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INYUVESI YAKWAZULU-NATALI

Impact on intestinal epithelial and stromal cells in people living with HIV infection

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AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cells
ARV	Anti-retroviral
ART	Anti-retroviral Therapy
BMP	Bone Morphogenetic Protein
BREC	Biomedical Research Ethics Committee
CBC	Crypt-based Columnar Cells
CBF	Crypt-based Fibroblasts
CI	Clinically Indicated
CSC	Crypt-based Stromal Cells
DNA	Deoxyribonucleic Acid
EF	Epithelial Fraction
EEC	Enteroendocrine Cells
GALT	Gastrointestinal Associated Lymphoid Tissue
GI	Gastrointestinal
HIV	Human Immunodeficiency Virus
IEL	Intestinal Epithelial Cells
ISC	Intestinal Stem Cells
LF	Lymphocyte Fraction
NCI	Non-Clinical, Research Elected
PLWH	People Living With HIV
RNA	Ribonucleic Acid
WNT	Wingless-related Integration Site

ABSTRACT

The gastrointestinal (GI) tract is the largest immune organ in the human body and a critical site for HIV pathology. Dysregulation of gut homeostasis and depletion of GI tissue-resident $CD4^+$ T-cells remain permanent regardless of antiretroviral therapy (ART) and recovery of $CD4^+$ T-cells in circulation. The irreversible depletion of GI tissue-resident $CD4^+$ T-cells may contribute to the dysregulation of gut homeostasis by impacting intestinal stem cells (ISC) and stromal cells through impaired immune signalling. To address this question, flow cytometric analysis of duodenum, colon, and ileum pinch biopsies obtained from uninfected controls and people living with HIV (PLWH) was performed. Flow cytometric analysis of epithelial cells ($CD45^-EpCAM^+$) showed an increase in intestinal stem cells (ISC) ($CD44^+EpBH2^+$) in the colon, duodenum, and ileum of PLWH. Flow cytometric analysis of stromal cells ($CD45^-EpCAM^-CD235a^-CD38^-CD19^-$) showed a significant change in the $CD31^-PDPN1^+$ stromal fibroblast population. Across intestinal compartments, PLWH showed increased fibroblast frequencies compared with uninfected controls that were not directly linked to $CD4^+$ T-cell depletion in the gut or blood viremia status. Overall, these results indicate that HIV infection increases the amount of ISCs and fibroblasts in the gut, which may contribute to the overall HIV-associated dysregulation in the GI tract. Further investigation is required to determine the mechanisms by which HIV impacts non-hematopoietic cellular compartments in the gut.

CHAPTER ONE

1.1. Introduction

Human immunodeficiency virus (HIV) targets the host's immune system, predominantly through the infection and destruction of $CD4^+$ T-cells[1]. Transmission of HIV between different hosts occurs when there is an exchange of bodily fluids that contain infectious viruses. Without treatment, $CD4^+$ T-cell levels become critically low and HIV progresses to acquired immunodeficiency syndrome (AIDS). People with AIDS no longer have a functional immune system making them vulnerable to opportunistic infections with fatal outcomes.

As of 2023, 39.9 million people worldwide were infected with HIV[2]. Nineteen percent of them live in South Africa[3]. More people are living with HIV (PLWH) today, than in previous years of the HIV epidemic. Fortunately, this is not correlated with more infections. Instead, it is due to improved treatment strategies. When comparing new HIV infections and AIDS-related deaths over the past two decades, there have been 1.5 million fewer new infections and 1.17 million fewer AIDS-related deaths. In contrast, since 2000 30.19 million more people are accessing treatment[2].

HIV is treated using anti-retroviral therapy (ART) though this is not a cure. ART functions to reduce the mass replication of HIV [4] and enables partial restoration of $CD4^+$ T-cells thus preventing the disease progressing to AIDS. However, despite ART, HIV continues to persist in its host through evasion of immune detection and the presence of viral reservoirs[5, 6, 7, 8, 9]. To maintain the function of their immune system, a person must continue with ART for life. The stopping of ART results in viral rebound due to the presence of viral reservoirs established during the initial infection of HIV[6, 7, 10, 11, 12].

A major early target for HIV is the gastrointestinal (GI) tract and gastrointestinal-associated lymphoid tissues (GALT)[1, 13, 14, 15]. Patients suffering from HIV/AIDS are known to experience GI disorders[16, 17, 18] and disruption in gut homeostasis. Gut homeostasis refers to the balance between immune tolerance and immune activation in the gut. An imbalance in gut homeostasis is associated with inflammation of the gut. This inflammation is believed to be caused by the movement of microorganisms across the intestinal epithelium[19]. It has been proposed that PLWH experience a leaky gut[15] enabling microbial translocation. Some studies have shown damage to the intestinal epithelium in PLWH[20, 21] which may lead to a leaky gut. However, the intestinal epithelium is a robust barrier with self-repair mechanisms under normal conditions. The repair of the intestinal epithelium and maintenance of intestinal epithelial integrity are upheld through a balance in the proliferation and differentiation of intestinal stem cells (ISCs)[22]. The proliferation and differentiation of ISCs are influenced by signals sent by epithelial,

stromal, and immune cells[23, 24, 25, 26, 27, 28, 29]. In this study I hypothesise that cytokine signalling between these cells is impaired due to HIV-depletion of gut resident $CD4^+$ T-cells, thus preventing proper epithelial repair of the leaky gut.

1.2. Background

The global HIV epidemic began in 1981 and was first recognized through its advanced disease state AIDS[30]. People were succumbing to opportunistic infections that are easily overcome by a functional immune system. Four years after the identification of AIDS it was found that the lentivirus HIV-1[31], and later HIV-2 was responsible for causing the disease. Once identification of the cause of AIDS was identified, research into treatments and a cure for HIV began in earnest. In 1995 ART began to be used to treat HIV[32]. In 2004, ART was formally rolled out by the South African government to combat HIV in the country – an epicentre of the epidemic[33]. This treatment for HIV has resulted in increasing the life expectancy of those infected from 19 years (in 1996) to 53 years (in 2011)[34]. Despite nearly 40 years having passed since the identification of HIV and AIDS with continued investments in treatment, HIV remains an incurable disease.

1.2.1. HIV Progression

Transmission of HIV can occur through sexual intercourse, the exchange of drug equipment, or from mother to child[35]. Once HIV has been transmitted to a person, the disease progresses along four distinct disease phases: eclipse, acute, chronic, and progressive[12, 35, 36].

The eclipse phase starts from the moment of infection. In the eclipse phase and throughout HIV infection, HIV uses its envelope gp120 proteins to bind the CD4 receptor[37, 38], and CCR5 or CXCR4 co-receptors of T-helper cells and macrophages[39]. HIV R5 isolates bind CCR5 and HIV X4 isolates bind CXCR4. The binding of gp120 to CD4 and CCR5 or CXCR4 enables the fusion of HIV's viral membrane with the plasma membrane of the host cells, which in turn allows transference of single-stranded RNA and enzymes – such as reverse transcriptase, integrase, and protease – into the host cells. In the host cell, reverse transcriptase produces viral error-filled double-stranded DNA from the viral single-stranded RNA[40]. The double-stranded viral DNA enters the host cell's nucleus and can become incorporated with the host's DNA by the enzyme integrase[41]. The host cell's RNA polymerase then transcribes the host cell's DNA into RNA, now including the viral DNA. The RNA is subsequently translated into a polyprotein. The polyprotein is cleaved by HIV protease[42]. The viral proteins and RNA then migrate to the periphery of the host cell where they assemble to form new HIV virions[43]. The new virions bud off the infected host cell and continue HIV infection and replication. The infection and

replication of HIV, however, do not go unnoticed. In response to HIV infection and replication, destruction of infected and uninfected $CD4^+$ T-cells occurs. Infected $CD4^+$ T-cells are destroyed via caspase-3-mediated apoptosis. The majority of $CD4^+$ T-cells destroyed, however, are uninfected. These uninfected $CD4^+$ T-cells are destroyed via caspase-1-mediated pyroptosis[44]. It is this depletion of $CD4^+$ T-cells throughout the progression of HIV that is responsible for the harm done to the host. The eclipse phase ends once HIV spreads from the mucosal site of infection to other lymphoid tissues[45].

The acute phase of HIV occurs after the eclipse phase and lasts for 3 to 9 weeks[12, 36, 46]. The acute phase of HIV infection is characterized by detectable viremia in the blood, peak viremia, and symptoms[12, 36, 46, 47]. From the eclipse phase into the acute phase, viremia increases as $CD4^+$ T-cells deplete. The innate immune system responds to the increased viremia by producing a variety of chemokines and cytokines that combat HIV and enhance the host's immune response[47, 48]. Fortunately, the immune response becomes effective and HIV viremia begins to decline largely due to $CD8^+$ T-cells' cytotoxic activity[49, 50, 51, 52]. With this decrease in viremia, PLWH in the acute phase can experience a brief increase in $CD4^+$ T-cells in the blood, however, depletion remains irreversible in mucosal sites, particularly GALT[13, 15, 53, 54, 55].

The slowed rate of infection and replication generally causes a stabilization of viremia known as the viral set point[36, 47] which lasts about 10 years, without treatment[56]. This decrease and then stabilization of viremia is referred to as chronic HIV infection. A person with chronic HIV infection usually does not exhibit symptoms[46] and continues to experience a consistent decline in $CD4^+$ T-cells. Furthermore, HIV continues to replicate and evade the immune[36, 47, 57, 58, 59].

HIV avoids immune detection by mutating rapidly[58, 59, 60]. This means that $CD8^+$ T-cells, which combat HIV, fail to select for HIV escape mutants. The continuous HIV replication and immune evasion leave the immune system in an active state, ensuring constant HIV infection and depletion of immune cells. With the immune system exhausted and no longer able to produce more $CD4^+$ T-cells than those lost to infection, HIV is left unchecked. Once the body has a non-functional immune system, evidenced by less than 200 $CD4^+$ T-cells/μL in the blood, HIV enters the progressive phase known as AIDS[46]. In addition to a low blood $CD4^+$ T-cell count, AIDS is identified by the presence of AIDS-defining illnesses. AIDS-defining illnesses are infections that a functional immune system can easily overcome (such as *Pneumocystis jirovecci*, *Cryptococcus neogormans*, and *Cytomegalovirus*), but which a devastated immune system is overwhelmed. People with AIDS generally exhibit rapid weight loss, extreme fatigue, and symptoms of opportunistic infections they have, like sores and diarrhoea. Without treatment, patients with AIDS typically succumb to their infections and are only able to survive for a few years[61].

1.2.2. Treatment of HIV

The main treatment for HIV is ART. ART makes use of administering 2 to 3 anti-retroviral drugs (ARVs) at the same time. ART prevents viral replication and keeps HIV plasma viral load low to undetectable and non-transmissible levels enabling improvement and restoration of patients' immune systems and quality of life. However, ART must be taken for the rest of a person's lifespan. If a person stops taking their ARVs, they will experience a viral rebound, where a high HIV viral load is re-established, due to the establishment of an HIV reservoir[4, 5, 6, 7, 8, 9]. Conversely, ARVs can also negatively impact the body. Side effects for ARVs include an increase in risk for cardiac disease, organ toxicity, reduction in bone density, psychiatric function, GI dysfunction[62, 63, 64], and immune reconstitution inflammatory syndrome[65]. Thus, although ART greatly improves the quality of life of PLWH, they face continued health issues. This has become more obvious as the life expectancy of those with HIV, due to ART, increases.

1.2.3. GI Tract

GI issues such as diarrhoea, abdominal pain, and vomiting are hallmarks of HIV pathology[17, 61] and ART side effects[62, 64]. The GI tract is responsible for breaking down substances, obtaining nutrients for survival, and excreting the waste by-products. Due to the nature of digestion, the GI tract must maintain a balance between commensal bacteria that aid in digestion and invading microorganisms entering the body. This balance is enforced by the large number of immune cells, protected by the intestinal epithelium, in the lamina propria. An imbalance in maintaining the commensal bacteria and invading microorganisms can cause great discomfort and health issues such as those seen in HIV pathology.

The main components of the GI tract are the: mouth, oesophagus, stomach, small intestine, and large intestine[66]. The mouth and stomach function to break down food to release nutrients. The small intestine breaks down the food further and absorbs the nutrients released from the broken-down food. The large intestine functions to absorb water and electrolytes to convert the liquid waste into stool that can be expelled from the body through the anus[67]. Accessory organs that help the GI tract with digestion are the gall bladder, the liver, and the pancreas[66].

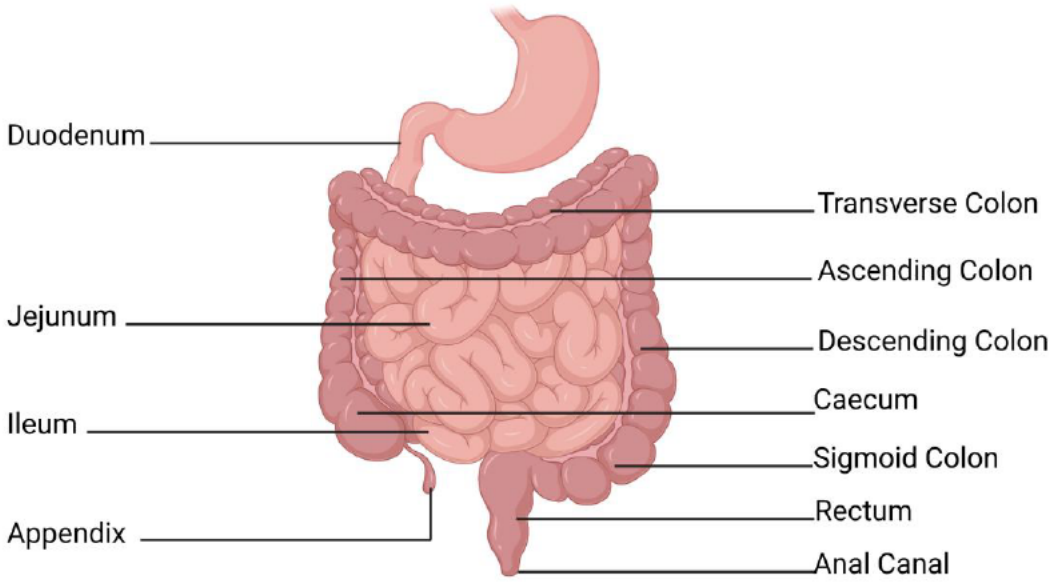

Figure 1: **an image of the small and large intestines with each intestinal segment labelled.** Image constructed using BioRender.

1.2.3.1. The Small Intestine

The small intestine is roughly 6 meters long with a small diameter of 3.0cm. The small intestine contains villi. Villi are protrusions of intestinal epithelia that absorb most nutrients released during digestion[68, 69, 70]. Minerals, vitamins, short-chain fatty acids, sugars, amino acids, and lipids are some important nutrients that are absorbed by the small intestine[71].

The small intestine consists of 4 layers of tissue. The mucosa is the innermost layer of the small intestine. The mucosa is exposed to the lumen; the cavity in which digested products are passed through the small intestine. The mucosa's foremost function is to absorb nutrients due to the proximity of the mucosa to the digested products. The submucosa (the layer beneath the mucosa) is a connective tissue that contains blood vessels, lymphatic vessels, and nerves. The next layer below the submucosa is the muscularis. The muscularis is the muscle of the small intestine and consists of two layers spanning the whole of the small intestine. The muscularis controls the constriction of the small intestine via the layer of circular muscle fibres. The muscularis controls the shortening and elongation of the small intestine via the layer of longitudinal fibres. The shortening, elongation, and constriction of the small intestine facilitate the movement of digested products across it and into the large intestine. The outermost layer of the small intestine is the serosa. The serosa consists of a mesothelium and epithelium layer[72, 73].

The small intestine has three segments from its connection to the stomach to the large intestine: the duodenum, the jejunum, and the ileum (see Figure 1)[72]. The duodenum is the most proximal segment of the small intestine; connected to the pylorus of the stomach. The duodenum receives partially digested food and gastric juices from the stomach, enzymes from the pancreas, and bile from the liver and gall bladder[72]. The duodenum absorbs most of the minerals and water-soluble and fat-soluble vitamins. Water-soluble minerals and vitamins, the duodenum absorbs include iron, calcium, magnesium, phosphorus, niacin, and biotin. Fat-soluble vitamins, the duodenum absorbs include retinol, alpha-tocopherol, and calciferol[74]. The jejunum is the middle segment of the small intestine joining the duodenum and the ileum. It is also the largest section of the small intestine. Like the duodenum, the jejunum absorbs water-soluble and fat-soluble vitamins not absorbed by the duodenum. In addition to these vitamins, the jejunum is also responsible for absorbing monosaccharides, small peptides, amino acids, and lipids[74]. The ileum is the most distal segment of the small intestine connected to the jejunum and the caecum of the large intestine. The ileum is responsible for absorbing final nutrients not absorbed by the duodenum and the ileum as well as bile acids, ascorbic acid, and cobalamin[72, 74].

1.2.3.2. The Large Intestine

The large intestine, also known as the colon, is shorter than the small intestine, with a length of approximately 1.5 meters[75]. The large intestine is called such as it has a large diameter. The diameter of the colon ranges from 3cm to 9cm depending on the segment of the large intestine[76]. The large intestine has 7 segments; caecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum and anus[74]. The colon has the same four layers of tissue as the small intestine: the mucosa, submucosa, muscularis, and serosa. There are, however, differences in these layers between the small and large intestine, mainly the mucosa and muscularis layers. The mucosa layer of the large intestine differs from the small intestine in two major components: the absence of villi and the presence of saccules known as haustra. Haustra are sacculated structures in the colon in which undigested materials move through the colon. Haustral contractions move the undigested materials throughout the colon[75]. The muscularis layer of the large intestine also has a circular and longitudinal fibre layer . However, unlike the small intestine, the large intestine's longitudinal layer is clustered to form 3 bandlike structures known as the taenia coli instead of spanning the whole of the intestine. The Taenia coli results in the formation of the haustra in the mucosa[77].

The first segment of the colon is the caecum. Undigested material moves from the ileum of the small intestine into the caecum through the ileocecal valve. The caecum is followed by the ascending colon, the transverse colon, and the descending colon, which are named by how they span the abdomen (see Figure 1). Most of the remaining water from the undigested material is absorbed in the ascending and transverse colon to produce stool. Once stool has formed, it moves from the transverse colon to the

descending colon. The descending colon stores the stool. The sigmoid colon, which connects the descending colon and the rectum, pushes stool from the descending colon into the rectum. The rectum then stores the stool until it can be expelled from the anus as faeces[75].

1.2.4. Mucosal Immunity and HIV

Early in HIV infection, there is severe, irreversible depletion of immune cells in the gut[13, 15, 53, 54, 55]. A better understanding of how the gut's mucosal immunity impacts and is impacted by HIV is imperative to understand and treat HIV infection as a whole.

1.2.4.1.Intestinal Mucosal Immunity

A major site of mucosal immunity is the GI tract. As mentioned previously, the GI tract must balance commensal bacteria and invading micro-organisms entering the body. The intestinal mucosal immunity must therefore accurately determine dangers and act quickly. To enable accurate and quick assessments of all the antigens and microorganisms the mucosal immune system encounters, it has inductive and effector sites with no clear distinction[25].

Inductive sites are lymphoid tissues that contain naïve, non-activated, adaptive immune cells and antigen-presenting cells (APCs). If naïve immune cells are not activated by APCs, like dendritic cells, in the inductive site, they will re-circulate throughout the body to another inductive site. If an antigen or pathogen is present, epithelial cells will secrete cytokines to inform APCs that antigen sampling is required. The APCs will bind an antigen or pathogen through their pattern recognition receptors[25] and determine if a tolerogenic or an adaptive immune response is required. An adaptive immune response signal is initiated by co-stimulation of a T-cell. Co-stimulation of a T-cell occurs when T-cells recognize the antigen/pathogen and its ligands CD80 or CD86 presented by the APC[78]. If there is no co-stimulation, the T-cell becomes tolerogenic[78, 79].

Activated $CD4^+$ T-cells (known as T-helper) proceed to activate B-cells and macrophages. These activated immune cells upregulate homing receptors, such as $\alpha 4\beta 7$ integrins. The activated immune cells via their homing receptors migrate to effector sites expressing cytokines and proteins, such as MAdCAM-1, that attract said homing receptors. At effector sites and sites of infection, these immune cells confront and destroy the foreign antigen or pathogen[25].

Under normal conditions, a few days after T-cell activation the pathogen is cleared. Pathogen removal causes a decrease in the expression of immune checkpoint inhibitors such as cytotoxic T lymphocyte

antigen-4 (CTLA-4), lymphocyte activation gene protein 3 (LAG-3), and programmed cell death receptor 1 (PD-1). A decrease in these proteins' expression levels results in a reduction of T-cell activation. Issues arise when pathogens cannot be fully removed, resulting in T-cells remaining active due to constant stimulation until they become exhausted, like in the case of a non-treated HIV infection[78].

1.2.4.2.Intestinal Mucosal Immunity in PLWH

The lamina propria is an effector site in the GI tract. The lamina propria is a loose connective tissue found on the basal side of the intestinal epithelium. As an effector site with a high frequency of $CD4^+$ T-cells, the lamina propria is an ideal target for HIV to infect and replicate[15, 80]. It has been shown that the intestinal lamina propria is a major site of HIV replication regardless of the location of the initial site of infection[18, 81, 82]. The intestinal epithelium acts as a barrier against infection and the movement of microorganisms from the lumen to the lamina propria. The lamina propria is a major site of HIV replication in PLWH, so it is clear that the intestinal epithelium is no longer able to act as an effective, protective barrier.

