Assessment of helminth-HIV co-infection as a potential risk factor for colorectal cancer in South African adult subpopulation

by

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DECLARATION

I, **Botle Dirontso Precious Damane**, declare that the thesis entitled "**Assessment of helminth-HIV co-infection as a potential risk factor for colorectal cancer in South African adult subpopulation**" is the result of my original work, except where otherwise indicated. I completed this research under the supervision of Prof Zilungile L. Mkhize-Kwitshana and Dr P Naidoo from the University of KwaZulu-Natal's College of Health Sciences, and Prof Zodwa Dlamini from the University of Pretoria, Faculty of Health Sciences.

I also declare that this thesis has not been submitted to UKZN or other tertiary institutions to obtain an academic qualification, whether by me or any other party. This thesis does not contain other persons' data, pictures, graphs, or additional information unless specifically acknowledged as being sourced from other persons. This thesis does not contain other persons' writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, their words have been restructured, but the general information attributed to them has been referenced. This thesis does not contain text, graphics, or tables copied and pasted from the internet unless specifically acknowledged, and the source is detailed in the thesis and the references section.

Signed: ……[redacted]…… **Date**: 31 July 2025

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Prof Zilungile L Mkhize-Kwitshana

Supervisor

DEDICATION

To my beloved parents, whose lives were both claimed by cancer, your absence fuels my purpose. You are the reason I chose this path, and the quiet strength behind every page of this thesis. This work is for you, and for every family touched by this disease.

To my amazing brothers and sisters, my constant anchors through every storm. And to Mabonza, my sister and my best friend, my fierce supporter and safe place, you have been my laughter, my courage, and my reminder that I never walk alone.

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Here's to breaking generational curses. Here's to doors opening wide, to seizing opportunities without hesitation, and to meeting challenges with grace and courage, for this generation, and for generations to come.

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The candidate used ChatGPT (OpenAI, 2025) to improve the grammar, phrasing, and readability of the thesis. No content was generated or interpreted by AI, and all final text was reviewed and approved by the supervisors.

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CONFERENCE PRESENTATIONS

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LIST OF ABBREVIATIONS

ACTB	β-actin
APC	Adenomatous Polyposis Coli
BAT26	Microsatellite Marker BAT26
BRAF	B-Raf Proto-Oncogene
BMI	Body Mass Index
BMP3	Bone Morphogenetic Protein 3
BTLA	B and T Lymphocyte Attenuator
CEA	Carcinoembryonic Antigen
CIMP	CpG Island Methylator Phenotype
CMV	Cytomegalovirus
CRC	Colorectal Cancer
CTLA-4	Cytotoxic T Lymphocyte Antigen 4
DCs	Dendritic Cells
DDR	DNA Damage Response
DEGs	Differentially expressed genes
DRGs	DNA Repair Genes
EBV	Epstein-Barr Virus
FDA	Food and Drug Administration
FOBT	Fecal Occult Blood Test
GITR	Glucocorticoid-Induced TNFR-Related Protein
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HPV	Human Papillomavirus
HVEM	Herpesvirus Entry Mediator
IDO	Indoleamine 2,3-Dioxygenase
IL	Interleukin
JCV	John Cunningham Virus
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
LAG-3	Lymphocyte-Activation Gene 3
LMICs	Low-Middle-Income Countries
LOH	Loss of Heterozygosity
MCPyV	Merkel Cell Polyomavirus
MSI	Microsatellite Instability
PD-1	Programmed Cell Death Protein 1

PD-L1	Programmed Death-Ligand 1
PD-L2	Programmed Death-Ligand 2
PLWHIV	People Living With HIV
PRRs	Pattern Recognition Receptors
SCFAs	Short-Chain Fatty Acids
STHs	Soil-Transmitted Helminthiasis
TGF-β	Transforming Growth Factor-β
Th1	Type 1 T Helper
Th2	Type 2 T Helper
TIM-3	T-cell Immunoglobulin and Mucin-Domain Containing-3
TME	Tumor Microenvironment
TP53	Tumor Protein P53
Tregs	Regulatory T Cells
WHO	World Health Organization

ABSTRACT

Background: Colorectal cancer (CRC) incidence is rising in sub-Saharan Africa, where HIV and helminth infections remain endemic. These chronic infections induce profound immune, microvirome, metabolic, and epigenetic perturbations, yet their integrated contribution to CRC susceptibility in African populations is poorly understood. This thesis presents the first comprehensive investigation linking immune checkpoint dysregulation, gut DNA virome driven functional shifts, and stool-based methylation biomarkers in HIV-helminth co-infection, providing a novel, multi-dimensional view of infection-associated cancer risk.

Methods: This retrospective observational study analysed stored samples from a peri-urban community south of Durban, South Africa, collected between March 2020 and May 2021. Multiplex assays were performed on 78 serum samples to profile immunoregulatory markers. Shotgun metagenomic sequencing of 62 stool samples was conducted to characterise the gut virome and assess epigenetic and metabolic signatures. Fecal occult blood testing was also evaluated as a potential non-invasive CRC screening tool.

Results: Multiplex immune assays showed that HIV infection elevated PD-1 (adjusted $\beta = 0.12$, $p = 0.034$) and TIM-3 ($\beta = 23.15$, $p = 0.052$) while reducing CD28 ($\beta = -651.95$, $p = 0.010$). Helminth infection increased TIM-3 ($\beta = 20.98$, $p = 0.020$), and co-infection elevated PD-1 ($\beta = 0.18$, $p = 0.0046$) and PD-L2 ($\beta = 7.95$, $p = 0.033$), with marked CD28 downregulation across all infected groups, indicating a sustained immunosuppressive state conducive to tumor escape. Stool DNA showed strong virome convergence between CRC and HIV-helminth co-infected groups ($p < 2.2 \times 10^{-16}$). Functional enrichment included tRNA modification GTPase (K03650) and adenine-specific DNA methylase (COG2189) as shared viral epigenetic signatures, alongside distinct metabolic reprogramming, particularly butyrate metabolism, oxidative stress response, and polyamine biosynthesis. These pathways were consistently enriched across CRC, helminth-only, and HIV-helminth co-infected groups and are associated with promoting tumor initiation, immune evasion, and disease progression.and progression. Stool-based methylation assays detected vimentin methylation in 90% of CRC cases and at high frequencies in infected groups (HIV: 92.9%, helminth: 93.3%, HIV-helminth co-infected: 77.9%) compared to 10% in uninfected controls, indicating early epigenetic reprogramming before clinical disease onset.

Conclusion: By integrating immune, virome, metabolic, and epigenetic datasets, this thesis identifies convergent molecular signatures linking HIV-helminth co-infection to CRC. These findings advance mechanistic understanding of infection-driven oncogenesis and highlight the potential for non-invasive, multi-marker screening to guide precision cancer prevention in high-

burden African settings. This work establishes a new framework for cancer risk assessment in populations where infectious and oncologic burdens collide.

Keywords: HIV-helminth co-infection, Immune checkpoint markers, Gut DNA virome, Vimentin methylation, Metabolic reprogramming, Colorectal cancer risk

CHAPTER ONE

Introduction

One of the most common infections in developing countries is helminth infection. According to the World Health Organization (WHO) newsroom, January 2022, over 1.5 billion people worldwide are infected with soil-transmitted helminths [1]. These infections affect the nutritional status and host immunological responses. The parasites depend on the host for nutrition and thus feed on the host tissues, with some causing bleeding that leads to anemia. *Ascaris lumbricoides* and *Trichuris trichiura* are infections that are significantly associated with malnutrition in children [2]. A recent global meta-analysis reported that people living with human immunodeficiency virus (HIV) (PLWHIV) had a higher risk of *Strongyloides stercoralis* infection, while the likelihood of hookworm infection appeared to be lower in this population. Among the helminths evaluated, *Ascaris lumbricoides* had the highest prevalence, reaching 8%, with Sub-Saharan Africa showing the most significant burden [3]. A study by Kaneshiro *et al*., 2019 found the incidence of HIV-helminth co-infections remains high in adults at 35.4% [4]. It is not clear whether helminth infections increase susceptibility to HIV, or vice versa [5]. However, certain helminth-induced immunological phenotypes might suggest the former [6]. Whilst moving throughout the body, helminth infections can cause tissue damage by blocking organs and initiating an immune response. Their prolonged lifespan elicits chronic inflammatory reactions driven by the body's persistent allergic responses [7].

In HIV-helminth co-infections, immune alterations are amplified, primarily through Th2-skewed responses. The recruitment of alternatively activated (M2) macrophages shifts the immune balance from Th1 to Th2, promoting cytokine release and expansion of immunosuppressive regulatory T cells (Tregs) [8]. Tregs can suppress antiviral responses and regulate HIV replication [9,10], contributing to an immunosuppressive microenvironment that favors tumor development and progression [11]. These cells also express co-inhibitory molecules such as PD-1 and CTLA-4, which impair antitumor immunity and facilitate immune evasion by cancer cells, increasing the potential risk of malignancy in co-infected patients [12,13].

Moreover, infections generally trigger immune responses and cause disturbances of the normal flora. The shift in the gut microbial signatures (dysbiosis) is associated with cancer development [14-16]. A complex interrelationship between infection and immunity exists within susceptible hosts, particularly in LMIC countries. With colorectal cancer (CRC) being the second most deadly cancer, accounting for 9.4% of global cancer deaths recorded in 2020, with the incidence estimated to increase to ~3.15 million cases by 2040 [17]. The interaction between HIV and

helminth infections may inadvertently increase susceptibility to CRC, yet the combined effects of HIV and helminth co-infection on CRC risk remain poorly understood. In particular, according to our knowledge, no studies to date have comprehensively examined the interaction between HIV-helminth co-infection, gut microvirome dynamics, and cancer-associated DNA methylation patterns within African populations. This represents a critical knowledge gap, especially in regions where the burden of these infections is highest. Furthermore, it is important that the immunomodulatory effects of helminths, together with HIV co-infection and its consequences, are thoroughly evaluated. Consequently, the inclusion of CRC predictive biomarkers in these patients may provide critical insights into the potential risk of cancer development.

1.1 Study Rationale

According to the 2022 report by Statistics SA, the total number of PLWHIV increased from an estimated 3.68 to 8.45 million. Alarmingly, nearly one in four South African women aged between 15 and 49 years is HIV positive [18]. Helminth infections remain highly prevalent in South Africa, particularly in impoverished areas, where over 90% of children are affected. Soil-transmitted helminths infect an estimated 55.8% of school-aged children, with Ascaris lumbricoides being the most common [19]. Although prevalence has declined, rural adults continue to experience high infection rates (65.2%), with HIV/AIDS patients showing a 30% burden of intestinal parasites [20]. These neglected infections, while rarely fatal, cause significant morbidities such as growth stunting, cognitive impairment, blindness, and elephantiasis [21].

HIV is a known contributor to CRC development [22], and several helminthic parasites, including *Schistosoma haematobium*, *S. japonicum*, and *S. mansoni*, are associated with urinary bladder, colorectal, and hepatocellular cancers, likely through genotoxin-induced epithelial gene aberrations [23]. The interplay between helminths and the gut microbiome further influences CRC risk, with microbial metabolites from Helicobacter spp., Dietziaceae, Oxalobacteraceae, and Pseudomonadaceae linked to oncogenesis [23]. Emerging evidence also implicates the gut microvirome in CRC development, potentially promoting chronic inflammation, genetic mutations, and immune evasion [24]. Alterations in the gut microvirome may dysregulate immune checkpoints, including PD-1, CTLA-4, and TIM-3, undermining immune surveillance and fostering a pro-oncogenic environment [25,26]. Current CRC screening methods in resource-limited settings are invasive and costly [27]. Differentially methylated genes (DMGs), including Sept9, SDC2, MGMT, NDRG4, SNAP91, APC, FOXA1, RASSF1A, and vimentin, have been identified as potential non-invasive biomarkers that can be utilised for early CRC detection [28]. Understanding these complex interactions may inform novel screening strategies and therapeutic approaches, ultimately improving health outcomes for affected populations.

1.2 Hypothesis

We hypothesize that HIV-helminth co-infection increases susceptibility to CRC by modulating cancer-associated markers, including immune checkpoint expression, virome composition, and gene methylation patterns.

1.3 Aims

The overarching aim of the study was to assess the role of HIV-helminth co-infection as a potential risk factor for colorectal cancer in a South African adult subpopulation through the characterization of immunoregulatory pathways, gut virome dynamics, and epigenetic and metabolic alterations. Specifically, the study aimed:

- To determine the association between HIV-helminths co-infection and immunoregulatory pathways that modulate antitumor immune responses.
- To investigate the association between HIV-helminth co-infections and predictive CRC biomarkers, focusing on fecal occult blood and methylated vimentin gene previously associated with CRC.
- To characterise the gut DNA virome and identify shared taxonomic and functional signatures associated with colorectal cancer risk in HIV -helminth co-infection.
- To investigate gut virome-associated epigenetic and metabolic functional signatures linked to colorectal cancer susceptibility in HIV-helminth co-infection.

1.4 Objectives

- To assess the expression of immune regulatory molecules, including immune checkpoints/co-inhibitory molecules (BTLA, CD80, CD152 (CTLA4), IDO, LAG-3, PD-1, PD-L1, PD-L2, and TIM-3), and immune co-stimulatory molecules (CD27, CD28, CD137 (4-1BB), GITR, HVEM) in HIV and helminth uninfected, single-infected, and co-infected study participants using the ProcartaPlex Human Immuno-Oncology Checkpoint Panel 1, 14plex assay from Thermofisher Scientific.
- To analyze the gut DNA virome profiles in stool samples from uninfected, single-infected, and co-infected study participants with HIV and helminths, and from CRC-confirmed patients, using non-specific shotgun metagenomic sequencing.
- To assess the presence of fecal occult blood and vimentin methylation profiles in stool from study participants uninfected, single-infected, and co-infected with HIV and helminths, and CRC-confirmed patients using the FOBT and methylation-specific PCR, respectively.
- To investigate the differential expression levels of virome-related methylated genes and

cancer metabolic genes in the aforementioned study groups using statistical methods.

1.5 Overview of Study Design and Methodology

Study Design

This research was conducted as a retrospective cross-sectional sub-study nested within a larger ethically approved project (BE351/19). Additional ethical approval for this sub-study was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC/00005458/2023). The main study was conducted between March 2020 and May 2021 in a peri-urban area south of Durban, KwaZulu-Natal, South Africa.

Study Population and Recruitment

Participants were purposively selected from the main study cohort based on their HIV and helminth infection status. The study population comprised adults aged 18 years and above who provided written informed consent. Participants were excluded if they were under 18 years old, unable to provide informed consent due to impaired cognitive ability, refused HIV testing or counselling, had a CD4 count of less than 200 cells/mL, or presented with chronic conditions such as diabetes mellitus, known malignancies, or cardiovascular diseases. Individuals with a history of illicit drug or alcohol abuse, or those with clinical malnutrition (BMI < 10), were also excluded.

For the immunomodulatory molecule analysis, a total of 78 participants with complete datasets were included and stratified into four groups: HIV- and helminth-uninfected controls (n = 20), HIV-infected only (n = 20), helminth-infected only (n = 20), and HIV-helminth co-infected individuals (n = 18). For the stool methylation study, 62 participants were selected from the same parent cohort and stratified into the same four infection groups: HIV- and helminth-uninfected controls (n = 10), HIV-infected only (n = 14), helminth-infected only (n = 15), and HIV-helminth co-infected (n = 13). An additional 10 CRC-confirmed patients were recruited from Inkosi Albert Luthuli Central Hospital and Dr Pixley Ka Isaka Seme Memorial Hospital in Durban to serve as positive CRC controls.

Sample Collection

Whole blood and stool samples were collected from all participants in the main study and stored appropriately for later analyses. Serum samples were stored at -80 °C until use in immunological assays. Stool samples from both community participants and CRC patients were collected in sterile containers and processed promptly to ensure integrity for both parasitological and molecular analyses.

Parasite Detection

Helminth infection was diagnosed through both microscopic and serological techniques. Fresh stool samples were examined using the Kato-Katz method for quantifying helminth eggs. A modified fecal parasite concentrator method (mini Parasep® SF stool concentrator kit) was used to increase the sensitivity of parasite detection. Additionally, serum samples were tested for *Ascaris lumbricoides*-specific IgE (>0.35 kU/L) and IgG4 (>0.15 kU/L) antibodies using the Phadia™ 200 instrument.

HIV Detection

HIV serostatus was determined using the Alere Determine™ HIV-1/2 Ag/Ab Combo rapid test, with inconclusive results confirmed by the ICT HIV-1/2 Ag/Ab test kit. Whole blood was analysed using the AQUIOS CL Flow Cytometer System to obtain differential white blood cell counts.

Quantification of Immunomodulatory Molecules

Serum levels of key immune regulatory molecules, including CD27, CD28, CD137 (4-1BB), GITR, HVEM, BTLA, CTLA-4, CD80, IDO, LAG-3, PD-1, PD-L1, PD-L2, and TIM-3, were measured using the human ProcartaPlex immunoassay kit (Invitrogen, Thermo Fisher Scientific). Samples and standards were prepared according to the manufacturer's instructions, and analyte concentrations were determined using a Bio-Plex® 200 System with Bio-Plex Manager™ 4.0 software for analysis.

Stool DNA Extraction and Methylation Analysis

Approximately 200 mg of stool was used for genomic DNA extraction with the ZymoBIOMICS™ DNA Miniprep Kit. Mechanical lysis was performed using an Omni Bead Ruptor 4 Bead Mill Homogenizer, followed by inhibitor removal with a Zymo-Spin™ Humic Removal Column. High-purity DNA was used for downstream analyses, including sequencing and vimentin gene methylation assessment.

Bisulfite Conversion and High-Resolution Melting (HRM) Analysis

Extracted stool DNA was subjected to bisulfite conversion to enable methylation analysis. The methylation status of the vimentin gene was then assessed using High-Resolution Melting (HRM) analysis, with appropriate fully methylated and unmethylated controls included to ensure accuracy. Only samples with reproducible amplification and clear melt profiles were considered for downstream analysis.

Library Preparation, Sequencing, and Bioinformatics

DNA concentrations were quantified using the Qubit™ 4 Fluorometer and normalized for library construction with the Illumina® DNA Prep Kit, employing bead-linked transposome technology. Libraries underwent limited-cycle PCR for dual index addition, followed by purification and size selection. Sequencing was performed on the Illumina NextSeq 2000 platform (2 × 150 bp paired-end reads). Bioinformatics analysis included read trimming (TrimGalore), co-assembly (MEGAHIT), ORF prediction (Prodigal), and taxonomic/functional annotation using SqueezeMeta, with further diversity and differential abundance analyses conducted in R.

1.6 Thesis Outline

This thesis follows a publication/manuscript format in compliance with the University of KwaZulu-Natal's thesis submission guidelines. It consists of seven chapters, including two published manuscripts, one under peer review, and other chapters prepared in journal-ready formats. Each manuscript retains the structure and formatting required by its respective journal. The chapters are organised as follows:

Chapter 1: Introduction

This chapter outlines the background, rationale, and significance of the study, placing the research within the context of infection-associated CRC risk. It presents the study hypothesis, aims, and objectives, and provides an overview of the subsequent chapters.

Chapter 2: Inflammatory Signaling in HIV-Linked Colorectal Oncogenesis

This chapter provides a detailed literature review on inflammatory pathways implicated in HIV-associated CRC development. It includes the published review article:

Unravelling the Complex Interconnection between Specific Inflammatory Signaling Pathways and Mechanisms Involved in HIV-Associated Colorectal Oncogenesis, published in MDPI Cancers, IF = **4.4**.

Chapter 3: Immune Modulation in HIV and Helminth Co-infection and Implications for Colorectal Cancer Risk

This chapter investigates immune checkpoint alterations in HIV-helminth co-infection and their potential impact on CRC risk. It incorporates:

A conference paper: A Closer Look at Colorectal Cancer Risk: Insights from the Complex Relationship of HIV, Helminths, and Immune Responses, published in Cancer Research, IF = **16.6**.

A published research article: HIV-Helminth Co-Infections and Immune Checkpoints: Implications for Cancer Risk in South Africa, published in MDPI Viruses, IF = **3.5**.

Chapter 4: Screening of CRC-related Gut Microvirome as a Potential Prognostic Marker for CRC Risk in the HIV-Helminth Co-infected Group

This chapter presents original research on gut virome composition and its relationship to CRC risk in HIV-helminth co-infected individuals. The work produced a published conference paper: Gut virome remodeling in HIV-helminth co-infection reflects colorectal cancer-associated signatures in a South African cohort, published in Cancer Epidemiology, Biomarkers & Prevention, IF=**3.4**.

The work produced another published conference paper: Shared gut virome profiles highlight potential early colorectal cancer markers in HIV-helminth co-infection, published in Cancer Research, IF = **16.6**.

The work also includes the submitted manuscript: Gut Virome Alterations in HIV-Helminth Co-infection: Potential Links to Colorectal Cancer in South Africa, under peer review at Springer Nature, Archives of Microbiology, IF=**2.6**.

Chapter 5: Identification of Specific CRC-associated Methylated Genes in HIV-Helminth Co-infected Individuals as Potential Biomarkers

This chapter reports on the detection of stool-based vimentin gene methylation in CRC patients and infection-defined groups, assessing its potential as a non-invasive biomarker for CRC susceptibility in HIV-helminth co-infection. The work is titled Vimentin Methylation as a Potential Screening Biomarker for Colorectal Cancer in HIV-Helminth Co-Infected Individuals as a publication in MDPI, Microbiology Research, **IF=2.2**.

Chapter 6: Intersecting Pathways in Gut Virome, DNA Methylation, and Metabolism in Infection-associated CRC Risk

This chapter integrates metagenomic, methylation, and metabolomic datasets to explore the overlapping biological pathways that may contribute to infection-driven CRC development. This work, titled Virome Remodeling Rewires Epigenetic and Metabolic Pathways Linked to Infection-Associated Colorectal Cancer Risk is published in Elsevier, Aspects of Molecular Biology.

Chapter 7: Synthesis

This chapter synthesises findings from all preceding chapters, highlighting the overarching implications for infection-associated CRC risk in Africa. It discusses the novelty and translational relevance of the findings, outlines methodological and contextual limitations, and proposes directions for future research.

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CHAPTER TWO

Inflammatory Signaling in HIV-Linked Colorectal Oncogenesis

This chapter presents a published literature review that synthesizes current knowledge on the role of chronic inflammation as a driver of colorectal cancer (CRC) in the context of HIV infection, with a particular focus on the interplay between virus-induced immune activation, mucosal barrier dysfunction, and tumor-promoting pathways. The purpose of this work is to provide a conceptual framework for the current study. By outlining the inflammatory mechanisms already implicated in HIV-associated CRC, the review establishes a basis for exploring whether similar or synergistic immune signatures may also arise in helminth infections, given their known capacity to induce chronic immune modulation. This comparative perspective is central to the present research, which hypothesizes that overlapping inflammatory and immunoregulatory pathways in HIV and helminth co-infections may contribute to a tumor-permissive gut environment. The current chapter provides the scientific rationale for subsequent investigations into how co-existing infections influence colorectal cancer risk in a South African adult population.

Introduction

South Africa is reported to have the largest number of people living with HIV (PLWHIV) in the world [1]. Measures to ensure that more PLWHIV are on treatment and remain on treatment are in place. This aligns with the Paris Declaration on Fast-Track Cities: Ending the AIDS Epidemic [2]. However, HIV remains a significant challenge in sub-Saharan Africa, including South Africa [3]. The discovery of antiretroviral therapy has improved overall survival rates. Despite these advances, recent cuts to United States funding for HIV programs in sub-Saharan Africa [4, 5], including South Africa [6, 7], raise serious concerns about a potential resurgence of the HIV epidemic in the region [8].

People living with HIV have compromised immunity, making them vulnerable to various infections, including those caused by parasites. Helminth infections remain a significant public health concern, particularly in low- and middle-income countries (LMICs), where they contribute to high morbidity and, in some cases, mortality. While less common, these infections also occur in developed countries. Many cases remain undiagnosed, resulting in chronic infections and associated complications [9]. In addition to infectious diseases, PLWHIV are at increased risk for malignancies such as CRC [10].

Emerging evidence suggests that immunomodulatory pathways driven by chronic inflammation, triggered by HIV, bacterial, parasitic, and viral infections, may play a critical role in CRC

development. These mechanisms are discussed in the published literature review included in this chapter.

Review paper title: Unraveling the Complex Interconnection between Specific Inflammatory Signaling Pathways and Mechanisms Involved in HIV-Associated Colorectal Oncogenesis. Damane BP, Mulaudzi TV, Kader SS, Naidoo P, Savkovic SD, Dlamini Z, Mkhize-Kwitshana ZL. Unraveling the Complex Interconnection between Specific Inflammatory Signaling Pathways and Mechanisms Involved in HIV-Associated Colorectal Oncogenesis. Cancers. 2023; 15(3):748. https://doi.org/10.3390/cancers15030748

This paper reviews current evidence on core inflammatory signaling pathways, including toll-like receptor (TLR)/ nuclear factor kappa B (NF-κB)/ Interferon (IFN) molecular network and related cascades, and explores their relevance as potential therapeutic targets. The review also emphasizes the importance of considering co-existing parasitic and microbial infections in CRC development, which may influence disease progression and treatment responses in both HIV-infected and uninfected individuals. The full PDF version of the paper is included below.

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Review

Unraveling the Complex Interconnection between Specific Inflammatory Signaling Pathways and Mechanisms Involved in HIV-Associated Colorectal Oncogenesis

Botle Precious Damane [1,2], Thanyani Victor Mulaudzi [1], Sayed Shakeel Kader [3], Pragalathan Naidoo [2,4], Suzana D. Savkovic [5], Zodwa Dlamini [6,*] and Zilungile Lynette Mkhize-Kwitshana [2,4,*]

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Simple Summary: Despite the reduced death rate that comes with improved HIV treatment, chances of developing cancers in HIV infected individuals remains high. The main culprit here is exaggerated inflammatory responses, referred to as chronic inflammation. Inflammation happens when the tissue is damaged by an outside (e.g., injury by falling) or internal (e.g., infection by bacteria/parasites/viruses) sources. Normally, inflammatory responses assist with fighting off infections and promoting wound healing. Opportunistic infections are common in HIV and constantly trigger chronic inflammation responses and ultimately cancer development. Cancers are dubbed the wound that does not heal because cancers survive better in a chronic inflammatory state. Several inflammatory pathways are known to promote colorectal cancer initiation. These can be common in both colorectal cancers and HIV. Toll like receptor inflammatory pathways are important in the detection of injuries. Their role and therapeutic endeavors in HIV and cancers are well studied. These pathways connect to specific inflammatory pathways which in return interconnects with several other pathways that are involved in HIV-related colorectal cancers. Their contribution in colorectal cancers is impactful hence the suggestion to target specific or best, interconnected inflammatory pathways with the hope of halting cancer initiation, development and progression.

Abstract: The advancement of HIV treatment has led to increased life expectancy. However, people living with HIV (PLWH) are at a higher risk of developing colorectal cancers. Chronic inflammation has a key role in oncogenesis, affecting the initiation, promotion, transformation, and advancement of the disease. PLWH are prone to opportunistic infections that trigger inflammation. It has been documented that 15–20% of cancers are triggered by infections, and this percentage is expected to be increased in HIV co-infections. The incidence of parasitic infections such as helminths, with *Ascariasis* being the most common, is higher in HIV-infected individuals. Cancer cells and opportunistic infections drive a cascade of inflammatory responses which assist in evading immune surveillance, making them survive longer in the affected individuals. Their survival leads to a chronic inflammatory state which further increases the probability of oncogenesis. This review discusses the key inflammatory signaling pathways involved in disease pathogenesis in HIV-positive patients with colorectal cancers. The possibility of the involvement of co-infections in the advancement of the disease, along with highlights on signaling mechanisms that can potentially be utilized as therapeutic strategies to prevent oncogenesis or halt cancer progression, are addressed.

