individuals\(^{132,134}\). In peripheral blood, telomere shortening was observed immediately after HIV seroconversion but not at later time points\(^{135}\). Therefore the shortened telomere length demonstrated in the lung of HIV-positive donors may be due to attrition right after HIV infection occurs.
Analysis of the effect of HIV infection on the relationship between aTL and age was not significant in both the lung and small intestine. These results were similar to a study in peripheral lymphocytes where Liu et al demonstrated that slopes of aTL versus age did not differ between HIV-infected and uninfected patients. In the small intestine, these results agree with the previous analysis in which HIV infection did not lead to differences in aTL. In the lung, increased telomere attrition has been shown to occur early on in HIV infection and not in later stages which could be a factor in why the slopes of aTL versus age did not differ when comparing HIV-positive and HIV-negative donors.

5.3 Experiment 3: 16S MiSeq Sequencing

Alpha diversity measures of observed ASVs, Shannon and Simpson indices did not differ when HIV-positive and HIV-negative donors were compared in both lung and small intestine. Analysis of microbial composition using PCoA plot with a weighted UniFrac distance matrix showed no significant difference in microbial composition of HIV-positive and HIV-negative donors in both lung and small intestine. Analysis of beta diversity based on telomere length, viral load, CD4 count and clinical site demonstrated no differences in the lung. The small intestine also demonstrated no differences in beta diversity when samples were categorized based on telomere length, viral load and CD4 count but PERMANOVA analysis on microbial composition categorized by clinical site had a p-value of 0.054. While clinical site may indeed have an effect on microbial composition, uneven sampling depth with only 2 samples from Texas and 27 samples from UCLA may have biased the results in an analysis that compares diversity.

As donors in this cohort were on ART, these results in alpha and beta diversity measures are consistent with existing literature on the HIV lung and gut microbiome. Microbiomes
of HIV-infected patients on ART are similar to HIV-negative individuals. ART inhibits viral replication and prevents CD4 count decline, helping to avert deterioration of the immune system. Maintenance of the immune response may factor into why the microbiome of ART-treated HIV-infected individuals are comparable to uninfected individuals.

Phyla composition in both lung and small intestine consisted primarily of Firmicutes and Proteobacteria with the remainder comprising mostly of Bacteroides, Actinobacteria, Fusobacteria and Verrucomicrobia. However, comparison of individual phylum in lung and small intestine revealed no significant differences in relative abundance between HIV-positive and HIV-negative donors. Only the Verrucomicrobia phylum in small intestine had a significant difference between HIV-positive and HIV-negative donors with a p-value of 0.022. However, the difference appeared to be driven by high relative abundance in a small proportion of samples. Results on phyla composition in this study are consistent with literature comparing ART-treated HIV patients and uninfected individuals. This suggests that ART is effective in maintaining an environment necessary for a “healthy” microbiome.

In the lung, comparison between HIV-positive and HIV-negative relative abundance of the top 15 genera revealed no significant differences in relative abundance. In the small intestine, out of the top 15 genera, the Bacteroides and Akkermansia genera were significantly decreased in relative abundance in HIV-positive donors with p-values of 0.021 and 0.022 respectively. However, the difference in Akkermansia may have been driven by high relative abundance in only a few samples.
In the lung, LEfSe analysis demonstrated four features in HIV-positive (Pasteurellales, Pastuerellaceae, Burkholderiaceae and Lactobacillus) and three features in HIV-negative (Ruminococcus torques, Eisenbergiella and Carnobacteriaceae) that would most likely explain differences between the two experimental groups. One feature (Ruminococcaceae) for HIV-positive and ten features for HIV-negative were identified through LEfSe analysis in the small intestine. In particular, Bacteroidaceae, Bacteroidetes, Bacteroidia and Bacteroidales were found to be significant through LEfSe, corroborating with the previous observation of decreased Bacteroides (genus) in HIV-positive small intestine as the 4 features previously mentioned are all upstream taxonomic classifications of Bacteroides. Previous studies have also found decreased Bacteroides in HIV-positive subjects in comparison to HIV-negative subjects\textsuperscript{69}. This suggests that changes due to HIV infection, potentially in immune function, lead to decreased Bacteroides although no mechanism has been described thus far. Although other features have been discovered through LEfSe to potentially explain difference between the two experimental groups, identification of the same features in separate studies would be required as many other studies have also identified potential bacterial markers but have not been able to replicate the results.

Average abundance of individual ASV was demonstrated to be correlated in the lung and small intestine in both HIV-positive and HIV-negative groups (p<0.0001 for both). One potential explanation may be the overlap of factors that influence microbial composition such as environment, host immune system and oral sources. Whether and how communication between the two physically separate microbiomes could result in a relationship between abundance of the same bacteria in the two sites has yet to be studied.
Chapter 6: Conclusion

This project identified correlations in microbiotas of the gut-lung axis in terms of bacterial load and abundance of individual ASVs. ART-treated HIV donors did not demonstrate a significant difference in microbial composition in comparison to HIV-negative donors and categorization by host characteristics such as telomere length, CD4 count and viral load also did not reveal differences in microbial composition between the two experimental groups in both the lung and small intestine. Shortened telomere length was shown in the HIV-positive lung in comparison to the HIV-negative group but no differences were seen in small intestine.

In terms of phyla composition, HIV-positive and HIV-negative groups did not demonstrate significant differences in phyla relative abundance in both lung and small intestine. Genus Bacteroides in the small intestine was found to be decreased in HIV-positive donors. Furthermore, LEfSe identified the family Bacteroidaceae (under phylum Bacteroidetes, class Bacteroidia and order Bacteroidales, all of which were identified in LEfSe) in the small intestine to be one of the features most likely to explain the differences between the HIV-positive and HIV-negative groups. These results corroborated with previous analysis of the genus Bacteroides being decreased in HIV-positive small intestine.

This study was limited in its cross-sectional nature, sampling only post-mortem samples. An improved study design would include serial samples from patients starting at a time point prior to HIV seroconversion. Tissue sampling was also a limitation in this study as tissues sampled may not have been from the same cross-section or anatomical location in the lung and small intestine. Procedural controls for tissue sampling detailing sample site and cross-sectional area would help overcome these limitations. However, random sampling location could result in
non-systematic bias, leading to overestimated p-values in this study. Changes in microbiome could occur due to post-mortem changes prior to storage in -80°C and differences in length of HIV-infection, ART regimen and concomitant medications.

Future studies with controlled sample collection, targeted sampling technique and increased sample size would be able to overcome some of the limitations in this project. Interventional studies in the microbiome are required to further the field in investigating the exact mechanisms in which the gut-lung axis communicate and affect each other. Furthermore, development of new statistical tools may be required for analysis of results exploring relationships between two separate ecosystems. Overall, the results from this thesis show that ART-treated HIV patients present microbiota similar to HIV-negative patients, demonstrating the ability of ART to provide the microenvironment necessary to maintain a “healthy” microbiome. Decreased Bacteroides in the small intestine may be a potential identifier of HIV infection and could be a result of immune dysfunction processes specific to HIV. Furthermore, microbiomes of the gut-lung axis have demonstrated a correlation in bacterial load and abundance of individuals ASVs, with the latter suggesting a strong relationship between abundances of overlap bacteria between the two sites.
Bibliography


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93. Larsen, J. M. The immune response to *Prevotella* bacteria in chronic inflammatory...


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## Appendix A  Sample Metadata

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