A recent study conducted by Krug et al.[83] compared the permeability of duodenal and sigmoid intestinal epitheliums to macromolecules between healthy people, viral suppressed PLWH on ART, and PLWH not on ART (described as treatment-naïve). In Krug et al's study, they found that the duodenal and sigmoid intestinal epithelium of treatment-naïve PLWH was more permeable than those of control healthy people. The permeability was more pronounced and significant in the duodenal epithelium than in the sigmoid epithelium. The increased permeability of the intestinal epithelium in treatment-naïve PLWH was not observed in those virally suppressed. This increased permeability was found to be due to apoptotic leaks and transcytosis. These apoptotic leaks may be caused by an increase in interleukin-18 (IL-18), known to occur during HIV infection, via activation of caspase-1 and caspase-3[84].

1.2.5. Intestinal epithelium

The intestinal epithelium is a one-cell layered lining of the gut. This lining is structured into protrusions known as villi and invaginations known as crypts. The villi extend into the lumen and absorb nutrients from digested food into the body[69, 70]. The crypts produce progenitor cells to replace cells on the villi once they have died and maintain the intestinal epithelium's barrier integrity[22].

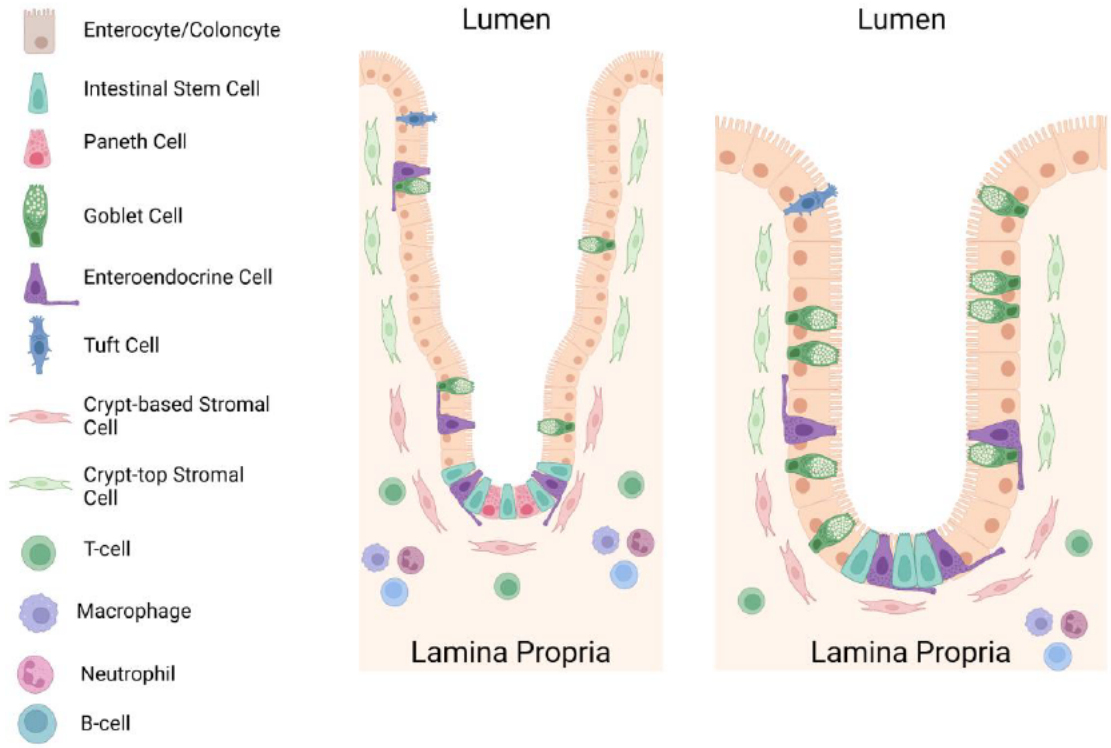

Figure 2: **an image depicting the small (left) and large (right) intestines' intestinal epithelium and the types of cells present in these structures.** Image constructed using BioRender,

As the main function of the villi is to absorb nutrients, these structures contain many enterocytes[70]. Enterocytes are responsible for absorbing nutrients released from digested food. In addition to enterocytes, the villi contain goblet cells, enteroendocrine cells (EECs), tuft cells, and microfold cells (M cells). Goblet cells are secretory cells that produce glycoproteins called mucins, particularly MUC2 in the intestine, to form a mucus layer[85]. The mucus layer is a physical layer protecting the intestinal epithelium from digestive enzymes and micro-organisms present. EECs are a small percentage, only 1%, of the epithelial cells in the intestinal epithelium. EECs co-ordinate digestion of food through the secretion of hormones and peptides[86, 87]. Tuft cells detect and react to a wide range of substances present in the lumen using their various apical receptors. Upon recognition and binding, tuft cells can summon an immune response to react appropriately to remove pathogen protozoa and parasitic worms[88]. M-cells are epithelial cells found in GALT tissue such as Peyer's patches. These microfold cells transport antigens from the intestinal lumen to the underlying immune tissue to initiate an immune response[25, 89].

The crypts contain two main types of cells: intestinal stem cells (ISCs) and Paneth cells. ISCs differentiate to replace lost enterocytes, goblet cells, EECs, and Paneth cells. Paneth cells maintain the stem cell niche and regulate gut microbiota. Paneth cells perform their function through the secretion of molecules and signals. Paneth cells secrete epithelial growth factor (EGF), Wingless/Integrated 3

(Wnt3), and Delta-like 4 (Dll4) activating various signal pathways, discussed later, to control the proliferation and differentiation of ISCs[90]. Paneth cells also secrete antimicrobial peptides to manage the gut microbiota[25].

1.2.6. Intestinal stem cells (ISCs)

The intestinal epithelial barrier performs its functions in its difficult environment due to its ability to regenerate every 4 – 5 days[25]. The epithelial barrier regenerates through the proliferation and differentiation of ISCs. ISCs are located at the bottom of intestinal epithelial crypts (see Figure 2). There are two main types of ISCs at the bottom of these crypts, crypt-based columnar cells (CBC), identifiable by their key marker Lgr5+[91], and +4 ISCs. CBCs are active ISCs responsible for proliferating and differentiating to replace lost cells and maintain barrier integrity. +4 ISCs are quiescence and are used to ensure the ISC pool is still present in cases where CBC ISCs become fully depleted[92, 93]. Under normal conditions, CBC ISCs will divide into two: one cell will remain an ISC while the other will become a transit-amplifying cell that will eventually differentiate. Interestingly, it has been found that differentiated epithelial cells can de-differentiate and migrate back to the crypts to act again as CBC ISCs[94, 95, 96].

The proliferation and differentiation of ISCs are dependent upon various signal pathways such as the Wingless-related Integration Site (Wnt) pathway, the Bone Morphogenic Protein (BMP) pathway, and the Notch pathway. These pathways function together to enable a homeostatic balance between the proliferation and differentiation of ISCs. A disruption in this balance can result in the loss of the intestinal epithelial barrier's integrity, causing subsequent health problems.

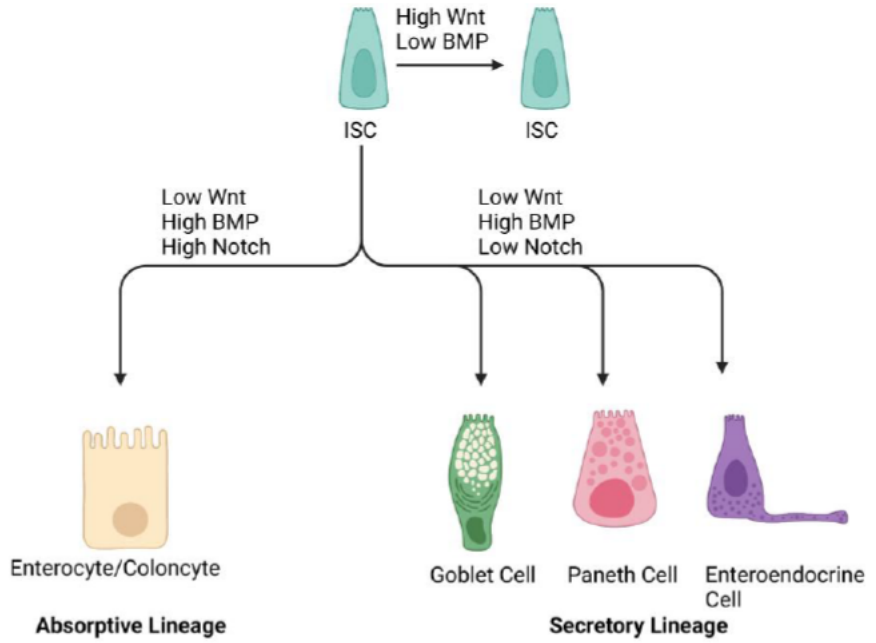

Figure 3: **an image depicting signalling pathways controlling ISC proliferation and differentiation.** Image constructed using BioRender.

1.2.6.1.Wnt Pathway

The Wnt pathway is the main signalling pathway that regulates ISC proliferation. In the Wnt canonical pathway, Wnt or R-spondin (Rspo) proteins[97] – secreted by Paneth cells and crypt-based stromal cells – bind to the Frizzled (FZD) receptor and low-density lipoprotein receptor-related protein (LRP5/6) co-receptor found on the surface of the ISCs. A subsequent signalling cascade prevents the phosphorylation and degradation of β-catenin[29]. β-catenin accumulates in the cytoplasm and eventually migrates to the cell's nucleus where it activates stem cell proliferation genes *Lgr5*, *Axin2*, *EphB2,* and *Cd44*[23]. Generally, Wnt signals are brief due to the E3 ligases Ring finger protein 43 (RNF43) and Zinc and ring finger 3 (ZNRF3). RNF43 and ZNRF3, when activated as negative regulators, place ubiquitin on the FZD receptor and LRP5/6 co-receptor[98,99]. This results in FZD, and LRP5/6 being removed from the cell's surface preventing continued Wnt signalling. Lgr5 receptor (a key marker of ISCs) can bind to RNF43 and ZNRF3 to retain FZD and LRP5/6 on ISCs' surface[99, 100, 101]. Retention of FZD and LRP5/6 on ISCs' surface enables continuous rapid proliferation of ISCs.

1.2.6.2.BMP Pathway

The BMP pathway is another important signalling pathway that regulates ISCs. Activation of the BMP pathway is used to prevent hyperproliferation and promote the differentiation of ISCs. Agonists of the BMP pathway bind to the BMP type II receptor (BMPRII). Once BMPRII is bound, BMPRII activates the type I receptor (BMPRI) via phosphorylation. The activation of BMPRI causes phosphorylation of Smad1, Smad5, and Smad8. Once Smad1, Smad5, and Smad8 are phosphorylated they form a complex with Smad4. This protein complex moves to the nucleus to prevent the transcription of signature ISC genes *Lgr5* and *Olfm4*[23]. The BMP pathway also stimulates the expression of cell cycle arrest genes like *Cdkn1a* and *Ovol2*[23].

1.2.6.3.Notch Pathway

A key differentiation pathway for ISCs is the Notch pathway. High Notch signalling encourages ISCs to differentiate along an absorptive lineage while low Notch signalling encourages ISCs to differentiate along a secretory lineage[29]. The Notch pathway is activated when a ligand, like Dll4, binds to the Notch receptor. The binding of a ligand to the Notch receptor results in conformational changes that activate γ-secretase. Activated γ-secretase cleaves the Notch intracellular domain (NID) releasing it from the plasma membrane allowing it to translocate into the cell's nucleus. The NID, in the nucleus, then forms a transcriptional activation complex and activates HES1 genes. HES1 inhibits the expression of atonal transcription factor 1 (ATOH1). A decrease in ATOH1 leads to the formation of absorptive lineages, i.e., enterocytes[29]. An increase in ATOH1 leads to the formation of secretory epithelial cells like Paneth, goblet, and EECs (see Figure 3).

These signalling pathways are produced as a gradient along the crypt-villus axis. The signalling pathways' gradient encourages the movement of differentiated cells out of the crypts and towards the tips of villi while Paneth and ISCs remain in the spatially limited crypt area (see Figure 2)[25]. Crypt-based stromal cells (CSCs) (Figure 2), also known in the literature as mucosa-associated fibroblasts, $CD90^{+}$ fibroblasts, and trophocytes ($CD81^{+}$ $PGFRA^{lo}$), encourage proliferation and impede differentiation of ISCs thus maintaining the ISC niche[102, 103, 104, 105]. These CSCs promote proliferation and inhibit differentiation by releasing Wnt ligands (Wnt3 and R-spo3) and BMP antagonists (Gremlin1)[106, 107, 108]. Crypt-top stromal cells (see Figure 2), also known in the literature as myofibroblasts and telocytes ($CD81^{-}$ $PGFRA^{high}$), on the other hand, produce BMP agonists (Bmp3 and Bmp7) activating the BMP pathway to inhibit ISC proliferation and encourage ISC differentiation[107]. Thus, ISCs are maintained in the crypts while differentiated cells are located towards the intestinal villi. The significance of these stromal cells' signals' ability to maintain the intestinal epithelium was

highlighted in a recent study conducted by Lin et al. Lin et al., successfully established a mature intestinal organoid when co-cultured with stromal cells without supplementation of Wnt agonists and BMP antagonists[109].

The ISC niche and these pathways are set and controlled by epithelial and stromal cells[110]. However, ISCs, epithelial cells, and stromal cells are also susceptible to pathogenic and immune signals. This means that immune responses, or lack thereof, may result in an improper proliferation and differentiation response. This in turn may negatively impact the intestinal epithelium and gut homeostasis.

1.2.6.4. ISCs and Immune Cells

Intestinal epithelial cells (IECs) act as APCs[111] to activate immune cells which combat injury and pathogenic threats. Immune cells can also release important cytokines that promote the proliferation, differentiation, and apoptosis of ISCs.

Recent studies have found that T-helper cells, a major target of HIV infection, produce cytokines that influence ISCs and their niche[111]. Saito et al. found that interleukin-4 (IL4) produced by T-helper 2 cells may decrease the expression of Paneth cells and in turn, hamper the proliferation of ISCs in enteroids[26, 27]. Lin et al. showed that T-helper 17 cells may promote ISC differentiation into secretory cells (like Paneth and goblet cells) through their production of interleukin 17 receptor A (IL-17RA)[27, 28] which induces the expression of ATOH1[28]. Takashima et al. showed that T-helper 1 cells produce interferon-gamma (IFNγ) activating the signal pathway JAK1/STAT1 and triggering apoptosis of ISCs[24, 27].

Other immune cells that have been shown to impact ISCs and their niche include macrophages and innate lymphoid cells (ILCs). Macrophages are an important source of Wnt agonists upon radiation damage when stromal and Paneth cells may become compromised[112]. Type 3 ILCs secrete interleukin-22 (IL-22). IL-22 promotes the formation of Paneth cells, which are known to be important epithelial cells that maintain the ISC niche[113].

The results from these recent studies motivate investigation into how HIV, through the depletion of immune cells, impacts ISCs. Investigation into if and how HIV impacts ISCs through immune cell depletion may provide crucial information as to how HIV can disrupt the intestinal epithelium and gut homeostasis.

1.2.6.5.ISCs and HIV

The intestinal epithelium is the frontline barrier against HIV's propagation and pathology in the gut of PLWH. Once HIV can penetrate the intestinal epithelium, it can interact with immune and stromal cells in the lamina propria, establishing HIV infection and reservoirs in the gut[8]. ISCs are not known to interact and be directly infected by HIV. IECs, however, are associated with HIV infection[114] as well as have pro-viral[115, 116], and anti-viral[117, 118, 119, 120, 121, 122, 123, 124, 125] influences on HIV.

A study by Liu et al.[114] using in situ hybridization and immunostaining, showed that IECs obtained from asymptomatic PLWH and people living with AIDS contained HIV nucleic acids. They were able to detect HIV nucleic acids in 83% of the asymptomatic samples and 96% of the AIDS samples. They also showed a positive correlation between decreasing $CD4^+$T-cells in blood and increasing $p24^+$ gastrointestinal epithelial cells.

In a study by Meng et al.[115] they showed that $CCR5^+$, jejunal IECs were able to proficiently transfer HIV R5 to $CCR5^+$ cells that then replicate and amplify the expression of HIV. This has important implications, as most of the $CD4^+$ T-cells in the mucosal immune system are $CCR5^+$[126]. $CD4^+$ $CCR5^+$ memory T-cells are known to facilitate mother-to-child HIV transmission[127]. The ability of IECs to trans-infect $CD4^+$ T-cells was also shown in a study by Wiche Salinas et al.[116]. In Wiche Salinas et al.'s study, they co-cultured HT-29 IECs with $CD4^+$ T-cells taken from uninfected people, aviremic PLWH on ART, and untreated PLWH. Their results showed that interleukin-17A (IL-17A) released by T-helper 17 cells, in conjunction with TNF, activated the IECs. Activated IECs then promoted transinfection of $CD4^+$ T-cells.

In a study conducted by Guo et al.[117] they were able to show that cultured IECs produce exosomes that have anti-viral HIV properties. These anti-viral HIV exosomes can be taken up by macrophages and used to inhibit HIV's ability to infect and replicate. In particular, they were able to show that once IECs were activated via their toll-like receptor 3 (TLR3) they released exosomes containing HIV restriction miRNAs, viperin, guanylate binding protein 5 (GBP5), and myxovirus resistance B proteins (MxB). The particular miRNAs shown to be produced by the IECs were miRNA-17, miRNA-28, miRNA-29, and miRNA-125. miRNA-17 and miRNA-28 bind to a host cell's DNA and inhibit the incorporation and transcription of HIV DNA[118, 119]. miRNA-28[120], miRNA-29[121], and miRNA-125[120] bind directly to HIV transcripts to inhibit translation[118]. Viperin has been shown to inhibit HIV production, though the exact mechanism by which it can do so remains unknown[122]. GBP5 can impair HIV-1 virions incorporation of gp120[124]. MxB has been shown to block the nuclear import of HIV-1 DNA[125].

1.2.7. Intestinal stromal cells

Intestinal stromal cells are a heterogeneous population of connective tissue cells between the intestinal epithelium and the lamina propria. Types of intestinal stromal cells include blood endothelial cells (BECs), intestinal endothelial cells, fibroblasts, myofibroblasts, and pericytes[128]. These stromal cells function to build the infrastructure of the GI tract, maintain tissue homeostasis, and mediate immune responses[128]. These stromal cells can affect tissue homeostasis and immune function through cell-to-cell interactions and the secretion of chemokines and cytokines. The importance of stromal cells in tissue homeostasis and immune response suggests that they may have an unknown role or are affected by inflammation and HIV pathogenesis.

1.2.7.1. Stromal Cells and ISCs

Three main lineages of fibroblasts are found close to and interact with the intestinal epithelium[105]. Two of the three lineages of fibroblasts function to maintain the intestinal epithelium[102, 103, 104, 105]. Crypt-bottom fibroblasts and crypt-top fibroblasts (see Figure 2) keep the localization and balance of ISCs and epithelial cells by secreting important agonists and antagonists of the Wnt and BMP pathways. The effect of these pathways on ISCs has been studied in-depth. However, the knowledge of the consequences of these pathways on stromal cells is lacking. In a recent study by Lin et al., using their assembloid model, they showed that BMP2, an agonist of the BMP pathway, influences the stromal and ISC niche[109]. The addition of BMP2 increased crypt-top stromal cell gene expression while reducing the gene expression of CSCs. Furthermore, whether BMPRI was knocked out from the epithelial cells or the stromal cells, a functional intestinal epithelium with crypts was unable to form[109]. These results emphasize the interwoven signalling between IECs and stromal cells in maintaining a functional intestinal epithelium and gut homeostasis.

In addition to the two groups of fibroblasts that maintain the intestinal epithelium, there is a third lineage of fibroblasts: interstitial fibroblasts[105]. Interstitial fibroblasts are located in the muscularis mucosa. Interstitial fibroblasts are suspected to have an important role in wound healing. In a study by Jasso et al.[105] these fibroblasts were shown to express genes related to extracellular matrix remodelling and immunomodulation.

1.2.7.2. Stromal Cells and Immune Cells

Stromal cells and immune cells communicate and influence each other. A major way these cells affect each other is to facilitate wound healing. Wound healing has four phases: haemostasis, inflammation,

re-epithelialization, and remodeling[129]. After bleeding has stopped, local immune cells secrete pro-inflammatory cytokines interleukin-1β and tumour necrosis factor-alpha (TNFα) to activate residential stromal cells. Activated stromal cells subsequently express other pro-inflammatory cytokines such as interleukin-6 and interleukin-8 which recruit and activate more immune cells to the site of damage where they may combat pathogens and clear cellular debris[130]. Proliferation and differentiation of epithelial cells occur to replace the damaged cells cleared and form new epithelial tissue. Stromal cells then perform extracellular matrix remodelling and production of collagen to support the epithelial tissue.