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Keywords: colorectal cancer; inflammation; metastasis; EMT; TLR; vimentin; HIV; NF-kB; inflammasome; epigenetics

1. Introduction

Colorectal cancer (CRC) is the third most common and second most deadly cancer worldwide. The disease affects more men than women, with a lifetime risk of 1 in 23 and 1 in 25, respectively. More younger adults (<50 years old) are seen, with an incidence of a 1.5% increase every year from 2014 to 2018 [1,2]. By, 2040, the estimated number of new CRC cases is projected to be around 3.2 million and thus poses a great burden and continuing global public health challenge [2]. Immunosuppression is the main factor that predisposes people living with human immunodeficiency virus (HIV) (PLWH) to infection-related cancers. A study by O'Neill et al. [3] found the incidence of CRC to be the same in HIV and non-HIV-infected individuals. The study included 27 studies performed in North America (18), Europe (7), the Pacific region (4), and South America (1) [3]. However, this study did not include subjects from the low-middle income countries (LMIC), particularly those from the Sub-Saharan African regions. The challenge with LMIC is the disparities in socioeconomic status. Poor living conditions expose PLWH to opportunistic infections which are exacerbated by malnutrition. Poor sanitation can lead to exposure to fecal parasites including soil-transmitted helminths [4]. These parasites can cause tissue damage to the colorectum as they migrate through the body [5]. Other factors such as microbial infections and chemical agents can also cause injury, which then triggers inflammatory responses [6,7].

Prolonged exposure to inflammatory mediators such as cytokines, chemokines, and growth factors expressed during inflammatory responses result in a chronic inflammatory state associated with the initiation of CRC [8]. This has led to the use of nonsteroidal anti-inflammatory drugs (NSAIDs) for the treatment of CRCs [9]. These drugs showed an improvement and even regression in some patients, including those diagnosed with familial adenomatous polyposis (FAP), with an inherited mutation in the tumor suppressor gene, adenomatous polyposis coli (APC) [10]. Proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin (IL)-1 trigger the activation of inflammatory signaling pathways including nuclear factor kappa B (NF-kB), which is needed for the transcription of cytokine genes [11]. The activation of the NF-kB signaling pathway also promotes CRC progression [12]. Inflammatory cytokines promote the growth of tumor cells [13]. Tumor cells release cytokines that activate the NF-kB pathway, pushing tumor progression towards metastatic disease [14]. The activation of the NF-kB signaling pathway is initiated by the toll-like receptors (TLRs). The TLRs are expressed on the surface of colonic and innate immune cells. Upon activation by a pathogenic antigen(s), a cascade of signaling molecules is stimulated to elicit immunity against the pathogen and to initiate tissue repair mechanisms [15]. Both the TLRs activation and the subsequent activation of the NF-kB signaling pathways are associated with oncogenesis. The TLR-2, 4, and 9 have been specifically correlated with CRC [16–18]. TLRs are also expressed by tumor cells [19], which prompted investigations for the use of TLRs antagonists as a treatment of choice for CRC [20] and other cancers [21]. Patients with HIV were found to be at a higher risk of developing CRC [22]. Therapeutic interventions for HIV-1 using TLRs have long been suggested [23] and have been successfully shown to eliminate HIV-infected cells in pre-clinical studies [24]. This effect on inflammatory signaling pathways links CRC and HIV, which could perhaps be a strategy for "killing two birds with one stone". This could therefore guide the efforts to develop therapeutic strategies for preventing CRC development and progression in PLWH who are on antiretroviral therapy. Herein, we discuss key inflammatory signaling pathways, with more focus on the TLRs- NF-kB/IFN and associated pathways and the possibility of targeting these pathways as a therapeutic strategy that could indiscriminately benefit HIV- and non-HIV-infected CRC patients.

These therapies should take into consideration the effect of parasitic or microbial infections associated with CRC development as well.

2. HIV in Colorectal Cancer

PLWH present with CRC at a younger age than their HIV-negative counterparts. The median age at CRC diagnosis was 55 years (32–73 years), and the median period of HIV infection prior to CRC diagnosis was 15 years. An HIV-infected group had more smokers or people with a previous history of smoking than the negative group. Furthermore, high levels of tumor-infiltrating lymphocytes (TILs) and a tendency of presenting with right-sided CRC was reported [25]. In concurrence with this study, HIV-infected CRC patients presented at a much younger age than those without HIV. The recurrence rate was also reported to be higher in HIV-positive (14.7%) CRC than in HIV-negative (6.8%) patients [26]. This study also supports the reports indicating that PLWH have a fourfold increased risk of developing CRC [27]. One of the mechanisms for the increased risk may be due to the Transactivator of transcription (Tat) protein, which modulates HIV-1 gene expression and facilitates the efficacy of its viral transcription. Tat is known to have oncogenic properties, and this effect was used to investigate its role in CRC oncogenesis. The main factors associated with Tat-induced CRC were its ability to significantly induce cancer cells migration, thus facilitating cancer invasiveness and metastasis. Furthermore, Tat inhibits epithelial cytodifferentiation and apoptosis, both of which are the hallmarks of cancer [27].

Conventional CRC therapy compromises immune responses and predispose patients to opportunistic infections. The use of opportunistic infection prophylaxis along with HAART was shown to decrease the risk of getting such infections [28]. Recently, HIV therapy lamivudine (3TC) was shown to attenuate CRC progression [29]. Lamivudine is a nucleoside analog and reverse transcriptase inhibitor [30]. Retrotransposon insertions [31] and repeating units [32] are associated with CRC development and progression; hence, treatment with lamivudine could halt CRC progression more profoundly in p53-mutant cell lines. The mechanism of this drug includes the induction of the interferon (IFN) response gene and the DNA damage response [29]. Xu et al. (2020) found IFNγ to play an important role in the induction of the immune checkpoint (IC) by the CRC tissue microenvironment. Apart from inducing classical ICs such as programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1), the study identified three potential novel IC-related genes induced by IFNγ. The IFNγ-inducible lysosomal thiol reductase (IFI30), guanylate binding protein1 (GBP1), and guanylate binding protein 4 (GBP4) were found to be highly expressed in CRC tumors compared to controls. The study indicated that the efficacy of IC inhibitors as cancer immunotherapy in CRC might improve by the addition of more than one IC inhibitor, with a consideration of higher instead of lower IFNγ expression levels [33].

3. Cytokine Gene Transcription Signaling in Relation to HIV-Associated CRC

Evidently, controlling HIV replication appears to play a crucial role in attenuating CRC progression [34,35]. Thus, in the efforts of finding curative HIV therapy, combination therapy with TLR agonists and broadly neutralizing antibodies (bNAbs) successfully blocked HIV-1 replication but failed to do so when used individually in people who were on anti-retroviral (ARV) therapy. Preclinical studies indicate that HIV remission could be sustained without the use of ARVs [24]. The use of HAART cannot completely eradicate HIV because of the virus's ability to hide in the immune cells and remain dormant (HIV latency) until such time that the treatment is ceased, allowing the virus to become active and replicate. Both cytokine IL-7 and C-C chemokine ligand (CCL) 19 could increase the rate of latent HIV infection threefold. In terms of productive HIV infection, IL-7 had a fivefold increase, whilst CCL19 had a twofold increase in productive infection [36]. Latency-reversing agents (LRA) could be used to reactivate HIV transcription in dormant cells, thus stimulating the expression of surface antigens that will trigger immune responses

and concurrently prevent the release of virions by HAART. However, the treatment of HIV-infected peripheral mononuclear cells (PBMCs) with TLR agonists, gene expression modulator 91 (GEM91), and a gag antisense phosphorothioate oligonucleotide showed an increased viremia caused by the induction of TLR 9 stimulation through its CpG motif. This effect was demonstrated in dose-escalation studies [37] indicating that TLR signaling contributes to challenges related to HIV treatment and possibly CRC in PLWH. Although TLRs can be beneficial in inducing immune reactivation to clear infections and prevent cancer development and progression, in certain instances, TLRs promote cancer survival and metastasis. Thus, the TLR pathway for cancer therapy is dependent on the type of TLR and the type of cell producing it. Under normal cancer immunosurveillance, TLR agonists can be beneficial to cancer cells as well. Therefore, the design of TLR-targeting agonists as well as antagonists is a promising immunotherapeutic approach to cancer [38].

TLR Signaling

Toll-like receptor family proteins are part of the innate immune system that initiates a cascade of inflammatory responses against microbial [39], parasitic [40], and viral [41] infections. There are 250 TLRs in total that have been identified thus far, with TLR 1–10 being identified in humans. The TLR family members are divided into vertebrate type (V-Type) and protostome type (P-Type). The V-Type are characterized by single-cysteine-cluster TLRs, while P-Type TLRs have several cysteine clusters. These TLRs can further be categorized into cellular (TLR 1, 2, 4, 5, 6, and 10) and intracellular membrane (TLR 3, 7, 8, and 9) TLRs (Figure 1). The TLRs are expressed on the surfaces of antigen-presenting cells (APCs) and recognize pathogen-associated molecular patterns (PAMPs) on various microorganisms. They are critical in sensing the microbiota and maintaining tolerance or triggering an immune response to pathogens. The TLRs, therefore, contribute to the prevention of gut microbial dysbiosis, associated with CRC initiation and progression [42].

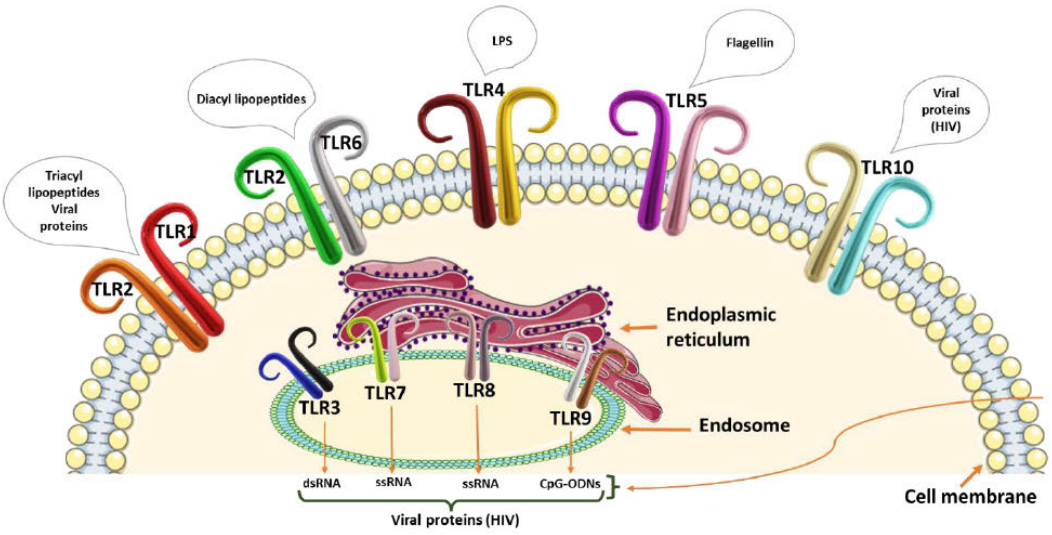


Figure 1. Representation of the human toll-like receptors on the cell membrane and the endosome with stimulatory pathogen-associated molecular patterns or danger-associated molecular patterns. Toll-like receptors 1 and 2 recognize triacyl lipopetides and viral proteins, whilst TLR2 and TLR6 recognize diacyl lipopeptides. The lipopolysaccharide (LPS) is recognized by TLR4 and flagellin by TLR5. TLR10 is specific for viral proteins including HIV protein receptors. The endosome TLR 3 recognizes double-stranded RNA (dsRNA). TLR 7 and 8 recognize single-stranded RNA (ssRNA) molecules, and TLR9 recognizes unmethylated CpG motifs. All endosome TLRs have the ability to recognize viral proteins.

4. Nuclear Factor Kappa B (NF-kB) Signaling

All TLRs subsequently end in the activation of NF-kB, which will guide the type of innate immune response elicited based on a specific pathogen [43]. Dysregulations in the NF-kB family of transcription factors are associated with inflammation in most cancers [44]. The NF-kB family consists of five connected components, the NF-κB1 and 2 (p50 and p52), RelA and B, and c-Rel [45]. These components form a homodimer or heterodimer transcriptional complex through the Rel homology domain (RHD). The RHD facilitates dimerization, binding to the promoter region of the DNA strand, and interaction with IκBs and RelA. The composition of the RelA subunit allows it to be the only member that can initiate DNA transcription. The inhibitor proteins IκBs interact with NF-kB as an on-and-off switch. This group consists of IκBα, IκBβ, IκBγ, IκBε, Bcl-3, IκBζ, and IκBNS. The Bcl-3, IκBζ, and IκBNS are responsible for transcriptional regulation within the nucleus, whilst IκBα, IκBβ, IκBγ, and IκBε are cytoplasmic inhibitors that assist in keeping NF-kB in its inactive form [46,47].

The IκBα binds as a monomer to other dimeric cytoplasmic inhibitors via their RHD blocking the DNA sequence needed for nuclear localization. Upon the activation of NF-kB by pathogen stimuli or other factors such as chemical agents, IκBα gets phosphorylated. However, the phosphorylation of IκBα is not sufficient for dissociation from the complex to allow for the mobilization of NF-kB into the nucleus to be initiated. Phosphorylation only marks IκBα for degradation by a specific ubiquitin-proteasome system that requires ATP. Complete degradations take place when sufficient ubiquitin multimers have been accumulated, and the phosphorylated IκBα polyubiquitin conjugates serve as a substrate for the 26S proteasome (Figure 2). This will then allow for the subsequent translocation of NF-kB into the nucleus [48].

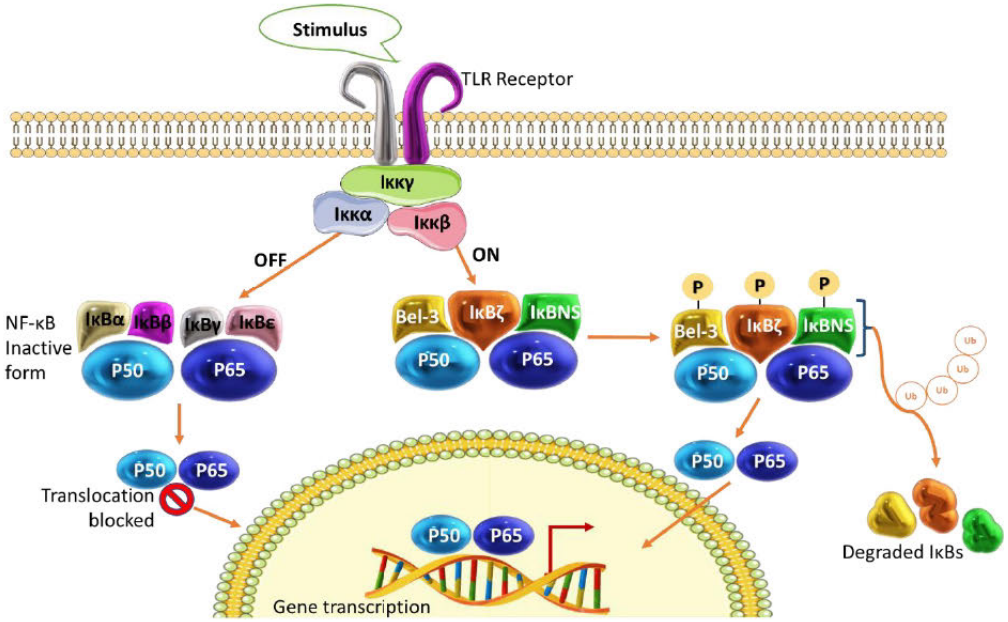


Figure 2. Representation of the NF-κB signaling pathway. The IκBα, IκBβ, IκBγ, and IκBε inactivate and prevent NF-kB from translocating into the nucleus, thus switching off the transcription of inflammatory genes. The Bcl-3, IκBζ, and IκBNS are mainly associated with facilitating transcription within the nucleus. The phosphorylation of these IκBs marks them for degradation by ubiquitinase, which then allows NF-kB to be translocated into the nucleus for the induction of cytokine gene transcription.

A study by Jana et al. suggested that blocking NF-kB along with its activator, Activin, can reduce the chances of CRC metastasis, with the possibility of even halting cancer growth. Mechanistically, Activin-NF-kB induced an increase in the mouse double minute 2 homolog (MDM2) ubiquitin ligase and the subsequent degradation of p21 via phosphatidylinositol 3-kinase (PI3K) activation [49]. The p21, also known as cyclin-dependent kinase inhibitor 1, promotes cell cycle arrest, and its dysregulation is associated with oncogenesis. The maintenance of a homeostatic balance between cell proliferation and death is crucial for keeping cancer cells at bay. The p21 can serve as a tumor suppressor or oncogene. It can either promote or halt cancer proliferation by inhibiting or promoting apoptosis [50,51]. The progression of CRC in a metastatic stage was inhibited via the induction of lipocalin2 (LCN2) and the concomitant inhibition of the NF-κB/snail signaling pathway, which promotes EMT, migration, and invasion in CRC [52]. Treatment of colon cancer xenografts with prolyl hydroxylase 2 (PHD2) could inhibit metastasis through the inhibition of the NF-κB signaling pathway and the downregulation of EMT-related proteins including vimentin. Furthermore, PHD2 regulates inflammatory responses by the suppression of pro-tumorous immune cells such as myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs). This induces the production of anti-tumor cytokines including G-CSF, TNF-α, IL-6, IL-8, IL-1β, and IL-4 [53].

4.1. Notch-Mediated NF-kB Inflammatory Response

It is important to note that the TLR4 signaling discussed earlier upregulates the activation of the Notch signaling pathway. Jagged1 (a ligand for Notch) is a direct TLR target gene that is overstimulated by IFN-γ [54]. In return, the overexpression of Notch1/2 suppresses the expression of TLR4-induced proinflammatory cytokines, TNF-α, and IL-6 and favor cancer-promoting anti-inflammatory cytokine IL-10. This results in an ultimate reduction in NF-κB transcription activity through extracellular signal-regulated kinase (ERK)-dependent signaling pathways [55]. The ERK fall under the mitogen-activated protein kinase (MAPK) family of signaling pathways, which are important in cell proliferation and differentiation. The dysregulation of this signaling cascade is implicated in oncogenesis [56]. Components of the NF-kB signaling pathway associate with the Notch signaling pathway, to promote inflammatory responses in cancer. The incorporation of Notch with TNF-α and κB kinase 2 (Ikk2) blocks anti-inflammatory machinery and promote oncogenesis [57] (Figure 3). Treatment with fructus aurantii extracts can also upregulate Notch/NF-κB/IL-1 signaling, leading to inhibition of colitis-associated CRC proliferation [58]. In CRC, active IKKα phosphorylates, silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) along with specific Notch genes (*hes1, hes5, and herp2/hrt1*) facilitate its translocation into the nucleus, where cancer-favoring gene expression takes place. A compound referred to as BAY11-7082 dysregulates IKKα activity and significantly reduces cancer proliferation [59]. Interestingly, a study by Ahmed et al. [60] found that crosstalk between the Notch and NF-κB signaling pathways has a regulatory role in inflammatory responses and stem cell function during transmissible murine colonic hyperplasia (TMCH). Chronic administration of a Notch blocker dibenzazepine (DBZ) inhibited Notch and NF-κB signaling, resulting in the abrogation of hyperplasia. A significant expression of the cytokines IL-1α, granulocyte colony-stimulating factor (GCSF), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 2 (MIP-2), and keratinocyte-derived chemokine (KC) was observed. The authors noted that the Notch and NF-kB signaling pathways are associated with epithelial cancers and chronic intestinal inflammation, respectively. These pathways are commonly coactivated, making them a potential target for therapeutic endeavors [60].

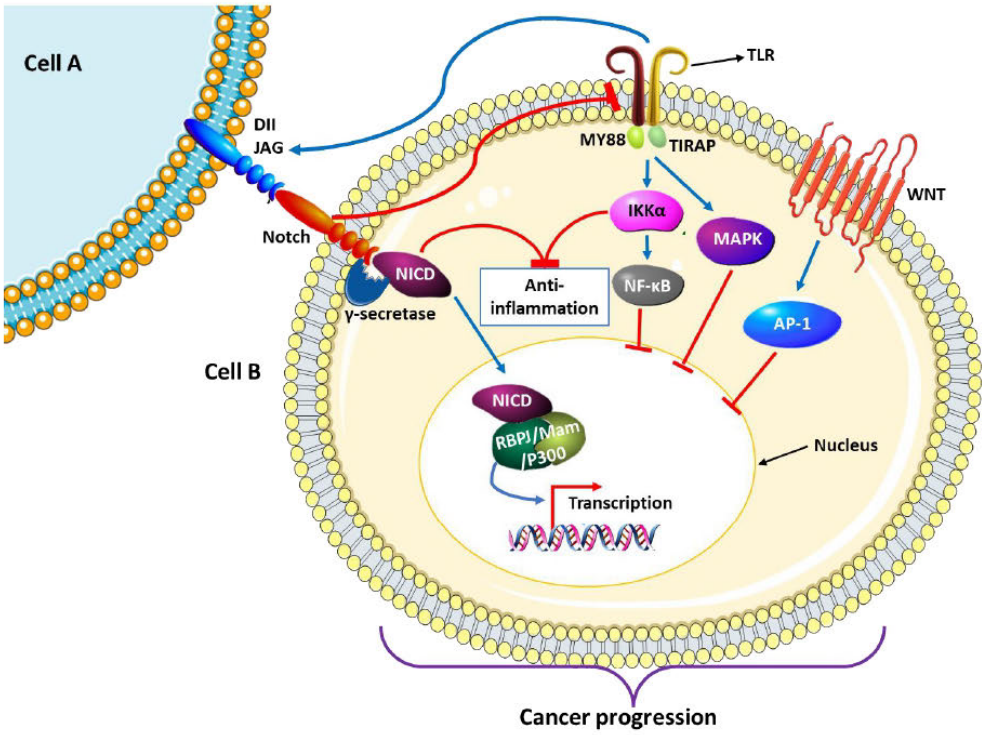


Figure 3. Notch works in synchrony with TLR-NF-κB signaling to control inflammatory responses that are central to cancer development. The TLRs can activate Notch via jagged proteins, and in return, the overexpression of Notch negatively regulates TLRs, resulting in a reduction in NF-κB signaling transcription activity, thus favoring cancer progression. The synergy between the components of the Notch and the NF-κB signaling leads to cancer progression by blocking anti-inflammatory protein expression. Mutations of the components of the AP-1 pathway promote cancer progression during viral infections. This pathway can be activated through the Wnt pathway, known for its ability to promote CRC progression.

Similarly, coactivation of the Notch and NF-kB signaling pathways plays a major role in HIV-related disease, including cancer. Notch4 was found to be responsible for tubulointerstitial injury and inflammation in HIV-associated nephropathy. The deletion of Notch4 resulted in a significant reduction in the inflammatory cytokines IL-6 and CCL2 and NF-κB transcription factors [61]. HIV-1 viral protein U (Vpu) was shown to inhibit the activation of NF-κB signaling to avoid anti-viral immune responses [62]. On the other hand, an HIV-1 accessory protein called viral protein R (Vpr) associates with transforming growth factor-β-activated kinase 1 (TAK1) to activate NF-κB and activator protein 1 (AP-1) signaling pathways, giving the virus access to host cell gene expression machinery [63]. AP-1 is responsible for the transcription of certain genes, including cytokines. One of the subcomponents of AP-1, c-jun gene, gets mutated during retroviral transduction and develops carcinogenic properties such as the ability to convert normal fibroblasts into cancer-associated fibroblasts (CAFs). Furthermore, mutated c-jun renders CAFs resistant to apoptosis by alkylating agents. The KRAS pathway is the major activator of the AP-1 pathway in CRC. The AP-1 signaling is activated through the induction of MAPK and the Wnt signaling pathway [64] (Figure 3).

Other studies have also reported a crosstalk between the Wnt/β-catenin and NF-κB signaling pathways in inflammation. These pathways can both regulate the expression of genes responsible for cell differentiation, proliferation, and survival. Individually, NF-κB is

known for its capacity to control inflammatory responses, whilst Wnt/β-catenin signaling is more important in tissue development and regeneration. These pathways have been shown to regulate each other via positive or negative feedback mechanisms depending on the type of the cell or tissue being targeted [65]. Several Wnt signal pathway-activated intestinal stem cells markers (Lgr5, EphB3, Ets2, Sox9 61, and Smoc2) have a significant role in promoting CRC metastasis. CRC cells with strong nuclear β-catenin localization are prone to undergoing epithelial mesenchymal transition (EMT) processes, thus initiating cancer invasiveness and metastasis. The EMT processes are responsible for the development of cancer stem cells in CRC and promote drug resistance [66].

4.2. Vimentin Gene Expression in HIV-Related CRC

The association between vimentin and cancer metastasis has been thoroughly studied and evaluated [67,68], leading to the acceptance and establishment of the use of vimentin as a marker for EMT-mediated metastasis [69,70]. High levels of vimentin were associated with poor prognosis and progression in CRC [71]. The immune expression of vimentin was associated with a high tumor grade in CRC. A significant correlation of vimentin and distant metastasis was also observed [72]. Blocking the NF-kB pathway was shown to inhibit the stemness, self-renewal, and migration capabilities of cancer cells [73]. Vimentin was identified as a regulator of NF-kB [74], suggesting that one of the mechanisms of vimentin-associated EMT is through the induction of the NF-kB signaling pathway (Figure 4). The correlation between high expression levels of vimentin, the NF-kB pathway, and related transcription factors is associated with poor prognosis in breast cancer patients [75,76]. Another study showed that NF-κB-mediated transforming growth factor-beta (TGF-β) induced the expression of vimentin in prostate cancer and was responsible for progression into metastatic disease [77]. A novel NF-κB-inducing kinase (NIK) and IκB kinase β (IKKβ) binding protein, referred to as NIBP, is required for the activation of the NF-κB alternative pathway. The upregulation of NIBP expression in colon cancer seemed to inhibit the expression of E-cadherin (Figure 4), whilst the expression of vimentin and CD44 remained high. The inhibition of the NF-κB pathway could not suppress the expression of CD44 and vimentin. The authors suggest that the inhibition of the NIBP might serve as an effective treatment for CRCs [78].

The NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) inflammasome is responsible for defense against viral infections through the stimulation of IL-1β and IL-18 secretion [79]. Vimentin is required for the activation and regulation of the NLRP3. This concept was proven in mice studies in which wild-type mice succumbed to a systemic inflammatory response threefold more often than the vimentin-knockout mice [80]. The study indicates that vimentin is somewhat important in inflammatory responses known to influence oncogenesis. In a study by Yu et al., vimentin was shown to favor the secretion of a cancer-promoting cytokine, IL-10, through the stimulation of dendritic cells (DCs). Vimentin-stimulated DCs were also shown to have a decreased production of anti-cancer cytokines, IL-6 and IL-12 [81]. The expression of vimentin is itself regulated by interleukins. The expression of IL-2 was shown to induce vimentin 10 to 20-fold more, while IL-4 suppressed its expression by inhibiting IL-2-induced vimentin RNA [82]. The specific roles of these interleukins in CRC initiation, development, progression, and metastasis are reviewed in more detail by Li et al. [83].