Issues can arise when wound healing does not occur as expected. The co-dependence of immune and stromal cells in wound healing can have harmful consequences as seen with chronic inflammation and fibrosis. Inflammatory Bowel Disease (IBD) refers to two conditions characterized by chronic inflammation in the GI tract. Crohn's disease and ulcerative colitis are the two conditions that are grouped as IBD. Crohn's disease occurs anywhere in the GI tract while ulcerative colitis is limited to the colon. IBD, as well as other chronically inflamed conditions, is strongly associated with fibrosis. Fibrosis arises from an excessive accumulation of collagen and can restrict easy movement and function of the tissue. People suffering from IBD and intestinal fibrosis are known to experience narrowing and obstruction of their intestines resulting in abdominal discomfort and difficulty digesting foods. In extreme cases, these patients are required to undergo surgery[131].

The influence stromal cells and immune cells have on each other is important and necessary, however, it can also become harmful and dangerous. The possibility that HIV impacts how immune cells interact with stromal cells, and on stromal cells themselves is worthy of investigation to improve understanding of the relationship between these cells and HIV pathology.

1.2.7.3. Stromal Cells and HIV

As discussed previously, the lamina propria is a major site of HIV replication. The compromised intestinal epithelium layer enables HIV access to the lamina propria, and the immune cells residing therein. In this environment, HIV can infect $CD4^+$ T-cells directly or through trans-infection[132]. In a study conducted by Neidleman et al.[132], they found that stromal fibroblasts increase HIV infection of $CD4^+$ T-cells through trans-infection. These cells were more efficient at increasing HIV infection by trans-infection than dendritic cells and are particularly influential under low HIV viral conditions. Stromal fibroblasts are not the only stromal cells known to increase HIV infection of $CD4^+$ T-cells. A study by Eddy et al.[133], showed that intestinal endothelial cells can increase HIV infection of $CD4^+$ T-cells.

In addition to intestinal stromal cells increasing HIV infection, Asmuth et al.[134] proposed that these cells may also prevent immune reconstitution by promoting profibrotic, pro-inflammatory conditions. The authors were able to show that HIV participants have a pro-fibrotic condition in the duodenum before ART. They also showed a correlation between the pro-fibrotic condition and immune cell constitution in which expression of pro-fibrotic factors and the percentage of T-cells present were inversely proportional.

These studies show that differentiated stromal cells, particularly fibroblasts and endothelial cells, worsen HIV infection and pathology. Studies have also investigated the effects of undifferentiated stromal cells, known as mesenchymal stem cells (MSCs), on HIV. A study by Chandra et al. showed that adipose and bone marrow-derived MSCs can increase the HIV p24 levels and HIV-1 long terminal repeat directed gene expression in latent HIV T-cell and macrophage cell lines[135]. Chandra et al. were also able to show that common pathways for HIV reactivation (NFκB and PI3K pathways) were utilized by the adipose and bone marrow.

Interestingly, MSCs are also beneficial in the treatment of HIV. In a study by Zhang et al., the use of umbilical cord MSCs increased immune reconstitution in ART-treated HIV participants[136]. However, a phase I/II clinical trial conducted by Trujillo-Rodriquez et al., using adipose-derived MSCs, could not replicate the immune constitution[137].

These studies show that the relationship between stromal cells, inflammation, and HIV is a complex matter in which each can play a role in treating or progressing HIV infection. The relationship between stromal cells, inflammation, and HIV, both directly and indirectly, remains largely unknown and requires further investigation.

1.3. Problem Statement

The central organ implicated in HIV pathology is the gut compartment. PLWH suffers from a life-long disease that drastically impacts their health in various ways. Gut homeostasis is important for overall health and involves the epithelial barrier of the gut. A balance of ISC proliferation and differentiation is crucial in maintaining the intestinal epithelium. ISC proliferation and differentiation are controlled by signalling between intestinal epithelial, immune, and stromal cells. An imbalance in the proliferation and differentiation of ISCs can disrupt the epithelial barrier integrity, and cause bacterial translocation, inflammation, and gut pathology. This in turn leads to health complications. A better understanding of how ISCs and stromal cells are impacted by HIV can inform the design of host-directed therapies for

the gut and provide support to and enable those living with chronic HIV, an improved quality of life and health.

1.4. Rationale

People live longer with HIV due to effective, but not curable, ART, and therefore, will enter higher risk groups for comorbidities. ART, however, does not reverse HIV gut pathology despite plasma viral suppression. This study serves to better understand the impact of HIV infection on gut homeostasis and overall gut health by investigating the effect of HIV infection on ISCs and stromal cells that maintain the intestinal epithelium barrier and mucosal structure.

1.5. Aim

The overall aim of this study is to determine if HIV-associated intestinal immune dysfunction, influences ISCs and intestinal stromal cell frequencies that may contribute to HIV gut pathology.

1.6. Hypothesis

I hypothesise that HIV depletion of $CD4^+$ T-cells results in hyper-proliferation and impaired differentiation of ISCs, and that HIV increases the frequencies of intestinal stromal populations. Changes in ISC and stromal cell subsets may contribute to immune activation and pro-fibrotic conditions in the GI tract and ultimately prevent immune reconstitution in the gut.

1.7. Objectives

1.7.1. Identify ISCs in healthy uninfected people and PLWH.

1.7.2. Characterize HIV-associated changes in ISCs between uninfected people and PLWH who have a GI-indicated clinical condition.

1.7.3. Identify intestinal stromal populations in healthy uninfected people and PLWH.

1.7.4. Characterize HIV-associated changes in stromal populations in uninfected people and PLWH who are known to be experiencing health issues.

CHAPTER TWO

2.1. Methodology

2.1.1. Ethics Consideration

Ethics approval for this project was obtained from the Biomedical Research Ethics Committee. The reference number for the ethics approval is: BREC/00006916/2024

2.1.2. Study design and participants

The study population included participants from a clinically indicated (CI) cohort (Table 3)[55], and a non-clinical research-elected (NCI) cohort. The CI and NCI cohorts both have ethical approval (CI: BE 021/13, NCI: BREC/00001356/2020) and obtain written informed consent from all participants. The CI and NCI cohorts consist of participants undergoing endoscopy and colonoscopy in Kwa-Zulu Natal, South Africa, an HIV-endemic area[55]. Information on the participants' HIV status and ARV treatment is obtained by clinical staff through a health questionnaire and blood testing. The CI cohort has 5 arms: 1000, 2000, 3000, 5000, and 6000. Participants of the CI cohort in the 1000 arm undergo a colonoscopy. Participants of the CI cohort in the 2000 arm undergo an endoscopy. Participants of the CI cohort in the 3000 arm undergo an advanced endoscopy known as endoscopic retrograde cholangiopancreatography (ERCP). Unlike a standard endoscopy, which is limited to the upper GI tract, an ERCP enables medical professionals to inspect and biopsy the gallbladder, pancreas, and liver[138] in addition to the small intestine. Participants of 1000, 2000, and 3000 arms usually undergo a colonoscopy or an endoscopy (including ERCP) when experiencing symptoms such as severe abdominal pain, diarrhoea, or passing blood in their stool. Participants of the 5000 and 6000 arms undergo routine surgeries. Reasons for participants undergoing routine surgeries include cholelithiasis, obstructive jaundice, and the presence of tumours and cancer. Biopsies analysed in this study came from all arms of this cohort irrespective of the reasons participants were undergoing a procedure. The NCI cohort is research-elected. The NCI cohort participants have no underlying gastrointestinal complications and are considered overall healthy. These participants are recruited to undergo both an endoscopy and colonoscopy to obtain biopsy pinches of the duodenum, ileum, and colon.

2.1.3. Isolation of the epithelial fraction (EF)

The EF, containing ISCs, was isolated from pinches of gut tissue taken from the duodenum, ileum, and colon of PLWH and uninfected control participants. Pinches of gut tissue were incubated in 8nM EDTA

(Invitrogen) at either 4°C, for pinches of duodenum and ileum, or 37°C, for pinches of the colon, for an hour and 30 minutes. After incubation, the gut tissue was vortexed for 1 minute to ensure the release of the EF from the gut tissue into the EDTA supernatant. The supernatant containing the EF was removed and placed on ice. 1X PBS wash of the gut tissue was performed twice to ensure sufficient extraction of the EF. The EF was centrifuged at 500x g for 5 minutes at 4°C. The EF pellet was resuspended and incubated at 37°C for 5 minutes in TrypLEsolution (Thermo Fisher Scientific). After incubation, the EF was passed through a 100μm strainer. The strained EF was centrifuged at 500x g for 10 minutes at 4°C. The EF pellet was resuspended in 1ml of 1X PBS for cell counting using a BioRad TC20 and subsequent staining for flow cytometry analysis of ISCs using a BDFACS Symphony A5.

2.1.4. Isolation of lymphocyte fraction (LF)

The LF, containing the stromal and immune cells, was isolated from the same pinches of gut tissue as those used to isolate the EF. After the EF had been removed from the gut tissue, the gut tissue was incubated in digestion media containing 0.1% DNAse (Merck) and 0.5% Collagenase D (Roche) in R10 media at 37°C for 30 minutes. The digested gut tissue was vortexed to ensure the release of stromal and immune cells. The gut tissue and released cells were passed and mashed through a 100μm strainer. The strained LF was centrifuged at 500x g for 10 minutes at 4°C. The LF pellet was then resuspended in 1ml of 1X PBS for cell counting using a BioRad TC20 and subsequent staining for flow cytometry analysis using a BDFACS Symphony A5 and Aria Fusion.

2.1.5. Staining of intestinal stem and immune cells

ISCs underwent extracellular and intracellular staining. Two master mixes were made (Table 1), one for extracellular straining and one for intracellular straining. Master mix 1 (MM1) was used for the extracellular stain and master mix 2 (MM2) was used for the intracellular stain. MM1 contained the following conjugated mouse antibodies: BUV395-CD8 (BD Biosciences), BUV496-CD4 (BD Biosciences), V500-CD45 (BD Biosciences), BV650-CD117 (BioLegend), BV785-CD3 (BioLegend), FITC-CD44 (BD Biosciences), PE-HLA-DR (BioLegend), PE-CF594-EpCAM (BioLegend), PE-Cy7-CD24 (BD Biosciences), and APC-EpBH2 (BD Biosciences) in Brilliant Violet Buffer (BD Biosciences). MM2 contained a conjugated mouse antibody PerCP-Cy5-5-Ki67 (BD Biosciences) in 1X Permeabilization Wash Buffer (eBiosciences). 4X Foxp3/Transcription Factor Fixation/Permeabilization mixture was made by diluting the Foxp3/Transcription Factor Fixation/Permeabilization Concentrate with the eBioscience Fixation Perm Diluent. Once the EF was isolated, as described above, the EF was centrifuged at 2000rpm for 5 minutes at 18°C. Upon removal

of the supernatant, 0.2µL of IR Fluorescence Live/Dead (Invitrogen) was added directly to the cell pellet and incubated in the dark for 20 minutes at room temperature. After incubation, the stained cells were washed with 2ml of 1X PBS and centrifuged again at 2000rpm for 5 minutes at 18°C. As with the Live/Dead stain, 25µL of the MM1 was added to the EF pellet, incubated for 20 minutes in the dark at room temperature, washed with 2ml of 1X PBS, and centrifuged at 2000 rpm for 5 minutes at 18°C. The stained EF pellet was resuspended in 1mL of Foxp3/Transcription Factor (eBioscience) mixture and incubated in the dark for 45 minutes at 4°C to permeabilize the cells and prepare them for intracellular staining. After the incubation, the solution was washed in 2ml of 1X Permeabilization Wash Buffer (eBioscience) and centrifuged at 2000rpm for 5 minutes at 18°C twice. The pellet was then resuspended in 2µL of 2% goat serum (Thermo Fisher Scientific) and incubated in the dark for 15 minutes at room temperature to prevent non-specific antibody binding. After the incubation, the EF was again centrifuged at 2000rpm for 5 minutes at 18°C, and the goat serum was poured off. The EF pellet was then resuspended in 25µL of MM2 and incubated in the dark at room temperature for 30 minutes. The stained solution was washed for a final time in 2ml of 1X BD Perm Wash (BD Biosciences) and centrifuged at 2000rpm for 5 minutes at 18°C. After centrifugation, the EF pellet was resuspended in 150µL of 2% paraformaldehyde fixative (PFA) and stored at 4°C for running on the BD FACSymphony A5.

Table 1: Extracellular Mastermix Composition of the Intestinal Stem Cell (ISC) Panel.

Cell Marker	**Fluorophore**	**Vendor**	**Catalogue Number**	**Dilution Factor**	**Identifies**
Cellular amines	APC-Cy7	Invitrogen	L10119	1:2000	Dead Cells
CD45	V500	BD Biosciences	560777	1:100	Immune Cells
CD3	BV785	BioLegend	317330	1:50	T-Cells
CD4	BUV496	BD Biosciences	612936	1:50	$CD4^+$ T-Cells
CD8	BUV395	BD Biosciences	563796	1:50	$CD8^+$ T-Cells
EpCAM	PECF594	BioLegend	324232	1:2000	Epithelial Cells
CD44	FITC	BD Biosciences	560977	1:50	ISCs
EpBH2	APC	BD Biosciences	564699	1:25	ISCs
CD24	PE-Cy7	BD Biosciences	561646	1:25	Secretory Cells
CD117	BV650	BioLegend	313222	1:25	Secretory Cells
HLA-DR	PE	BioLegend	307606	1:100	Activation
Ki67	PerCP-Cy5.5	BD Biosciences	561284	1:50	Proliferation

2.1.6. ISC Flow Cytometric Gating Strategy

The ISC panel, as stated in Chapter 2, section 2.1.5, was performed on the EF extracted from the gut biopsies of uninfected controls and PLWH with a healthy gut or experiencing gut issues. The flow cytometric gating strategy of the ISC panel aimed to identify differentiated epithelial cells and ISCs involved in proliferation and inflammation.

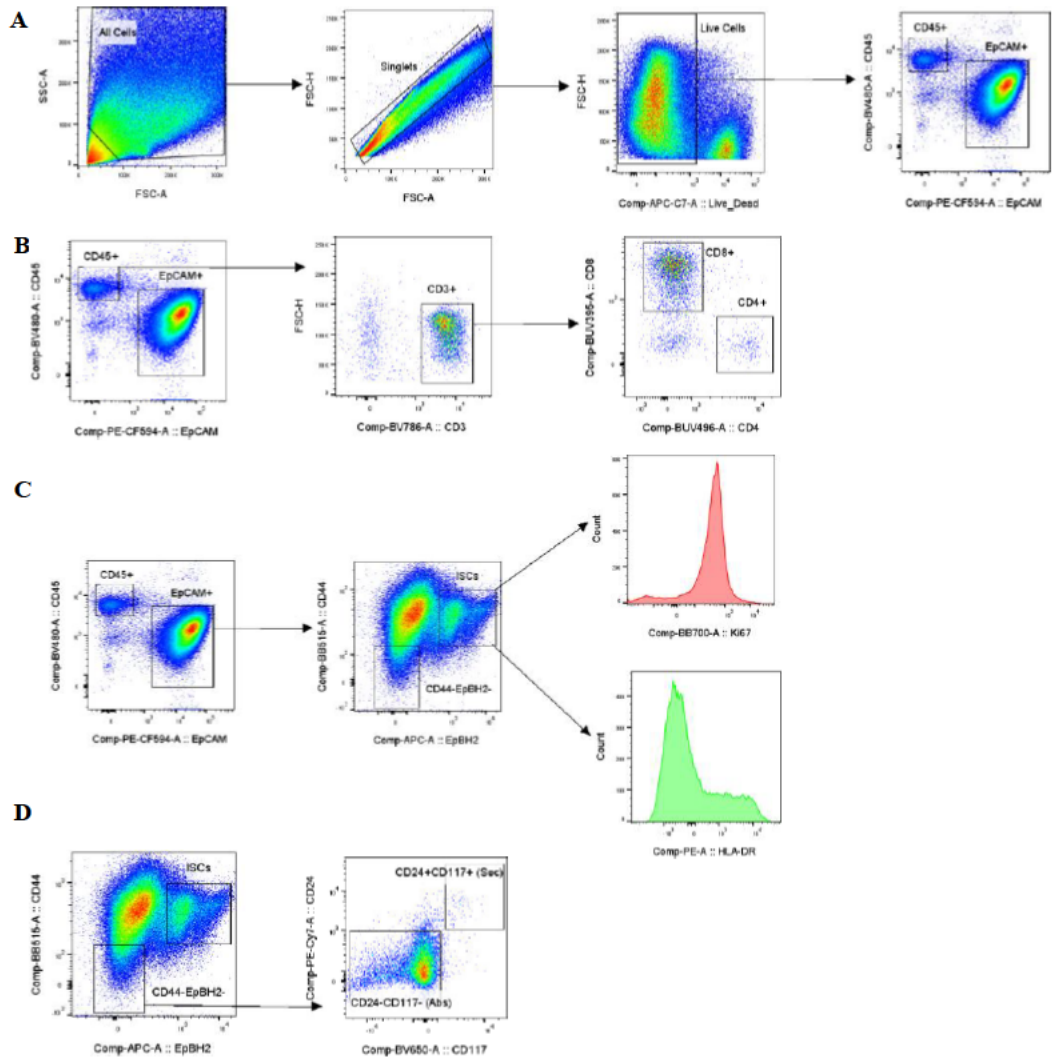

Figure 4: **flow cytometric gating strategy of the ISC panel**. A) Single, live cells are separated into immune ($CD45^+$) and epithelial ($EpCAM^+$) populations. B) $CD3^+$ T-cells and subsequently $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T-cells are derived from the immune population. C) Differentiated cells ($CD44^-EpBH2^-$) and ISCs ($CD44^+EpBH2^+$) are derived from the epithelial population. The ISC population subsequent expression of proliferation ($Ki67^+$) and inflammation ($HLA\text{-}DR^+$) are displayed as histograms D) Differentiated cells are separated into secretory ($CD24^+CD117^+$) and absorptive ($CD24^-CD117^-$) lineages.

The ISC panel first functioned to remove debris, clumped cells, and dead cells (Figure 4A). IR Fluorescent Dye, an APC-Cy7 fluorescent amine dye, was used to stain dead cells. IR Fluorescent Dye penetrates the compromised cellular membrane of dead cells and binds to free cytoplasmic amines. Live cells with intact cellular membranes protect their cytoplasmic amines from binding to the IR Fluorescent Dye. Therefore, in the ISC panel live cells were considered APC-Cy7$^-$. The live cells were subsequently gated to form 2 distinct populations: immune cells (CD45$^+$) and epithelial cells (EpCAM$^+$) (Figure 4A).

The immune cells were gated further to assess if HIV impacts intraepithelial T-cells in the gut. As detailed in section 2.1.5, Table 1 CD3 is commonly used in flow cytometry to identify T-cells. The ISC panel therefore selected for T-cells by selecting the CD45$^+$CD3$^+$ population. T-cells were gated into the T-cell's main groups: CD4$^+$ T-helper cells and CD8$^+$ cytotoxic T-cells (Figure 4B).

The epithelial cells (EpCAM$^+$) were gated further to separate them into ISCs and differentiated epithelial cells. CD44[139, 140] and EpBH2[141] are surface proteins found on ISCs that play a role in the proliferation, signalling Wnt pathway[81, 142] and can be used as markers to identify ISCs. The gating strategy therefore considered epithelial cells which were CD44$^+$EpBH2$^+$ as ISCs and CD44$^-$EpBH2$^-$ cells as differentiated. ISCs were subsequently gated against Ki67 (a proliferation marker) and HLA-DR (an inflammation and MHCII marker).[111] This enabled us to determine if ISCs were actively proliferating (Ki67$^+$) and if ISCs may contribute to or be affected by inflammation (HLA-DR$^+$) (Figure 4C). The differentiated epithelial cells were gated further to separate them into the absorptive or secretory lineages. CD24[143] and CD117[144] are markers known to be found on Paneth and goblet cells. The gating strategy therefore considered CD24$^+$ CD117$^+$ as secretory epithelial cells and CD24$^-$CD117$^-$ as absorptive epithelial cells (Figure 4D). The event cut-off applied to key source populations CD45$^+$, EpCAM$^+$ analyzed was a minimum of 1,000. Each phenotypic subset had an event cut-off of 100 events.

The gating strategy was determined using FMOs excluding: CD45, EpBH2, and CD117 (see Figure 5).