In addition, vimentin serves as a substrate for retroviruses including HIV [84,85]. HIV-1 uses vimentin to alter the chromatin organization and distribution in infected cells, thus ascertaining vimentin's contribution in HIV-1-associated cytopathogenesis and oncogenesis [86]. Vimentin was identified as a potential biomarker for HAART efficacy in PLWH [87] and a therapeutic target for HIV [88]. These findings provide a strong correlation between CRC-Vimentin-HIV and the potential use of vimentin in the effort to prevent and eradicate CRC in PLWH who are on long-term treatment with HAART.

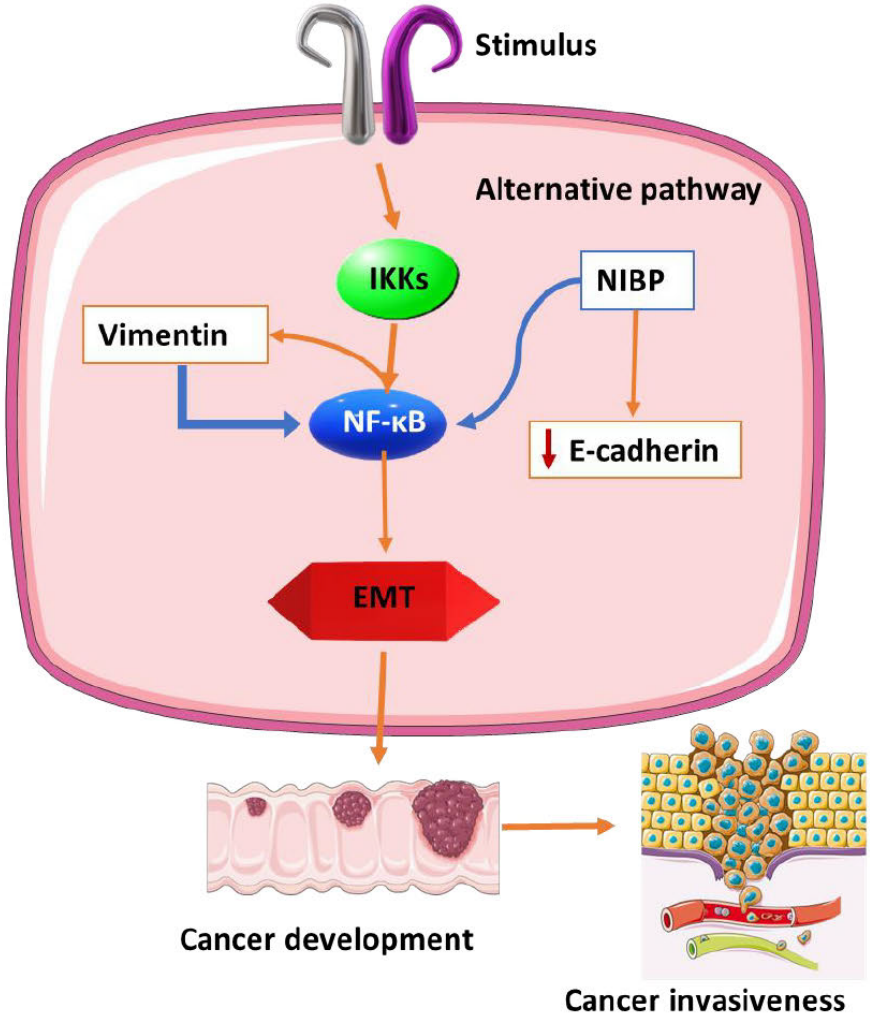


Figure 4. Vimentin as a regulator of the NF-κB pathway. Vimentin induces the NF-kB pathway to promote the stemness, self-renewal, and migration ability of cancer cells (EMT). In EMT, cells lose their adhesiveness and gain migratory and invasive capabilities. The NF-kB pathway can also induce the expression of vimentin, thus doubling up in promoting EMT in cancers. The alternative NF-κB-inducing kinase (NIK) and IκB kinase β (IKKβ) binding protein (NIBP) inhibits the expression of one of the EMT markers (E-cadherin) but fails to inhibit the expression of vimentin. EMT—epithelial–mesenchymal transition.

5. Interferon Signaling Pathways

Interferons are a family of inflammatory mediators secreted in response to various insults, including cancer cells and viruses. There are over twenty interferons identified thus far, categorized into three classes: IFN 1, 2, and 3 interferons. The IFN I are stimulated in response to viruses such as HIV. They primarily have a proinflammatory role, but prolonged exposure can lead to immune dysregulation. However, interferons have a contradictory role in cancer immunity [89] (Table 1).

Table 1. Interferons and their contribution towards HIV-related CRC.

Subgroups	Classes	Receptors	Role in HIV-Related CRC	References
IFN I	α β κ ε ω δ τ	IFNAR1/2	Pro-cancerous • Promote immune evasion • Susceptible to genetic mutations • Promote chronic inflammation • Facilitate cancer invasion and metastasis via EMT • Upregulate PDL-1- and IDO1-promoting metastasis • Regulatory T cells (Tregs) activation, thus suppressing CD8 T cells anti-cancer mechanisms Anti-cancerous • Inhibit angiogenesis • Facilitate anti-cancer immune mechanisms • Cancer cell cycle arrest • Inhibit cancer cell proliferation and promote apoptosis • Potential therapeutic efficacy in combinatorial therapies	[89–91]
IFN II	γ	IFNGR1/2	Pro-cancerous • IL-10 superfamily of cytokines (has cancer-promoting abilities) • Susceptible to genetic mutations • Upregulate PDL-1- and IDO1-promoting metastasis • Tregs activation, thus suppressing CD8 T cells anti-cancer mechanisms Anti-cancerous • Cancer cell cycle arrest • Facilitate natural killer cell activity • Facilitate antigen presentation and lysosome activity of macrophages • Increase specificity of MHC class I and II molecules	[90,92]
IFN III	λ1 λ2 λ3 λ4	IFN-λ R1/2	Pro-cancerous • Expression significantly correlated with immune checkpoints (CTLA4, PD1, PDL1, Tim3, IDO1, and LAG3) and poor survival • Gene mutations render susceptibility to CRC initiation and development Anti-cancerous • Regulate cancer cells apoptosis through IL-15 • Potential effective therapeutic target • Inhibits macrophage HIV infection, thus limiting the effects of HIV on CRC • Upregulates Th1 and downregulates Th2 response during infection	[33,93–98]

In CRC, both IFN 1 and 2 have been found to have a dual effect on cancer development and progression. This can take place, in part, through the expression of immunosuppressive Tregs and MDSCs which inhibit the expression of IFN by T helper 1 cells, thus blocking anti-cancer immunity [99]. The presence of IFN within the tumor microenvironment facilitates anti-cancer mechanisms in several ways. These include promoting the expression of major histocompatibility complex (MHC) by cancer cells; enhancing T-cells and natural killer cells trafficking; stimulating the expression of anti-cancer cytokines and favoring T helper 1 polarization.. Dysregulation in the IFN signaling pathways makes an immense contribution to immunotherapy resistance, hence the consideration to include IFNs in the development of CRC therapies [100]. However, in the case of CRC in HIV infection, the timing of therapy in terms of chronic versus acute infection seems to be of great importance if one is to benefit from IFN therapy and still manage to circumvent the deleterious effects thereof [101].

The manipulation of the IFN signaling pathway via the administration of IFN-α2a could temporarily upregulate antiviral genes and prevent simian immunodeficiency virus (SIV) infection in rhesus macaques. Continued treatment resulted in IFN-1 exhaustion and increased infection, which was accompanied by a low CD4 count [102]. New HIV infections turn out to be more resistant to IFN than the chronic infections. Infections are considered chronic six months from the initial infection. The IFN resistance was attributed to HIV-1 replicative fitness rather than resistance to a specific type of IFN [103]. Contrary to these findings, the initial viral infections were resistant but, with time, became susceptible, specifically to IFN-α, with no difference in the replicative fitness of the HIV-1 infection [104].

5.1. Indoleamine 2,3 Dioxygenase 1 (IDO1)

IDO1 regulates T cells' immune responses by degrading tryptophan in DCs and macrophages. During this process, T cells go through proliferation arrest, rendering them futile as a defense mechanism against cancer development [105]. The expression of IFN-γ induces the expression of both IDO1 (Figure 5) and the tryptophan selective transporter in the colon epithelial cell line [106]. Radiotherapy induces the expression of IDO1 via IFN 1 and 2 in CRC. As a result, the inhibition of IDO1 sensitizes CRC to radiotherapy and promotes radiation-induced apoptosis. Blocking IDO1-induced anti-cancer Th1 cytokines within the tumor microenvironment facilitates the overall eradication of cancer cells, even around the surrounding stroma. Consequently, a IDO1 blockade can serve as a protective mechanism against radiation-related adverse effects, particularly on the normal small intestinal epithelium [107]. The activity of IDO is reported to be high in PLWH that are on HIV treatment. This activity is observed from the initial stages of the infection and persists throughout the chronic stage. Furthermore, the IDO activity is associated with the size of the HIV reservoir, immune activation, and T cell exhaustion, thus indicating its role in immune-related metabolic aberrations in HIV persistence [108]. Because most cancers constitutively express IDO and its role in HIV has already been established, blocking IDO might be of great therapeutic importance, especially in PLWH. The inhibition of IDO restored the proliferation of tumor-specific T cells with less toxicity, indicating that IDO might be favorable more so in individuals who are already at risk of developing detrimental adverse effects, as in the case of HIV infection [105].

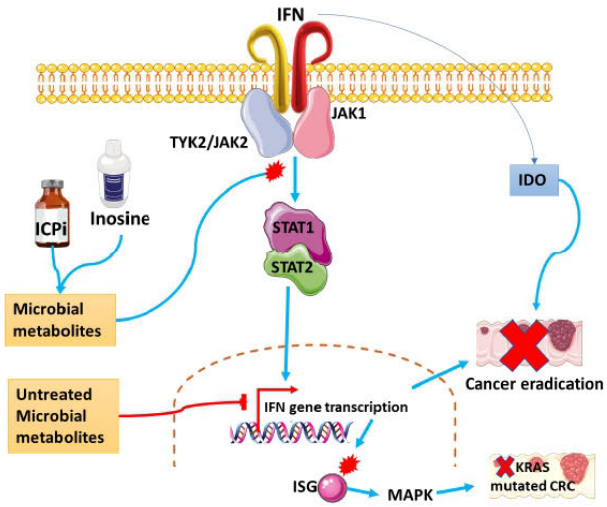


Figure 5. Interferon and related pathways involved in the regulation of HIV-related CRC mechanisms. The IFN-induced activation of the TLRs leads to downstream signaling which result in the expression of IFN genes and inflammatory cytokines. Microbial dysbiosis can regulate the expression of IFN cytokines to promote oncogenesis. The combination of inosine and ICPi can be used as a potential targeted therapy for microbial metabolites, thus blocking processes that lead to the induction of CRC. KRAS mutations are known for their ability to promote CRC induction. Treatment of CRC with KRAS mutations requires inhibition of both the MAPK and IFN signaling to halt cancer progression hence the encouragement to target interconnected signaling pathways Components of the IFN pathway, along with IDO, can sensitize cancer cells to radiation therapy and assist in the eradication of the disease. ICPi—Immune checkpoint inhibitor, ISP- Interferon stimulated gene.

5.2. Interferon-Induced KRAS Signaling and Related Pathways

The Kirsten rat sarcoma virus (KRAS) gene provides instructions for making the KRAS protein which activates the intracellular transduction signaling pathway known as

the RAS/MAPK pathway [109]. The KRAS mutations are the most common in cancers and affect 52% of CRCs. Oncogenic KRAS mutations mediate inflammation and promote immune evasion, leading to cancer initiation and progression. Proinflammatory cytokines such as IL-6 and 8 have been identified as transcriptional targets of the KRAS mutations which take place through the activation of the MAPK/PI3K signaling pathways. Inhibition of the MAPK pathway showed no clinical benefit in KRAS-mutant CRC, unless it was coupled with the suppression of IFN-stimulated gene (ISG) expression (Figure 5), leading to cancer cells apoptosis [110]. Further, KRAS mutations have been linked to the induction of PD-L1 expression, leading to immune evasion via the activation of the AKT/mTOR pathway [111]. Blocking Myc-inhibited $KRAS^{G12C}$ mutation, leads to the induction of the IFN signaling pathway and the resultant reduction in tumor-favoring immunosuppressive cells and upregulation of anti-tumor mechanisms. Contrary to the finding of the previous study, Mugarza et al. [112] observed no significant effect in cold tumors when using combinatorial treatment with both anti-PD-L1 and $KRAS^{G12C}$ inhibitors. Cetuximab is an anti-CRC drug that competitively inhibits binding of epidermal growth factor receptors (EGFR) to cancer cells thus preventing their proliferation and survival. Its efficacy is tested by the continual assessment of KRAS mutations. It has been determined that cetuximab is ineffective in metastatic CRC with KRAS mutations. A study found that one of the mechanisms of cetuximab is through the induction of the IFN/STAT1 signaling pathway, which promotes cancer survival rather than inhibition in tumor cells. This was due to cetuximab having a preference for IFN sensitive cells over tumor cells. The former cells upregulates IFN/STAT1 signaling and are resistant to treatment. The authors suggested that targeting the IFN/STAT1 signaling pathway, EGFR ligands, and related genes could serve as novel predictors of efficacy in KRAS mutations in cancer patients on cetuximab treatment [113].

The ArfGAP with dual PH domain-containing protein 1 (ADAP1) can upregulate certain T cell signaling programs in HIV-1 infection via the stimulation of KRAS/ERK/AP-1 signaling, leading to latent HIV-1 reactivation [114]. The study of the role of KRAS deficiency in CD8 T cell immune responses during acute viral infection indicated the importance of KRAS in the development and proliferation of both CD4 and CD8 T cells. KRAS was also found to modulate the TCR-induced activation of the Raf-1/MEK/ERK pathway in CD8 T cells [115]. All these are promising therapeutic approaches to deciphering the deleterious effects of KRAS mutations in HIV-related and metastatic CRC.

5.3. CRC and HIV-Associated Opportunistic Infections

Apart from viruses, other microbial organisms including bacteria and parasites have a way of manipulating the immune responses. Microbial dysbiosis in the gut induces CRC by modulating inflammatory responses, molecular networks, and the production of metabolites [116–119]. The microbiota composition in CRC is diverse and includes strains such as the *Bacteroides fragilis, Streptococcus gallolyticus, Enterococcus faecalis, Escherichia coli, Parvimonas, Peptostreptococcus, Prevotella, Alistipes, Akkermansia* spp., *Fusobacterium nucleatum, Porphyromondaceae, Coriobacteridae*, and *Methanobacterials* [118,120,121]. The abundance of the microbial species seems to differ according to the location, with the right-sided colon cancer predominately populated by *Firmicutes* and the sigmoid colon cancer populated by *Verrucomicrobia, Ruminococcaceae, Streptococcaceae, Clostridiaceae, Gemellaceae*, and *Desulfovibrio*. Similarly, the depletion of the gut bacteriome (*Akkermansia, Anaerovibrio, Bifidobacterium*, and *Clostridium*) was observed in chronically infected HIV-1 subjects. Depletion of these bacteriome is associated with metabolic disorders, CD8+ T cell responses, and chronic inflammation [122]. Another study showed the abundance of Proteobacteria—more predominantly, the Enterobacteriaceae family, which includes *Salmonella, Escherichia, Serratia, Shigella*, and *Klebsiella species*—in HIV. Additionally, *Staphylococcus, Pseudomonas*, and *Campylobacter* spp. are well studied opportunistic pathogens and sources of bacteremia in HIV-infected subjects [123].

Clay et al. identified inosine as a targetable microbial metabolite for 9potential therapeutic strategy for CRC antitumor immunity by looking at studies that correlated inosine with immune checkpoint inhibitors and the IFN-γ pathway (Figure 5) [121]. Moreover, microbiota can control the destiny of viral infections by the positive or negative regulation of the IFN signaling pathway. The eradication of intestinal and bile-derived microbiota can reduce the normal expression of IFN-λ, disrupting its antiviral activity. The same effect is observed with the impairment of the IFN-λ receptors [124]. The gut microbiota dysbiosis was also shown to influence the therapeutic response to HAART [125]. Targeting microbiota in HIV could decrease the chronic inflammation [126] related to CRC induction.

6. HIV and Parasitic Co-Infections and CRC Oncogenesis

The connection between parasitic infections and oncogenesis is well studied and established. Parasites and cancers share the ability to manipulate and evade the immune system. These abilities ensure that cancers and parasites survive and multiply [127–129]. Protozoa and helminths are the major classes of parasites and could result in asymptomatic colonization or cause several clinical outcomes including life-threatening illness [130]. Some of the important cancer-associated parasitic infections are noted as helminths [131]. These parasites can cause intestinal obstruction that might require surgery and also lead to infections such as sepsis [132]. The chronic inflammation induced by these parasites is the major cause of oncogenesis [133]. A constrictive lesion at the sigmoid colon was observed in a patient with intestinal obstruction caused by *Schistosoma mansoni* infection. Upon further evaluation, the patient was found to have CRC, which could be a result of the chronic inflammation induced by the parasitic infection [134]. Moreover, CRC has been associated with a common intestinal protozoan, *Blastocystis* sp. The prevalence of *Blastocystis* sp. had a fivefold increase in stool samples of CRC subjects [135]. Other parasites such as *Cryptosporidium* [136], *Schistosoma japonicum* [137–139], *Schistosoma mekongi* [140], and intestinal helminths [141] have also been linked to CRC oncogenesis [131,136]. There are currently clinical trial studies underway aimed at validating the link between intestinal helminths and protozoa with CRC pathogenesis [142].

Several protozoans such as *Plasmodium, Entamoeba, Toxoplasma*, and *Leishmania* trigger host inflammatory signaling pathways by the secretion of cytokine molecules that are structurally similar to those of humans. The macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine that induces acute inflammatory responses. The MIF binds to CD74 and induces the expression of TNF-α, IL-6, IL-8, and IL-12. These proteins have also been shown to activate the ERK1/2 and PI3K/Akt pathways [143], which are both implicated in oncogenesis [144] (vide supra). It is worth noting that the MIF can also promote the activation of the nucleotide-binding oligomerization domain-like receptor (NLR) pyrin domain-containing 3 (NRLP3) inflammasome [143], which is to be discussed in more detail in the next section.

7. Role of the Inflammasome Complex in HIV and CRC Oncogenesis

Inflammasome is one of the signaling pathways of the innate immune system that is induced by stimuli from microbial pathogens including viruses and the gut microbiota. The components of the inflammasome complex consist of the sensor proteins, NLRP 1-3, NLR family CARD domain-containing protein 4 (NLRC) 4, absent in melanoma 2 (AIM2), and pyrin. Caspase-1 is connected to the sensor proteins through the adaptor apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). ASC consists of the caspase activation and recruitment domain (CARD) and the pyrin domain of which the former is used to connect to the inflammasome sensor proteins. Cleavage of Caspase-1 is prompted by the connection of pro-caspase 1 to the complex through the CARD domain. This then leads to the activation of caspase-1-dependent pro-inflammatory cytokines, IL-1β and IL-18, and the induction of pyroptotic cell death. The ASC is important in the induction and facilitation of inflammatory responses [145,146].

Helminth infections can activate the NLRP3 inflammasome, resulting in the modulation of T helper 2 immunity [147]. The prevalence of HIV–helminths co-infections is high in the African population [148,149]. It is not clear whether helminths infections increase the susceptibility to HIV or whether HIV increases the susceptibility to helminths infections [150]. However, certain phenotypes of helminths suggest the former [151]. Helminth infections caused by *Schistosoma haematobium* and *Opisthorchis viverrine* have been shown to have carcinogenic properties. These parasites release metabolites such as oxysterols and catechol estrogens, which can integrate with the host cell DNA through the involvement of CYP450 enzymes. The ultimate formation of DNA-adducts is associated with helminth-associated oncogenesis [152]. Several cancers including colorectal hepatocellular carcinoma are associated with helminths infections [153]. Furthermore, activation of the NLRP3 inflammasome on its own is associated with cancers including the colon cancer. The NLRP3 complex can be activated via TLR- NF-κB signaling, which then induces the transcription and expression of the NLRP3 inflammasome cytokines, IL-1β, and IL-18. [154].

Dysregulation in the NLRP3-induced IL-1β and IL-18, which are critical for maintaining the integrity of the intestinal epithelial barrier, can result in CRC oncogenesis [155]. The level of IL-1β is upregulated in CRC, resulting in the activation of immunosuppressive cells such as MDSCs and promotion of cancer metastasis by facilitating the EMT processes. This cytokine can also induce angiogenesis and promote drug resistance in CRC. On the other hand, IL-18 has a pleiotropic effect, leading to both the suppression and promotion of CRC. IL-18 can suppress colitogenic microbiota associated with inflammasome-mediated intestinal dysbiosis but also promote endothelial cell stemness and angiogenesis [156]. Moreover, inflammasomes are the main inducers of inflammation, which promotes inflammatory diseases such as inflammatory bowel disease (IBD), which is associated with a high risk of CRC development [157]. A decrease in inflammasome-induced IL-18 can lead to the disruption of the IFN-γ pathway and ultimate microbial dysbiosis associated with CRC oncogenesis. Studies found these effects to be a result of a deficiency in inflammasome complex subunits including NLRP6. Taken together, the pro-inflammatory microenvironment generated by inflammasomes creates a suitable condition for CRC development and progression [155]. The most studied inflammasome, NLRP3, is the major culprit of chronic inflammation and microbial dysbiosis causing intestinal epithelial cells dysfunction and CRC oncogenesis; hence, there have been suggestions to use it as a potential therapeutic target for CRC [158]. NLRP3 was also shown to be increased in PLWH on HAART treatment. The inflammasome genes, NLRP3 and caspase-1 were found to be upregulated in immunological non-responders. The authors suggested that this could be the reason behind the constant immune activation that keeps these individuals immunocompromised [159]. The NLRP3 inflammasomehas a critical role in maintaining HIV-1 infection-associated inflammation and has been suggested as a therapeutic target for HIV as well. This serves as an indication that targeting NLRP3 might be efficacious in HIV-associated CRC [160].

8. Epigenetic Regulation of Inflammatory Signaling

The contribution of epigenetic dysregulations, such as microsatellite instability (MSI), caused by mutations in DNA mismatch repair genes in CRC is well established. These modifications happen in 10–15% of CRCs [161], whilst chromosomal instability (CIN) takes place in 65%–70% of sporadic CRCs [162], with approximately 65% of cases of CRCs being sporadic [163]. Epigenetic modifications are key regulators of inflammatory processes induced by inflammasomes, more so with the NLRP3 components. In preclinical studies, targeted therapeutic strategies for blocking NLRP3 inflammasome are performed using the diarylsulfonylurea-containing compound, MCC950. Alternatively, the inflammasome complex assembly and activation can be blocked with the use of NF-κB inhibitors, caspase-1 inhibitors, and the inhibition of the ATPase activity of the NLRP3 [164]. Recent advances have identified epigenetic mechanisms involved in the expression, assembly, and activation of the NLRP3 inflammasome as potential targets for therapeutic purposes [165]. However, when targeting the inflammasomes, caution should be taken to not disrupt their crucial

role in fighting cancers and infections. Thus, a homeostatic balance ensuring the continual clearance of newly mutated cells and infections should be retained, and the epigenetic drugs developed should be precise and disease-specific. Microbiota can induce epigenetic modifications at the CpG islands in the promoter region of the NLRP3 gene [166]. This leads to the exaggerated inflammatory responses seen in inflammatory bowel disease and an increased risk of CRC oncogenesis [167,168]. Treatment with inhibitors such as JQ1 can protect the colon and inhibit NF-kB activation and the expression of NLRP3-associated components [169]. The activation of the NF-κB signaling pathway can cause DNA damage and induce epigenetic modifications such as CIN, which promotes oncogenesis [170,171]. This is attributed to the crosstalk between NF-κB and STAT3 signaling that contributes to challenges in CRC therapy. Further, NF-kB/IL-6 induces the expression of DNA (cytosine-5)-methyltransferase 1 (DNMT1), which downregulates the suppressor of cytokine signaling 3 (SOCS 3) needed for the regulation of cytokine-induced STAT3 signaling, leading to CRC oncogenesis [172]. Moreover, several microRNAs (miRNA) are involved in the regulation of the NF-κB signaling pathway and related transcription proteins in cancers. Some oncogenic viruses utilize miRNAs to activate NF-κB signaling and regulate immune responses to promote oncogenesis [173]. MicroRNA 146 targets and activates NF-κB, which in return upregulates miR-146 gene expression. To control its own expression, miR-146 downregulates the expression of interleukin-1 receptor-associated kinase 1 (IRAK1) and TNFR-associated factor 6 (TRAF6), which have a crucial role in the activation of NF-κB. The activation of NF-κB by miR-21 in some cancers is associated with therapeutic resistance and metastasis. The miR-301a is the most potent activator of NF-κB that negatively regulates it through its target gene, NF-κB repressing factor (NKRF). The upregulation of miR-301a in CRC is associated with increased cancer proliferation, migration, and invasion. This takes place through the activation of the TGF-β/Smad pathway, which downregulates the SOCS 6 and Smad4. MiR-301a also promotes cancer progression into the metastatic stage through the hyperactivation of the Wnt/β-catenin signaling. On the other hand, miR-30b blocks CRC progression by regulating the expression of KRAS, and it has been suggested as a prognostic indicator and therapeutic target of CRC [174].

9. Compounds/Agents Targeting Inflammatory Signaling in CRC

Wang et al. [175] have identified natural compounds that can potentially be used to target signaling pathways in CRC. These includes phytochemicals such as flavonoids and polyphenolic compounds such as epigallocatechin gallate (EGCG), which are targeted at the NF-κB signaling pathway. In addition to flavonoids, alkaloids and terpenoids can be used to target the Wnt/β-catenin signaling pathway. The most common cancer-related pathway, the MAPK/PI3K/Akt/mTOR signaling pathway, might benefit from treatment with polyphenolic compounds in addition to flavonoids [175]. Phytochemicals induce or inhibit cytochrome P450 enzymes [176] that are essential for mitigating cancer development, metastasis, and the response to anticancer therapies [177]. In addition to phytochemicals, nanoparticles have been used as targeted therapy against inflammatory signaling pathways in cancers. Nanoparticles serve as delivery systems of phytochemicals that modulate the function of specific inflammatory signaling pathways. These pathways include NF-κB, MAPK, PI3K/AKT/mTOR, and Wnt pathways discussed in this paper [178].

Several other agents and inhibitory drugs have been developed against the Wnt signaling pathway as a targeted therapy for CRC [179]. Targeted Wnt therapies are developed depending on the regulatory level and specific ligands. These include the extracellular and cell membrane levels, the cytoplasmic level, and the nuclear level. Lastly, the interrelated signaling crosstalk also has different levels that regulate the Wnt pathway [180]. NSAIDs are commonly used to relieve pain and reduce inflammation but have been shown to have chemo-preventative capabilities due to the association of chronic inflammation with oncogenesis [181,182]. The NSAIDs block cyclooxygenase-2 (COX-2) and reduce prostaglandin E2 synthesis associated with cancer proliferation and angiogenesis. The mechanisms of NSAIDs are also implicated in promoting cancer cells apoptosis [183,184]. This has led to

the development of COX inhibitors for the treatment and prevention of cancers. The NSAID mechanism of action does not require COX for its chemo-preventative effect. NSAIDs can regulate several inflammatory pathways, including the NF-κB, the Wnt/β-Catenin, and the MAPK/Akt signaling pathways [185,186].