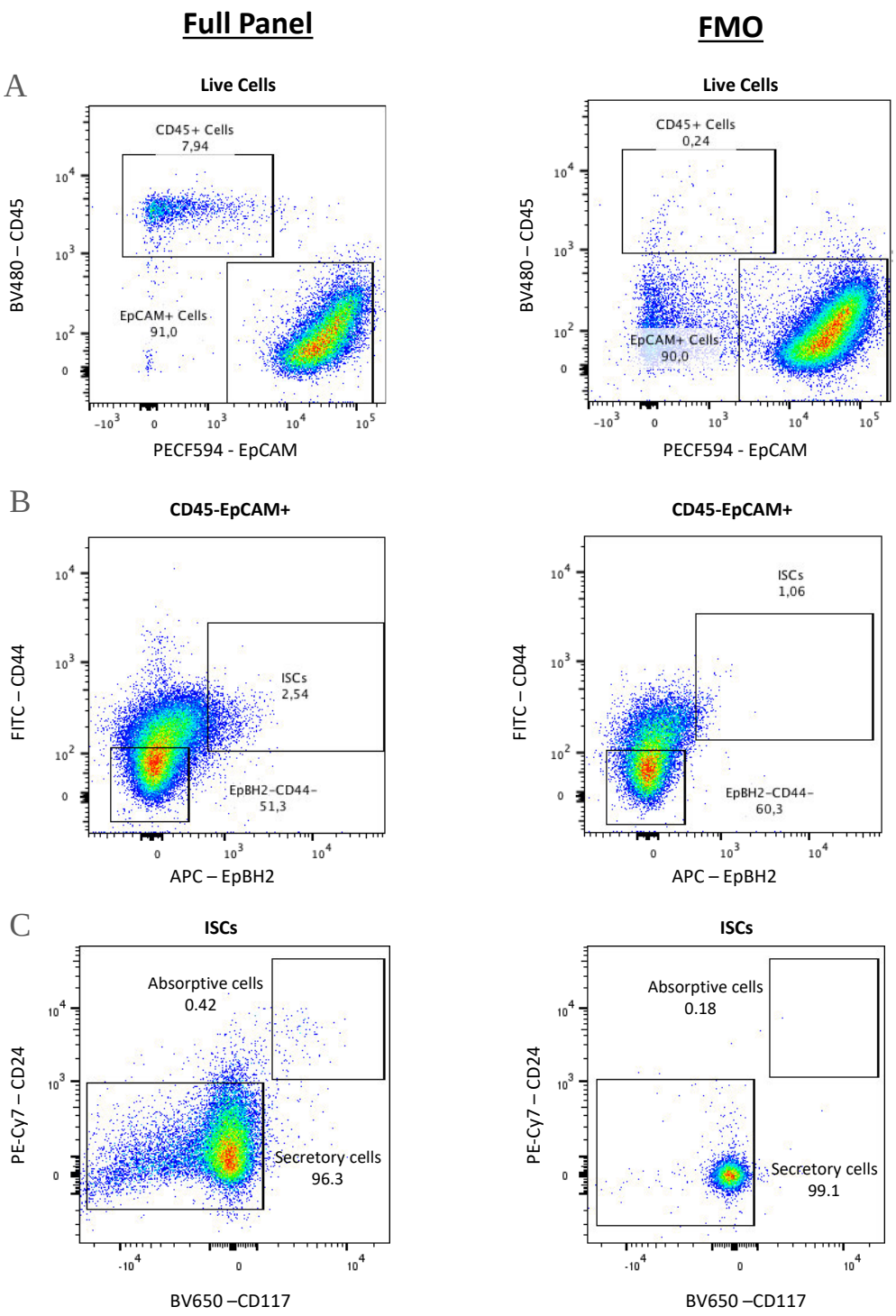

Figure 5: **FMOs of key markers used to establish the ISC gating strategy**. A) Comparison of gut tissue stained with the ISC panel including CD45 on V500 (left) and excluding CD45 on V500 (right). B) Comparison of gut tissue stained with the ISC panel including EpBH2 on APC (left) and excluding EpBH2 on APC (right). C) Comparison of gut tissue stained with the ISC panel including CD24 on PE-Cy7 (left) and excluding CD24 on APC (PE-Cy7).

2.1.7. Staining of stromal and immune cells

A stromal panel master mix (Table 2) was made in Brilliant Violet Buffer (BD Sciences) containing the following conjugated mouse antibodies: BUV395-CD8 (BD Biosciences), BUV496-CD4 (BD Biosciences), BV421-VCAM (BioLegend), V500-CD45 (BD Biosciences), BV605-CD19 (BioLegend), BV650-CD117 (BioLegend), BV711-ICAM (BD Biosciences), BV785-CD3 (BioLegend), FITC-CD235a/b (BD Biosciences), PE-HLA-DR (BioLegend), PE-CF594-EpCAM (BioLegend), PE-Cy7-CD34 (BioLegend), APC-CD31 (BD Biosciences), and AlexFlour700-CD38 (BioLegend). A conjugated rat antibody: PerCP-Cy5-5-Podoplanin (PDPN)(BioLegend) was also added to the master mix. Once the LF was isolated and the stromal panel master mix made up, as described above, the LF was centrifuged at 2000rpm for 5 minutes at 18°C. As with the EF upon removal of the supernatant, 0.2µL of IR Fluorescence Live/Dead (Invitrogen) stain was added to the pellet and incubated for 20 minutes in the dark at room temperature. The stained cells were washed with 2ml of 1X PBS and centrifuged at 2000rpm for 5 minutes at 18°C. 25µL of the stromal master mix was added to the LF pellet. The LF and stains were incubated for 20 minutes in the dark at room temperature. After incubation, the LF was washed with 2 ml of 1X PBS. After the wash step, 150µL of 2% PFA or 500µL of 1X PBS was added to the stained LF and kept at 4°C for running on the BD FACSymphony A5 or BD Aria Fusion.

Table 2: Extracellular Mastermix Composition of the Stromal Panel.

Cell Marker	**Fluorophore**	**Vendor**	**Catalogue Number**	**Dilution Factor**	**Identifies**
Cellular amines	APC-Cy7	Invitrogen	L10119	1:2000	Dead Cells
CD45	V500	BD Biosciences	560777	1:100	Immune Cells
CD3	BV785	BioLegend	317330	1:50	T-Cells
CD4	BUV496	BD Biosciences	612936	1:50	$CD4^+$ T-Cells
CD8	BUV395	BD Biosciences	563796	1:50	$CD8^+$ T-Cells
EpCAM	PECF594	BioLegend	324232	1:2000	Epithelial Cells
CD235a	FITC	BD Biosciences	559943	1:25	Erythrocytes
CD38	AlexaFluro700	BioLegend	356624	1:25	Plasma Cells
CD19	BV605	BioLegend	302244	1:1000	B-Cells
CD31	APC	BioLegend	303116	1:25	Endothelial Cells
PDPN	PerCP-Cy5.5	BioLegend	337012	1:25	Fibroblasts
CD34	PE-Cy7	BioLegend	343516	1:25	CSCs

HLA-DR	PE	BioLegend	307606	1:25	Activation
VCAM	BV421	BD Biosciences	744309	1:25	Activation
ICAM	BV711	BD Biosciences	564078	1:25	Activation

2.1.8. Stromal Gating Strategy

The stromal panel (as stated in Chapter 2, section 2.1.7) was stained on the LF of the gut biopsies taken from uninfected controls and PLWH with healthy guts and those experiencing gut issues. The flow cytometric gating strategy of the stromal panel aimed to identify stromal cells and their expression of inflammation markers.

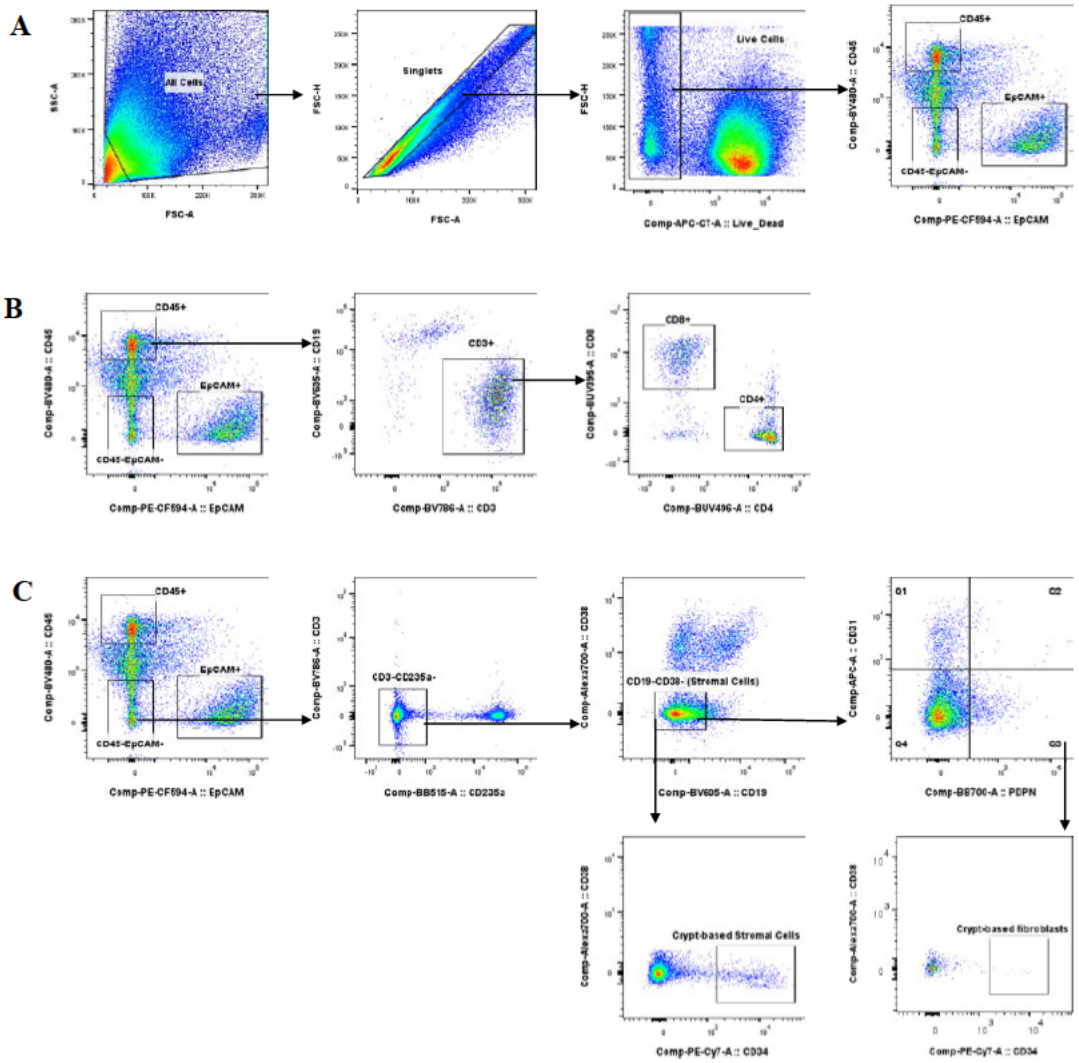

Figure 6: **the stromal panel's flow cytometric gating strategy**. A) Single, live cells are separated into immune ($CD45^+$), epithelial ($EpCAM^+$) and non-immune, non-epithelial ($CD45^-EpCAM^-$) populations. B) $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T-cells are derived from the immune population. C) Removal of erythrocytes ($CD235a^+$), plasma cells ($CD38^+$) and B-cells ($CD19^+$) from the non-immune, non-epithelial population identify the stromal cells. The stromal cells are then identified as: Q1 – blood endothelial cells ($CD31^+PDPN^-$), Q2 – lymphatic endothelial cells ($CD31^+PDPN^+$), Q3 –fibroblasts ($CD31^-PDPN^+$), Q4 – $CD31^-PDPN^-$ stromal cells, crypt-based stromal cells ($CD34^+$) and crypt-based fibroblasts (CD31-PDPN+CD34+).

The stromal gating strategy first removed debris, clumped, and dead cells (Figure 6A). As described previously the dead cells were identified as APC-$Cy7^+$, and the live cells as APC-$Cy7^-$. The live cells

were then separated into three populations: immune cells ($CD45^+$), epithelial cells ($EpCAM^+$), and non-immune ($CD45^-$), non-epithelial ($EpCAM^-$) cells (Figure 6A).

The immune cells were gated further to observe the impact of HIV on the $CD4^+$ T-cells in the gut. As stated in section 2.1.7, Table 3: CD3 is a T-cell marker and CD19 is a B-cell marker. The gating strategy therefore isolated T-cells ($CD45^+CD3^+CD19^-$) cells and excluded B-cells ($CD45^+CD19^+$). The T-cells were then gated to separate them into $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T-cells (Figure 6B).

The non-immune, non-epithelial cellular population was gated further to identify stromal cells. Stromal cells are a heterogeneous population with few distinct flow cytometric markers. To accurately identify stromal cells, the gating strategy first excluded non-stromal cells such as T-cells ($CD3^+$), erythrocytes ($CD235a^+$), plasma cells ($CD38^+$), and B-cells ($CD19^+$). The $CD45^-EpCAM^-CD3^-CD235a^-CD38^-CD19^-$ cellular population was therefore considered stromal cells by the gating strategy. These stromal cells were then gated further to identify how much of the stromal cells present were BECs ($CD31^+$ $PDPN^-$), lymphatic endothelial cells (LECs) ($CD31^+$ $PDPN^+$), fibroblasts ($CD31^-$ $PDPN^+$)[105], $CD31^+$ $PDPN^+$ stromal cells, and CSCs ($CD34^+$)(Figure 6C)[109]. The percentage of these stromal populations expressing the inflammation markers was also observed (Figure 8) though not analysed and discussed in this study. The event cut-off applied to key source populations $CD45^+$, $CD45^-EpCAM^-$ analyzed was a minimum of 1,000. Each phenotypic subset had an event cut-off of 100 events.

The stromal gating strategy was determined by analysing epithelial cells, known to be negative for key immune, blood, and stromal markers in the stromal panel (see Figure 7).

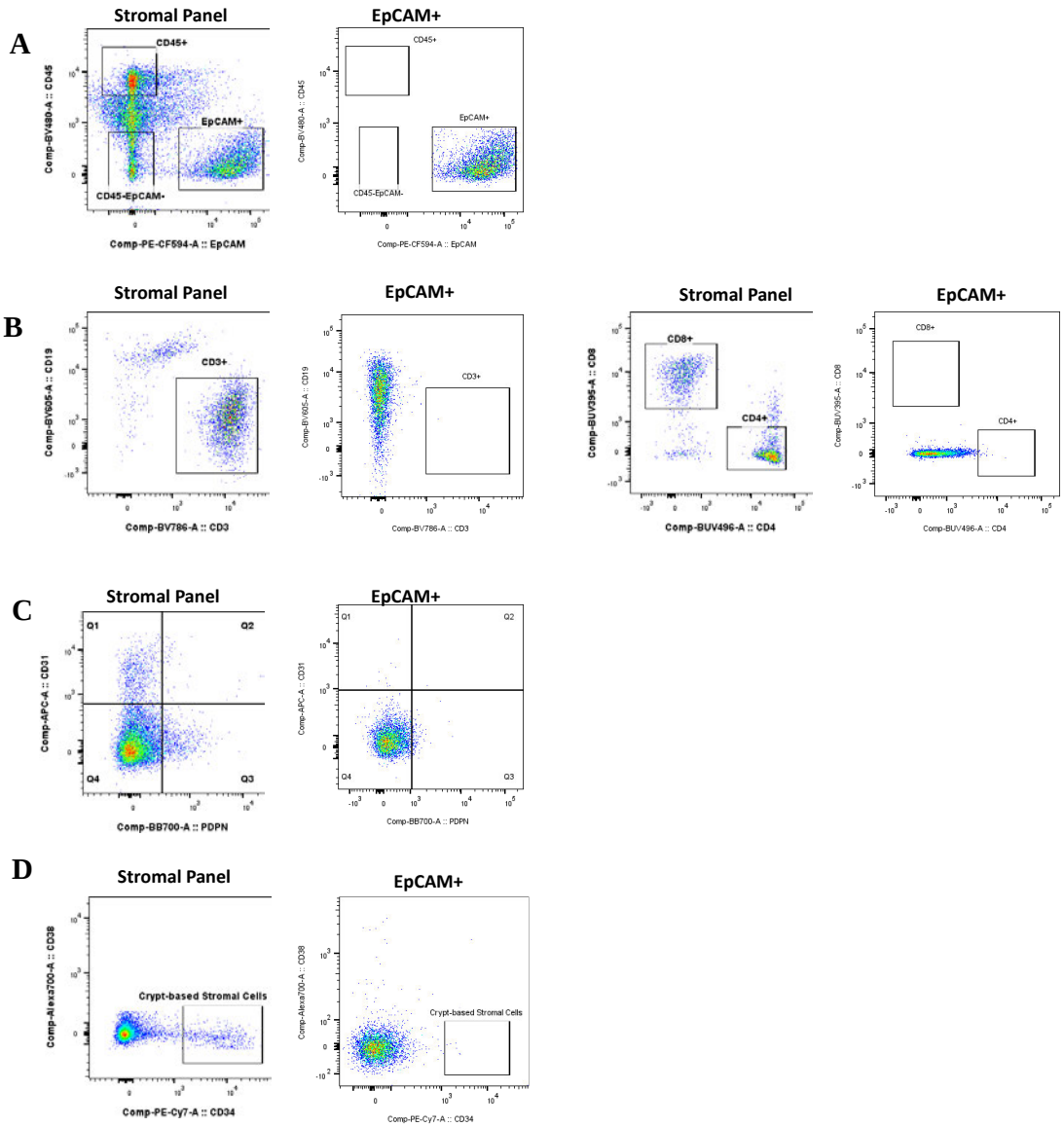

Figure 7: **Epithelial cells set against key immune and stromal markers.** A) Stromal panel (left) against epithelial cells known to be negative for CD45-V500 (right). B) Stromal panel staining of immune cells that express CD19 and CD3 (left) against epithelial cells known to be negative for CD3-BV785 (middle left). Stromal panel staining of immune cells that express CD8 and CD4 (left) against epithelial cells which are negative for CD8-BUV395 and CD4-BUV496 (middle left). C) Stromal panel staining of stromal cells that express CD31 and PDPN (left) against epithelial cells known to express are negative of CD31 and express little PDPN. D) Stromal panel staining of stromal cells expressing CD34 (left) against epithelial cells that express little CD34.

Epithelial cells (EpCAM^{+}) are known to not express, or express very little of the main immune (CD45, CD3, CD8, and CD4) and stromal markers (CD31, PDPN, and CD34). They were, therefore, used to set boundaries of the negative populations and to assist in gating positive populations for said markers in the Stromal Panel (see Figure 6).

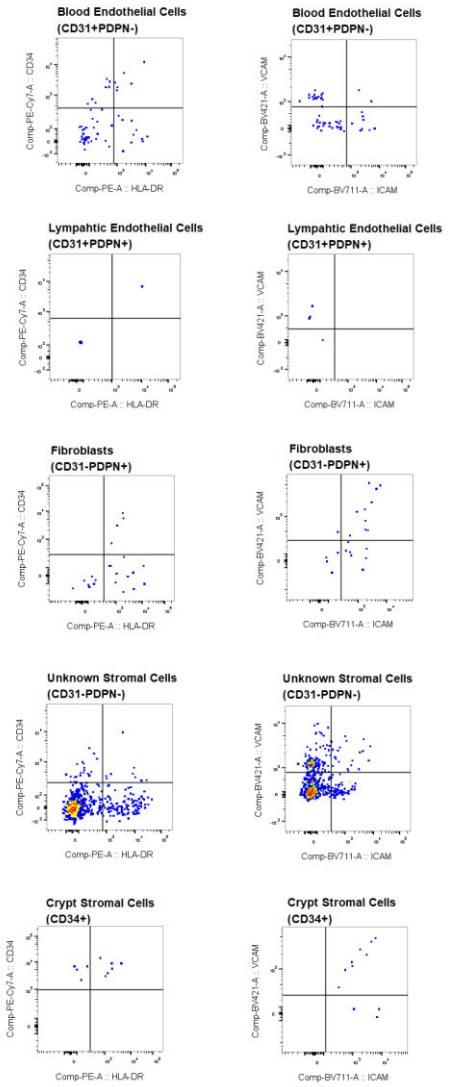

Figure 8: **the stromal panel's flow cytometric grating strategy used to determine the expression of various inflammation markers (VCAM, ICAM, and HLA-DR) by the populations identified.** The expression of HLA-DR (left) as well as VCAM and ICAM (right) by blood endothelial cells (CD31+PDPN-), lymphatic endothelial cells (CD31+PDPN+), fibroblasts (CD31-PDPN+), CD31-PDPN- stromal cells, and crypt-based stromal cells (CD34+) are depicted as pseudo-colour dot plots.

2.1.9. Data Analysis

Flow cytometric data was analysed per the gating strategies in Figure 4 and Figure 6 using FlowJo v10.0. Flow cytometric data from the CI cohort was separated into two groups: negative controls and PLWH. Flow cytometric data from the NCI cohort was divided into three groups based on the blood viremia of the participants. The “Negative” group is uninfected control participants who were confirmed to be HIV-negative. The “Aviremic” group participants are confirmed to be people living with suppressed HIV. Aviremic was defined as PLWH that have an undetectable viral load or a viral load of less than 250 HIV RNA copies per ml of plasma. The “Viremic” group was defined as PLWH that have a viral load of more than 250 HIV RNA copies per ml of plasma. Mann-Whitney and Kruskal-Wallis tests were used for statistical analysis of all data obtained in GraphPad Prism 10.

CHAPTER THREE

3.1. Participant Data and Gating Strategies Used

3.1.1. Background Participant Data from the CI and NCI Cohorts

The prevalence of HIV and its disease progression are impacted by a variety of patient factors, including viral load, $CD4^+$ count, use of ART[36, 47], age, and gender. The population group in South Africa with the highest percentage of PLWH are females between the ages of 15 and 49[3]. Around 77% of PLWH are on ART while 71% have a suppressed viral load. In general, although more South African females have HIV than males, they are also more likely to take ART than their male counterparts living with HIV[3].

Table 3: CI participant data at the time duodenum and colon biopsies were taken and analysed in this study.