10. Conclusions

Improvements in CRC screening methods in Western/high-income countries allowed for much earlier detection, but the current treatment strategies are not beneficial to all individuals diagnosed with CRC. The introduction of HAART has come with the benefit of improving the lifespan of PLWH but with the disadvantage of rendering PLWH susceptible to opportunistic infections, microbial dysbiosis, and cancers, which are all associated with the induction of inflammatory responses. There are several signaling pathways associated with CRC, with the most important being those that regulate inflammatory responses. Herein, the review discussed specific inflammatory signaling pathways intertwined by different mechanisms and pathways. These includes but are not limited to microbial-regulated signaling pathways that contribute to CRC oncogenesis and progression. Cancer and HIV take advantage of these pathways, working in concert to ensure their survival and spreading. In this manner, the culprits always have an alternative loop way that they can fall into should their common pathway be blocked by certain inhibitors. Notably, in most cases, cancer/HIV would manipulate the mechanisms of a pathway that favors its initiation to interaction with a pathway that antagonizes it thus increasing its chances of survival and progression.

This could also be through the regulation of the expression of genes involved in anti-inflammatory signaling cascades. This includes related transcription factors involved in controlling and dampening anti-viral/tumor pro-inflammatory signaling pathways. Epigenetic modifications are well recognized in CRC, and these play a crucial role in regulating inflammatory mechanisms. Evidence shows that microbiota contribute to oncogenesis through the expression of biological molecules such as inflammasomes and short-chain fatty acids (SFCA). One of the mechanisms of SFCA action is the regulation of the histone deacetylases involved in inflammatory responses, cell proliferation, and migration. These biological molecules are also associated with CRC; however, this is beyond the scope of this paper and will not be discussed any further. These molecules trigger specific inflammatory signaling pathways which further contribute to the advancement of CRC. Previously, HIV was only associated with specific cancers such as Kaposi sarcoma, but over the years, its contribution to non-AIDS defining cancers is unquestionable. Thus, uncovering the complexity of several intertwined exits that HIV and related cancers manipulate to evade the immune system without severe adverse effects would be of great benefit, especially in aggressive and hard-to-treat cancers such as CRC. It is hoped that this approach can provide a fast and effective response that could also prevent metastasis and recurrence.

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CHAPTER THREE

Immune modulation in HIV and helminth co-infection and implications for colorectal cancer risk

Building on the inflammatory framework established in Chapter 2, this chapter investigates the role of immune modulation in HIV-helminth co-infection as a potential contributor to colorectal cancer (CRC) risk. While chronic inflammation is a recognised driver of oncogenesis in HIV-associated CRC, helminth infections are also known to induce long-lasting immunoregulatory states that may dampen antitumor immunity. The convergence of these immune-altering conditions raises the possibility that co-infection may create a tumour-permissive gut microenvironment. This chapter aimed to determine the association between HIV-helminth co-infection and key immunoregulatory pathways that modulate antitumor immune responses. The results presented here provide novel insight into the immunoregulatory landscape of HIV-helminth co-infection and its potential implications for CRC pathogenesis in a South African adult population.

Introduction

Chronic inflammation is a well-established driver of cancer, and infections such as HIV and helminths are major contributors to dysregulated immune responses in populations with high colorectal cancer (CRC) burden. Helminth infections, which remain highly prevalent in regions also affected by HIV, induce a range of immunomodulatory effects that can significantly alter host immunity. While these effects may assist in regulating autoimmunity and allergic reactions, they can also disrupt vaccine pharmacodynamics and increase susceptibility to co-infections [1]. Helminths manipulate immune responses through several mechanisms, including targeting pattern recognition receptors, expanding immunosuppressive regulatory T cells (Tregs), shifting T helper (Th) cell responses from Th1 to Th2, inducing cytokine production, and orchestrating immune cell apoptosis [2].

Beyond its effects on T cells, HIV uses monocytes as a vehicle for tissue dissemination [3], and the HIV-1 protein has been shown to polarize macrophages toward the M2 phenotype [4]. These M2-like macrophages, also referred to as tumor-associated macrophages (TAMs), are activated by Th2 cytokines, particularly IL-4 and IL-13 [5]. Helminth infections similarly induce M2 macrophage polarization, a process strongly implicated in tumor initiation and progression [6]. The gastrointestinal tract, which contains abundant resident macrophages to maintain immune homeostasis, becomes a key site of vulnerability. CRC tumors secrete chemoattractants that

recruit monocytes, which differentiate into TAMs in the tumor microenvironment (TME). These TAMs, along with Tregs, exert immunosuppressive effects by producing anti-inflammatory cytokines such as IL-10, IL-4, and transforming growth factor-β (TGF-β) [7].

In addition to their immunosuppressive cytokine profiles, both Tregs and TAMs express a range of immune checkpoints (ICs) and co-inhibitory molecules, including leukocyte immunoglobulin-like receptor B1 (LILRB1), sialic acid-binding Ig-like lectin 9 (Siglec-9), and programmed death-ligand 1 (PD-L1), which further suppress antitumor immunity and facilitate tumor progression [8, 9]. Given that helminth infections also drive chronic immune activation and suppression via overlapping pathways, studying these mechanisms offers important insights into how persistent parasitic infections may shape cancer risk in vulnerable populations.

This study is the first, to our knowledge, to examine immunoregulatory molecules, including immune checkpoint expression, during helminth-induced inflammation in the context of HIV co-infection. These findings offer new perspectives on the immunological crosstalk between parasitic and viral infections in CRC development. The immunomodulatory effects identified in this study were presented as a poster at the American Association for Cancer Research (AACR), with the abstract published in a high-impact journal (IF = 16.6). The results emanating from this study were also published in MDPI Viruses, IF = 3.5.

Conference paper title: A Closer Look at Colorectal Cancer Risk: Insights from the Complex Relationship of HIV, Helminths, and Immune Responses. Damane BP, Mulaudzi TV, Kader SS, Ogunsakin RE, Naidoo P, Dlamini Z, Mkhize-Kwitshana ZL. Abstract 120: A closer look at colorectal cancer risk: Insights from the complex relationship of HIV, helminths, and immune responses. Cancer Res. 2024;84(6_Suppl):120. doi:10.1158/1538-7445.AM2024-120.

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Abstract 120: A closer Look at colorectal cancer risk: Insights from the complex relationship of HIV, helminths, and immune responses FREE

Botle P. Damane; Thanyani V. Mulaudzi; Sayed S. Kader; Ropo E. Ogunsakin; Pragalathan Naidoo; Zodwa Dlamini; Zilungile L. Mkhize-Kwitshana

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Abstract

The advent of antiretroviral therapy has markedly increased the survival rates of individuals with human immunodeficiency virus (HIV). However, this demographic, particularly in low-middle-income countries (LMICs) where HIV-helminth co-infections are prevalent, remains susceptible to infections. The intricate interplay between helminth infections and HIV susceptibility, often inducing immunological phenotypes that heighten vulnerability, remains poorly understood. Studies have shown the association between HIV-helminth co-infections and elevated susceptibility to colorectal cancer (CRC), introducing an additional layer of health concerns for this already vulnerable population.

This retrospective observational study delves into the profound effects of HIV-helminth co-infections on immune responses, with a specific focus on type 2 T helper (Th2) cell immunomodulation and susceptibility to CRC. Utilizing samples from a master project (n=414), 80 systematically randomized individuals were categorized into uninfected control, HIV-infected only, helminth-infected only, and HIV-helminth co-infected groups. Parasite detection employed the Kato-Katz technique, and serum testing was conducted for helminth-specific antibodies. Serological HIV testing, viral load categorization, white blood cell counts, and differential analyses were performed. Human ProcartaPlex Panel 1 was used for cytokine profiling of immune stimulatory and inhibitory soluble proteins.

A significant reduction in B and T lymphocyte attenuator (BTLA) (p=0.049) in helminth infections compared to the control. The association of BTLA with its ligand, herpesvirus entry mediator (HVEM), establishes a direct link between the CD28 and tumor necrosis factor receptor (TNFR) families, triggering wide-ranging and robust immune responses. Subsequently, a notable reduction in CD28 was observed in HIV-infected subjects (p=0.003), followed by helminth-infected and HIV-helminth co-infected individuals (p=0.03) compared to the control. Furthermore, programmed death ligand-2 (PD-L2) expression, a major contributor to alternatively activated macrophage polarization during helminth infections, was significantly increased in the HIV-helminth co-infected group (p = 0.05), favoring a shift towards Th2 immune responses.

These observed alterations underscore the complexity of the immunological interplay in co-infected individuals, emphasizing an additional risk factor for colorectal cancer development. This necessitates a comprehensive understanding, providing valuable insights for the development of therapeutic strategies in LMICs and similar settings, addressing the heightened risk of CRC within this population.

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HIV-Helminth Co-Infections and Immune Checkpoints: Implications for Cancer Risk in South Africa

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Abstract: South Africa has the highest HIV prevalence globally, often co-occurring with helminth infections in impoverished regions. The coexistence of these infections leads to immunological interactions, potentially enhancing oncogenesis by upregulating immune checkpoint molecules (ICs) among other effects. Notably, most ICs are overexpressed in cancer and correlated with its progression. Helminth infections trigger Th2-type immunity, increasing immunosuppressive M2 macrophages, regulatory T cells, and associated IC molecules. PD-L2 is reported to contribute to Th2-type immunity induced by helminth infections. Similarly, TIM-3, elevated during chronic viral infections, induces a similar immunosuppressive profile. CTLA-4 and PD-1 impact T-cell function by interacting with CD28, crucial for T-cell function. CD28 is downregulated in chronic infections and cancer. This study investigated the impact of HIV-helminth co-infection on co-stimulatory and co-inhibitory molecule profiles associated with antitumor immunity. Using 78 serum samples collected from March 2020 to May 2021, participants were categorized into uninfected control (no HIV and helminth infections), HIV-infected, helminth-infected, and HIV-helminth co-infected groups. Multiplex immune regulatory molecule assay analysis was conducted. The data were analyzed using multivariate regression analysis and adjusted for confounders (age, gender, BMI, ART, supplements, and other chronic diseases). The uninfected control group was used as the baseline reference group for analysis. HIV-infected individuals had higher PD-1 (adjusted $\beta = 0.12$, $p = 0.034$) and TIM-3 (adjusted $\beta = 23.15$, $p = 0.052$) levels, with the latter showing a trend toward significance. However, lower CD28 levels (adjusted $\beta = -651.95$, $p = 0.010$) were observed. Helminth-infected individuals had higher TIM-3 levels (adjusted $\beta = 20.98$, $p = 0.020$). The co-infected group had higher PD-1 (unadjusted $\beta = 0.18$, $p = 0.0046$) and PD-L2 (adjusted $\beta = 7.95$, $p = 0.033$) levels. A significant decrease in CD28 profile was observed across all infected groups: HIV-infected (adjusted $\beta = -651.95$, $p = 0.010$), helminth-infected (adjusted $\beta = -674.32$, $p = 0.001$), and co-infected (adjusted $\beta = -671.55$, $p = 0.044$). The results suggest that HIV-helminth co-infections alter immune checkpoint markers, potentially increasing cancer risk by promoting an immunosuppressive microenvironment that hinders anti-cancer

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immunity. CD28's downregulation underscores immune inefficiency in chronic diseases. Addressing these co-infections is crucial for improving HIV care and potentially reducing cancer risks through targeted strategies.

Keywords: HIV-helminth co-infection; immune checkpoint molecules; immunosuppressive microenvironment; CD28 downregulation; Th2-type immunity; PD-1 and TIM-3; chronic infections and cancer; regulatory T cells (Tregs); antitumor immunity dysfunction

1. Introduction

People living with human immunodeficiency virus (HIV) are particularly susceptible to various infections [1], including helminths, which are common in children and adults residing in poorly resourced regions of low-middle-income countries (LMICs) [2–4]. The immunological and biological interaction between HIV and helminth infections is complex, and it remains unclear whether helminth infections definitely increase susceptibility to HIV and pathogenesis or vice versa [5]. Certain helminth-induced immunological phenotypes might suggest that helminths increase susceptibility to HIV [6]. Helminth infections have been shown to have negative effects on HIV immune responses, predominantly modulating type 2 T helper (Th2) cell responses. Mulu et al. (2015) noted that 12 weeks after deworming and antiretroviral therapy (ART), there was a significant reduction in serum IgE levels among symptomatic HIV-helminth co-infected individuals, indicating a downregulation of the Th2 immune response [7]. The ability to polarize Th1 into Th2 immunity at the early stages of an insult is what helminths have in common with cancer. Secretory molecules from helminths can also recruit immunosuppressive and cancer-favoring regulatory T cells (Tregs), which aid in the sustenance and survival of chronic infections [8] and cancer cells [9].

A systematic review by Alghanmi et al. (2024) [10] demonstrated that helminth-derived proteins can influence the host's immune response, serving as an adaptive strategy for helminth survival. In mice models of colitis, the administration of these proteins was found to suppress the production of Th1/Th17 cytokines, such as IL-1β, IL-6, TNF-α, IL-17, and IFN-γ, while enhancing the levels of Th2/Treg cytokines, including IL-4, IL-13, TGFβ, and IL-10, in serum, colon tissue, and spleen samples [10]. Helminth infections induce the recruitment of immune cells, such as eosinophils and basophils, to sites of helminth infection which is mediated by Th2 cytokines. This is an attempt to expel the invading worms. However, the helminths have developed mechanisms to evade the immune attack, and eventually a modified Th2 response ensues, to facilitate the parasite's survival [11,12]. The modified Th2 response is accompanied by the production of alternatively activated macrophages (AMMs or M2) among others. Th2 response assists in controlling excessive inflammation, thereby creating a more hospitable environment for the helminths to persist within the host [13].

Helminth-derived vesicles also have a major contribution to immune regulation. Their intracellular effect is mostly achieved through helminth-derived products (HDPs), which interact with toll-like receptors (TLRs) in antigen-presenting cells (APCs), not only modulating TLR expression, but also skillfully influencing their intracellular signaling pathways. Certain HDPs can redirect TLR4 signaling toward the MAPK pathway and ERK1/2 activation, thereby promoting the induction of Treg and Th2 responses [14,15]. Although not well understood, helminth infections have been shown to promote myeloid-derived suppressor cells (MDSCs) and alternatively activated macrophages (AAMs), also known as M2 macrophage-mediated immunosuppression [16], Table 1. Furthermore,

helminths induce increased numbers of M2 macrophages, that, among other functions of driving the host defense, prevent type 2 immunopathology. They mitigate and repair parasite-induced tissue damage and generate an anti-inflammatory environment through the production of anti-inflammatory mediators such as interleukin 10 or prostaglandin E2 which assist the parasite to evade host immunity [17,18].

The co-stimulatory molecule, CD28, plays a significant role in generating, activating, and regulating antigen-specific T cells [19,20]. However, helminths and HIV have been shown to decrease the cellular expression of CD28 while increasing the expression of immune checkpoints, such as cytotoxic T-lymphocyte Antigen-4 (CTLA-4) and programmed cell death 1 (PD-1), aiding in the reduction of T cell activation and proliferation [21]. In addition, Tregs and M2 macrophages express co-inhibitory receptors CTLA-4, the lymphocyte activation gene-3 (LAG3), the T cell immunoglobulin and mucin domain-containing molecule 3 (TIM-3) [22], and PD-1 and its ligands, PD-L1/2 [23] (Table 1). Helminth and other parasitic infections can induce the expression of PD-L2 from activated macrophages, which bind to PD-1 on activated T cells, leading to anergy and reduced activation [24]. The expression of PD-L2 on peritoneal macrophages has been linked to increased susceptibility to *Fasciola hepatica* infection, as demonstrated in PD-L2 deficient mice. The authors found that PD-L2 expression is crucial for M2 macrophage polarization during helminth infections and is necessary to maintain the balance between Th1- and Th2-type immune responses [24]. Another related immune-inhibitory molecule, with similar functions to both PD-1 and CTLA-4, is the B and T lymphocyte attenuator (BTLA) receptor [25]. An infection with the helminth *Strongyloides ratti* has been shown to upregulate the BTLA pathway. Conversely, reducing BTLA or its ligand, the herpes virus entry mediator (HVEM), impairs the helminth's ability to evade the host's immune responses, thus compromising its survival [26]. The helminth-induced upregulation of immune checkpoints and reduction of costimulatory molecules, such as CD28 (as shown in Table 1), share similarities in immune modulation observed in cancer with a potential significant impact on cancer immunotherapy. Dual infection with helminths and HIV may further amplify immunosuppressive effects, potentially creating a favorable environment for cancer development and progression.

Table 1. Shared immunosuppressive mechanisms between helminths and cancer.

Mechanism	Helminths	Cancer	References
PD-1/PD-L1 Upregulation	Helminths upregulate PD-1 and PD-L1 on T cells and antigen-presenting cells, leading to immune suppression.	Tumors express PD-L1 to evade immune detection.	[24,27]
Treg Expansion and Th2 Skewing	Helminths induce Treg expansion and shift immune responses from Th1 to Th2, dampening pro-inflammatory responses.	Tumors recruit Tregs to suppress anti-tumor immunity.	[28,29]
MDSC and M2 Macrophage Induction	Helminths promote MDSC accumulation and M2 macrophage polarization, which suppress immune responses.	Tumors recruit MDSCs and polarize macrophages to M2 phenotype, aiding tumor progression.	[16,30]
Secretion of Immunomodulatory Molecules (IL-10, TGF-β)	Helminths secrete IL-10 and TGF-β, leading to immune suppression and tissue remodeling.	Tumors secrete IL-10 and TGF-β to create an immunosuppressive microenvironment.	[31,32]
CTLA-4 Expression	CTLA-4 expression in helminth-infected individuals, suggesting a role in immune regulation might be mainly from immunosuppressive cells such as Tregs.	Tumors exploit CTLA-4 checkpoints to suppress T-cell activation.	[27,32,33]

Programmed cell death protein 1 (PD-1), Programmed cell death ligand 1 (PD-L1), T helper (Th), Myeloid-derived suppressor cells (MDSC), M2 macrophages (M2), Interleukin (IL), Transforming growth factor-beta (TGF-β), Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4).

The benefits of blocking the expression of ICs such as CTLA-4 and PD, along with their ligands (CD80/86 and PD-L1/2, respectively) in cancer [34] have been extensively investigated. Given the profound immunomodulatory effects of helminths and HIV co-infection, it is imperative to investigate the potential risks these co-infections may pose in cancer initiation and development. Understanding these interactions can aid in the development of comprehensive treatment and prevention strategies that address the dual burden of these infections and their possible role in cancer pathogenesis. This study, therefore, aimed to investigate the association between HIV-helminth co-infection and the expression of co-stimulatory and co-inhibitory molecules involved in immunoregulatory pathways that influence antitumor immune responses.

2. Materials and Methods

2.1. Study Design

This was a cross-sectional observational sub-study based on the main project with ethics approval number BE351/19. A separate approval (BREC/00005458/2023) for this study was received from the Biomedical Research Ethics Committee of the University of KwaZulu Natal (UKZN), Durban, South Africa.

2.2. Study Population and Sampling

The present sub-study used stored samples collected for the main project conducted in a peri-urban area south of Durban, KwaZulu-Natal, South Africa, from March 2020 to May 2021. Whole blood and stool samples were collected from consenting participants for the master study and utilized in the retrospective cohort. The recruitment of participants has been described in detail elsewhere [3]. A total of 78 participants were purposively selected based on their infection status. Those with complete datasets for additional analyses were then divided into four groups [HIV and helminth uninfected control (n = 20), HIV infected (n = 20), helminth infected (n = 20), and HIV-helminth co-infected (n = 18)]. Every second participant was selected from each pool in the respective group until there were 20 participants per group. Only 18 were HIV and helminth co-infected; thus, by default, this group comprised 18 participants.

2.3. Parasite Detection

Collected stool samples were processed using the Kato–Katz technique for detecting intestinal helminths. A modified fecal parasite concentrator technique was performed using a mini Parasep® SF stool concentrator kit to concentrate potential parasite ova and cysts for increased detection sensitivity. To further enhance the sensitivity of coproscopy, serum samples were tested for the presence of Ascaris lumbricoides-specific IgE (>0.35 kU/L) and IgG4 (>0.15 kU/L) antibodies using a Phadia™ 200 instrument (Phadia AB, Thermo Fisher Scientific, Uppsala, Sweden). This method is described in more detail elsewhere [3].

2.4. Full Blood Cell Count and Detection of HIV Status

Serological HIV testing of the participants utilized the Alere Determine™ HIV-1/2 Ag/Ab Combo rapid test, manufactured by (Abbott Diagnostics Scarborough, Inc., Scarborough, ME, USA), with inconclusive results confirmed using the ICT HIV-1/2 Ag/Ab test kit (ICT diagnostics, Cape Town, South Africa). Whole blood was analyzed using the full blood count analyzer (AQUIOS CL Flow Cytometer System, Beckman Coulter Life Sciences, Brea, CA, USA) to determine differential white blood cell counts. A previous publication provides a more detailed description of the methods [35].

2.5. Analysis of Immune Regulatory Molecules

Serum stored at −80 °C was thawed at 4–8 °C overnight. From a human ProcartaPlex immunoassay kit (Invitrogen, Thermo Fisher Scientific, MA, USA), we analyzed CD27, CD28, CD137 [4-1BB], BTLA, CTLA, CD80, IDO, LAG-3, PD-1, PD-L2, and TIM-3 immunoregulatory profiles. The experimental procedure, standard samples, magnetic beads, and patient samples were prepared according to the manufacturer's instructions. Upon the addition of diluted serum samples (1:2) and standards, an antibody specifically directed against each target is covalently coupled to color-coded polystyrene beads, which react with the serum samples containing unknown analyte concentrations or with standard solutions of known concentrations. After a washing step to remove unbound proteins, a biotinylated detection antibody specific to a different epitope on the analyte is added to form a sandwich of antibodies around the analyte. Thereafter, a biotinylated detection antibody is added, followed by another washing step. Streptavidin-R-Phycoerythrin is added to bind to the detection antibody. The concentration of each analyte in a single well was read on a Bio-Plex® 200 System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the concentration of the target analyte was calculated by Bio-Plex Manager™ 4.0 software using a standard curve derived from the supplied recombinant standard; the results are reported in pg/mL.

2.6. Statistical Analysis

Multivariate regression analysis was performed on the IC profiles. The results are reported as unstandardized beta coefficients (β) along with their 95% confidence intervals (CIs). In Table 2, for each IC marker, data in row "A" represent the unadjusted data, while data in row "B" represent data adjusted for age, gender, BMI, vitamin and nutrient supplement intake, antiretroviral treatment, and chronic illnesses. A p-value < 0.05 was considered statistically significant. In this analysis, the uninfected control group served as the baseline reference for comparison. Statistical analysis was conducted with Stata Statistical Software: Release 17 (StataCorp LLC, College Station, TX, USA, 2019).

Table 2. Participants' demographics and blood cell counts (n = 78).

Parameters	Uninfected Controls (n = 20)	HIV-Infected Only (n = 20)	Helminth-Infected Only (n = 20)	HIV + Helminth Co-Infection (n = 18)	p-Value
Age (years)	46.4 ± 17.3	42.9 ± 12.3	37.1 ± 16.4	39.4 ± 10.0	0.200
Gender, n (%) Males Females	 6 (30) 14 (70)	 9 (45) 11 (55)	 7 (35) 13 (65)	 6 (30) 14 (70)	0.725
BMI (kg/m^2)	28.0 ± 6.9	26.0 ± 7.0	29.6 ± 8.3	27.0 ± 5.4	0.418
CD4 count (u/L)	996 ± 375 *	629 ± 448 #	892 ± 197 *	579 ± 370 #	0.001
CD8 (u/L)	781 ± 341	910 ± 455	724 ± 299	742 ± 354	0.3801
CD4/CD8 ratio	1.57 ± 0.92 *	0.75 ± 0.44 $	1.43 ± 0.60 #	0.90 ± 0.62 #,$	0.0004
Viral load (copies/mL) n (%) <20 >20	 	 13 (65) 7 (35)	 	 11 (61) 7 (39)	1.000
White cell count (×10^9/L)	6.52 ± 1.96	5.65 ± 2.06	6.07 ± 1.83	5.16 ± 1.81	0.163
Neutrophils (×10^9/L)	3.61 ± 1.50	3.05 ± 1.41	3.19 ± 1.34	2.65 ± 1.04	0.180
Lymphocytes (×10^9/L)	2.27 ± 0.61	2.02 ± 1.00	2.21 ± 0.50	1.79 ± 0.66	0.164
Monocytes (×10^9/L)	0.42 ± 0.12	0.40 ± 0.12	0.47 ± 0.15	0.39 ± 0.11	0.206

The different superscript values for a given parameter between the different groups denote statistical significance.

3. Results

3.1. Patient Demographics

The demographics of study participants are shown in Table 2. Study participants (n = 78) were divided into four groups: the uninfected control (or no HIV or helminth infection) (n = 20), HIV infection only (n = 20), helminth infected only (n = 20), and HIV-helminth co-infections (n = 18).

The average age of participants across the groups was as follows: Controls had a mean age of 46.4 years (±17.3); HIV-infected only had a mean age of 42.9 years (±12.3); Helminth-infected only had a mean age of 37.1 years (±16.4); and those with HIV and helminth co-infection had a mean age of 39.4 years (±10.0). The differences in age between these groups were not statistically significant (p-value = 0.200). Most participants were female, and there was no significant difference in gender distribution between the groups. A slight variation in BMI was observed, with the helminth-infected only group having the highest mean BMI (29.6 ± 8.3 kg/m²) and the HIV and helminth co-infection group having the lowest (27.0 ± 5.4 kg/m²). Notably, the CD4 count was significantly lower in the HIV-infected only (629 ± 448 u/L) and HIV and helminth co-infection (579 ± 370 u/L) groups compared to the other groups, with a p-value of 0.001. Regarding the CD4/CD8 ratio, the HIV-infected (0.75 ± 0.44) and HIV-helminth co-infected (0.90 ± 0.62) groups had significantly lower ratios compared to the uninfected control group. Furthermore, the helminth-infected (1.43 ± 0.60) group had significantly higher ratio values compared to the HIV group (0.75 ± 0.44). The white cell and neutrophil counts were slightly lower in the HIV-infected only and HIV and helminth co-infection groups. This trend is consistent with the lower lymphocyte counts observed in the HIV and helminth co-infection group compared to other groups. Additionally, a slight increase in monocyte counts was observed in the helminth-infected only group compared to the other groups.

3.2. The Prevalence of Parasites

Figure 1 below indicates the prevalence of helminth infections in the study population.

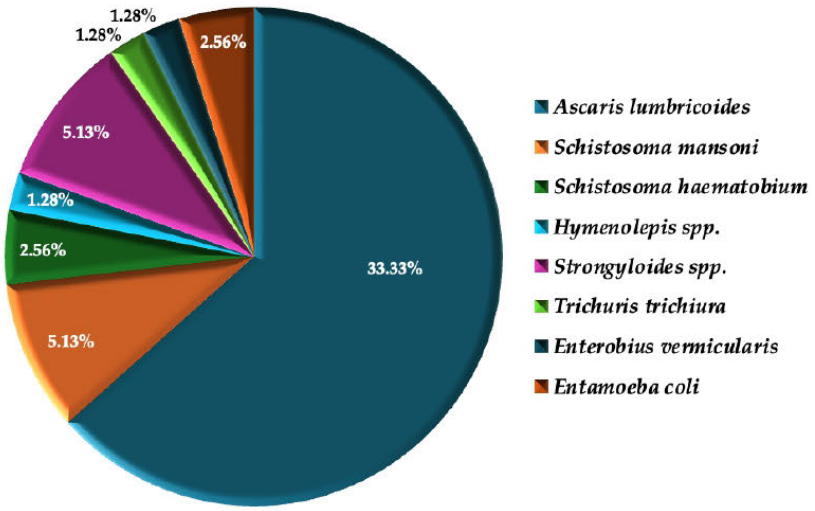


Figure 1. The prevalence of species-specific helminth infections [n (%)].