	PLWH (n = 24)	Viremic (n = 3)	Aviremic (n = 21)	HIV - (n = 38)
Age	57 (30 - 63)	55 (52 - 62)	45 (30 - 63)	56 (22 - 88)
Males	6	1	5	15
Females	18	2	16	23
Viral Load (cps/mL)	900 (<20 - 149 657)	5 296 (282 - 1 4967)	<20	N/A
Blood CD4+ count (cells/uL)	551 (167 - 1 152)	226 (167 - 272)	597 (206 -1 152)	663
Blood CD4:CD8 ratio	0,81 (0,11 - 1,65)	0,5 (0,11 - 1)	0,86 (0,25 - 1,65)	1,57
Time on ART (years)	8,2 (0 - 18)	5 (0 - 10)	8,6 (1 - 18)	N/A

Participants from the CI cohort all had diverse GI issues[55] at the time duodenal and colon biopsies were taken. These duodenum and colon biopsies were obtained from different participants based on their GI issues and are therefore unmatched. A total of 24 duodenum biopsies and 24 colon biopsies were obtained from CI participants and analysed on the ISC panel (see Figure 4). Forty-two percent of both duodenum and colon biopsies were from PLWH and 58% were from uninfected controls. No duodenum biopsies analysed on the ISC panel were from viremic PLWH. All HIV-positive duodenum biopsies analysed on the ISC panel were from aviremic PLWH. Unlike the duodenum biopsies, 8% of the HIV-positive colon biopsies analysed were from viremic PLWH. Around 70% of both duodenum and colon biopsies obtained from PLWH were female and 30% male. The ISC results obtained from the CI cohort are therefore skewed toward aviremic females. The same number of duodenum and colon biopsies on the ISC panel was analysed on the Stromal panel (see Figure 6) with a skewing of PLWH data towards aviremic females.

Table 4: NCI participant data at the time intestinal biopsies were obtained and analysed in this study.

	PLWH (n = 17)	Viremic (n = 7)	Aviremic (n = 10)	HIV - (n = 6)
Age	30 (20 - 50)	30 (20 - 40)	43 (25 - 50)	24 (23 - 26)
Males	5	4	1	5
Females	12	3	9	1
Viral Load (cps/mL)	38 633 (<20 - 210 000)	38 633 (230 - 210 000)	<20	N/A
Blood CD4+ count (cells/uL)	655 (11 - 1 372)	359 (11 - 554)	862 (529 - 1 372)	933 (676 - 1 307)
Blood CD4:CD8 ratio	0,88 (0,06 - 1,96)	0,45 (0,06 -0,92)	1,18 (0,33 - 1,96)	1,45 (1,33 - 1,96)
Time on ART (years)	4,24 (0 - 17)	0,43 (0 - 3)	6,9 (0 - 17)	N/A

Participants from the NCI cohort had no GI issues when the endoscopies and colonoscopies were performed. Doctors were instructed to obtain biopsies of the duodenum, ileum, and colon from all participants to enable the matching of data across intestinal compartments. A total of 23 participants were recruited for which duodenum, ileum, and colon biopsies were processed and analysed on the ISC panel (Figure 4). Twenty-seven percent of the participants were uninfected controls. Seventy-two percent of the participants were PLWH. Forty-one percent of the participants were aviremic, while 32% were viremic. A higher percentage of PLWH recruited were female, similar to the CI cohort. The ISC results obtained from this population were similarly distributed across uninfected controls, aviremic PLWH, and viremic PLWH. This similar distribution allowed the comparison of data across HIV viremia. However, there was a gendered bias towards males in the uninfected controls and females in aviremic PLWH. This bias could unintentionally affect the results observed.

A total of 19 participants undergoing a colonoscopy and endoscopy to obtain pinches from the duodenum, ileum, and colon biopsies were analysed on the Stromal panel (Figure 6). Thirty-two percent of the participants were uninfected controls. The remaining 68% were PLWH. Of the 68% PLWH, 46% were aviremic and 54% viremic. All uninfected controls were males. All aviremic PLWH were females. Fifty-seven percent of viremic PLWH were male and 43% were female. As with the ISC data, the stromal data was evenly distributed across HIV viremia and is skewed with the sex of the participants.

3.2. Intestinal Stem Cell Results

3.2.1. ISC Results from a CI Cohort

The gut biopsies analysed from the CI cohort (Table 3) were obtained from uninfected controls and PLWH with ongoing GI issues and analysed on the ISC panel (Figure 4). The data obtained from the CI cohort only relates to unmatched duodenum and colon biopsies.

3.2.1.1. Comparison of Epithelial Cell Populations in PLWH and Controls

ISC proliferation and differentiation are normally balanced to maintain the intestinal epithelium. This study investigated whether HIV compromises the intestinal epithelium's integrity by impacting ISCs and IECs. This was done by staining gut biopsies of uninfected controls and PLWH using the ISC panel (see Figure 4). Differences in the percentage of epithelial cells that are ISCs and differences in differentiated epithelial populations in uninfected controls and PLWH were assessed.

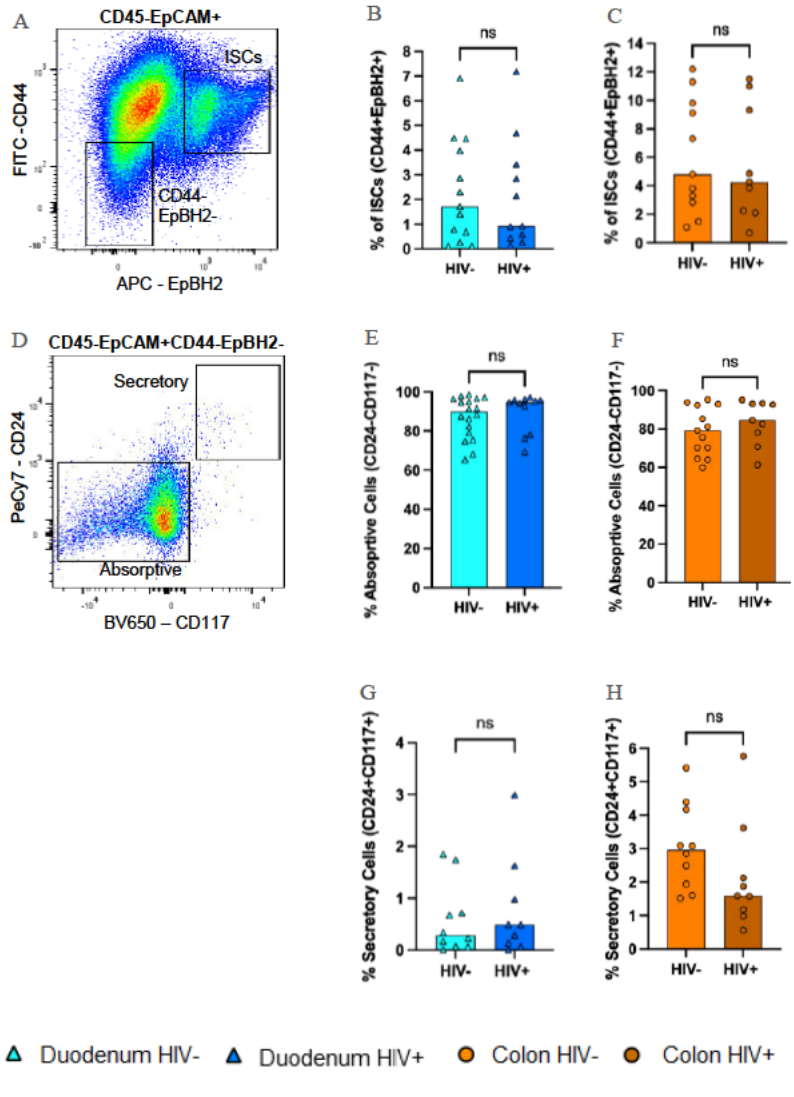

Figure 9: **comparison of epithelial populations in uninfected controls and PLWH experiencing gut issues.** A) Representative flow cytometry plot depicting ISCs ($CD44^{+}Epbh2^{+}$) and differentiated epithelial cells ($CD44^{-}Epbh2^{-}$). B – C) Comparison of the percentage of epithelial cells that are ISCs in the duodenum (B) and colon (C) of controls and PLWH. D) Representative flow cytometry plot of differentiated epithelial cells separated into absorptive ($CD24^{-}CD117^{-}$) and secretory lineages ($CD24^{+}CD117^{+}$). E – H) Comparison of the percentage of differentiated epithelial cells that are absorptive and secretory in the duodenum (E & G) and colon (F & H) of controls and PLWH. Statistical *P*-values were calculated using the Mann-Whitney test NS (*P*-value >0.05).

Contradictory to this study's hypothesis, HIV did not appear to have a higher frequency of ISCs in PLWH (Figure 9B & C). There were no statistically significant differences in ISCs, absorptive cells, and secretory cells between uninfected controls and PLWH in the duodenum or colon of clinically indicated participants (Figure 9E – H). As the main function of the intestines is to absorb nutrients, most of the differentiated epithelial cells in uninfected controls and PLWH were absorptive (Figure 9E & F). At the same time, a small percentage were secretory (Figure 9G & H). Overall, contradictory to this

study's hypothesis, there were no significant differences in the frequency of ISCs or changes in epithelial populations between uninfected controls and PLWH in the CI cohort.

3.2.1.2. Comparison of ISCs Proliferative and Inflammatory Expression in PLWH and Controls

A study by Batman et al.[145] showed hyperproliferation in the crypts of HIV-infected intestinal tissue. A more recent study by Biton et al.[111] showed (using a mouse model) that ISCs and IECs can act as non-conventional APCs with $CD4^+$ T-helper cells. They also showed that the interaction between ISCs and IECs with $CD4^+$ T-helper cells can impact ISC proliferation. This study therefore analysed the expression of a proliferation marker (Ki67) and a major histocompatibility complex II (MHC II) marker (HLA-DR) by ISCs.

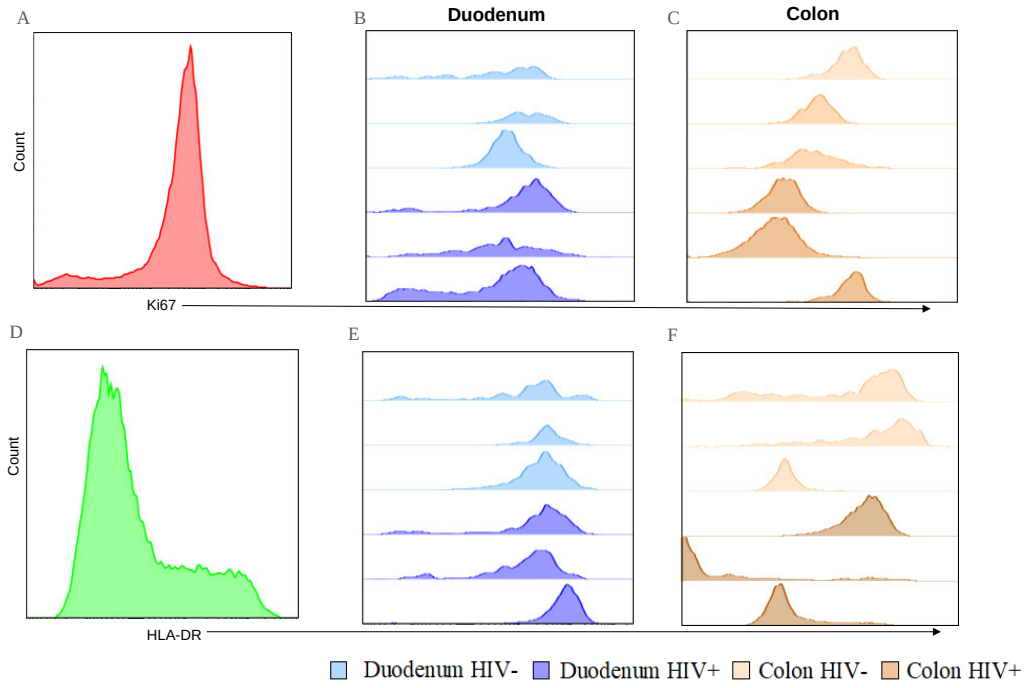

Figure 10: **histograms displaying the brightness of positive Ki67 and HLA-DR signals in the duodenum and colon of uninfected controls and PLWH.** A & D) representative flow cytometric histograms of ISCs expressing Ki67 (A) and HLA-DR (D). B & E) histograms displaying the brightness of the positive Ki67 (B) & HLA-DR (E) signals expressed by ISCs isolated from the duodenum of controls and PLWH. C & F) histograms displaying the brightness of the positive Ki67 (C) & HLA-DR (F) signals expressed by ISCs isolated from the colon of controls and PLWH. The brighter the signal the more right the peak is. Light blue represents control duodenum samples. Dark blue represents PLWH duodenum samples. Light orange represents control colon samples. Dark orange represents PLWH colon samples.

Comparison of Ki67 and HLA-DR positive signals between uninfected controls and PLWH (Figure 10) were variable. There was no significant trend or shift of Ki67 and HLA-DR signals being more or less bright between uninfected controls and PLWH in the duodenum and colon. Therefore, it appears that within people experiencing gut issues, there is no significant increase in ISC proliferation or hyper-proliferative ISCs, nor was there a decrease in ISCs' ability to interact with immune cells and differentiate. This is supported by the lack of significant differences in epithelial populations observed in Figure 9. Further analysis of the mean fluorescence intensity (MFI) for the expression of Ki67 and HLA-DR was performed to confirm this finding.

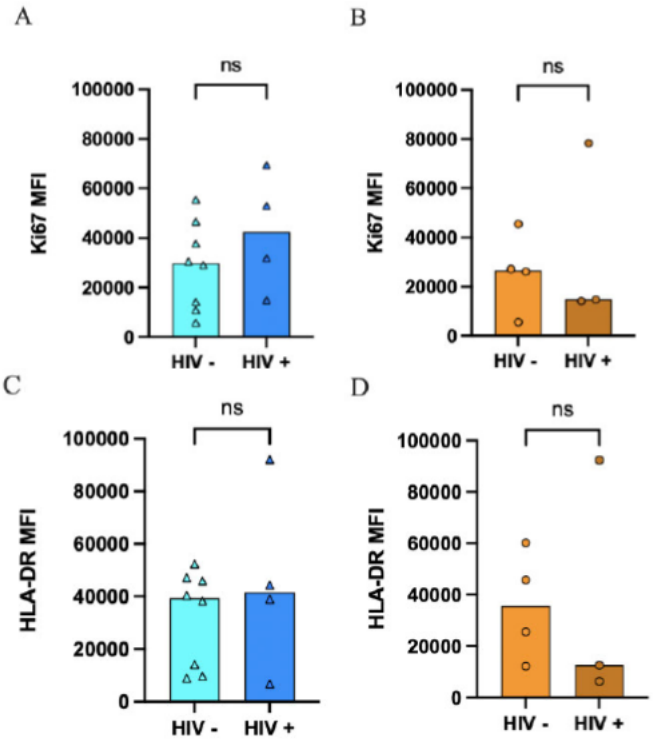

Figure 11: **Comparison of MFI for Ki67 and HLA-DR by ISCs in the duodenum and colon of uninfected controls and PLWH.** A & B) MFI, calculated using the geometric mean, for Ki67 expression by ISCs in the duodenum (A) and colon (B) of uninfected controls, and PLWH. C & D) MFI, calculated using the geometric mean, for HLA-DR expression by ISCs in the duodenum (C) and colon (D) of uninfected controls and PLWH. Statistical *P*-values were calculated using the Mann-Whitney test. NS (*P*-value > 0.05).

Comparison of MFI for Ki67 and HLA-DR between uninfected controls and PLWH remained similar (Figure 11). This further supports the findings of Figure 9 and 10.

3.2.2. ISC Results from a NCI Cohort

The gut biopsies for the NCI cohort participants (Table 4) were obtained from people volunteering to undergo an endoscopy and colonoscopy and analysed on the ISC panel (see Figure 4). The data pertains to matched duodenum, ileum, and colon biopsies.

3.2.2.1. Comparison of Epithelial Cell Populations in PLWH and uninfected controls.

The results from the CI cohort did not align with this study's hypothesis and findings from other studies [111, 145]. To ensure that the diseased state of the participants from the CI cohort did not mask the visible impact HIV has on intestinal epithelial populations, this study repeated its analysis in an NCI cohort. All participants from the NCI cohort (Table 4) were recruited to either be uninfected people, aviremic PLWH, or viremic PLWH with no underlying GI issues.

There is a strong correlation between HIV viremia and the severity of symptoms PLWH can experience[142]. Furthermore, HIV viremia is linked to the progression of HIV to AIDs [12, 36, 47]. This study, therefore, re-analysed the ISC data per blood viremia. The ISC data was separated into participants who were uninfected (Negative), aviremic PLWH (Aviremic), or viremic PLWH (Viremic) when the intestinal biopsies were obtained.

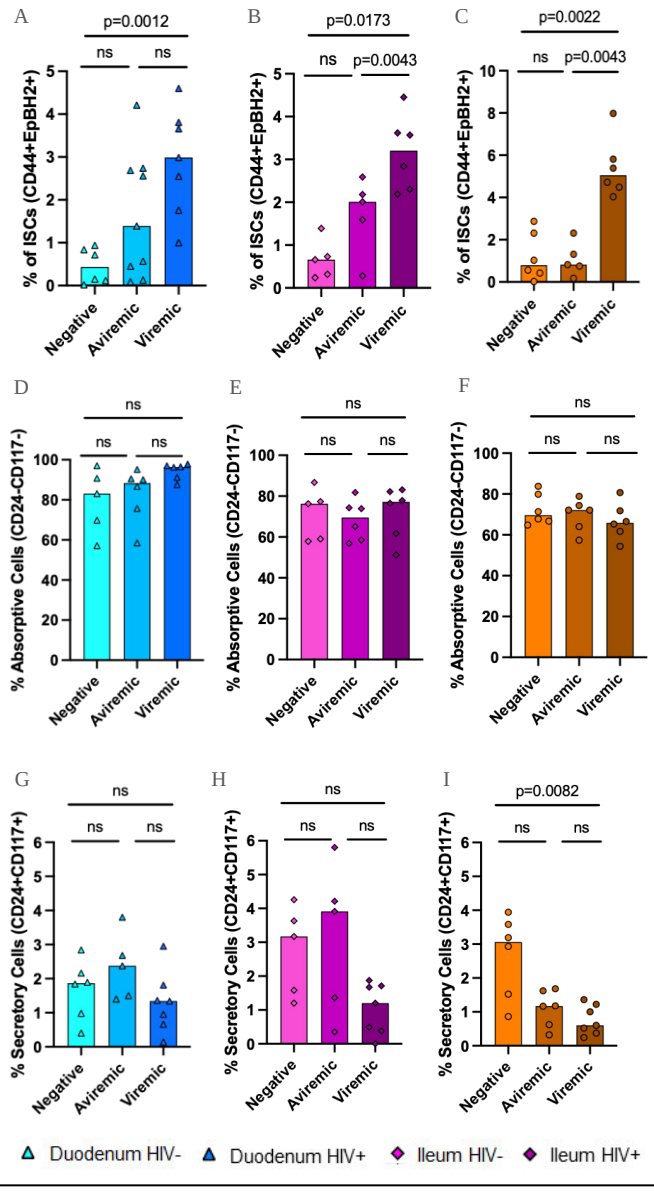

Figure 12: **comparison of the epithelial populations: ISCs, absorptive cells and secretory cells in the duodenum, ileum, and colon of uninfected controls and PLWH separated according to viremia**. A – C) Comparisons of the percentage of epithelial cells that are ISCs in the duodenum (A), ileum (B), and colon (C) of controls, aviremic and viremic. D – F) Comparisons of the percentage of differentiated epithelial cells that are absorptive in the duodenum (D), ileum (E), and colon (F) of control, aviremic and viremic. G – I) Comparisons of the percentage of differentiated epithelial cells that are secretory cells in the duodenum (G), ileum (H), and colon (I) of control, aviremic and viremic. Statistical *P*-values displayed were calculated using the Mann-Whitney test. NS (*P*-value > 0.05).

There was a higher frequency of ISCs in the duodenum, ileum, and colon of viremic PLWH compared with healthy, uninfected controls (Figure 12A – C). The frequency of ISCs in uninfected controlswas around 1 to 2% in all intestinal compartments. In viremic PLWH the ISCs had a higher frequency of up to 5% in the duodenum and ileum and up to 8% in the colon. Interestingly, although aviremic PLWH showed a higher frequency of ISCs compared with uninfected controls, particularly the duodenum (*P* = 0.1810) and ileum (*P* = 0.0952) (Figure 12A – B), it was not considered statistically significant. There were, however, statistically significantly more ISCs in the ileum and colon (*P* = 0.0043) of viremic PLWH compared with aviremic PLWH (Figure 12B & C).

There was no significant changes in the frequency of absorptive and secretory cells in the duodenum and ileum of PLWH compared with uninfected, healthy people (Figure 12D; E; G; H). Unlike the duodenum and ileum, the colon showed a significant decrease in secretory cells when comparing healthy, uninfected controls to viremic PLWH (Figure 12I).

Altogether, there was a trend for ISCs to be present at a higher frequency as viremia increases in the duodenum and ileum.The colon may require a high HIV viral load to trigger an increase in ISCs. To assess this assumption the correlation between viral load in the blood and a higher frequency of ISCs in the gut was analysed.

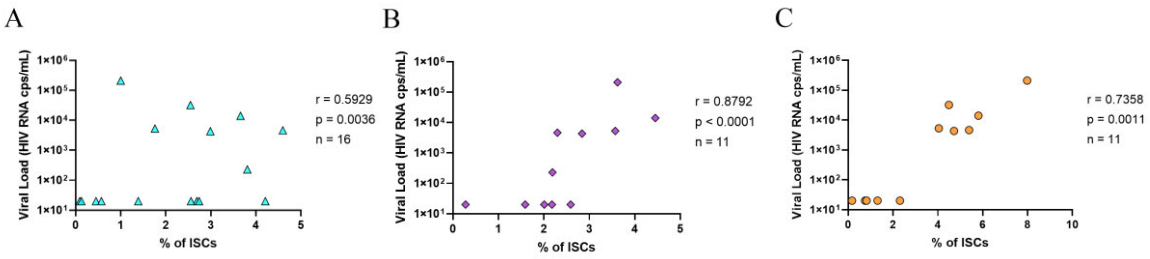

Figure 13: **Correlation graphs of viral load in the blood to the frequency of ISCs in the duodenum, ileum, and colon**. A) The correlation of viral load in the blood to the frequency of ISCs in the duodenum. B) The correlation of viral load in the blood to the frequency of ISCs in the ileum. C) The correlation of viral load in the blood to the frequency of ISCs in the colon. Statistical r and *P*-values were calculated using the Spearman test.

The correlation between viral load in the blood and a higher frequency of ISCs was considered moderate in the duodenum (r = 0.5929; *P* = 0.0036) (Figure 13A), very strong in the ileum (r = 0.8610; *P* = <0.0001) (Figure 13B) and strong in the colon (r = 0.7358; *P* = 0.0037) (Figure 13C).

3.2.2.2. Comparison of ISCs Proliferative and Inflammatory Expression in PLWH and negative controls.

To determine if the higher frequency of ISCs observed (Figure 12A – C) was due to hyper-proliferation, an increase in hyper-proliferative ISCs or a reduced capacity to differentiate, this study investigated the expression of Ki67 and HLA-DR by ISCs. A brighter Ki67 signal expressed by ISCs would indicate that ISCs are hyper-proliferating. An increase in the HLA-DR signal expressed by ISCs could indicate an increase in hyper-proliferative ISCs. A decrease in the HLA-DR signal expressed by ISCs could indicate that ISCs have reduced differentiation capacity[111].

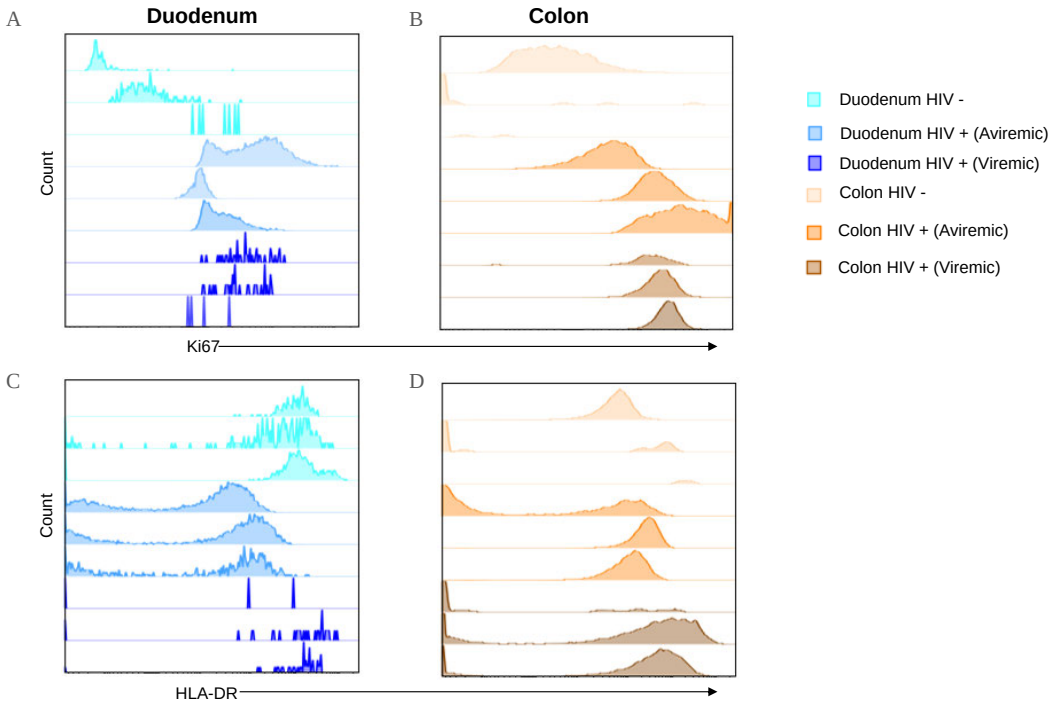

Figure 14: **histograms showing the brightness of positive Ki67 and HLA-DR signals by ISCs in the duodenum and colon of uninfected controls and PLWH.** A & C) histograms displaying the brightness of the positive Ki67 & HLA-DR signals expressed by ISCs isolated from the duodenum of uninfected controls and PLWH. B & D) histograms displaying the brightness of the positive Ki67 & HLA-DR signals expressed by ISCs isolated from the colon of controls and PLWH. The brighter the signal the more right the peak is. Light blue (first 3 rows) represents uninfected duodenum samples. Pale dark blue (middle 3 rows) represents aviremic duodenum samples. Dark blue (last 3 rows) represents viremic duodenum samples. Pale orange (first 3 rows) represents uninfected colon samples. Bright orange (middle 3 rows) represents aviremic colon samples. Dark brown (last 3 rows) represents viremic colon samples.

There seemed to be a brighter signal for the proliferation marker Ki67 in the duodenum and colon of PLWH (both aviremic and viremic) (Figure 14A & B). This indicates that the higher frequency of ISCs (see Figure 12A & C) is due to hyper-proliferation. There appeared to be no obvious changes in the brightness of ISCs expressing HLA-DR. These results suggest that the frequency of ISCs in the duodenum and colon is not caused by a reduced capacity for ISCs to differentiate.

Further analysis into the MFI of both Ki67 and HLA-DR by ISCs was undertaken.

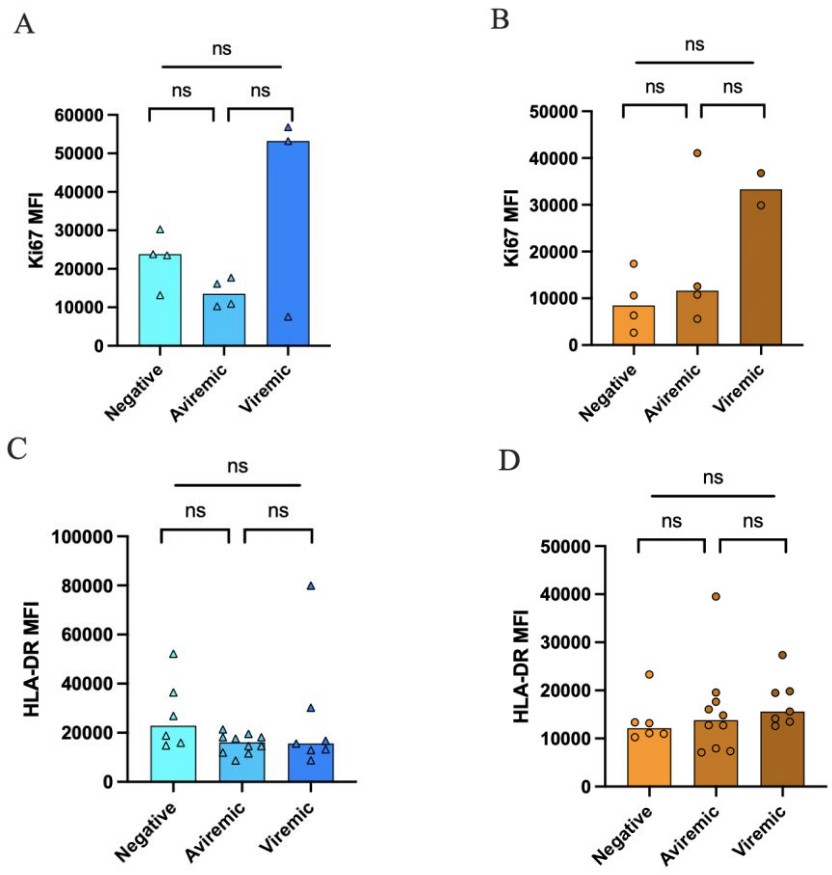

Figure 15: **Comparison of MFI for Ki67 and HLA-DR by ISCs in the duodenum and colon of uninfected controls and PLWH.** A & B) MFI, calculated using the geometric mean, for Ki67 expression by ISCs in the duodenum (A) and colon (B) of uninfected controls, aviremic PLWH, and viremic PLWH. ISCs in the duodenum. C & D) MFI, calculated using the geometric mean, for HLA-DR expression by ISCs in the duodenum (C) and colon (D) of uninfected controls, aviremic PLWH, and viremic PLWH. ISCs in the duodenum. Statistical *P*-values were calculated using the Mann-Whitney test. NS (*P*-value > 0.05).

There were no significant changes in the MFI expression of Ki67 and HLA-DR by ISCs in the duodenum and colon (Figure 15) when comparing uninfected controls and PLWH. The lack of an increase in the MFI for Ki67 is unexpected and contradictory to this study's hypothesis and findings in Figures 12 A & C, and Figure 14.

3.2.3. ISC Results across the CI and NCI Cohorts

Analysis of epithelial populations differed between the CI cohort and the NCI cohort. It was suspected that the diseased state of participants in the CI cohort may have caused the discrepancy in the results observed. Reanalysis and comparison of the epithelial populations between the cohorts in uninfected controls and PLWH was performed to quantify how much of a difference the diseased state of the gut obscures the impact of HIV.

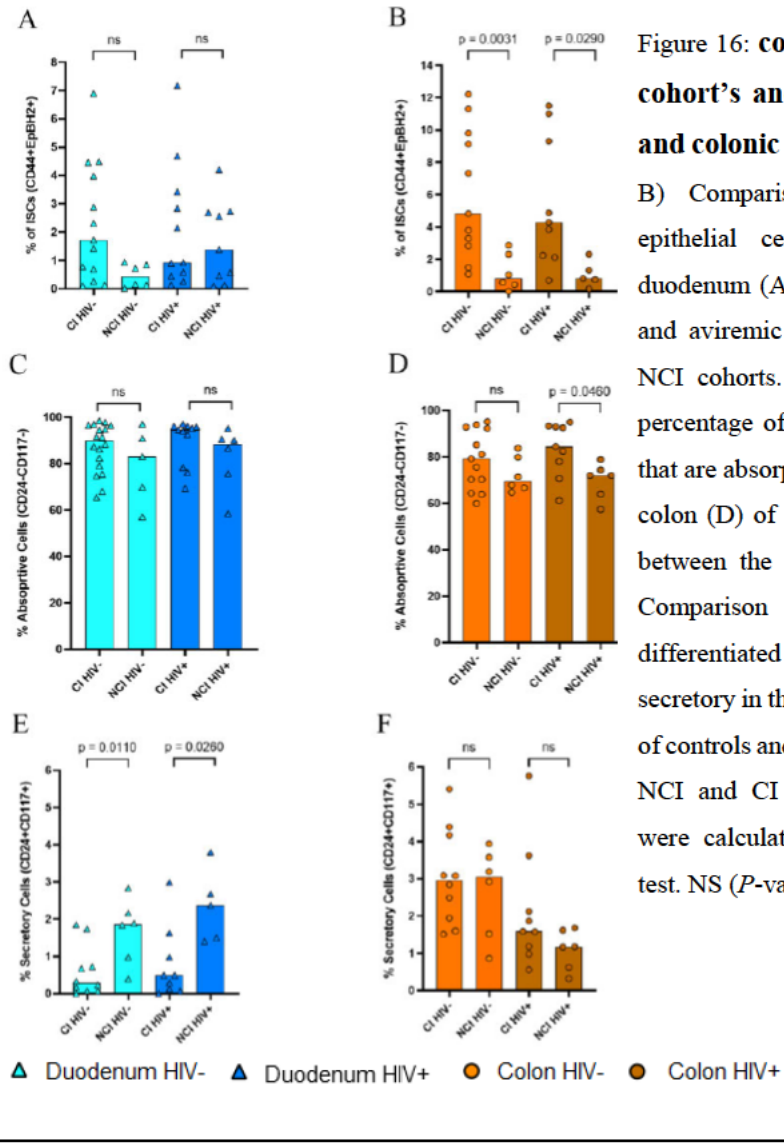

Figure 16: **comparison between the CI cohort's and NCI cohort's duodenal and colonic epithelial populations.** A – B) Comparison of the percentage of epithelial cells that are ISCs in the duodenum (A) and colonic (B) of controls and aviremic PLWH between the CI and NCI cohorts. C – D) Comparison of the percentage of differentiated epithelial cells that are absorptive in the duodenum (C) and colon (D) of controls and aviremic PLWH between the CI and NCI cohorts E – F) Comparison of the percentage of differentiated epithelial cells that are secretory in the duodenum (E) and colon (F) of controls and aviremic PLWH between the NCI and CI cohorts. Statistical *P*-values were calculated using the Mann-Whitney test. NS (*P*-value > 0.05).

The frequency of ISCs in the duodenum of aviremic PLWH was similar, regardless of whether they were from the CI or NCI cohort (Figure 16A). There was no difference in the frequency of absorptive cells in the duodenum in either the CI or NCI cohort (Figure 16C). Secretory cells were significantly more in the duodenum of healthy people, both those uninfected ($P = 0.0110$) and aviremic PLWH ($P = 0.0260$), when compared with those experiencing gut issues (Figure 16E). Intriguingly, the colon appears to be affected differently than the duodenum in all epithelial populations analysed. The colon had a higher frequency of ISCs in the CI cohort compared with the NCI regardless of HIV infection ($P = 0.0031$ and $P = 0.0290$) (Figure 16B). There were significantly fewer absorptive cells in the colon of healthy, aviremic PLWH ($P = 0.0460$) compared with those in the CI cohort (Figure 16D). There were no significant differences between the CI and NCI cohorts in the secretory cells of uninfected and aviremic PLWH (Figure 16F).

In conclusion, injury or disease, whether caused by HIV or not, increases the presence of ISCs in the duodenum and colon. A diseased state or HIV infection of the gut does not appear to impact the number of absorptive cells in the duodenum, though a diseased state (and not HIV) can reduce secretory cells. The colon, however, experiences a reduction in absorptive , which is more pronounced in a healthy gut than one experiencing GI issues. These results suggest the mechanism by which ISCs are increasing due to HIV infection is likely to be multifaceted and differ between intestinal compartments. Furthermore, the microenvironment of the gut may counteract or enhance the impact HIV has on intestinal epithelial populations.

3.3. Intestinal Stromal Cells Results

3.3.1. Stromal Results from a CI Cohort

The CI cohort participants (Table 3) underwent clinical procedures for GI-related complications. Duodenum and colon biopsies from different participants were processed and analysed on the Stromal panel (see Figure 6).

3.3.1.1. Comparison of Stromal Cell Populations in PLWH and uninfected people.

The stromal populations investigated using the Stromal panel (see Figure 6) were: blood endothelial cells (BECs), lymphatic endothelial cells (LECs), fibroblasts, and CSCs. BECs and LECs play an important role in migrating immune cells to and from a site of infection. Fibroblasts play an integral role in maintaining intestinal structure and disease pathology. Crypt-based fibroblasts (CBFs) and CSCs have been indicated to play an important role in influencing the proliferation and differentiation of ISCs.

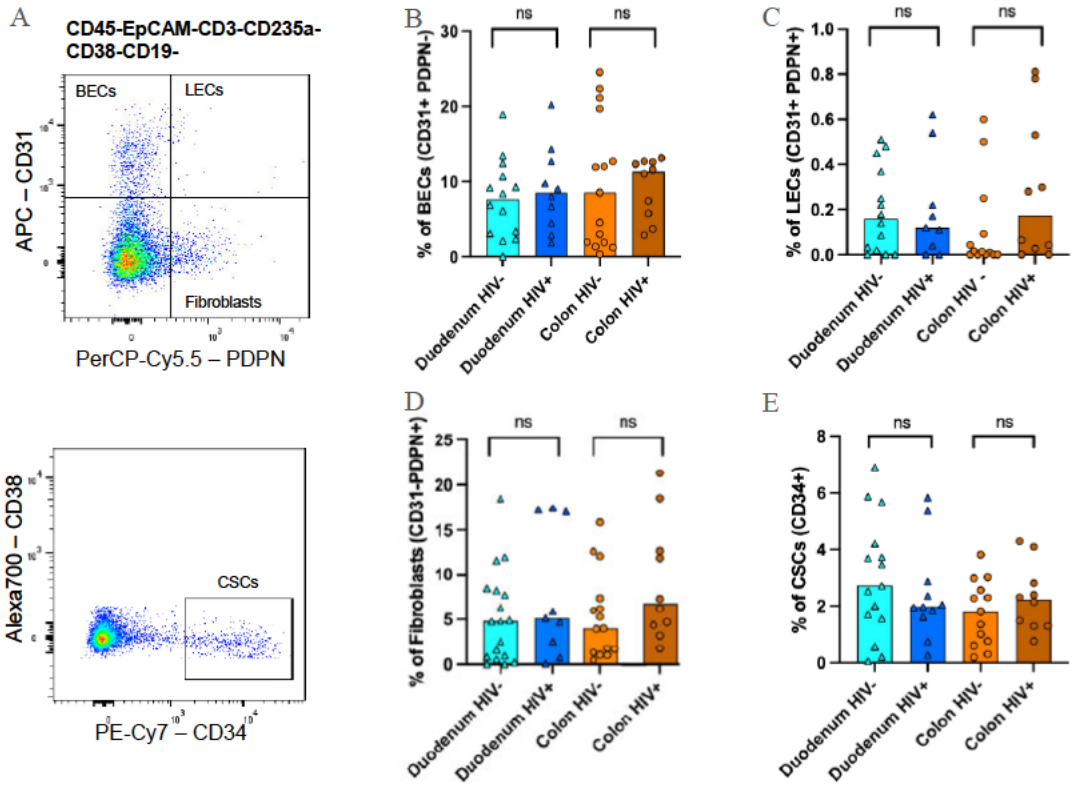

Figure 17: **comparison of stromal populations in the duodenum and colon of uninfected controls and PLWH experiencing gut issues**. A) Representative flow cytometry plots of stromal cells ($CD45^-EpCAM^-CD3^-CD235a^-CD38^-CD19^-$) identified as BECs ($CD31^+PDPN^-$), LECs ($CD31^+PDPN^+$), fibroblasts ($CD31^-PDPN^+$), and CSCs ($CD34^+$). Percentage of stromal cells that are B) BECs, C) LECs, D) fibroblasts, and E) CSCs in the duodenum and colon of controls and PLWH experiencing gut issues. Statistical *P*-values displayed were calculated using the Mann-Whitney test. NS (*P*-value > 0.05).

There was no statistically significant difference in the frequency of BECs, LECs, fibroblasts, and CSCs in the duodenum or colon of PLWH and uninfected controls experiencing GI issues (Figure 17). Overall, for all CI participants, the percentage of stromal cells that were BECs, LECs, fibroblasts, and CSCs varied but showed no trend to increase or decrease based on HIV infection.

3.3.2. Stromal Results from the NCI Cohort

The NCI participants were recruited to undergo a colonoscopy and endoscopy to obtain healthy gut (duodenum, ileum, and colon) samples. Matched duodenum, ileum, and colon data enabled us to see how HIV impacts all intestinal compartments in one participant.

3.3.2.1. Comparison of Stromal Cell Populations in PLWH and uninfected people

The obscuration of important differences between epithelial populations in the CI cohort motivated further exploration into the stromal populations in the NCI cohort. The same analysis of stromal cells performed in the CI cohort was repeated in intestinal biopsies from the NCI cohort (Table 4).

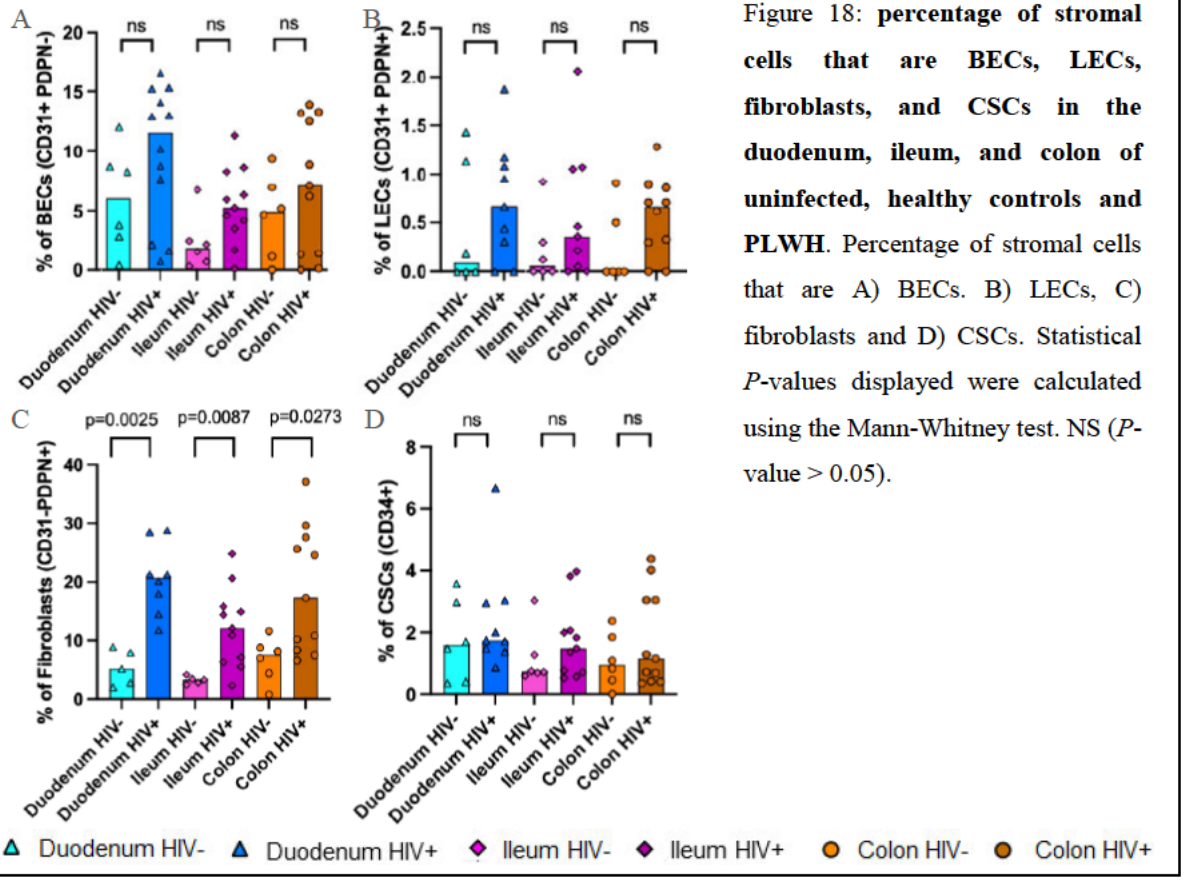

Figure 18: **percentage of stromal cells that are BECs, LECs, fibroblasts, and CSCs in the duodenum, ileum, and colon of uninfected, healthy controls and PLWH**. Percentage of stromal cells that are A) BECs. B) LECs, C) fibroblasts and D) CSCs. Statistical *P*-values displayed were calculated using the Mann-Whitney test. NS (*P*-value > 0.05).