Ascaris lumbricoides was the most prevalent parasitic infection (n = 26, 33.33%). Other helminth species identified included *Strongyloides species* (n = 4, 5.13%), *Schistosoma mansoni* (n = 4, 5.13%), and *Schistosoma haematobium* (n = 2, 2.56%). Additionally, *Hymenolepis*

species, *Trichuris trichiura,* and *Enterobius vermicularis* were each detected (*n* = 1, 1.28% each). Notably, some individuals harbored multiple species, such as two participants co-infected with *Ascaris lumbricoides* and *Entamoeba coli.* However, infections with *Entamoeba coli* alone were excluded from the analysis. Percentages are based on the number of participants infected with each species, not the total number of infections, as some individuals harbored multiple species.

3.3. The Profile of Immune Checkpoints/Co-Inhibitory Molecules, Immune Co-Stimulatory Molecules, and Immunosuppressive Enzymes

The association of immune regulatory molecule profiles during HIV and helminth single infection and co-infection is presented in Table 3. Data were adjusted for age, gender, BMI, nutrient supplement intake, ARVs, and chronic illnesses.

Table 3. Multivariate association of immune regulatory molecule profiles during HIV and helminth single infection and co-infection.

		Unstandardized β–Coefficient Values (Reference Group: Uninfected Controls)					
Parameters		**HIV Infected**		**Helminth Infected**		**HIV and Helminth Co-Infected**	
		β (95% CI)	***p***	**β (95% CI)**	***p***	**β (95% CI)**	***p***
Immune Checkpoints/Co-Inhibitory Molecules							
Programmed cell death (PD)-1	A	0.05 (−0.04–0.14)	0.251	0.13 (−0.11–0.37)	0.266	0.18 (0.00–0.35)	0.046
	B	0.12 (0.01–0.23)	0.034	0.15 (−0.16–0.45)	0.335	0.19 (−0.13–0.51)	0.235
PD-ligand (L)2	A	0.57 (−12.41–13.54)	0.929	6.29 (−9.95–22.52)	0.436	7.95 (0.67–15.23)	0.033
	B	7.68 (−9.11–24.480	0.351	12.53 (−8.38–33.45)	0.228	6.96 (−3.73–17.66)	0.193
B and T lymphocyte attenuator (BTLA)	A	0.79 (−1.24–2.83)	0.431	2.26 (−1.70–6.22)	0.253	15.15 (−17.03–47.33)	0.342
	B	1.96 (−0.51–4.42)	0.114	3.66 (−1.07–8.39)	0.124	44.05 (−10.47–98.57)	0.107
Cytotoxic T-lymphocyte Antigen-4 (CTLA-4)	A	4,03 (−20.65–28.71)	0.743	4.59 (−16.74–25.93)	0.665	12.03 (−33.70–57.77)	0.597
	B	3.99 (−31.11–39.09)	0.813	5.05 (−15.93–26.04)	0.626	33.97 (−37.38–105.33)	0.339
T cell immunoglobulin and mucin domain containing molecule 3 (TIM-3)	A	13.58 (−5.73–32.90)	0.161	6.02 (−10.38–22.43)	0.459	12.58 (−8.68–33.84)	0.235
	B	23.15 (−0.20–46.50)	0.052	20.98 (3.52–38.45)	0.020	18.11 (−13.02–49.25)	0.238
Immunosuppressive Enzymes							
Indoleamine 2,3-dioxygenase (IDO)	A	−9.47 (−31.92–12.98)	0.394	−5.84 (−27.65–15.97)	0.589	−10.39 (−32.94–12.16)	0.352
	B	−9.44 (−39.65–20.77)	0.522	−2.40 (−26.65–21.84)	0.840	−6.64 (−47.18–33.89)	0.735
Immune Co-Stimulatory Molecules							
CD27	A	803.69 (−310.51–1917.89)	0.150	−23.60 (−544.54–497.34)	0.927	213.92 (−403.73–831.57)	0.483
	B	911.70 (−535.26–2358.66)	0.204	427.14 (−156.24–1010.53)	0.144	511.32 (−349.16–1371.80)	0.229
CD28	A	−594.33 (−946.57–−242.10)	0.002	−590.42 (−902.25–−278.59)	0.001	−594.01 (−958.93–−229.08)	0.003
	B	−651.95 (−1126.74–−177.15)	0.010	−674.32 (−1054.48–−294.16)	0.001	−671.55 (−1322.53–−20.58)	0.044
CD80	A	−0.93 (−2.44–0.59)	0.220	0.81 (−2.58–4.21)	0.629	2.32 (−2.84–7.48)	0.363
	B	−0.86 (−2.94–1.22)	0.398	1.06 (−3.15–5.28)	0.608	5.92 (−3.42–15.26)	0.200
CD137	A	0.27 (−0.14–0.689)	0.190	1.40 (−4.63–3.26)	0.136	1.18 (−0.14–2.50)	0.077
	B	0.51 (−0.43–1.06)	0.069	1.48 (−0.94–3.90)	0.219	2.31 (−0.129–4.74)	0.062

The uninfected control group (study participants with no HIV and no helminth infections) was the reference group for multivariate analysis. A: Unadjusted data. B: Data were adjusted for age, gender, BMI, vitamin and nutrient supplements intake, antiretroviral treatment, and chronic illnesses.

The study observed a significant increase in PD-1 levels in HIV-infected (adjusted β = 0.12, $p = 0.034$) and co-infected (unadjusted β = 0.18, $p = 0.046$) individuals. A significant increase in PD-L2 expression was observed between the uninfected control and co-infected groups (unadjusted β = 7.95, $p = 0.033$). A marginally significant increase in the expression of TIM-3 was noted in the HIV singly infected group (adjusted β = 23.15, $p = 0.052$) and a significant increase was detected in the helminth singly infected group (adjusted β = 20.98, $p = 0.020$). The expression levels of CD28 were decreased across all groups: HIV-infected (adjusted β = -651.95, $p = 0.010$) (unadjusted β = -594.33, $p = 0.002$); helminth-infected (adjusted β = -674.32, $p = 0.001$) (unadjusted β = -590.42, $p = 0.001$), and co-infected (adjusted β = -671.55, $p = 0.044$) (unadjusted β = -594.01, $p = 0.003$) groups.

4. Discussion

The present study aimed to explore the impact of HIV-helminth co-infection on immune checkpoint molecules. Data were adjusted for variables such as age, gender, BMI, ART status, supplement use, and other chronic diseases. The results show distinct alterations in immune checkpoint profiles among the groups. HIV-infected and co-infected individuals had significantly increased levels of PD-1. TIM-3 expression was marginally increased in HIV-infected individuals and significantly increased in the helminth-infected group. Notably, CD28 expression was significantly decreased across all infected groups. The observed downregulation of CD28 suggests a reduced T cell co-stimulatory capacity, which is associated with impaired immune responses in chronic infections. This reduction in CD28 expression could also be due to immunosenescence, further contributing to an immunosuppressive environment that facilitates cancer initiation. Taken together, these findings suggest that HIV-helminth co-infection modulates immune checkpoint markers, potentially fostering an immunosuppressive environment that may impair anti-cancer immunity and increase the risk of cancer.

4.1. CD28 Dysregulation and PD-1 Expression as Potential Pathways to Oncogenesis

In this study, the expression levels of CD28 were significantly downregulated across all groups: HIV-infected [A ($p = 0.002$); B (0.010)], helminth-infected [A ($p = 0.001$); B ($p = 0.001$)], and HIV-helminth co-infected [A ($p = 0.044$); B ($p = 0.003$)] in both adjusted and unadjusted data. A study showed that a decrease in CD28 + CD8 + cells in helminth-infected individuals normalized after anti-helminth treatment compared to the untreated infected individuals [36]. Ndlovu (2013) highlighted that CD28 is necessary for the induction of Th2 immunity against the helminth, *Nippostrongylus brasiliensis*, associated with impaired memory $CD4^+$ T cell responses [37]. CD28 is crucial not only for anti-tumor T cell proliferation but also for developing and maintaining Tregs [38]. Cellular senescence arises in cells with irreversible damage, halting their ability to divide and often triggering the persistent release of pro-inflammatory molecules, a phenomenon known as the senescence-associated secretory phenotype. In T cells, the downregulation of CD28 has been closely linked to the onset of cellular senescence, contributing to their diminished functionality and impaired immune responses [39,40]. CTLA-4 is an inhibitory molecule that competes with the co-stimulatory molecule CD28 for binding, thereby regulating T-cell responses against pathogens and cancer [41]. Blocking CTLA-4 in helminths (*Nippostrongylus brasiliensis*) improved CD28 T cell activation, resulting in clearance of the parasite and normalization of immune responses [42]. In this study, CTLA-4 levels were increased in the HIV-infected, helminth-infected, and HIV-helminth co-infected groups compared to the uninfected control group; however, the differences were not statistically significant.

Studies have shown that PD-1 uses CD28 post-TCR activation to block immune responses, although PD-1 does not entirely depend on CD28 for immunosuppression [43]. In

the current study, a significant increase in PD-1 expression (a cancer-favoring phenomenon) was observed in HIV individuals ($p = 0.034$) compared to controls. Blocking the PD-1/PD-L1 pathway has been shown to restore the activity of exhausted virus-specific CD8 + T cells, resulting in regulated chronic HIV infection [44] and enhanced anti-cancer immunity [45]. The increase in PD-1 levels observed in the current study correlates with a decrease in CD28 in the HIV singly infected group, which potentially contributes to immunosuppression and susceptibility to cancer initiation. Furthermore, Vivar and colleagues reported that T cells lacking CD28 expression in HIV patients were found to be prone to apoptosis and exhibited a low proliferation rate compared to HIV patients on ARVs who had a controlled chronic viral infection [46]. This is exacerbated by HIV's ability to reduce or impair CD28 + T cells [47]. However, while polymorphisms in the CD28 co-signaling molecule have been shown to contribute to cancer initiation and progression, significant work remains to fully develop its potential as both a screening/diagnostic tool and a therapeutic marker [48].

4.2. PD-L2 Expression in a HIV-Helminth Co-Infected Group and Its Potential Role in Oncogenesis

The current study found a significant increase in PD-1 ligand (PD-L2) expression in the helminth-HIV infection ($p = 0.033$) group. Imai et al. (2023) found the expression of PD-L2 to be higher in metastatic cancer cells (47%) compared to PD-L1 (11%), with no PD-L1 expression observed in tumor-infiltrating lymphocytes (TILs) (0%), whereas 64% of TILs expressed PD-L2 [49]. Yuki et al. reported different findings, showing PD-L2 expression in cancer tumors as 0% and in associated lymphocytes as 20.3%, in contrast to PD-L1, which was expressed at 0.8% in tumors and 46.9% in lymphocytes [50]. Despite conflicting findings, these studies emphasize the importance of this underexplored immune checkpoint in cancer and call for further research to clarify its role in oncogenesis. Similarly, while helminth infections can induce PD-L2 expression in macrophages [51], additional research is required to fully understand the role of the PD1-PD-L1/2 axis in oncogenesis before considering it as a potential prognostic biomarker.

4.3. TIM-3 Increase and Its Implications for Cancer Onset in HIV- and Helminth-Infected Groups

A significant increase in TIM-3 levels, a molecule known for its role in cancer initiation, was observed in both the HIV-infected (marginally significant $p = 0.052$) and helminth-infected ($p = 0.020$) groups compared to the controls. Prévost et al. report that the HIV-1 protein Vpu decreases the surface expression of TIM-3 on infected CD4 + T cells, indicating that TIM-3 may contribute to suppressing viral replication. By downregulating TIM-3, Vpu counteracts this inhibitory effect, thereby facilitating increased viral replication and spread [52]. However, changes in TIM-3 levels during parasitic infections appear to be cell-specific. Hou et al. reported that *Plasmodium falciparum* downregulated TIM-3 expression in the circulating monocytes of infected patients, with an even more pronounced effect in splenic macrophages in mice studies. Blocking TIM-3 enhanced monocyte/macrophage activity, aiding in parasite clearance [53]. Similarly, Hou et al. observed differential TIM-3 expression in immune cells across various organs in mice infected with *Schistosoma japonicum*. The study found a higher infiltration of immune cells, CD4/8 T cells, natural killer (NK) cells, and monocytes in the liver with lower TIM-3 levels in CD8 T cells and monocytes in the spleen. Helminth infection upregulated TIM-3, leading to poor NK cell functionality. Notably, blocking TIM-3 improved the NK cell function and inhibited cancer-favoring M2 macrophages, also known as tumor-associated macrophages (TAMs) [54]. Additionally, increased TIM-3 expression in the tumor microenvironment is associated with a higher metastatic stage ($p < 0.05$) and decreased overall survival [55]. Thus, taken together, TIM-3 might potentially serve as a screening marker for cancer risk in HIV-helminth co-infected patients.

4.4. Implications for Colorectal Cancer (CRC) Risk

The gastrointestinal mucosa plays a very central role in HIV and parasitic infections because (i) early HIV initial replication and immune response establishment start in the gut-associated lymphoid tissues (GALTs) [56], and (ii) intestinal parasites reside in the same environment, establishing chronic low-grade inflammation while modulating the immune response. Coincidentally, this is also the same anatomical site for colorectal cancer (CRC) origin. It is therefore biologically plausible that these immunological interactions (Figure 2) accentuate the risk of cancer development in the colon relative to other cancers.

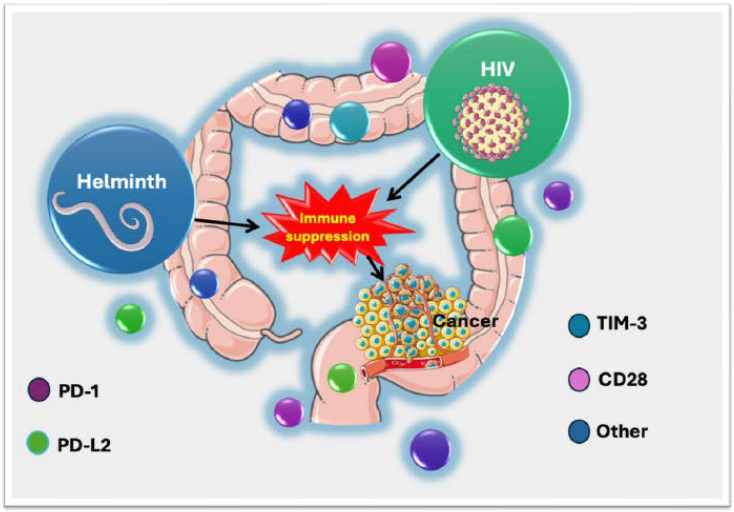


Figure 2. Implications of HIV and helminth co-infection in altering immune checkpoint profiles and subsequent CRC risk. The gastrointestinal mucosa is the primary site for HIV and parasitic infections because (i) HIV infection and immune response establishment begin in the gut-associated lymphoid tissues (GALTs), and (ii) intestinal parasites reside in the same environment, which is also the site of CRC origin. HIV-helminth infections promote the immuno-suppressive microenvironment via the secretion of molecules that promote anti-tumor immune responses including immune checkpoints PD-1/PD-L2 and TIM-3. Downregulation of co-stimulatory molecules such as CD28 is an attempt to prevent T cell activation and evade immunological attack. Other than chronic inflammation induced by helminths, other molecules/cells, generally known to be favored by both helminths and cancer cells increase the chances of individuals with HIV-co-infections developing CRC.

Helminth infections are known for promoting intestinal Th2-mediated inflammation through suppression of Th1 responses, causing cellular damage and an increased risk of co-infections [57]. A previously reported study in the same cohort found HIV-helminth co-infection is associated with elevated pro-inflammatory immune responses, which indicates that helminths have a deleterious effect on HIV immune responses [28]. Both HIV [58] and parasite [27] single infections are associated with dysregulated immune checkpoint profiles, with no reported studies on co-infected individuals. These alterations suggest a dysregulated immune response that could contribute to chronic inflammation, a known factor in the initiation of CRC. Helminth immune dysregulation is implicated in CRC initiation [59]. Intestinal helminth infections have been reported to drive carcinogenesis in colitis-associated colon cancer [59]. Helminth also promotes microbial dysbiosis [60], which is associated with oncogenesis and CRC risk [61].

As mentioned, in the present study, TIM-3 expression was elevated in the HIV and helminth singly infected groups, PD-1 and PD-L2 expression was elevated in the co-infected

group, and CD28 was lower in all study groups. Elevated TIM-3 and PD-1 expression in CRC tumors was associated with a higher metastatic stage and a decline in overall survival rate [62]. Elevated PD-1 [44] and decreased CD28 levels in [62] HIV-infected individuals contribute to immunosuppression and susceptibility to CRC initiation. Helminth infections play a role in inducing PD-L2 expression in macrophages [51]. Significantly higher PD-L2, and not PD-L1, expression was reported in metastatic CRC cells [63]. Therefore, populations affected by both HIV and helminth infections may face a heightened risk of CRC compared to those without such co-infections.

5. Challenges and Limitations

The sample size for this study may limit the statistical power to detect subtle differences between the groups, and a larger size might increase the robustness of the results and improve generalizability. The cross-sectional design further restricts the ability to infer causality between HIV-helminth co-infection and the immune profiles assessed. Longitudinal studies would be needed to track changes over time and better understand the temporal dynamics and causality of these immune alterations. Another limitation of the study is the unknown duration of both HIV and helminth infections since the acute and chronic phases of both infections are accompanied by distinct immunological changes as the diseases progress from acute to chronic [21]. Likewise, since the implementation of the "test and treat" HIV regulation in South Africa in 2016, the duration of HIV treatment is also unknown [64,65]. Finally, while the study focused on co-stimulatory and co-inhibitory molecules, other immune markers known to play a role in modulating antitumor immunity should be explored in future research.

6. Conclusions

This study's findings suggest that HIV and helminth co-infections impact immune checkpoint signaling, particularly through the reduction of CD 28 expression, potentially increasing cancer risk due to immune response inefficiency. The study highlights that immune homeostasis, which is important for eliminating cancer cells and pathogens, is disrupted under these co-infection conditions. The changes in immune checkpoint markers observed in HIV-helminth co-infected individuals may elevate the risk of developing cancer. Several negative immunoregulators, such as CTLA4, PD1, and its ligands, modulate the immune response to maintain homeostasis and prevent hyperactivation or autoimmunity. Blocking these T cell immune checkpoint molecules has revolutionized immunotherapy, significantly advancing treatment for several cancers [66]. Co-infections of HIV and helminths have been shown to alter immune checkpoint signaling pathways by regulating markers such as PD-1, PD-L1/2, [67] and TIM-3 [68]. These alterations suggest a dysregulated immune response that could contribute to chronic inflammation, a known factor in the initiation of cancer. Chronic inflammation, associated with immune dysregulation, is implicated as a potential mechanism linking these infections to cancer initiation [69]. Therefore, populations affected by both HIV and helminth infections may face a heightened risk of cancer compared to those without such co-infections. Addressing HIV-helminth co-infections through comprehensive treatment and prevention strategies is crucial. Effective management of these infections not only improves HIV outcomes but also potentially mitigates the associated risk of developing cancer [70,71]. Integrating parasitic infection control measures into HIV care programs is essential, especially in regions where both diseases are prevalent [72]. This study underscores the broader public health implications of HIV and helminth co-infections, particularly in regions with high prevalence rates. By addressing these co-infections, healthcare systems can enhance overall health outcomes and potentially reduce the burden of cancer within these populations. Lastly, this study advocates for inte-

grated approaches in healthcare policies and practices to effectively manage co-infections and improve long-term health outcomes in affected communities.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data generated from this study can be accessed from the corresponding author upon reasonable request.

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CHAPTER FOUR

Screening of CRC-related gut microvirome that can be used as a potential prognostic marker for CRC risk in the HIV-helminth co-infected group

The immunoregulatory alterations identified in Chapter 3 suggest that HIV-helminth co-infection may foster a gut environment permissive to tumour initiation and progression. However, immune modulation does not occur in isolation; it interacts closely with the gut microbiome and, in particular, the gut virome. Viruses within the gut microbiota, including bacteriophages and eukaryotic viruses, can influence microbial community structure, host immune tone, and even modulate metabolic pathways linked to colorectal oncogenesis. In HIV infection, gut dysbiosis and virome expansion are well-documented, while helminth infections have been shown to shape viral communities through immune tolerance mechanisms. The combined impact of these two infections on the gut virome, and its potential role in CRC risk remains poorly understood. The aim of this chapter was therefore to determine the profile of the gut DNA virome in individuals with HIV and helminth infections, with stool samples from CRC-confirmed patients serving as positive controls. By comparing virome compositions across infection groups, this study sought to identify viral taxa or community patterns that could serve as prognostic markers of CRC risk, particularly within the HIV-helminth co-infected subgroup. The findings from this work represent an important step toward linking host immune modulation, viral ecology, and CRC risk in the South African context.

Introduction

The intricate interplay between viral infections and immune dysregulation has long been recognized as a pivotal driver of oncogenesis. The present study sought to reaffirm this link; it builds on a substantial body of evidence highlighting how viruses shape the tumor microenvironment and contribute to malignant transformation. Notably, HIV's disruption of immune homeostasis creates a pro-oncogenic milieu, increasing susceptibility to cancer development [1]. Beyond HIV, oncogenic viruses such as Epstein-Barr virus, human papillomavirus, and John Cunningham virus have been implicated in CRC, acting as both initiators and enablers of tumorigenesis [2, 3]. Compounding this is the emerging recognition of the gut virome as a dynamic ecosystem whose composition shifts significantly in cancer. Recent studies have demonstrated a loss of key viral and bacterial species following CRC surgery, reinforcing the idea that tumor presence is tightly coupled with microbial signatures [4]. Preclinical virome mapping from marine fecal samples has uncovered novel CRC-associated

viruses such as *Brunovirus* and *Hpunavirus*, suggesting that certain bacteriophages may act as yet-unexplored modulators of carcinogenesis [5]. Comparative analyses have revealed profound differences in the gut virome profiles of CRC patients versus healthy individuals, particularly the enrichment of *Microviridae*, *Podoviridae*, *crAss-like*, *Quimbyviridae*, *Siphoviridae*, and *Myoviridae* families, hallmarks of a dysbiotic viral environment linked to disease progression [6, 7]. In this chapter, we interrogated this viral landscape within the context of HIV-helminth co-infection, an underexplored intersection in virome research. Using shotgun metagenomics, we profiled stool-derived viral communities and compared them to those in CRC patients. The resulting patterns not only mirrored known CRC-associated virome alterations but also revealed novel signatures unique to HIV-helminth co-infected individuals.

These findings are published in a conference paper in the American Association of Cancer Research (AACR) journal, Cancer Epidemiology, Biomarkers & Prevention: Botle Precious. Damane, Thanyani Victor. Mulaudzi, Jonathan Featherston, Sayed Shakeel. Kader, Pragalathan Naidoo, Zodwa Dlamini, Zilungile Lynette. Mkhize-Kwitshana; Abstract C035: Gut virome remodeling in HIV-helminth co-infection reflects colorectal cancer-associated signatures in a South African cohort. Cancer Epidemiol Biomarkers Prev 1 September 2025; 34 (9_Supplement): C035. https://doi.org/10.1158/1538-7755.DISP25-C035

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Abstract C035: Gut virome remodeling in HIV–helminth co-infection reflects colorectal cancer–associated signatures in a South African cohort FREE

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Cancer Epidemiol Biomarkers Prev (2025) 34 (9_Supplement): C035.

https://doi.org/10.1158/1538-7755.DISP25-C035

Split-Screen Share Tools Versions

Abstract

Background:

Colorectal cancer (CRC) incidence is rising in sub-Saharan Africa, where immune-modulating infections such as HIV and helminths remain prevalent. These chronic infections may disrupt gut microbial ecosystems, including the virome, thereby contributing to cancer risk. However, virome alterations linked to HIV-helminth co-infection and their association with CRC-associated signatures remain poorly characterized in African populations.

Methods:

We conducted untargeted shotgun metagenomic sequencing of fecal DNA from 62 South African adults across five clinical groups: uninfected controls (n=10), HIV-only (n=14), helminth-only (n=15), HIV-helminth co-infected (n=13), and CRC-confirmed patients (n=10). Sequencing reads were quality filtered, taxonomically profiled, and functionally annotated using Prodigal and DIAMOND. Diversity indices and differential abundance were computed via SQMtools, DESeq2, and vegan in R.

Results:

Shannon diversity was significantly higher in CRC samples compared to uninfected and helminth-only groups ($p<0.05$). Beta diversity analysis showed strong clustering between CRC and HIV-helminth groups ($p<2.2\times10^{-16}$), indicating convergent gut virome restructuring. Among 26 shared viral species, nine were unique to HIV-helminth co-infection and eight to HIV-only. Notably, the HIV-helminth group displayed increased abundance of *Myoviridae* and *Podoviridae*,

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including cyanophages previously implicated in gut inflammation. This taxonomic shift is consistent with studies that showed significant virome alterations in CRC patients, including changes in *Siphoviridae* and *Myoviridae*. Functionally, adenine-specific DNA methylase (COG2189), involved in DNA replication and methylation, was significantly enriched in CRC (p=2.98e-06), helminth-only (p=1.30e-07), and HIV-helminth groups (p=6.11e-06), suggesting a shared microbial epigenetic signature potentially linked to CRC oncogenic processes.

Conclusion:

HIV-helminth co-infection is associated with a distinct virome that mirrors CRC in both diversity and functional gene enrichment. This overlap reinforces the notion that chronic infections shape the virome in ways that may promote tumorigenesis. Functional markers such as adenine-specific DNA methylase may serve as early indicators of CRC risk in co-infections. This study emphasizes the need to integrate virome ecology into cancer surveillance strategies in high-burden, underrepresented regions.

Citation Format:

Botle Precious. Damane, Thanyani Victor. Mulaudzi, Jonathan Featherston, Sayed Shakeel . Kader, Pragalathan Naidoo, Zodwa Dlamini, Zilungile Lynette. Mkhize-Kwitshana. Gut virome remodeling in HIV–helminth co-infection reflects colorectal cancer–associated signatures in a South African cohort [abstract]. In: Proceedings of the 18th AACR Conference on the Science of Cancer Health Disparities; 2025 Sep 18-21; Baltimore, MD. Philadelphia (PA): AACR; Cancer Epidemiol Biomarkers Prev 2025;34(9 Suppl):Abstract nr C035.

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The work produced another published conference paper in the American Association of Cancer Research (AACR) journal, Cancer Research: Botle Precious Damane, Thanyani V. Mulaudzi, Jonathan Featherston, Sayed Shakeel Kader, Pragalathan Naidoo, Zodwa Dlamini, Zilungile Lynette Mkhize-Kwitshana; Abstract 1414: Shared gut virome profiles highlight potential early colorectal cancer markers in HIV-helminth co-infection. Cancer Res 1 April 2026; 86 (7_Supplement): 1414. https://doi.org/10.1158/1538-7445.AM2026-1414

ARTICLE NAVIGATION

POSTER PRESENTATIONS - PROFFERED ABSTRACTS | APRIL 03 2026

Abstract 1414: Shared gut virome profiles highlight potential early colorectal cancer markers in HIV-helminth co-infection.

Botle Precious Damane; Thanyani V. Mulaudzi; Jonathan Featherston; Sayed Shakeel Kader; Pragalathan Naidoo; Zodwa Dlamini; Zilungile Lynette Mkhize-Kwitshana

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Cancer Res (2026) 86 (7_Supplement): 1414.

https://doi.org/10.1158/1538-7445.AM2026-1414

Split-Screen Share Tools Versions

Abstract

Background:

In regions with high HIV and helminth prevalence, co-infection may reshape the gut virome in ways that promote oncogenic processes. Characterizing these alterations may identify early colorectal cancer (CRC) biomarkers in high-risk populations.