There was no statistically significant difference in the percentage of stromal cells that were BECs ($CD31^+PDPN^-$) and, LECs ($CD31^+PDPN^+$) in the duodenum ($P = 0.1505$), ileum ($P = 0.0782$), and colon ($P = 0.3121$) of PLWH (Figure 18A & B). There was no statistically significant difference in the percentage of CSCs ($CD34^+$) between PLWH and uninfected, healthy controls. (Figure 18D) The only stromal population that appeared to be affected by HIV in a statistically significant manner was fibroblasts. There was a statistically significant higher frequency ($P_{duodenum} = 0.0025$, ($P_{ileum} = 0.0087$, and $P_{colon} = 0.0273$) (Figure 18C) in all intestinal compartments of PLWH compared with healthy, uninfected participants.

3.3.2.2. Impact of viremia HIV on Fibroblast population

The only stromal population which appeared to be affected by HIV was fibroblasts. As seen in Figure 16C, there was a larger percentage of fibroblasts in PLWH than in healthy, uninfected people.

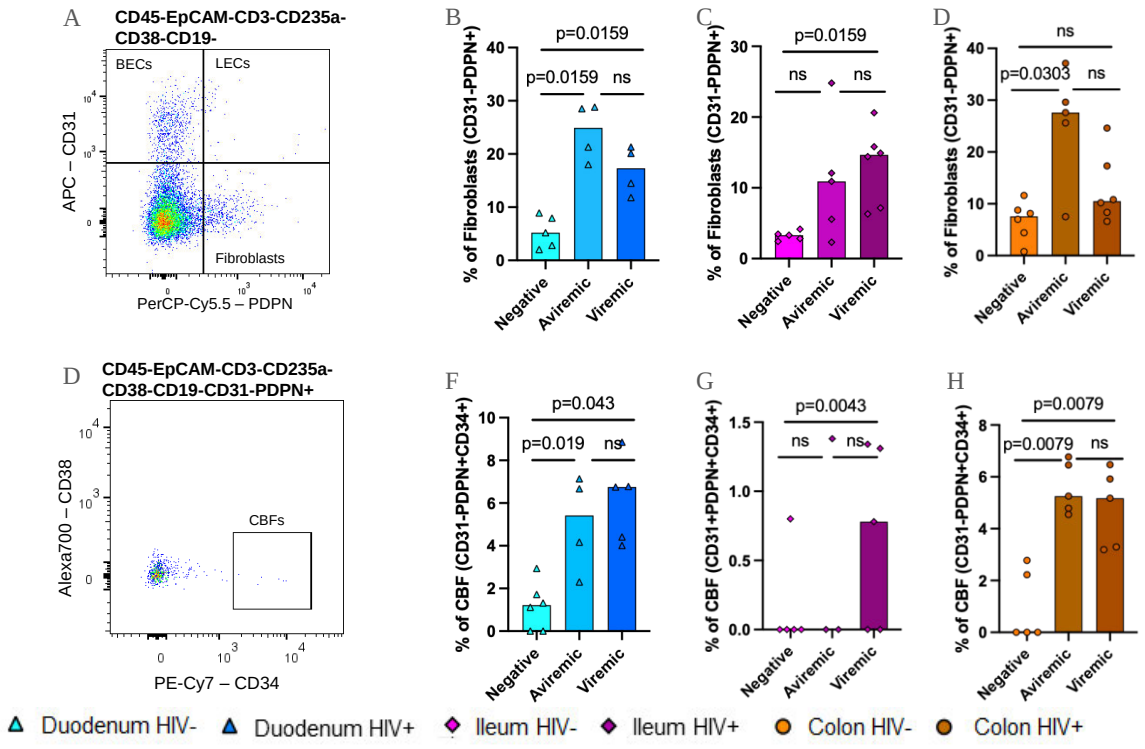

Figure 19: **percentage of stromal cells that are fibroblasts and the percentage of fibroblasts that are crypt-based in the intestinal compartments of uninfected controls, aviremic PLWH and viremic PLWH.** A) Representative flow cytometry plot of stromal cells separated into BECs ($CD31^+$ $PDPN^-$), LECs ($CD31^+$ $PDPN^+$), and fibroblasts ($CD31^-PDPN^+$). B – D) Comparison of the percentage of fibroblasts found in the duodenum (B), ileum (C), and colon (D) of controls, aviremic PLWH and viremic PLWH. E) Representative flow cytometry plot of CBFs ($CD31^-PDPN^+CD34^+$). F – H) Comparison of percentage of CBFs found in the duodenum (F), ileum (G), and colon (H) of controls, aviremic PLWH and viremic PLWH. Statistical *P*-values displayed were calculated using the Mann-Whitney test. NS (*P*-value > 0.05).

There was no direct correlation between HIV viremia and the higher frequency of fibroblasts seen in PLWH compared with healthy, uninfected controls (Figure 18C and Figure 19). This was evident as there was no significant difference in the percentage of stromal cells being fibroblasts when comparing aviremic and viremic PLWH ($P_{duodenum} = 0.1429$, $P_{ileum} = 0.4286$ $P_{colon} = 0.0823$). A significantly higher frequency of fibroblasts could be seen between aviremic PLWH and uninfected controls in the duodenum ($P = 0.0159$) (Figure 19B) and colon ($P = 0.0303$) (Figure 19D). Only in the ileum did there appear to be a higher frequency of fibroblasts from uninfected controls, aviremic PLWH to viremic

PLWH, though the differences between each group are statistically insignificant (Figure 19C). The higher frequency of fibroblasts between viremic PLWH and healthy controls was reflected in CBFs. There are statistically significantly more CBFs in viremic PLWH in all intestinal compartments ($P_{duodenum} = 0.043$, $P_{ileum} = 0.0159$, $P_{colon} = 0.0079$) (Figure 19F – H). The higher frequency of fibroblasts in all intestinal compartments of aviremic PLWH did not correspond to a higher frequency of CBF. Overall, the data suggests that infection with HIV can increase the presence of fibroblasts, including CBFs, in the duodenum, colon, and ileum, particularly in viremic PLWH.

3.3.2. Stromal Results across the CI and NCI Cohorts

The higher frequency of fibroblasts observed in the NCI cohort was not apparent in the CI cohort when comparing uninfected controls and PLWH. This motivated the comparison of stromal populations between the two cohorts. Reanalysis and comparison of the percentage of stromal cells that are BECs, LECs, fibroblasts, and CSCs between the clinically indicated cohort and the healthy, research-elect cohort were performed to provide insight as to how a diseased gut can combat or augment how HIV impacts the stromal populations.

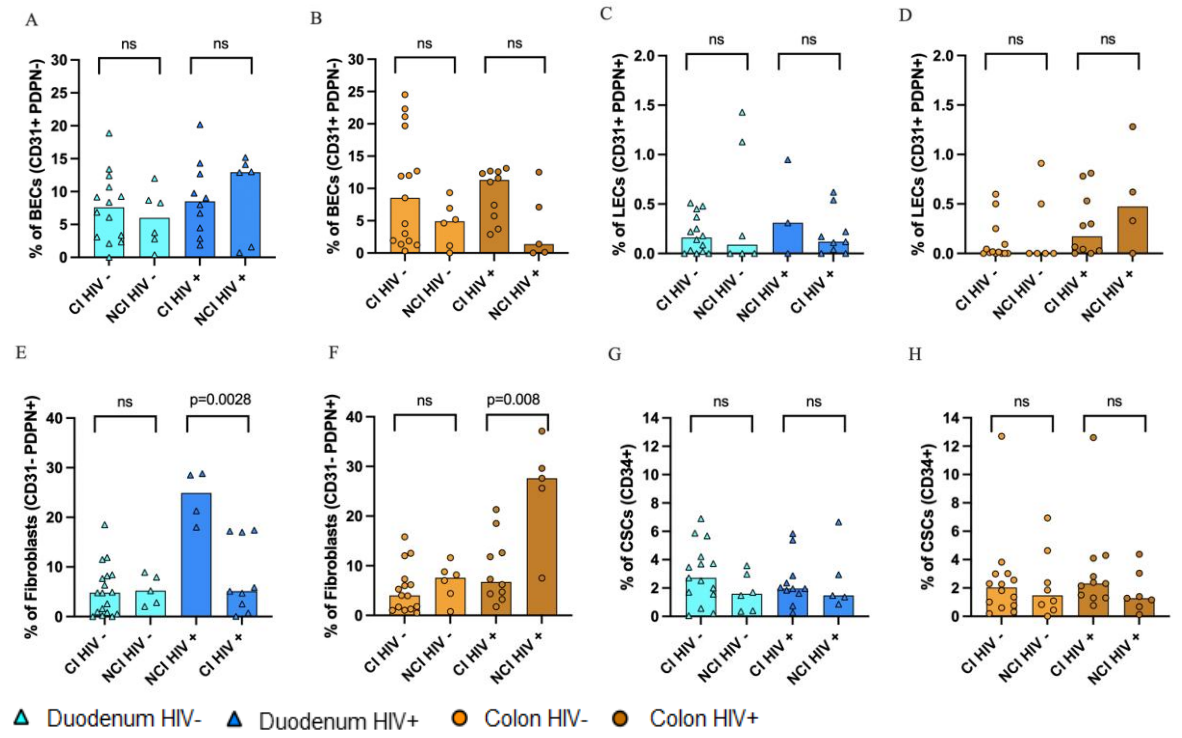

Figure 20: **comparison between the CI cohort's and NCI cohort's duodenal and colonic stromal populations.** A & B) Comparison of the percentage of stromal cells that are BECs in the duodenum (A) and colon (B) between the CI and NCI cohorts. C & D) Comparison of the percentage of stromal cells that are LECs in the duodenum (C) and colon (D) between the CI and NCI cohorts. E & F) Comparison of the percentage of stromal cells that are fibroblasts in the duodenum (E) and colon (F) between the CI and NCI cohorts. G & H) Comparison of the percentage of stromal cells that are CSCs in the duodenum (G) and colon (H) between the CI and NCI cohorts. Statistical *P*-values were calculated using the Mann-Whitney test. NS (*P*-value > 0.05).

The percentage of stromal cells that were BECs, LECs, and CSCs in controls and aviremic PLWH when comparing the CI and NCI remained similar (Figure 20A – D & G – H). Only fibroblasts showed a significant difference between the cohorts. Fibroblasts consisted of a larger percentage of stromal cells in the duodenum ($P = 0.0028$) and colon ($P = 0.008$) of aviremic PLWH in the NCI cohort compared with the CI cohort (Figure 20E & F). There was no significant difference in the percentage of stromal cells that are fibroblasts in uninfected controls regardless of the diseased state of the gut (Figure 20E & F). Taken together, these results indicate HIV increases the presence of intestinal fibroblasts in a healthy gut. The frequency in fibroblasts caused by HIV may be impacted by other health issues in the gut.

3.4. T-cell Depletion in Gut Tissue

It is well-known that HIV severely depletes $CD4^+$ T-cells in the gut early on in HIV infection[1, 47, 53, 54]. The central hypothesis of this study is that $CD4^+$ T-cell depletion will result in a higher frequency of ISCs and stromal cells. To assess the association of $CD4^+$ T-cells depletion to changes in intestinal epithelial and stromal populations, analysis of $CD4^+$ T-cells and $CD8^+$ T-cells in the biopsies shown to have changes in their epithelial and stromal populations was performed.

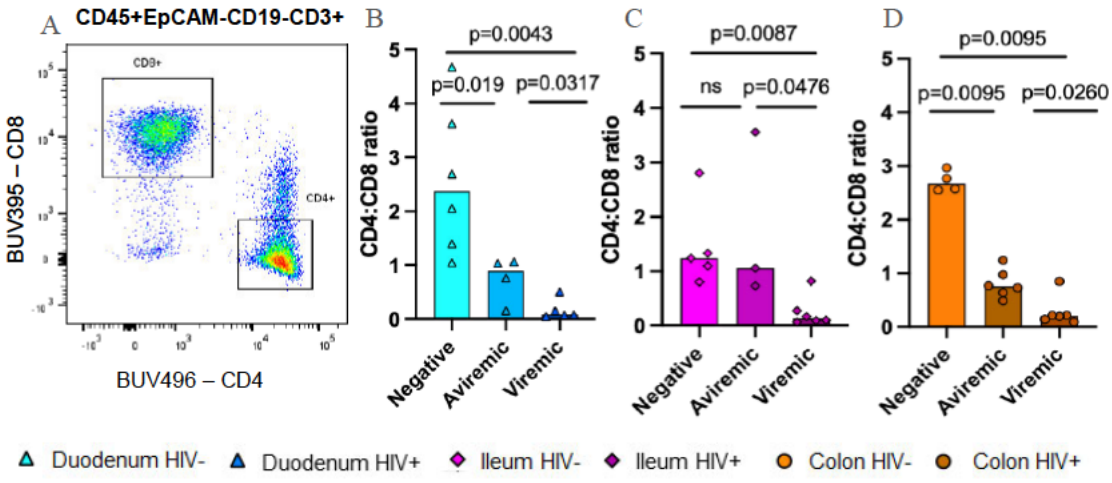

Figure 21: **comparison of tissue-resident CD4:CD8 ratios obtained from the duodenum, ileum, and colon biopsies of healthy uninfected controls and PLWH separated according to HIV blood viremia.** A) Representative flow cytometry plot depicting $CD8^+$ and $CD4^+$ T-cells in intestinal tissue. Tissue-resident CD4:CD8 ratios present in the duodenum (B), ileum (C), and colon (D) of healthy controls and PLWH separated according to HIV blood viremia. Statistical *P*-values were calculated using the Mann-Whitney test NS (*P*-value >0.05).

The CD4:CD8 ratios were obtained from the lymphocyte fraction, processed from the gut biopsies of participants listed in Table 4, and run on the Stromal Panel Figure 6). The frequencies of $CD4^+$ T-cells and $CD8^+$ T-cells (Figure 21A) for each sample were analysed and the CD4:CD8 ratio was calculated (see Supplementary Information, Table 5). The duodenum (Figure 21B), ileum (Figure 21C), and colon (Figure 21D) all had a significantly lower CD4:CD8 ratio in viremic PLWH compared with uninfected controls and aviremic PLWH. As per other studies[1, 47, 53, 54, 55, 146], $CD4^+$ T-cells remained depleted in the gut despite $CD4^+$ T-cell levels restoring in the blood. This can be seen particularly in the duodenum ($P = 0.019$) and the colon ($P = 0.0095$) where the CD4:CD8 ratio in aviremic PLWH remains below 1 in contrast to uninfected controls which have a CD4:CD8 ratio above 1 (Figure 21B &D). Interestingly CD4:CD8 ratios of $CD4^+$ T-cells in the ileum of aviremic PLWH appear similar to uninfected controls (Figure 21C).

CHAPTER FOUR

4.1. Discussion

The GI tract is a large immune organ and a major site of HIV replication regardless of the initial site of infection[18, 81, 82]. As a major site of HIV replication and pathology, it is not surprising that PLWH (both aviremic and viremic) are known to experience GI health issues and a compromised quality of life[12, 17, 61]. A key issue in PLWH experiencing gut issues is the impairment of the intestinal epithelium which enables the translocation of foreign micro-organisms, viruses, and substances from the lumen into the body. However, the underlying mechanisms remain unknown. The intestinal epithelium and gut homeostasis, under healthy conditions, is maintained through signalling between epithelial cells, immune cells[112, 113], and stromal cells[102, 103, 104, 105]. Therefore, I proposed that HIV-depletion of $CD4^+$ T-cells may impact intestinal epithelial and stromal cells resulting in the dysfunction of the intestinal epithelium and gut homeostasis in PLWH. This study aimed to determine the frequencies of epithelial and stromal cells, particularly those maintaining the intestinal epithelium, in intestinal biopsies obtained from uninfected people and PLWH.

Flow cytometric analysis of gut biopsies obtained from the NCI cohort indicated that PLWH have distinct differences in gut epithelial populations compared with uninfected controls. In particular, the results showed that in all intestinal compartments of PLWH, there is a higher frequency of ISCs (see Figure 12A – C). In the duodenum and ileum, parts of the small intestine, there was a higher frequency of ISCs in aviremic and viremic PLWH compared with uninfected controls (see Figure 12A & B). Analysis of ISC frequency and viral load showed a moderate positive correlation in the duodenum and a very strong positive correlation in the ileum (see Figure 13). Comparison of CD4:CD8 ratios, used to assess $CD4^+$ T-cell depletion in the gut, did not show such a clear correlation to the higher frequency of ISCs observed. In both the duodenum and ileum of viremic PLWH the significantly higher frequency of ISCs ($P_{duodenum} = 0.0012$; $P_{ileum} = 0.0173$) (see Figure 12A – B) corresponded to lower CD4:CD8 ratios in the gut ($P_{duodenum} = 0.0043$; $P_{ileum} = 0.0476$) (see Figure 21B – C). However, the lower CD4:CD8 ratios did not correspond to a higher frequency of ISCs in the duodenum and ileum of aviremic PLWH. In the duodenum of aviremic PLWH, which had a significantly lower CD4:CD8 ratio ($P = 0.019$) (see Figure 21B), the higher frequency of ISCs was not significant ($P = 0.1810$) (see Figure 12A). In contrast, the ileum of aviremic PLWH did not have a lower CD4:CD8 ratio (see Figure 21C) but did have a statistical trend to have a higher frequency of ISCs ($P = 0.0952$) (see Figure 12B). The connection between a lower CD4:CD8 ratio in the gut and a higher frequency of ISCs in viremic PLWH but not aviremic PLWH is uniquely reflected in the colon. As seen in the duodenum and ileum, in the colon of viremic PLWH a higher frequency of ISCs ($P = 0.022$) (see Figure 12C) corresponded to a

significantly lower CD4:CD8 ratio (P = 0.0095) (see Figure 21D). Interestingly, a significantly lower CD4:CD8 ratio in the colon of aviremic PLWH (P = 0.0095) (see Figure 21D) did not reflect a significantly higher frequency of ISCs (see Figure 12C). This could indicate two things. One, it could indicate that although $CD4^+$ T-cell depletion in the gut may play a role, causing a higher frequency of ISCs observed in PLWH, it is not the sole mechanism. Two, using CD4:CD8 ratios to assess $CD4^+$ T-cell depletion in the gut is not ideal as the ratio can lower due to $CD4^+$ T-cell depletion and/or $CD8^+$ T-cell expansion which are known to both occur in HIV infection[147]. Performing a longitudinal study assessing changes in $CD4^+$ T-cell and ISC frequencies could provide a better insight into how $CD4^+$ T-cell depletion impacts ISCs in the guts of PLWH. This study should be done in all intestinal compartments as the data shown in this study clearly indicates that the mechanisms behind a higher frequency of ISCs are not necessarily the same between intestinal compartments even within the same intestinal organ.

There are two means by which ISCs increase: dedifferentiation[94, 95, 96] and proliferation. There were no significant differences in absorptive and secretory cells in the duodenum and ileum when comparing uninfected controls and PLWH (see Figure 12D –E; G – H). The colon showed no difference in absorptive cells (see Figure 12F) but did show a significant decrease in secretory cells (P = 0.0082) (see Figure 12I). The reduction of secretory cells corresponded with blood viremia and a low CD4:CD8 ratio (see Figure 12I & Figure 21D). However, the decrease in colonic secretory cells between uninfected controls and aviremic PLWH (P = 0.0649), and between aviremic and viremic PLWH (P = 0.2949), were not considered statistically significant (see Figure 12I). Overall, the change, or lack of, in differentiated IECs could indicate that the main mechanism by which ISCs occur in a higher frequency in the duodenum and ileum of PLWH is by hyper-proliferation while in the colon the higher frequency of ISC may be due to dedifferentiation of IECs and proliferation. Usually, dedifferentiation occurs when the ISC pool is depleted. In cases where the ISC pool is depleted IECs, both secretory and absorptive, begin to re-express the $Ascl2^+$ gene – a known transcriptional factor involved in dedifferentiation[96]. The transcriptional analysis of IEC expression of $Ascl2^+$ combined with an HIV infection model or $CD4^+$ T-cell depletion model should be performed. This analysis could help determine if the reduction in secretory cells observed is truly dedifferentiation.

Investigation into the proliferation of ISCs was limited to the duodenum and colon due to difficulty obtaining ileum samples. in both the duodenum and colon of aviremic and viremic PLWH, ISCs showed a much brighter proliferation signal than uninfected controls (see Figure 14A &B). However, a comparison of the MFI for Ki67 expression by ISCs did not show a significant change (Figure 15A & B). The lack of increase in the proliferation of ISCs in PLWH is contradicted by the higher frequency of ISCs observed in this study and by results found by Batman et al.[145] and Somsouk et al.[148]. Continued analysis of the MFI of ISCs for Ki67 in the duodenum and colon should be

performed to confirm this contradictory finding. In addition, microscopic staining of crypt-based ISCs and their expression Ki67 could help determine if the ISCs are hyper-proliferating or if ISCs occur at a higher frequency in PLWH by other means as the MFI results suggest. Further transcriptional analysis could provide support for and insight into the mechanisms causing the results observed in this study.

In a study by Biton et al.[111], they found that there were highly proliferative ISCs enriched for MHC II. Additionally, they showed ISCs increased in cases of $CD4^+$ T-cell depletion or when MHCII was genetically ablated from ISCs. They concluded that a lack of signalling between immune cells and ISCs, whether due to a lack of immune cells or ISCs' ability to interact with said immune cells, reduces ISCs' capability to differentiate. To assess if the higher frequency of ISCs observed was due to more hyper-proliferative ISCs or if there is a reduction in MHCII expression by ISCs, the expression of HLA-DR (an MHCII marker) was analysed. There was no significant difference in the HLA-DR signal between uninfected controls and PLWH (see Figure 14C & D and Figure 15C & D). This could indicate that MHCII expression by ISCs remains stable, and does not contribute to the higher frequency in ISCs observed. Nor does the higher frequency in ISCs observed correlate to an increase in hyper-proliferative ISCs specifically. Future spatial transcriptional analysis and immunohistochemistry (IHC) of the ISCs could provide insight into the higher frequency of ISCs observed in this study.

Overall differences observed in the NCI cohort were not replicable in the CI cohort. A comparison of epithelial populations between the CI and NCI cohorts showed differences in the duodenum and colon (see Figure 16). There was a lower frequency of ISCs in the duodenum and colon of healthy, uninfected controls compared with clinically indicated, uninfected controls, particularly the colon (see Figure 16A &B). PLWH, however, had a similar percentage of epithelial cells that are ISCs regardless of GI health in the duodenum. The frequency of ISCs was lower in uninfected or infected healthy people in the colon. This may suggest that HIV is causing intestinal injury, which in turn causes a higher frequency of ISCs observed. The participants from the CI cohort experienced a variety of gastrointestinal issues. A future study comparing a specific gastrointestinal disease (like colon cancer or IBS) in uninfected people and PLWH compared with the NCI cohort could elucidate the differences observed in this study. No difference was observed in the frequency of absorptive cells in the duodenum (see Figure 16C). The colon did show a higher frequency of absorptive cells in PLWH experiencing gut health issues compared with healthy PLWH. The lack of differences in and between both cohorts strongly indicates that HIV infection does not impact absorptive cells in the duodenum but gut disease may impact them in the colon. There were significantly more secretory cells in the duodenum of participants from the NCI cohort, uninfected and PLWH, compared with the CI cohort (see Figure 16E). This difference observed in the duodenum was not reflected in the colon (see Figure 16F). This could mean that the diseased and inflamed gut may impact the secretory cells or the lack of secretory cells observed may lead to the intestinal epithelium becoming compromised and inflamed, establishing a diseased gut. An

important thing to note is that "secretory cells" is a broad term and does not distinguish which secretory cells are more present in a healthy gut. Spatial transcriptional analysis, further flow cytometric analysis, IHC, or immunofluorescence (IF) to analyse the type of secretory cells reduced in a diseased gut could provide insight into the impact general disease, and HIV specifically, has on these cells and the intestinal epithelium. The comparison between the CI and NCI in this study was limited to aviremic PLWH due to the limited number of viremic biopsies obtained in the CI cohort. Continued analysis of how these epithelial populations differ in viremic PLWH is essential considering how significant differences in epithelial populations observed were between uninfected people and viremic PLWH.

It is well-known that HIV results in endothelial dysfunction[149, 150, 151, 152, 153, 154, 155]. Endothelial dysfunction associated with HIV includes increased adhesiveness[156, 157], permeability[158, 159], cell proliferation[160, 161], and apoptosis[162, 163, 164, 165]. It has been proposed that endothelial dysfunction caused by HIV is a contributing factor to PLWH being more susceptible to cardiovascular disease[149, 166]. Considering the dangers endothelial dysfunction poses, and the gut being a major site of HIV infection this study aimed to identify changes in BECs and LECs in the gut. In the NCI cohort, all intestinal compartments of PLWH contained more BECs and LECs than uninfected controls (see Figure 18A & B). This higher frequency, however, was not statistically significant. It may be that there is endothelial dysfunction occurring in the gut, however, the rates of cell proliferation and apoptosis result in the negligible effect observed in this study. Or endothelial cells in the gut may change, but their frequency remains consistent. In the CI cohort, the difference in BECs and LECs between uninfected controls and PLWH were minor (see Figure 17B & C). As seen with the fibroblasts, the diseased state of the gut samples taken from the CI cohort may be obscuring HIV's effect on BECs, LECs, and endothelial dysfunction. The stromal gating strategy used in this study is currently being optimized, with the release of a new study by Kvedaraite et al.[167] as a guide, and may be expanded to assess the proliferation and apoptosis of endothelial cells. Additionally, the inflammation gating strategy (see Figure 8) may be used to determine if there are increases in adhesiveness known to arise during endothelial dysfunction. Transcriptional analysis may also provide more detailed information on differences between the BECs and LECs in PLWH compared with healthy, uninfected people.

The only statistically significant stromal population shown to be affected by HIV in this study is fibroblasts. In the NCI cohort, fibroblasts constituted more of the total stromal cells in the duodenum, ileum, and colon in PLWH than uninfected controls (see Figure 18C). This higher frequency of fibroblasts in the intestines of PLWH was independent of viremia (see Figure 18B – D), CD4:CD8 ratio (see Figure 21), inflammation, and immune reconstitution (see Supplementary Information, Supplementary Figure 1). The higher frequency of fibroblasts in PLWH were obscured under clinical conditions as these results were not reflected in the CI cohort (see Figure 17D). When comparing stromal populations between the NCI and CI cohorts, only fibroblasts differed significantly. In the

duodenum and colon of PLWH, there were more fibroblasts in the NCI cohort than in the CI cohort (see Figure 19E & F). These results taken together suggest that HIV is directly linked to a higher frequency of fibroblasts. Furthermore, these results indicate that a diseased gut may hinder the mechanism by which HIV causes this higher frequency or may be masked by other clinical indications. A study by Estes et al.[168, 169], showed fibrosis was more extensive in Peyer patches of the ileum of PLWH than in uninfected people. Furthermore, initiation of ART for 6 months did not reduce collagen deposition. This increase in collagen deposition may be linked to the gain in fibroblasts in this study. If the gain in fibroblasts is connected to fibrosis and excessive collagen deposition these results may have implications for the limited immune reconstitution observed in the gut[168]. The higher frequency of fibroblasts in PLWH is not only a concern because of gut pathology and immune reconstitution but also HIV infection. Fibroblasts are known to promote inflammation[130, 170]. These fibroblasts could ensure continued inflammation in the gut and provide target cells for HIV to infect. Additionally, as mentioned in the literature review, fibroblasts have been able to trans-infect $CD4^+$ T-cells – particularly under low viral conditions[132]. Therefore, the higher frequency of fibroblasts observed in this study may contribute to the maintenance of HIV infection in the gut.

In addition to a higher frequency of fibroblasts in PLWH, this study also showed a higher frequency of CBFs (see Figure 19F – H). The higher frequency of CBFs may be linked to the hyper-proliferation of ISCs in viremic PLWH observed in this study and others[145, 148]. CBFs provide important Wnt agonists and BMP antagonists to encourage ISC proliferation and maintain the stem cell niche[102, 103, 104, 105]. A higher frequency of CBF may mean an overabundance of Wnt agonists and BMP antagonists leading to hyper-proliferation of ISCs (Figure 22). Despite this study showing a higher frequency of CBFs, there were no significant differences in CSCs overall (see Figure 18D). Stromal cells are heterogeneous and difficult to easily distinguish using only flow cytometry. Spatial transcriptional analysis of isolated fibroblasts from the gut biopsies of uninfected people and PLWH should be performed. This analysis would enable us to determine which specific fibroblasts are increasing in PLWH and how HIV affects their gene expression. In particular, analysis of their expression of Wnt and BMP pathways may provide further insight into how HIV affects stromal cells and the effect observed with ISCs. Transcriptional data may provide avenues for preventing and treating gut issues experienced by PLWH.

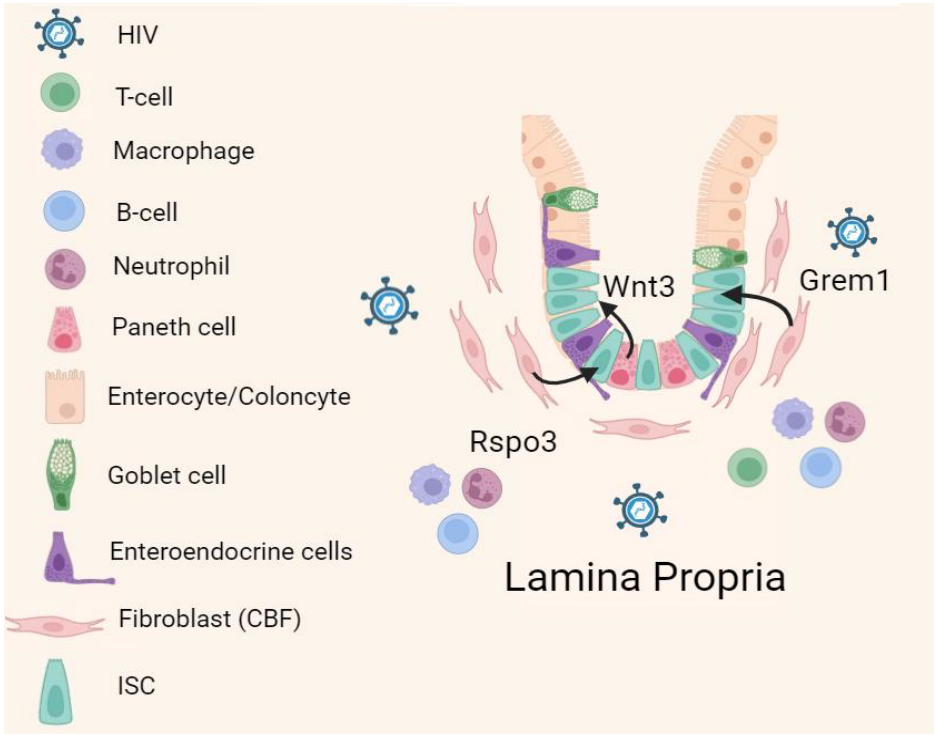

Figure 22: **an image depicting the effect HIV has on the intestinal epithelium's crypt**. PLWH has more ISCs and fibroblasts than uninfected people. Image constructed using BioRender.

The stromal panel used in this study is limited. The largest portion of stromal cells identified were $CD31^{-}PDPN^{-}$ or $CD34^{-}$. A decrease in this population led to an increase in the identifiable stromal cells detailed above. It is therefore a great limitation of this study to have not clarified this population of stromal cells. Cell sorting using the stromal panel described in this study and subsequent transcriptional analysis of this unknown population of stromal cells is vital to provide context for the observations made in this study. Furthermore, spatial transcriptomics and microscopy focused on CBFs and CTFs could confirm the increase in CBFs observed in this study and provide information about whether and how CTFs are affected in an HIV environment. This could help inform how the stem cell niche may be altered in PLWH.

Further limitations include this study's population and use of tissue biopsies. The data was skewed towards uninfected controls being males and PLWH being females. Continued collection of intestinal biopsies and analyses of ISCs and stromal populations evenly distributed across the sexes is essential to ensure no sex bias skewing the results. The population was also more skewed towards aviremic PLWH representing PLWH in the CI cohort. Continued analysis of viremic PLWH experiencing gut health issues could provide an ability to compare how general clinical pathology of the gut can affect HIV pathology. Intestinal biopsies are known to be representative of the part of the gut they are obtained from, and this study made an effort to obtain data from different parts of the gut. However, biopsies are

small pieces of tissue, and obtaining a decent number of cells to analyse can be difficult. This was apparent in the low event cut-off used by this study, hindering the robustness of the data. It was also particularly apparent in the ileum samples as doctors struggled to obtain more than a few, if any, gut pinches.

Taken together, this study shows that HIV impacts the epithelial and stromal cells present in the GI tract; particularly ISCs and fibroblasts. The effect of HIV on these cellular populations may contribute not only to HIV pathology but also to continued infection and infiltration of HIV in the gut. This study is, however, limited and functions more as a stepping stone for further analysis of these key populations in an HIV context. It contributes to an understudied area of HIV gut pathology that also has relevance for other chronic inflammatory gut settings, i.e. IBD, as the interplay between immune cells, intestinal stem cells, and stromal cells may be overlapping. The mechanisms by which these populations change in PLWH, remain unknown and require further studies.

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SUPPLEMENTARY INFORMATION

Fibroblasts, T-cell depletion, and Inflammation

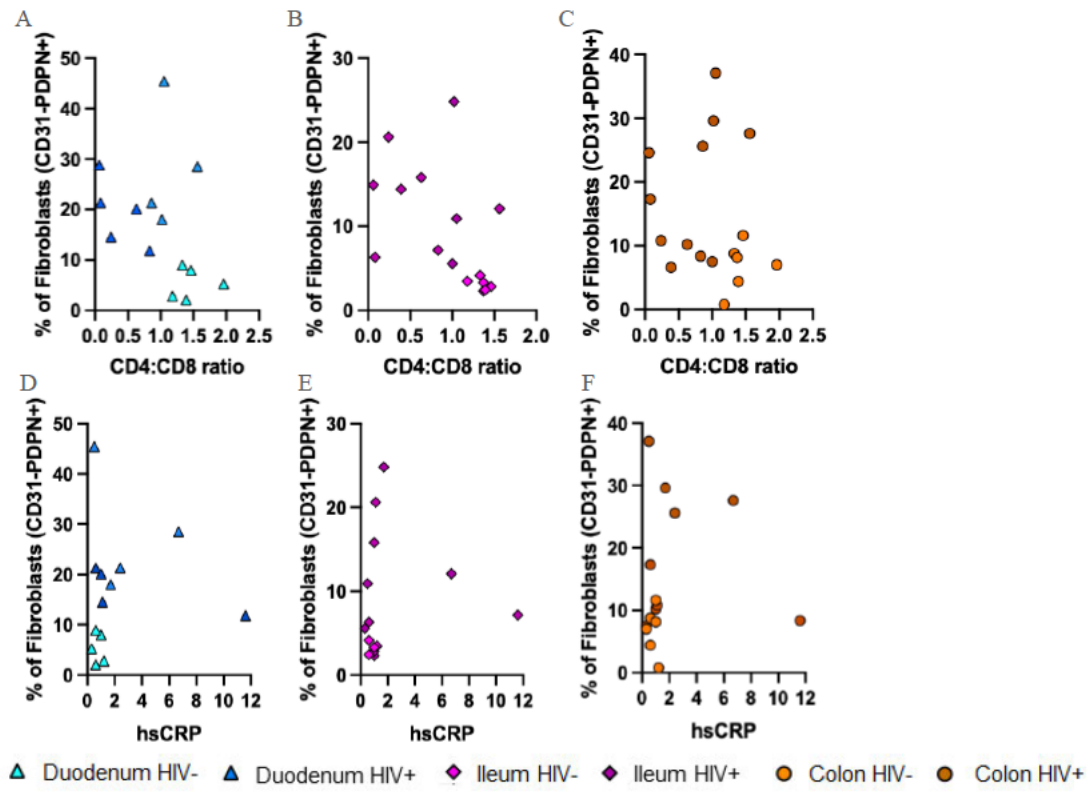
Table 5: $CD4^+$ T-cell and $CD8^+$ T-cell Frequencies used to calculate CD4:CD8 ratios in intestinal biopsies which had differences in epithelial and stromal populations.

PID	Tissue Biopsy	Viremia	Total Number of Events	CD4+ T-cell Frequency	CD8+ T-cell Frequency	CD4:CD8 ratio
041-18-0022	Duodenum	Negative	7 425	67,30	25,00	2,69
041-18-0017			5 620	66,90	14,30	4,68
041-18-0019			29 370	66,70	18,40	3,63
041-18-0018			3 833	62,70	30,50	2,06
041-18-0022			4 620	48,50	34,70	1,40
041-24-0028			3 578	47,40	45,20	1,05
041-18-0021		Aviremic	1 580	38,00	50,10	0,76
041-18-0020			2 750	11,60	74,90	0,15
041-18-0023			1 372	44,60	43,10	1,03
041-24-0022			1 580	42,50	40,10	1,06
041-18-0016		Viremic	30 885	7,31	47,60	0,15
041-18-0012			14 879	6,25	75,00	0,08
041-18-0013			11 148	6,49	88,10	0,07
041-18-0011			11 000	4,16	84,50	0,05
041-18-0014			3 630	31,40	62,00	0,51
041-18-0022	Ileum	Negative	213	42,70	34,50	1,24
041-18-0017			106	45,30	34,00	1,33
041-18-0018			247	34,80	31,70	1,10
041-24-0028			18 969	37,90	47,30	0,80
041-18-0015			5 707	58,90	21,00	2,80
041-18-0020		Aviremic	500	29,70	40,50	0,73
041-24-0030			7 867	73,00	20,50	3,56
041-24-0022			2 589	39,90	37,60	1,06
041-18-0003		Viremic	2 549	4,68	72,90	0,06
041-18-0016			703	5,72	56,40	0,10
041-18-0012			17 197	11,80	69,60	0,17
041-18-0013			32 835	20,00	72,30	0,28
041-18-0011			8 949	7,24	76,40	0,09
041-18-0014			5 520	41,20	50,40	0,82
041-18-0017	Colon	Negative	2 141	57,70	22,40	2,58
041-18-0019			7 483	60,00	20,20	2,97
041-18-0022			2 383	55,10	19,90	2,77
041-24-0028			8 025	61,00	26,10	2,34
041-18-0015			4 218	26,30	10,30	2,55
041-18-0021		Aviremic	1 828	44,40	45,60	0,97
041-18-0020			6 264	29,70	60,00	0,50
041-18-0023			1 895	49,00	39,40	1,24
041-24-0030			10 706	28,20	43,70	0,65
041-24-0022			2 435	35,50	46,20	0,77

041-24-0005			16 419	33,50	45,70	0,73
041-18-0003		Viremic	1 523	15,50	70,80	0,22
041-18-0016			12 024	39,70	46,80	0,85
041-18-0012			33 222	10,60	74,60	0,14
041-18-0013			13 105	6,77	73,10	0,09
041-18-0011			10 150	14,00	68,90	0,20
041-18-0014			13 476	16,40	75,10	0,22

The frequency of CD45$^+$ immune cells which were CD4$^+$ T-cells were lower in PLWH compared with uninfected controls in all intestinal compartments. The frequency of CD45$^+$ immune cells which were CD8$^+$ T-cells were higher in PLWH compared with uninfected controls, particularly in the duodenum and colon. Frequencies of CD4$^+$ T-cells are similar to those found in Aswota et al[55]. A decrease in CD4$^+$ T-cells and an expansion of CD8$^+$ T-cells are in line with HIV infection[147].

Fibroblasts are associated with inflammatory pathology[130, 131]. PLWH are known to experience elevated levels of inflammation markers regardless of viremia[171]. The restoration of CD4:CD8 ratios in the blood is associated with immune reconstitution and the suppression of HIV once ART is initiated. In some cases



Supplementary Figure 1: **correlation of inflammation and immune cell depletion to gut fibroblasts.** A – C) Correlation of intestinal fibroblasts found in the duodenum (A), ileum (B), and colon (C) of uninfected controls and PLWH to blood CD4:CD8. Two-tailed non-parametric correlation test was performed. D – F) Correlation of intestinal fibroblasts found in the duodenum (D), ileum (E), and colon (F) of uninfected controls and PLWH to inflammation marker hsCRP.

PLWH on ART experience immune reconstitution inflammatory syndrome[65].[65] The correlation of inflammation and immune reconstitution to the percentage of fibroblasts across all intestinal compartments in uninfected controls and PLWH was investigated.

There was no significant correlation between the increased presence of fibroblasts and inflammation in all intestinal compartments (Supplementary Figure 1). Although uninfected controls had high CD4:CD8 ratios and reduced fibroblasts compared with PLWH which had a lower CD4:C8 ratio, changes in the CD4:CD8 ratio and hsCRP did not correlate with a change in fibroblasts present in all intestinal compartments.