Methods:

Stool-derived metagenomic data from CRC patients, HIV-infected, helminth-infected, HIV-helminth co-infected individuals, and uninfected controls were analyzed. Viral taxa were quantified at family and genus levels using a metagenomic coassembly pipeline. Hierarchical clustering and heatmap visualisation compared abundance patterns across groups.

Results:

CRC samples formed distinct clusters with altered abundance of Siphoviridae (−1.6 ± 0.41; q = 0.0013), Podoviridae (2.3 ± 0.83; q = 0.031), unclassified Caudovirales (−1.6 ± 0.51; q = 0.010), Virus sp. ctWxR2 (−14.7 ± 5.04; q = 0.019), and CrAss-like virus sp. ctt4r3 (22.9 ± 5.00; q = 9.9×10^{-5}). HIV-helminth co-infected samples formed a subcluster overlapping CRC, showing similar enrichments in Siphoviridae (−2.0 ± 0.39; q = 5.3×10^{-6}), unclassified Caudovirales (−2.6 ± 0.48; q = 4.7×10^{-6}), Virus sp. ctWxR2 (−21.1 ± 4.79; q = 0.00011), and CrAss-like virus sp. ctt4r3 (24.8 ± 4.78; q = 2.2×10^{-7}). HIV-only samples resembled controls, while helminth-only samples displayed intermediate profiles.

Conclusion:

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HIV-helminth co-infected individuals showed closely aligned profiles with CRC, indicating that chronic immune perturbation may create virome states resembling early CRC-associated dysbiosis. These shared viral signatures could serve as potential non-invasive biomarkers for CRC risk stratification in high-burden settings. Longitudinal analyses are needed to clarify temporal dynamics and identify the bacterial hosts and functional pathways through which viral/phage restructuring may promote CRC development.

Citation Format:

Botle Precious Damane, Thanyani V. Mulaudzi, Jonathan Featherston, Sayed Shakeel Kader, Pragalathan Naidoo, Zodwa Dlamini, Zilungile Lynette Mkhize-Kwitshana. Shared gut virome profiles highlight potential early colorectal cancer markers in HIV-helminth co-infection [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2026; Part 1 (Regular Abstracts); 2026 Apr 17-22; San Diego, CA. Philadelphia (PA): AACR; Cancer Res 2026;86(7 Suppl):Abstract nr 1414.

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The submitted under review manuscript is titled: Gut Virome Alterations in HIV-Helminth Co-infection: Potential Links to Colorectal Cancer in South Africa. Damane BP, Mulaudzi TV, Featherston J, Kader SS, Naidoo P, Dlamini Z, Mkhize-Kwitshana ZL. Gut virome alterations in HIV-helminth co-infection: Potential links to colorectal cancer in South Africa. The paper was withdrawn from the journal. As with most free APC Springer journals, the paper was taking too long for consideration. It went through a couple rounds of reviews and has now been submitted to a more relevant journal, Archives of Microbiology, Ref: Submission ID 150242d2-5e7d-4ff6-894e-cb7b6ccae8ea.

By providing novel, functional-level insights into how chronic infections may influence virome-host dynamics to promote oncogenesis, this chapter contributes to a deeper understanding of the molecular and ecological underpinnings of CRC development in high-burden settings.

Gut Virome Alterations in HIV-Helminth Co-infection: Potential Links to Colorectal Cancer in South Africa

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Abstract

Background: The human virome is increasingly implicated in disease modulation, including cancer. In immunocompromised conditions such as HIV and helminth infections, virome alterations may contribute to immune dysregulation and cancer risk. However, the role of the gut DNA virome in HIV-helminth co-infection and its intersection with colorectal cancer (CRC) remains poorly characterized, particularly in African populations.

Methods: Shotgun metagenomic sequencing was performed on stool samples from 62 South African adults stratified into five groups: uninfected controls, HIV-only, helminth-only, HIV-helminth co-infected, and CRC-confirmed patients. Viral taxonomic and functional annotations were assessed to evaluate diversity and viral gene expression. Alpha and beta diversity were calculated using Shannon and Wilcoxon metrics; functional comparisons used KEGG orthologs.

Results: CRC samples showed significantly higher Shannon diversity than uninfected and helminth-only groups ($p < 0.05$). Beta diversity showed notable clustering between CRC and HIV-helminth co-infected groups (Wilcoxon $p < 2.2 \times 10^{-16}$). A significant enrichment in the MnmE helical domain (PF12631) and KEGG ortholog, tRNA modification GTPase (K03650) [MnmE/TrmE], was observed in both HIV-helminth and CRC groups. These enzymes mediate GTP-dependent wobble base modifications essential for translation under stress. The HIV-helminth group also showed enrichment of *Myoviridae* and *Podoviridae*, including cyanophages previously linked to gut inflammation.

Conclusion: These findings indicate shared functional virome signatures between CRC and HIV-helminth co-infection, highlighting alterations in viral functions that may be linked to CRC risk, particularly in populations burdened by endemic infections.

Keywords: Gut virome, Colorectal cancer, HIV-helminth co-infection, Metagenomic sequencing, Virome dysbiosis, Immune modulation, Viral functional signatures, Cancer pathogenesis

Introduction

Viruses are ubiquitous biological entities that infect virtually all forms of life, including bacteria, archaea, plants, arthropods, mammals, and even other viruses. The majority are bacteriophages, which significantly influence ecosystems by regulating bacterial abundance, diversity, and metabolism [1, 2]. Alterations in viral community structure within the human body have been linked to various diseases, including cancer [3]. Viruses are implicated in approximately 10-20% of human cancers globally, underscoring their central role in oncogenesis [4]. Oncogenic viruses contribute to tumorigenesis by integrating their genomes into host cells, altering cell cycle control, inhibiting apoptosis, and inducing chronic inflammation or immune suppression [5, 6]. In the gut, such viral interactions may disrupt host-microbe homeostasis and influence key features of cancer biology, including genomic instability and evasion of immunosurveillance. For example, pro-apoptotic B-cell lymphoma 2 (BCL-2) family proteins, BCL-2-associated X (BAX) and BCL-2 antagonist/killer (BAK), trigger host antiviral responses by promoting cell death to limit viral replication. However, certain viruses express viral BCL-2 homologs (v-BCL-2s) that inhibit apoptosis, enabling viral persistence and potentially supporting tumor progression, especially when tumor suppressors like retinoblastoma protein (RB) and tumor protein p53 (TP53) are inactivated [7].
HIV infection further complicates this landscape. Beyond its well-established immunosuppressive effects, HIV disrupts gut homeostasis by damaging mucosal immunity and compromising the epithelial barrier, facilitating microbial translocation, dysbiosis, and systemic inflammation, all of which contribute to increased cancer susceptibility, including colorectal cancer (CRC) [8, 9]. Importantly, co-infection with helminths, a common occurrence in sub-Saharan Africa, adds another layer of immune modulation, further weakening anti-tumor immunity through chronic immune suppression and skewed cytokine profiles [10, 11]. These compounded disruptions create a microenvironment conducive to malignant transformation [12].

Although the role of the human virome in cancer development is increasingly recognized, its contribution in immunocompromised settings such as HIV and helminth co-infection remains poorly understood, particularly in African populations. Given this gap, our study focused specifically on the gut DNA virome using shotgun metagenomic sequencing, rather than metatranscriptomics. Shotgun metagenomic sequencing enables comprehensive analysis of all genetic material within a microbial community, offering insights into both its taxonomic composition and functional capacity. Unlike targeted sequencing approaches, it bypasses the need for specific gene markers by fragmenting and sequencing the entire DNA content. The resulting protein-coding sequences are then aligned with reference databases to predict the biological functions present. This technique is also capable of identifying viruses and viroids, making it particularly useful for broad-spectrum microbial profiling [13].

Shotgun metagenomic sequencing allowed us to comprehensively characterize virome composition and potential functional capacity in 62 South African adults, stratified into five clinically relevant groups: HIV and helminth uninfected controls, HIV-infected only, helminth-infected only, HIV-helminth co-infected, and CRC-confirmed patients. By comparing viral diversity, taxonomy, and gene function across these groups, this study aimed to explore how chronic infection-associated virome restructuring in HIV-helminth co-infected individuals overlaps with virome profiles observed in CRC, providing insight into infection-linked viral community shifts that may contribute to tumour-promoting conditions. Our findings shed light on the shared and distinct virome signatures in HIV-helminth co-infection and CRC, providing a foundation for understanding virus-parasite-host interactions in cancer pathogenesis.

2. Materials and Methods

2.1. Study Design and Setting

This retrospective cross-sectional sub-study was nested within a larger ethically approved project (BE351/19). The Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC/00005458/2023) granted additional approval for this sub-study. The main study was conducted in a peri-urban area south of Durban, KwaZulu-Natal, South Africa, between March 2020 and May 2021.

2.2. Study Population and Sampling

This sub-study utilized stored stool samples collected from consenting participants enrolled in the main study. Study participants (n = 62) were purposively selected based on their HIV and helminth infection status. Thereafter, participants were stratified into four groups: HIV and helminth uninfected controls (n = 10), HIV-infected only (n = 14), helminth-infected only (n = 15), and HIV-helminth co-infected (n = 13) groups. Stool samples were also collected from consenting CRC confirmed patients (n = 10) recruited at Inkosi Albert Luthuli Central Hospital (IALCH) and Dr Pixley Ka Isaka Seme Memorial Hospital situated in Durban, KwaZulu-Natal, and were used as positive CRC controls. CRC confirmed samples were obtained preoperatively, 24 hrs before any bowel preparation or antibiotic administration. Samples from HIV-infected, helminth-infected, and HIV-helminth co-infected participants were collected non-invasively. The interval between collection and analysis ranged from approximately 2-3 years. All samples were immediately aliquoted and stored at -80 °C until DNA extraction to preserve nucleic acid integrity. A comprehensive description of the study design, setting, and participant demographics, including inclusion and exclusion criteria, has been previously published [14]. Detailed methodologies for stool sample collection, as well as procedures for HIV and helminth parasite detection, have also been previously described [15].

2.2.1. Helminth Detection

Collected stool samples were processed using the Kato-Katz technique for the detection of intestinal helminths. To increase diagnostic sensitivity, a modified fecal parasite concentrator technique was applied using the mini Parasep® SF stool concentrator kit to concentrate parasite ova and cysts. In addition, serum samples were analyzed for Ascaris lumbricoides-specific IgE (>0.35 kU/L) and IgG4 (>0.15 kU/L) antibodies using the Phadia™ 200 instrument (Phadia AB, Thermo Fisher Scientific, Uppsala, Sweden). Multiple species infections were observed in some individuals.

2.3. Experimental procedures

2.3.1. DNA Extraction from Stool Samples

Genomic DNA was extracted from approximately 200 mg of stool using the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Irvine, CA, USA; Cat. No. D4300), designed specifically for high-quality microbiome and metagenome applications. Briefly, stool samples were added to the ZR BashingBead™ lysis tubes containing 750 µl of lysis solution and mechanically disrupted using the Omni Bead Ruptor 4 Bead Mill Homogenizer (Lasec, Johannesburg, South Africa) on 6 meters per second (m/s) for 90 seconds, followed by a 5 minute rest on ice and the circle repeated thrice. Following centrifugation at 10,000 × g for 1 minute, the supernatant was processed through Zymo-Spin™ filters bound to silica columns using the DNA binding buffer. The bound DNA was washed with the kit's proprietary buffers and eluted in 100 µl DNase/RNase-free water. To eliminate PCR inhibitors, the eluted DNA was passed through a Zymo-Spin™ Humic Removal Column (HRC) column pre-treated with 600 µl HRC prep solution and centrifuged at 16,000 × g for 3 minutes, yielding high-purity DNA suitable for downstream analyses.

2.3.2. Library Preparation and Sequencing

Following DNA extraction, DNA concentrations were quantified using the Qubit™ 4 Fluorometer with the Qubit™ dsDNA High Sensitivity (HS) Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA; Cat. No. Q32851), to ensure accurate input levels, especially for low-concentration DNA samples. DNA was then diluted to match the input concentration requirements of the Illumina® DNA Prep Kit (Illumina Inc., San Diego, CA, USA; Cat. No. 20060060), which was used to construct sequencing libraries according to the manufacturer's protocol. This kit utilizes bead-linked transposome technology, which enables simultaneous fragmentation and adapter tagging of the genomic DNA. Following this, limited-cycle PCR was performed to amplify the library and add sample-specific dual indexes. Magnetic bead-based purification steps were included to remove excess reagents and size-select the DNA fragments. Automated library preparation was then conducted using the NGS Star Plus Workstation (Hamilton Company, Bonaduz, Switzerland). Final libraries were sequenced using the NextSeq 2000 sequencing platform (Illumina Inc., San Diego, CA, USA) on a P2 flow cell, generating 2 × 150 bp paired-end reads. Each sample produced approximately 20 million read pairs, allowing sufficient coverage for comprehensive metagenomic analysis.

2.3.3. Bioinformatics analysis

Poor read quality ($q < 20$) and adapter filtering was performed using TrimGalore v0.6.2 (https://github.com/FelixKrueger/TrimGalore), and after trimming, reads shorter than 50bp were discarded. The metagenome analysis pipeline, SqueezeMeta v1.6.2 [16], was used to perform 21 steps from assembly to taxonomic and functional annotation. The pipeline was run in "coassembly" mode with concatenated reads assembled using MEGAHIT v1.2.9 [17]. Open-reading frames (ORFs) in the co-assembly were predicted by Prodigal v2.6.3 [18], and DIAMOND [19] was subsequently used to query protein sequences against different databases. Assignment of gene function was derived from queries to the Pfam-A [20], eggNOG [21], and KEGG databases [22]. The SqueezeMeta pipeline consolidated outputs from various searches such as DIAMOND queries

against the NCBI's non-redundant (nr) protein database [23], RDP-classifier [24] of 16S sequences against the SILVA 132 database [25], and scans of hidden Markov models for archaea and bacteria to provide taxonomic assignment at the contig-level.

The outputs from SqueezeMeta were summarised into an SQMtools v1.6.3 [26] object and analysed in R v4.4.2. The SQMtools object was filtered at the super-kingdom level for viruses. To reduce the impact of low-abundance and potentially spurious detections, viral taxa were further filtered based on abundance and prevalence criteria. A taxon was retained for downstream analyses only if it reached a minimum threshold of ≥10 mapped reads (or ≥0.01% relative abundance) in a given sample and was detected in at least 20% of samples within at least one study group. Taxa not meeting these criteria were considered absent and excluded from comparative analyses.

Thereafter, DESeq2 [27] was used to compare annotations (KEGG, Pfam) and taxonomic abundance at the phylum, family, genus, and species levels between all groups. Read abundances at the genus level were used as input to vegan v2.6-4 [28], which was used to calculate alpha (Shannon entropy, observed richness, Chao1, and evenness) and Bray-Curtis beta-diversity. For both alpha and beta diversity, the Kruskal-Wallis test [29] was used for statistical comparisons across groups, while the Wilcoxon Signed-Rank test [30] with Benjamini-Hochberg correction [31] was used for pairwise statistical tests. Age and BMI were expressed as mean ± SD, with P-values derived from one-way ANOVA. Gender, chronic illness, and parasite prevalence were expressed as n (%), and P-values were obtained using the chi-squared test.

3. Results

3.1. Patient Characteristics

The study participants (n = 62) were divided into five groups: the HIV and helminth uninfected control (n = 10), HIV infection only (n = 14), helminth infection only (n = 15), HIV-helminth co-infection (n = 13), and CRC confirmed patients (n = 10). The average age of participants across the groups was as follows: Controls had a mean age of 40.9 ± 19 years; HIV-infected only had a mean age of 44.4 ± 13.8 years; helminth-infected only had a mean age of 36.5 ± 15.3 years; and those with HIV and helminth co-infection had a mean age of 40 ± 8 years. The differences in age between these groups were not statistically significant. Most participants were female, and there was no significant difference in gender distribution between the groups (Table 1). All HIV individuals were on ARTs and presenting with viral loads <20,000 copies/mL.

Table 1. Baseline Demographic and Clinical Characteristics

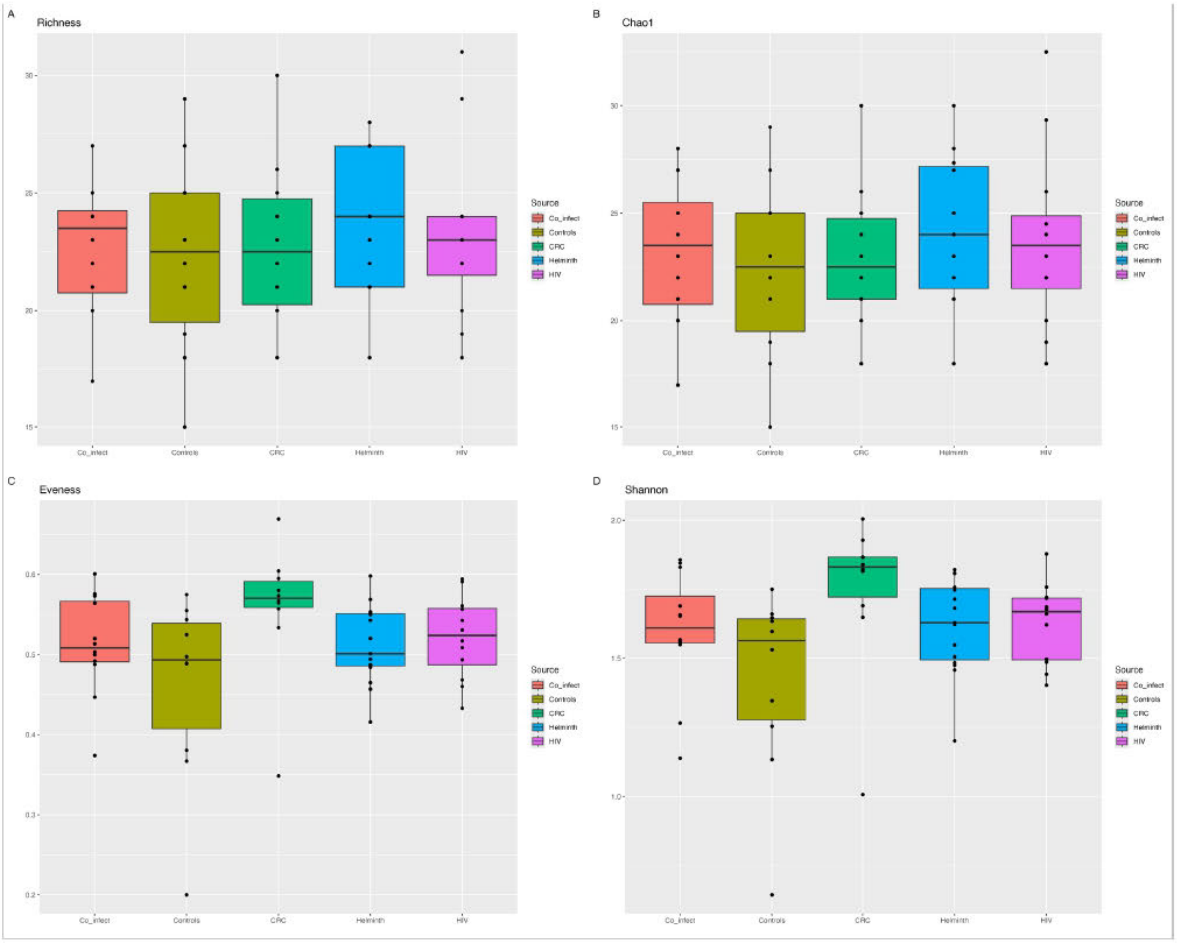
Parameter	Control	HIV	Helminth	Coinfection	p-Value
Age (years)	40.9 ± 19	44.4 ± 13.8	36.5 ± 15.3	40 ± 8	0.5303
Gender, N (%)					
Male	4 (40)	7 (50)	5 (33.3)	5 (38.5)	0.8331
Female	6 (60)	7 (50)	10 (66.7)	8 (61.5)	
BMI (kg/m^2)	26.3 ± 4.1	25.2 ± 6.2	30.2 ± 7.2	25.8 ± 4.8	0.1036
Chronic illness, N (%):					
Yes	2 (20)	3 (21.4)	3 (20)	2 (15.4)	0.9812
No	8 (80)	11 (78.6)	12 (80)	11 (84.6)	
Antiretroviral therapy (ART)	All study participants in the HIV-infected and HIV-Helminth coinfected groups were on ART.				
Parasite prevalence, N (%)					
A. lumbricoides			9 (60)	7 (53.8)	
T. trichiura			1 (6.7)	0 (0)	
Entamoeba coli			4 (26.7)	2 (15.4)	
Hymenolepis species			1 (6.7)	0 (0)	0.3389
Enterobius vermicularis			0 (0)	1 (7.7)	
S. mansoni			0 (0)	2 (15.4)	
Strongyloides species			0 (0)	1 (7.7)	

3.2. Alpha and Beta Diversity Analyses Reveal Distinct Microvirome Community Structures Across Clinical Groups

The alpha and beta diversity of microvirome communities was evaluated across five study groups: HIV and helminth uninfected controls (Control), HIV infection only (HIV), helminth infection only (Helminth), HIV-helminth co-infected (Co-infect), and CRC confirmed patients (CRC). The Richness index (Figure 1A) and Chao1 estimates (Figure 1B) were highest in the helminth-infected only group, indicating a greater numerical diversity of microvirome species. However, Kruskal-Wallis testing revealed no statistically significant differences between groups for either Richness ($\chi^2 = 0.5635$, $p = 0.967$) or Chao1 ($\chi^2 = 1.6484$, $p = 0.8001$). Evenness analysis (Figure 1C) showed that the CRC group had the highest Evenness values, suggesting a more uniform distribution of species compared to other groups. A borderline statistical difference was detected between groups for Evenness ($\chi^2 = 9.4556$, $p = 0.05067$). Shannon diversity analysis (Figure 1D) similarly showed that the CRC group had the greatest diversity, characterized by both Richness and Evenness. Shannon diversity differed significantly across groups ($\chi^2 = 11.722$, $p = 0.01954$). The Pairwise Wilcoxon rank sum tests corrected using the Benjamini-Hochberg method indicated that CRC patients had significantly higher Shannon diversity compared to HIV and helminth uninfected controls ($p = 0.029$) and helminth-infected only individuals ($p = 0.048$).

The Beta diversity was assessed using Bray-Curtis dissimilarity indices across the five groups (Figure 1E). Boxplots showed that the median Bray-Curtis dissimilarity was highest in the HIV-helminth uninfected control

infected only and HIV-helminth co-infected groups. The Kruskal-Wallis test identified highly significant overall differences in beta diversity ($\chi^2 = 118.51$, df = 4, $p < 2.2 \times 10^{-16}$). Subsequent pairwise Wilcoxon rank sum tests with Benjamini-Hochberg adjustment demonstrated significant differences between multiple group pairs: HIV and helminth uninfected controls vs HIV-helminth co-infected group ($p = 2.5 \times 10^{-6}$), HIV and helminth uninfected controls vs Helminth infected only ($p = 5.1 \times 10^{-10}$), HIV and helminth uninfected controls vs HIV infected only ($p = 0.0033$), CRC confirmed vs HIV-helminth co-infected group ($p = 4.9 \times 10^{-15}$), CRC confirmed vs Helminth infected only ($p < 2 \times 10^{-16}$), CRC confirmed vs HIV infected only ($p = 3.8 \times 10^{-10}$), and Helminth infected only vs HIV infected only ($p = 0.0010$).



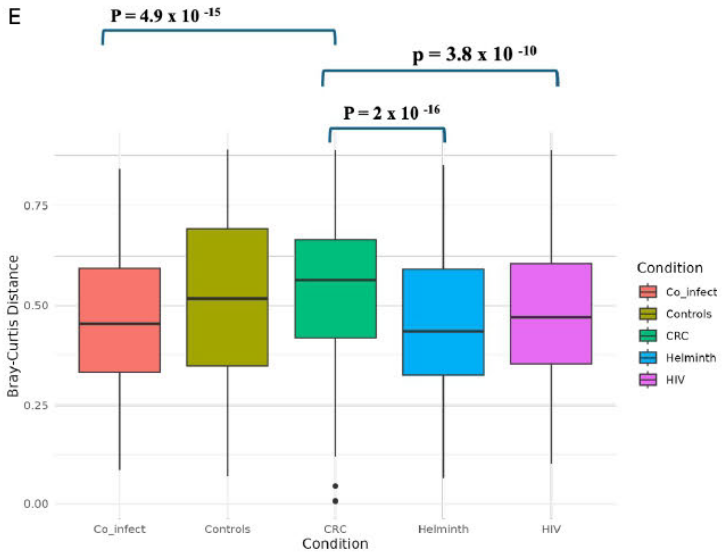


Fig 1. Alpha diversity was evaluated using Richness (A), Chao1 (B), Evenness (C), and Shannon diversity (D) indices across HIV-helminth co-infected, HIV and helminth uninfected controls, colorectal cancer (CRC) confirmed patients, helminth-infected only, and HIV- infected only groups. (E) Beta diversity was evaluated using Bray-Curtis dissimilarity indices to compare virome community structures among the study groups. Statistical comparisons were performed using Kruskal-Wallis and pairwise Wilcoxon rank sum tests with Benjamini-Hochberg correction.

To further understand differences in gut virome composition across study groups, we performed non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities. This ordination technique provides a visual summary of beta diversity, where the spatial proximity of points reflects the similarity of viral community structures. Each point represents a stool sample, color-coded by clinical group (HIV and helminth uninfected controls, HIV-infected only, helminth-infected only, HIV-helminth co-infected, and CRC confirmed patients). Notably, CRC confirmed and HIV-helminth co-infected samples plotted in partially overlapping regions of NMDS space, indicating partial visual clustering. In contrast, helminth-only, HIV-only, and HIV-helminth uninfected control samples were more dispersed and distinct from the CRC group (Figure 2). Ellipses around each group represent 95% confidence intervals. Consistent with the ordination, analysis of similarity (ANOSIM) indicated a small but statistically significant overall difference between groups ($R = 0.094$, $p = 0.001$). Pairwise comparisons revealed that CRC samples were most distinct, showing significant separation from HIV-helminth co-infected samples ($R = 0.282$, $p = 0.001$), HIV-only ($R = 0.275$, $p = 0.002$), and Helminth-only samples ($R = 0.204$, $p = 0.01$). This convergence may reflect early pre-malignant shifts in microvirome function driven by chronic infection. Together, these findings point to a scenario in which long-term immune modulation in co-infected individuals produces virome patterns resembling those of CRC confirmed patients, potentially indicating a shared ecological or functional remodeling process. In contrast, differences between HIV-helminth co-infected samples, HIV-helminth uninfected control, Helminth-only, and HIV-only groups were not statistically significant (all $p > 0.05$), indicating substantial compositional overlap.

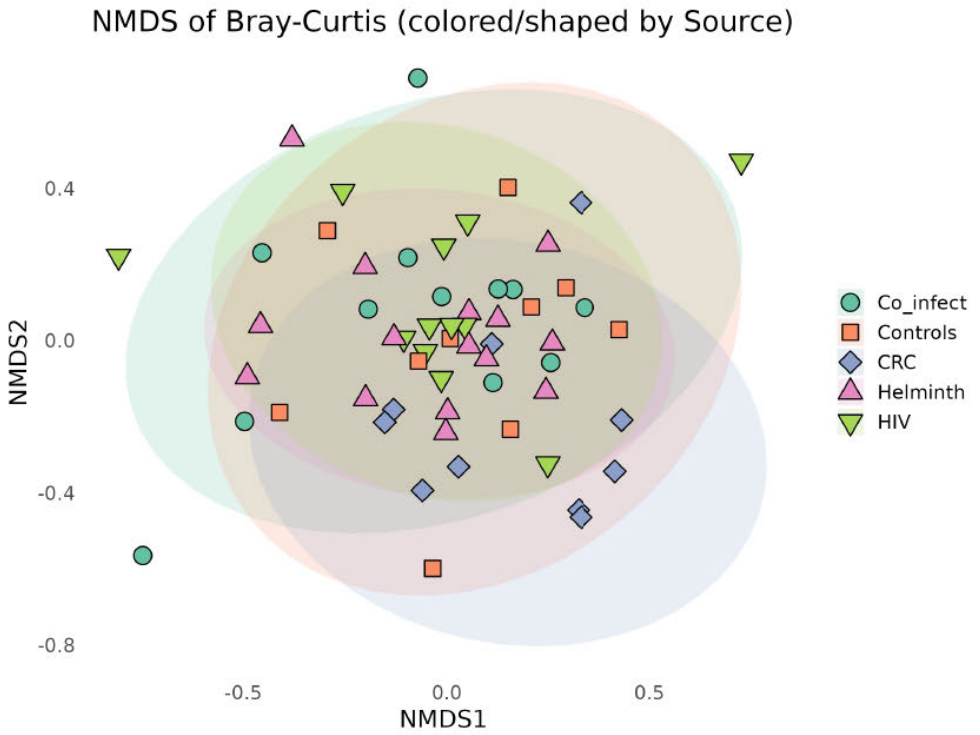


Fig 2. Non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarity illustrating gut virome beta diversity across clinical groups. Each point represents an individual stool sample, and colors/shapes indicate study groups: HIV-helminth co-infected (green circles), colorectal cancer (CRC) confirmed (purple diamonds), helminth-only (pink triangles), HIV-only (light green triangles), and HIV- and helminth-uninfected controls (orange squares).

3.3. Taxonomic composition

The relative abundances of viral taxa are shown at the phylum, family, genus, and species levels (Figure 3). The species labeled other represents low-abundance taxa grouped together for clarity. Several viral taxa, including unclassified viruses and bacteriophages not assigned to any known phylum, family, or genus in the NCBI database, were also detected.

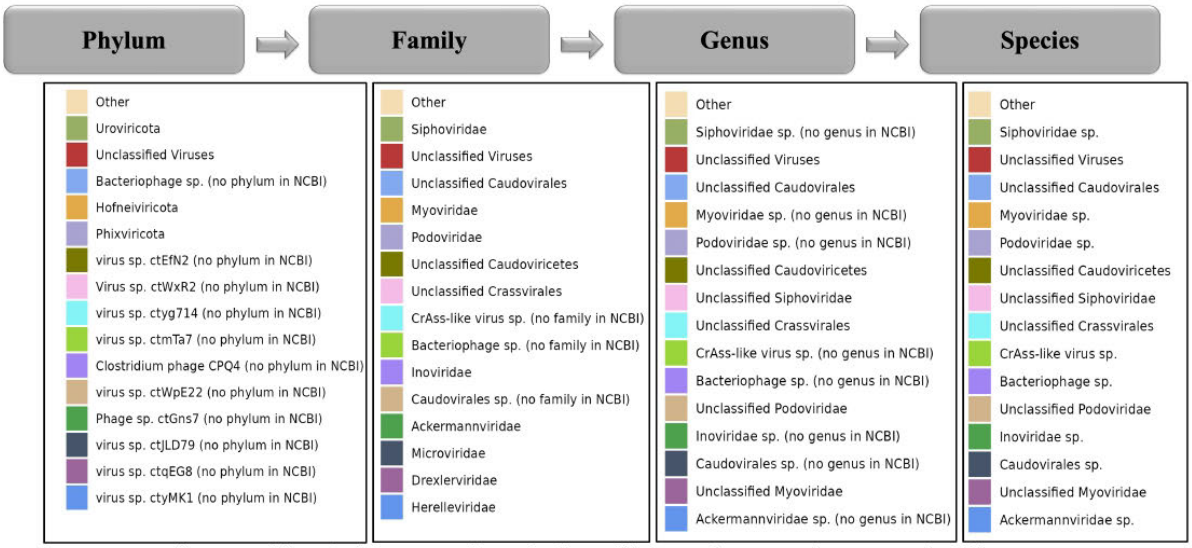


Fig 3. Taxonomic composition of viral communities. The figure illustrates the progressive taxonomic refinement from broader (Phylum) to more specific (Species) classification, highlighting the diversity and dominance patterns of the microvirome across all groups.

To explore functional shifts in the gut microvirome associated with disease, we examined differentially enriched protein families (PFAMs) protein domains across comparisons involving HIV-infected only, helminths-infected only, HIV-helminth co-infection, and CRC confirmed patients. Several PFAMs were significantly enriched (adjusted $p < 0.001$), including enzymes involved in folate metabolism, DNA replication, transposition, and RNA processing, many of which are implicated in oncogenic transformation, immune modulation, or genomic instability. Table 2 below has a curated list of PFAMs with known or potential relevance to cancer biology, supported by peer-reviewed references. Group comparisons are primarily made relative to HIV and helminth uninfected controls unless otherwise specified. In some cases, study groups are compared directly to one another (e.g., CRC confirmed vs HIV-infected only). Where multiple adjusted p-values are provided for a single PFAM, the relevant comparison group is indicated adjacent to its value.

Table 2. Differentially Abundant Cancer-Associated PFAM Domains Identified in Microvirome Contigs

PFAM ID	Gene Name/ Annotation	Group Comparison	Adjusted p-value	Role in Cancer	Reference
PF01261	Xylose isomerase-like Triosephosphate Isomerase (TIM) barrel	HIV-infected only	0.00023	TIM barrel enzymes are involved in metabolic reprogramming in cancer	[32, 33]
PF00773	RNB domain (exoribonuclease)	HIV-infected only	0.00015	Impacts RNA stability; dysregulation associated with altered gene expression in cancer	[34, 35]
PF13638	PilT N-terminus (PIN) domain (nuclease)	CRC confirmed	0.00024	Involved in RNA decay and surveillance pathways; dysregulation or mutation of PIN-domain-containing proteins is linked to impaired RNA turnover and tumor progression	[36, 37]
PF12631	MnmE helical domain	HIV-helminth co-infection CRC confirmed	0.00270 0.00054	Dysregulated tRNA-modifying enzymes may reprogram codon-biased translation, facilitating oncogenic proteins while suppressing tumor-protective pathways. Some may act cytoprotectively against ROS-induced DNA damage, and others may drive pro-tumorigenic translation programs.	[38]
PF10396	GTP binding protein	HIV-helminth co-infection CRC confirmed	0.00316 0.00075	The GTP-binding domain is shared among small GTPases, such as Ras, Rho, etc., proteins. Ras GTPases are dysregulated in several cancers including CRC	[39, 40]
PF02371	Transposase IS116/110/902	HIV-helminth co-infection CRC confirmed	0.00391 0.01424	Transposases contribute to insertional mutagenesis and chromosomal instability	[41, 42]
PF01051	Initiator replication protein	HIV-helminth co-infection CRC confirmed	0.00021 0.00012	Implicated in uncontrolled replication and genomic instability	[43, 44]
PF00763	Tetrahydrofolate dehydrogenase/ cyclohydrolase	HIV-infected only HIV-helminth co-infection	0.00080 0.00075	Essential for folate metabolism; linked to DNA synthesis and methylation in cancer	[45, 46]
PF00078	Reverse transcriptase/ RNA-dependent DNA polymerase	CRC confirmed vs HIV-helminth co-infection	0.00070	Key enzyme in retrotransposons and retroviruses; linked to insertional mutagenesis	[47, 48]

PF03167	Uracil-DNA glycosylase superfamily	CRC confirmed vs Helminth-infected only	0.00013	Initiate base excision repair to correct oxidative DNA damage. Their dysfunction increases mutational burden	[49, 50]
PF12733	Cadherin-like beta sandwich domain	CRC confirmed vs HIV-infected only	0.00044	Involved in cell-cell adhesion; loss promotes metastasis	[51, 52]

A total of 15 viral taxa were identified across all study groups (Figure 4). At the genus/species level, *Siphoviridae species (sp)*. was the most abundant taxon across all groups, followed by Unclassified Viruses and Unclassified *Caudovirales*. Other frequently detected taxa included *Podoviridae sp., Myoviridae sp., Bacteriophage sp.*, and Cross-Assembly Phage-like (*CrAss-like) virus sp*. Both the HIV and helminth uninfected controls and infected groups shared most of the dominant taxa; however, differences in relative abundance were observed. *Siphoviridae sp*. and Unclassified Viruses were predominantly detected in the HIV and helminth uninfected control group, while *Unclassified Caudovirales* and *Podoviridae sp*. showed higher relative abundance in the HIV-Helminth co-infection and helminth-only groups. *Myoviridae sp*. and *CrAss-like virus sp*. were moderately represented across all groups, whereas less abundant taxa such as *Ackermannviridae sp., Inoviridae sp., Caudovirales sp., Unclassified Podoviridae,* and *Skunavirus* were more sporadically distributed with lower relative abundances. The overall results showed that the HIV and helminth uninfected control samples displayed a higher abundance of *Siphoviridae sp.,* while HIV-Helminth co-infected samples showed a broader diversity of bacteriophages, suggesting possible shifts in virome structure linked to infection status.

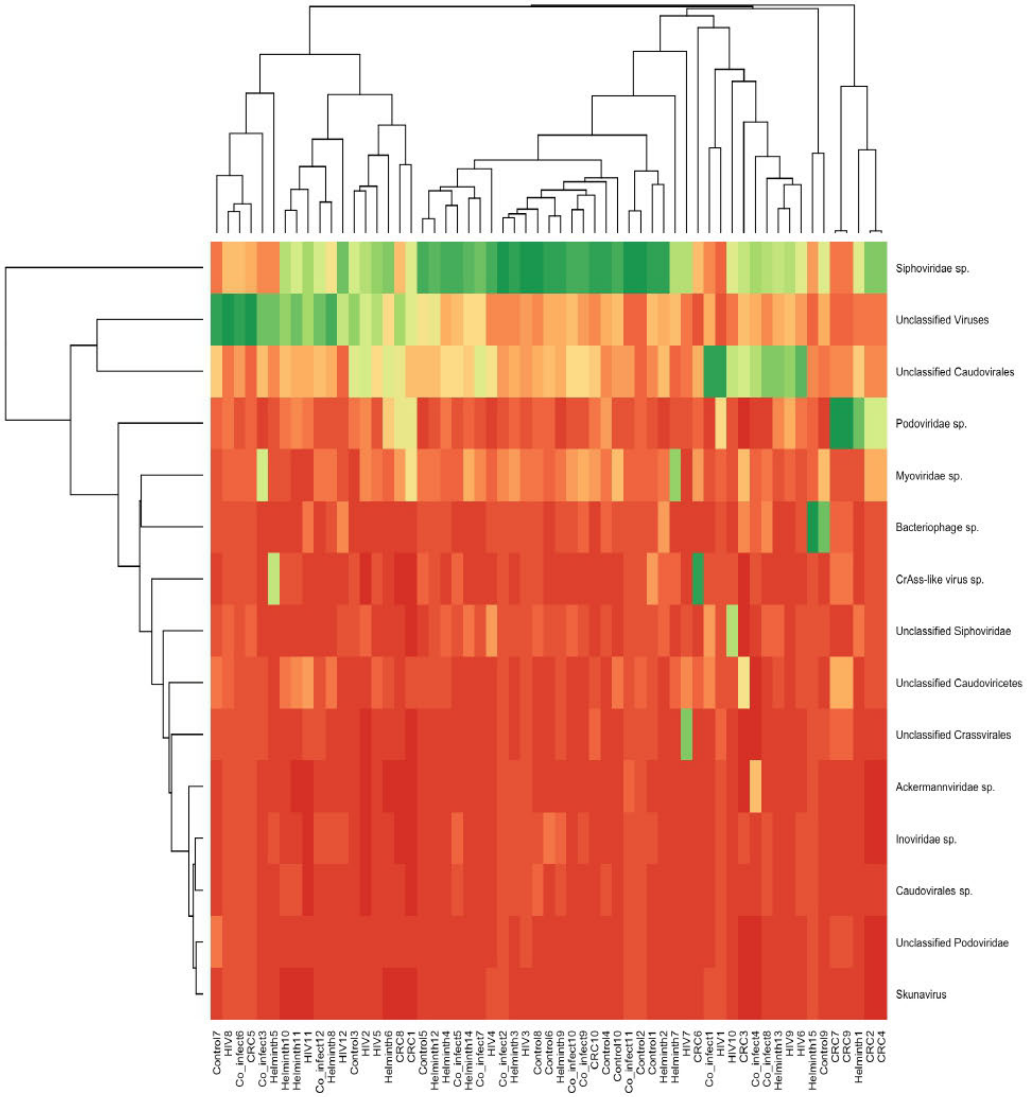


Fig 4. Differentially Abundant Viral Taxa. Heatmap showing the relative abundance of the top 15 viral taxa across all study groups. Viral taxa are presented on the y-axis and individual samples on the x-axis, ordered by hierarchical clustering. Colour intensity represents relative abundance, with green indicating higher and red indicating lower abundance. Taxa include *Siphoviridae sp*., unclassified viruses, and unclassified *Caudovirales*.

The HIV-helminth co-infected group in Figure 5 shows moderate viral diversity, with some samples having higher *Siphoviridae sp*. (coral pink) and Unclassified Viruses (orange) components. The CRC group displayed generally lower viral abundances compared to other groups. The HIV only group had one sample with a distinctive mustard segment (likely *Unclassified Caudovirales sp*.) that is not prominent in other groups. The helminth only group shows moderate viral diversity with no single species strongly dominating. Overall, the plot highlights inter-sample and inter-group variation in gut virome composition. However, it is important to note that the missing data are from other lineages, such as prokaryotes.

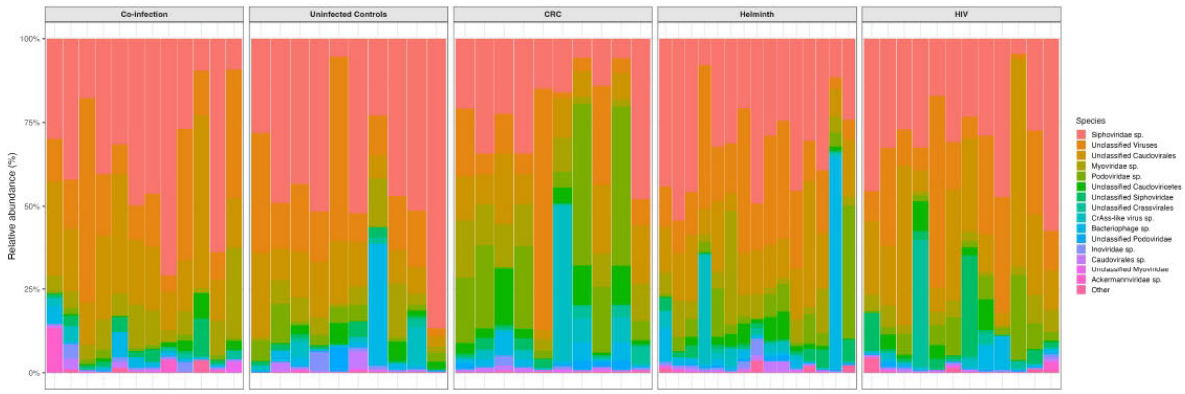


Fig 5. Group-Specific Abundance Patterns. The stacked bar plot shows the relative abundance of viral species across individual samples, grouped by infectious status: HIV and helminth uninfected controls, Helminth infected only, HIV-infected only, HIV-helminth co-infection, and colorectal cancer (CRC) confirmed patients. Each bar represents a single sample, and coloured segments within each bar indicate the proportion of different viral species as detailed in the legend on the right. The y-axis shows the percentage abundance of each species per sample, while the x-axis lists individual samples. Viral species include classified and unclassified members of major viral families (e.g., *Siphoviridae, Podoviridae, Myoviridae, Bacteriophage sp.*, and others).

A comparative analysis of the gut microvirome using a five-set Venn diagram (Figure 6) was used to illustrate how viral species are distributed across different groups. By examining the overlapping and non-overlapping sections, we aimed to identify a potential core virome shared across all groups, as well as distinct viral signatures associated with specific infections or disease states. A total of 26 viral species were found to be common across all five groups, constituting a potential core gut virome. The HIV-helminth co-infection group (9 unique species) and the HIV-infected only group (8 unique species) showed the most distinct virome profiles, suggesting co-infection and HIV alone may exert strong selective pressures on virome composition. In contrast, the CRC confirmed, helminth-infected only and HIV-helminth uninfected control groups showed fewer unique species (6, 5, and 4, respectively). This suggests that both shared and condition-specific alterations within the gut microvirome may occur, indicating that viral communities can shift in response to infection status and disease state. Zeros within the diagram indicate the absence of shared viral species between those specific group combinations, while smaller intersecting values highlight limited or unique overlaps that may suggest selective microvirome signatures or disease-driven viral shifts. The analysis provides a foundational view of virome dynamics across infectious states and disease in this cohort.

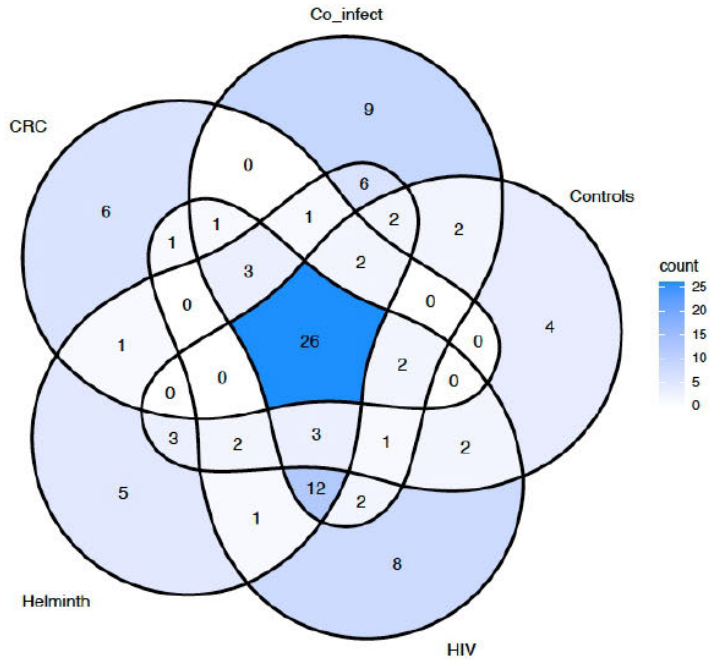


Fig 6. The Venn diagram depicting shared and unique viral species across groups.
The shared and unique viral species among participants with confirmed CRC, HIV-only infection, helminth-only infection, HIV-helminth co-infection, and those with no infection (HIV and helminth uninfected controls) are indicated. The number of shared and unique viral species across groups is shown, with each intersection representing species common to the overlapping categories. Colour intensity reflects the number of viral species within each segment, with darker blue indicating a higher count.

Pathway-Level Analysis of Viral-Associated Differentially Expressed Genes Using the Kyoto Encyclopedia of Genes and Genomes

A heatmap showing the relative abundance of the top 15 Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs (KOs) that are significantly different across all study groups is presented in Figure 7. While no clear clustering by study group was observed, whether healthy uninfected controls, CRC patients, HIV-only, helminth-only, or HIV-helminth co-infected individuals, notable patterns in functional viral gene expression emerged. For instance, Glutamate synthase [NADPH] large chain (K00568) and RNA polymerase sigma factor RpoE (σ^E) (K07741) were highly abundant in distinct sample clusters. These genes, commonly encoded by bacteriophages, suggest virome-driven metabolic reprogramming and stress response activation, which may shape host-microbe-virus interactions under immunosuppressed or tumor-associated conditions.

In contrast, genes like DNA polymerase III gamma/tau subunit (K03111) and phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase (K00509) displayed more heterogeneous expression, possibly reflecting context-dependent viral replication dynamics or microbial co-evolution in different disease or infection states.

Importantly, catalase-peroxidase (K01366), an enzyme involved in oxidative stress resistance and found in some viral genomes, was consistently expressed at low abundance across multiple samples. This may imply a reduced virome capacity to counteract reactive oxygen species, which could further exacerbate host mucosal damage, DNA instability, and inflammatory conditions conducive to tumorigenesis. The absence of distinct clustering by

disease group suggests that while specific functional virome genes are differentially abundant, virome-associated metabolic potential is not uniformly defined by infection status or cancer alone. Instead, it likely reflects complex interactions between host immunity, microbial ecology, and viral functional plasticity, particularly under the overlapping influences of chronic parasitic infections, HIV-induced immunosuppression, and colorectal cancer-associated dysbiosis.

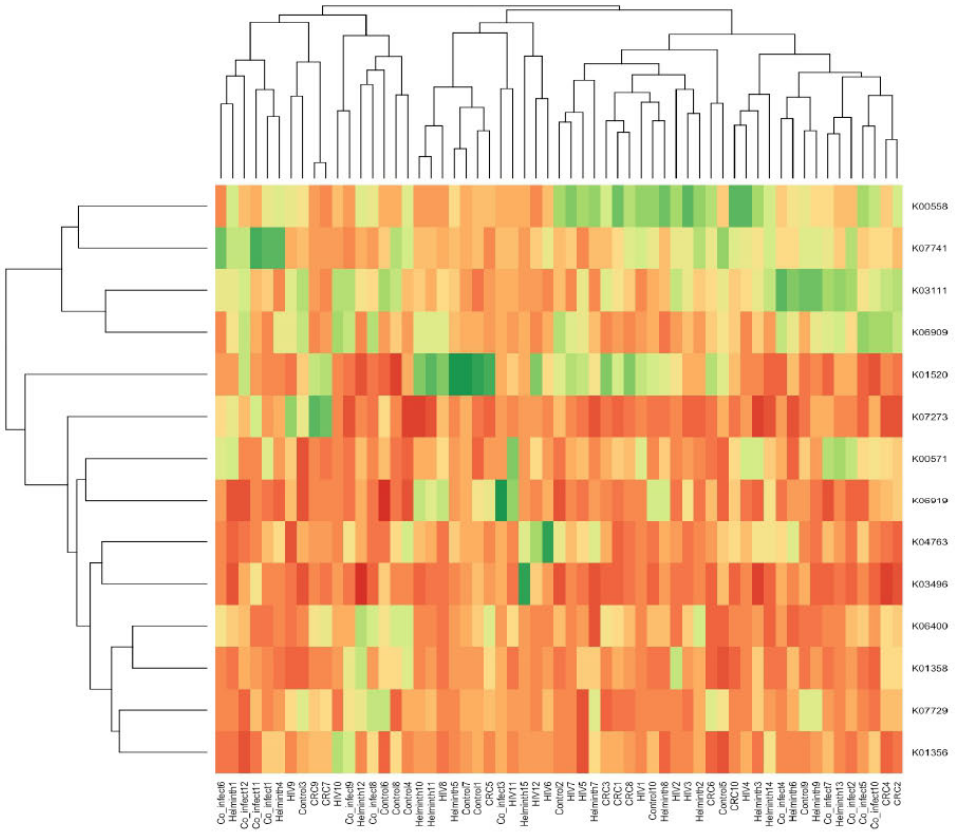


Fig 7. Heatmap of top KEGG Orthology KO relative abundance across study samples. The heatmap illustrates the relative abundance of the top 15 KEGG Orthology identifiers (rows) across individual study samples (columns). Each cell represents the abundance of a specific KO in a given sample, with colour gradients ranging from green (low abundance) through yellow/orange (intermediate abundance) to red (high abundance). Hierarchical clustering was applied to both KOs (rows) and samples (columns) using Euclidean distance and complete linkage, as indicated by the dendrograms. KOs with similar abundance profiles cluster vertically, while samples with comparable functional signatures cluster horizontally. Sample labels correspond to experimental groups: healthy uninfected controls, HIV only, helminth only, HIV-helminth co-infected, and colorectal cancer (CRC) positive individuals.

Compared to HIV and helminth uninfected controls, the HIV-helminth co-infected group displayed several differentially expressed viral functional genes that were also present in the CRC group. This pattern may indicate underlying virome-mediated mechanisms contributing to chronic inflammation and cancer-related pathways. The results showed downregulation of the transcriptional regulator PuuR (K14056), a helix-turn-helix repressor that modulates genes involved in putrescine metabolism and broader nitrogen regulatory processes in HIV-helminth co-infection [-7.35 (SE = 1.96, q = 0.003)] and CRC confirmed [-6.57 (SE = 2.04, q = 0.016)]. Its reduced expression in both groups could reflect disrupted nitrogen homeostasis, potentially linked to microbial dysbiosis or altered metabolic demands during co-infection and tumor development. In contrast, there was consistent upregulation of tRNA modification GTPase (K03650) [4.69 (SE = 1.20, q = 0.002)] and 3.91 (SE = 1.23, q = 0.016) respectively. This enzyme plays a vital role in modifying tRNA wobble bases to ensure accurate decoding during protein translation. Elevated expression might disturb translational fidelity, possibly increasing the frequency of misfolded or altered proteins, which could assist viral persistence or support tumor-promoting immune evasion strategies.

Additionally, uridine phosphorylase (K00757), which participates in the pyrimidine salvage pathway by catalyzing the reversible phosphorolysis of uridine, was also upregulated in HIV-helminth co-infection [4.38 (SE = 1.12, q = 0.002)] and CRC [4.00 (SE = 1.15, q = 0.010)]. This shift in nucleotide recycling suggests enhanced demand for nucleotides, potentially supporting increased viral replication or the proliferative needs of transformed host cells. The upregulation of a transposase (K07488) in HIV-helminth co-infection [7.67 (SE = 2.49, q = 0.020)] and CRC [8.31 (SE = 2.60, q = 0.017)], an enzyme facilitating the movement of genetic elements, may indicate active genomic rearrangements within the virome or host-associated mobile genetic elements. This could represent an adaptive response to immune stress or a mechanism for viral or microbial survival under inflammatory pressure, as commonly observed in chronic infections and tumorigenic microenvironments.

In the helminth-only group [4.41 (SE = 1.05, q = 0.001)], functional similarities with the CRC confirmed [3.97 (SE = 1.05, q = 0.010)] group were observed, including upregulation of P-type calcium-transporting ATPase type 2C (K01537), an enzyme that regulates calcium ion transport across membranes. This may contribute to microbial or host calcium imbalance, potentially influencing intestinal signaling pathways related to inflammation, barrier permeability, or epithelial repair. Notably, a shared expression pattern was seen in both HIV-only and helminth-only groups, characterized by downregulation of ferrous iron transport protein B (K04759) [-6.72 (SE = 1.41, q = 6.40e-05)]; [-6.63 (SE = 1.436, q = 3.87e-05)], nitrogen fixation protein NifB (K02585) -5.37 (SE = 1.20, q = 0.000); -5.83 (SE = 1.15, q = 1.48e-05), and large ribosomal subunit protein L7/L12 (K02935) -20.2 (SE = 4.23, q = 6.38e-05); -13.05 (SE = 4.07, q = 0.010). These changes suggest a suppression of microbial functions related to iron acquisition, nitrogen metabolism, and ribosome assembly, pathways often impacted by host immune pressures or nutrient competition during infection. At the same time, both groups displayed upregulation of S-adenosylmethionine synthetase (K00789) 4.37 (SE = 1.08, q = 0.001), an enzyme involved in methyl group donation for DNA and RNA methylation. This shift may reflect virome-driven efforts to influence host or microbial epigenetic regulation.

Each infection group also displayed distinct virome expression signatures. The HIV-helminth co-infection group uniquely showed downregulation of integrase/recombinase XerD (K04763) -2.50 (SE = 0.59, q = 0.000), a gene required for site-specific DNA integration and recombination, potentially indicating reduced mobile genetic element activity in this group. Moreover, SsrA-binding protein (K03664) -3.99 (SE = 1.09, q = 0.0049), which rescues stalled ribosomes and promotes quality control in translation, was absent in this group, possibly signifying impaired stress response and translational fidelity under dual infection. In the CRC confirmed group, unique upregulation of Bordetella phage reverse transcriptase (K21038) 3.58 (SE = 1.07, q = 0.013), lysozyme (K01185) 2.86 (SE = 0.90, q = 0.017), and 8-oxo-dGTP diphosphatase (K03574) 6.60 (SE = 2.07, q = 0.017) was observed. These genes may reflect elevated phage replication, microbial cell wall degradation, and oxidative damage repair, consistent with the tumor-promoting, inflamed gut environment of CRC.

The helminth-only group also exhibited exclusive downregulation of several genes involved in microbial stress adaptation and DNA repair: DnaJ homolog subfamily A member 2 (K09503) -6.23 (SE = 1.51, q = 0.001), a heat shock chaperone that assists protein folding; carbohydrate diacid regulator (K02647) 4.05 (SE = 1.23, q = 0.004), involved in metabolic stress sensing; phage terminase large subunit (K06909) -1.80 (SE = 0.50, q = 0.004), essential for viral DNA packaging; and tRNA-splicing ligase RtcB (K14415) -4.48 (SE = 1.21, q = 0.003), important for maintaining tRNA integrity. This may indicate reduced resilience of the microbial community under helminth-induced immune modulation. Lastly, in the HIV-only group, strong downregulation of DNA-processing enzymes such as exodeoxyribonuclease V alpha subunit (K03581) -4.50 (SE = 1.17, q = 0.002) and deoxyribonuclease IV (K01151) -4.05 (SE = 0.95, q = 0.000) was observed, suggesting impaired microbial DNA degradation and recombination capacity. Interestingly, upregulation of ferritin (K02217) 8.93 (SE = 2.54, q = 0.007), a key iron storage protein, may reflect viral or microbial adaptations to limit oxidative stress or enhance iron acquisition in a chronically inflamed gut environment (Figure 8).

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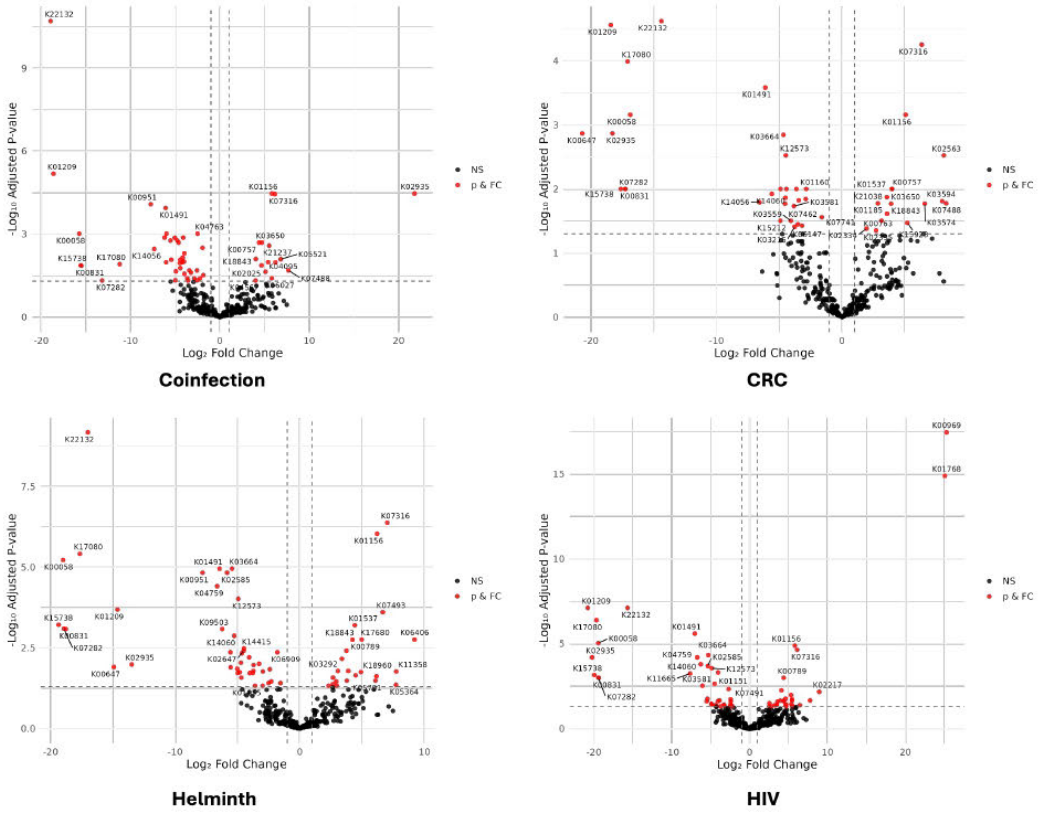


Fig 8. Volcano plots showing differentially abundant KEGG pathways across study groups compared with HIV and helminth-uninfected controls. Each point represents a KEGG pathway, with $\log_2$ fold change on the x-axis and $-\log_{10}$ adjusted p-value on the y-axis. Pathways enriched in the study group relative to controls are displayed on the right, while pathways reduced relative to controls appear on the left. Significant pathways (adjusted $p < 0.05$) are highlighted.

When comparing virome functional profiles between each infected group and the CRC-confirmed group (Figure 9A), distinct expression trends emerged, suggesting that viral communities may undergo different functional adaptations under various infection states. In the HIV-helminth co-infected group, downregulation of K06919 [-2.98 (SE = 0.75, q = 0.020)], a gene component of bacterial toxin-antitoxin (TA) systems, may indicate altered phage-mediated stress responses. TA modules encoded by temperate phages can support microbial survival by stabilizing lysogeny under stress. Reduced expression of this gene could suggest a shift away from these protective mechanisms, potentially reflecting a disrupted microbial ecosystem under the combined immune pressures of HIV and helminth infections. Additionally, upregulation of K15773 [5.53 (SE = 1.42, q = 0.021)], which encodes an ATP-binding cassette (ABC) transporter subunit, may signal altered microbial or viral transport activity. This could influence the virome's ability to modulate nutrient acquisition or efflux toxic compounds, potentially reshaping nutrient dynamics at the host-microbe interface in pro-inflammatory or tumor-permissive environments.

The helminth-only group did not show significant differences in viral functional gene expression compared to the CRC confirmed group, which may imply a more stable or conserved virome composition. This lack of differential

expression could suggest that, in isolation, helminth infections do not induce major shifts in virome functionality, or that their effects are more subtle and possibly dependent on co-existing immunosuppressive conditions. Conversely, the HIV-only group displayed distinct modulation of viral functions. There was downregulation of K11665 [-7.34 (SE = 1.77, q = 0.007)], a chromatin-remodeling ATPase (INO80), and K02334 [-2.71 (SE = 0.67, q = 0.007)], a bacteriophage-type DNA polymerase associated with DNA synthesis and ribosomal regulation. These changes may point to impaired virome replication dynamics or altered host-microbe gene expression control in the presence of HIV. In contrast, this group showed upregulation of several metabolic and signaling genes, including nicotinate-nucleotide adenylyltransferase (K00969) 21.47 (SE = 2.70, q = 1.72e-12) and adenylate cyclase (K01768) 20.51 (SE = 2.91, q = 7.48e-10), enzymes integral to energy production and lipid biosynthesis. Additionally, increased expression of K21528 (a serine recombinase from Enterobacteriaceae phage) 5.63 (SE = 1.37, q = 0.007) and K15773 (HipB, a transcriptional regulator and antitoxin) 5.28 (SE = 1.43, q = 0.028) points to increased activity in genetic regulation and transport systems. These shifts may reflect an adaptive virome that responds to HIV-induced immune dysregulation by enhancing energy metabolism and stress resilience.

Further comparative analysis between infection groups (Figure 9B) revealed additional virome functional divergence that may reflect infection-specific microbial or host-microbe interactions. When comparing the helminth-only group vs HIV-helminth co-infection group, there was upregulation of several key genes: ribosomal subunit protein L7/L12 (K02935) 35.27 (SE = 3.86, q = 5.45e-17), which plays a role in protein synthesis and translation efficiency; 3-oxoacyl-[acyl-carrier-protein] synthase I (FabB) (K00647) 27.63 (SE = 4.36, q = 9.63e-08) and transposase (K07486) 2.93 (SE = 0.59, q = 0.000), associated with genetic mobility and potential horizontal gene transfer. In contrast, K03091, encoding an RNA polymerase sigma factor (SigE_F_G) -4.83 (SE = 1.28, q = 0.035), was downregulated, possibly indicating altered transcription initiation in the co-infected state. The helminth-only vs. HIV-only comparison showed downregulation of DNA repair protein SbcC (K03546) -5.83 (SE = 1.43, q = 0.004), a gene involved in maintaining genome integrity, as well as chromatin-remodeling ATPase INO80 (K11665) -5.69 (SE = 1.60, q = 0.020) and bacteriophage-type DNA polymerase (K02334) -2.16 (SE = 0.60, q = 0.020), both of which are essential for DNA replication and remodeling. These findings suggest reduced virome replication capacity or impaired genetic repair in helminth-infected individuals compared to those infected with HIV alone. On the other hand, the HIV-only group displayed broad upregulation of genes involved in metabolic regulation and intracellular processing. These included nicotinate-nucleotide adenylyltransferase (NadD) (K00969) 21.47 (SE = 2.70, q= 1.72e-12) and adenylate cyclase (K01768) 20.51 (SE = 2.91 =, q = 7.48e-10) highlighting enhanced fatty acid biosynthesis. Additionally, Enterobacteriaceae phage serine recombinase (Gin) (K21528) 5.63 (SE = 1.37, q= 0.007), involved in site-specific recombination, was also upregulated.

Finally, the HIV-only vs HIV-helminth co-infected groups revealed a mixed expression pattern. There was downregulation of NadD (K00969) -23.50 (SE = 2.58, q = 1.51e-17), adenylate cyclase (K01768) -22.32 (SE = 2.78, q = 1.02e-13), DNA topoisomerase IV subunit A (K04769) -6.62 (SE = 1.83, q = 0.016), and ribonucleoside-diphosphate reductase alpha chain (K00525) -4.24 (SE = 1.21, q = 0.018) in the co-infected group, which may suggest suppressed replication and metabolic activity under dual infection. Meanwhile, upregulation was noted for ribosomal protein L7/L12 (K02935) 42.0 (SE = 4.03, q = 5.92e-23), chromatin-remodeling ATPase INO80 (K11665) 7.36 (SE = 1.70, q = 0.001), and voltage-gated potassium channels (K10716) 4.94 (SE = 1.40, q= 0.012),

pointing to altered translation regulation, signal transduction, and membrane potential control in the co-infected state.

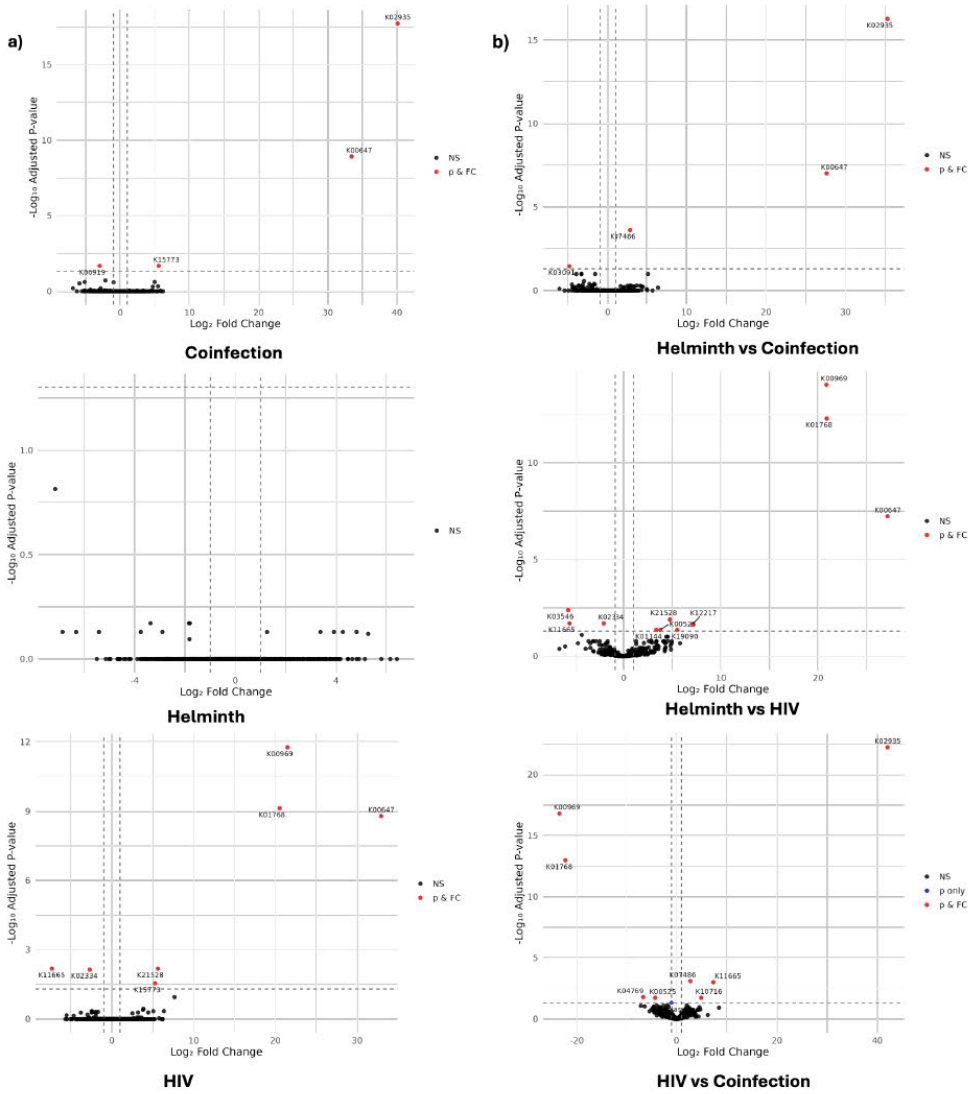


Fig 9. Volcano plots depicting differentially abundant KEGG pathways or biological processes across study groups. (A) shows comparisons between colorectal cancer (CRC) positive controls and all infected groups, (B) shows comparisons among all infected groups. Each point represents a pathway or biological process, with $\log_2$ fold change on the x-axis and $-\log_{10}$ adjusted p-value on the y-axis. Pathways increased in abundance relative to the comparison group are displayed on the right, and those decreased in abundance are shown on the left. Significant pathways (adjusted $p < 0.05$) are highlighted.

Overall, the KEGG pathway enrichment analysis revealed consistent alterations in pathways involved in lipid metabolism, transcriptional regulation, nucleotide metabolism, ion transport, and methylation processes shared by

both HIV-helminth co-infection and CRC confirmed groups (Table 3). These overlapping disruptions underscore potential mechanistic links through which co-infection may influence CRC susceptibility.

Table 3. Summary of Most Commonly Expressed KOs in CRC and Infected Groups

KO ID	Gene/Protein name	Functional role
K14056	PuuR; HTH-type transcriptional regulator, repressor for puuD	Transcriptional repressor involved in amino acid metabolism regulation
K03650	MnmE/TrmE/MSS; tRNA modification GTPase	Enzyme involved in tRNA modification, affecting translation fidelity
K00757	Uridine phosphorylase (Udp/UPP)	Enzyme involved in pyrimidine metabolism upregulated in cancer [49, 50]
K07488	Transposase	Enzyme facilitating transposition, genomic rearrangements
K01537	P-type Ca2+ transporter type 2C	Calcium ion transporter involved in cellular ion homeostasis
K04759	FeoB; Ferrous iron transport protein B	Iron uptake protein, important for microbial iron acquisition
K02585	NifB; Nitrogen fixation protein NifB	Protein involved in nitrogen fixation
K02935	Large ribosomal subunit protein L7/L12 (RP-L7/MRPL12/rplL)	Ribosomal protein critical for protein synthesis
K00789	MetK/MAT; S-adenosylmethionine synthetase	Enzyme producing methyl donor SAM, critical in methylation processes

The Uridine phosphorylase (K00757; EC 2.4.2.3) was present in the viral gene pool and significantly upregulated in HIV-helminth co-infected individuals (adj $p = 0.00201$) as well as in CRC confirmed patients (adj $p = 0.01690$) compared to HIV and helminth uninfected controls. This enzyme catalyzes the reversible phosphorolysis of uridine to uracil and ribose-1-phosphate, thereby feeding into nucleotide recycling and energy production pathways under stress or nutrient-limited conditions. The pathway visualization (Figure 10) illustrates the central role of K00757 in supporting metabolic flexibility, especially in inflammatory or tumor-prone gut environments. The presence of this KO in both HIV-helminth co-infection and CRC confirmed groups may indicate convergent viral strategies that support cell proliferation or immune evasion, potentially linking virome activity to CRC susceptibility. The permissive nature of the enzyme in supporting tumor metabolism by catalyzing the breakdown of uridine into ribose-1-phosphate may provide an alternative substrate that can feed into the glycolytic pathway. This would allow cancer cells to bypass glucose scarcity and continue producing energy, thereby promoting cell survival and sustained tumor growth under metabolically challenging conditions [53].

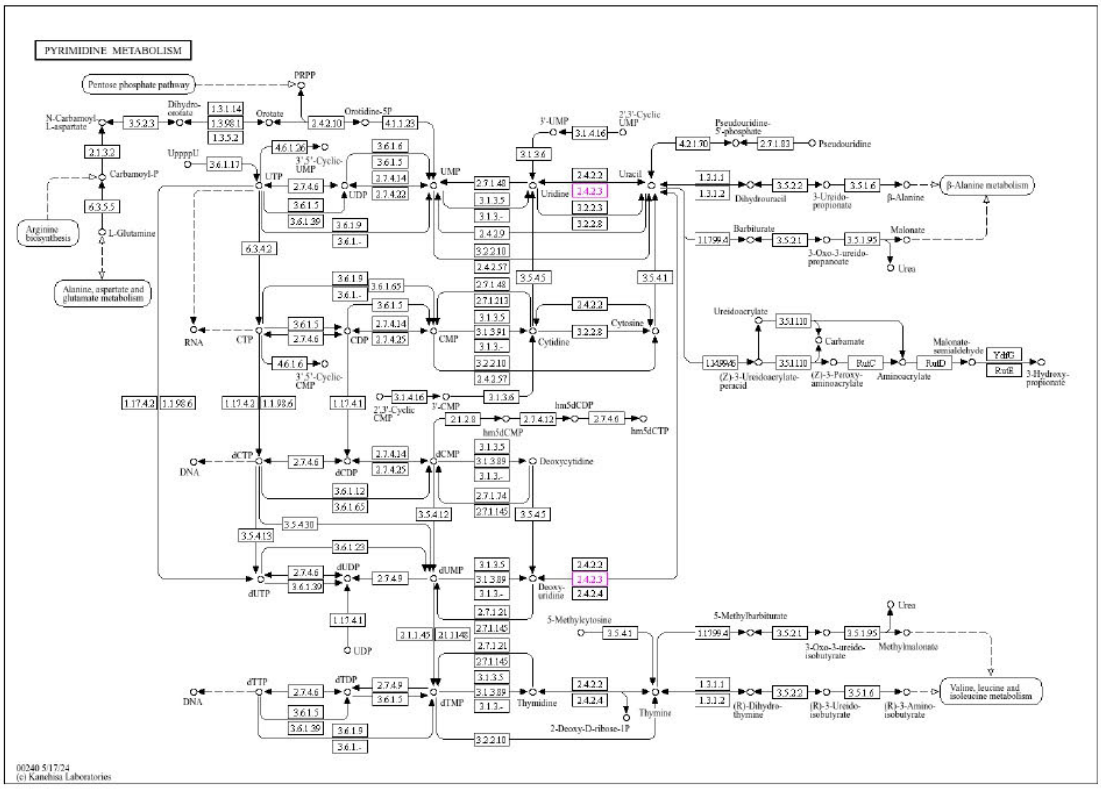


Fig 10. KEGG Pyrimidine metabolism pathway (map00240) with viral gene contributions identified in gut virome metagenomes.
The figure highlights the uridine salvage pathway, specifically the enzyme uridine phosphorylase (EC 2.4.2.3, KO: K00757), which catalyzes the reversible conversion of uridine to uracil and ribose-1-phosphate. This reaction enables pyrimidine recycling without requiring ATP and contributes to alternative energy production under metabolic stress. Pink-highlighted KO nodes in the pathway represent enzymes that were identified in the gut viral metagenomic dataset from at least one of the study groups which in this case is uridine phosphorylase (EC 2.4.2.3; KO: K00757).

Co-occurrence network analysis of dominant Gut Viral species

To better understand the structural and functional organization of the gut virome in the context of HIV and helminth co-infection, we performed a co-occurrence network analysis using metagenomic linkage groups (MLGs). These MLGs group viral contigs exhibiting similar abundance patterns across samples, enabling reconstruction of ecologically meaningful viral communities and their potential links to immune status, microbial dynamics, and disease progression. A co-occurrence network was constructed based on statistically significant correlations (Spearman's $|r| \geq 0.7$, $p < 0.05$) in abundance between MLGs and viral taxa within stool samples of HIV-helminth co-infected individuals (Figure 11).

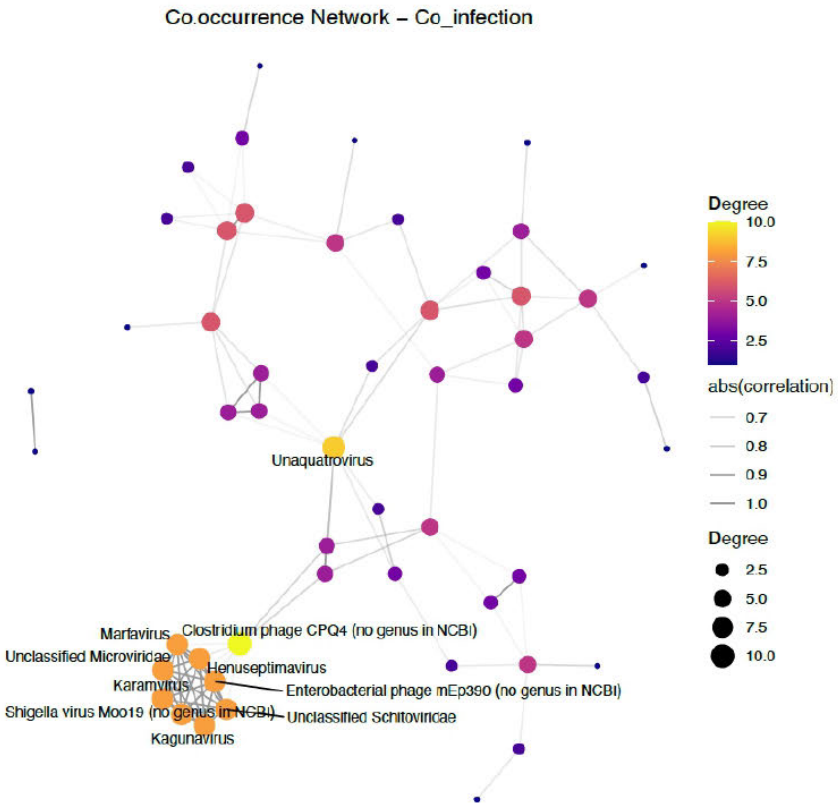


Fig 11. Co-occurrence Network of Gut Viral in HIV-Helminth Co-Infected Individuals. This network illustrates statistically significant correlations among viral taxa identified in the gut microvirome of individuals co-infected with HIV and helminths. Each node represents a distinct viral genus or unclassified viral taxon. Node colour and size correspond to degree centrality (i.e., the number of connections to other nodes), reflecting the relative prominence of each virome within the network. Edges indicate positive or negative correlations in abundance between taxa, with edge thickness proportional to the strength of the correlation.

4. Discussion

The aim of this work was to characterize the gut DNA virome in individuals with HIV and helminth co-infections and to explore its relationship with CRC. We sought to understand how co-infection impacts viral community structure, diversity, and functional gene expression, and whether these virome changes mirror those observed in CRC, thus potentially linking chronic infections to cancer risk. Our key findings reveal significant remodeling of the gut virome in HIV-helminth co-infected individuals, with viral diversity and functional profiles resembling those in CRC patients. We observed considerable inter-individual and inter-group variability in viral species abundance, with most individuals harboring diverse but low-abundance viral communities. Consistent with previous reports [54], bacteriophages classified into *Podoviridae*, *Siphoviridae*, and *Myoviridae* families dominated, highlighting their role in shaping bacterial populations. Importantly, increased viral evenness and diversity were noted in CRC-confirmed samples compared to HIV and helminth uninfected controls and helminth-only individuals. Previous studies have suggested that tumor-induced immune modulation may impair viral control mechanisms and foster complex viral ecosystems [3, 55]. The viral community balance, rather than species richness alone, emerged as a critical factor distinguishing disease states. Helminth-infected individuals trended

towards increased viral species richness, but overall virome diversity did not significantly change, aligning with previous work indicating that viral dysbiosis contributes to CRC pathogenesis through fostering immunomodulatory viral environments [56]. Core virome analysis identified 26 viral species shared across all groups, suggesting a stable viral consortium, while unique viruses in co-infected and infected groups indicate disease-specific viral enrichments, as described by others studying infection-associated microbiome shifts [1].

Functional gene expression profiles further corroborated these ecological observations. Elevated activity of the transposase family PF02371 in HIV-helminth co-infected individuals (adj p = 0.0039) was observed. These genes are reported to have an important role in insertional mutagenesis and chromosomal instability, key mechanisms implicated in CRC development [48, 57]. These studies align with broader cancer literature highlighting transposable elements as drivers of genomic instability [58]. Of particular interest, PF10396, a GTP-binding protein domain, was upregulated in both HIV-helminth co-infected (adj p = 0.00316) and CRC-confirmed (adj p = 0.00075) groups. Studies have reported aberrant RAS signaling pathways to be central to CRC progression and treatment resistance [59]. While RAS mutations remain consensus markers in advanced CRC, Yıldırım *et al*., 2025 argue that they also hold value in early-stage disease, especially for risk stratification and guiding adjuvant therapy, though their prognostic power varies by microsatellite status [40].

In addition to these findings, PF12631 was also significantly upregulated in both HIV-helminth co-infected individuals (adj p = 0.00054) and CRC-confirmed cases (adj p = 0.00270) relative to HIV and helminth uninfected controls. PF12631, the MnmE helical domain, forms part of the tRNA modification GTPase MnmE/TrmE complex, a key enzyme responsible for wobble-position tRNA modifications that enhance translational fidelity and support cellular adaptation to stress. A growing body of evidence links dysregulation of tRNA-modifying enzymes to tumorigenesis. These enzymes are essential for maintaining the accuracy of protein synthesis, particularly under oxidative or proliferative stress conditions commonly observed in both chronic infection and malignancy [60]. The KEGG Orthology gene K03650, also encoding tRNA modification GTPase (MnmE/TrmE), was upregulated in HIV-helminth co-infected (adj p = 0.00201) and CRC confirmed (adj p = 0.01690) groups. This KO gene also functions by catalyzing GTP-dependent modifications at the tRNA wobble base. Recent studies have shown that cancer cells co-opt these tRNA-modifying pathways to sustain high protein synthesis rates and adapt to hostile microenvironments. Impairment of such mechanisms can compromise translation fidelity and stress-response protein production, thereby promoting genomic instability and cancer progression [61, 62]. The concurrent upregulation of PF10396 (GTP-binding domain) and along with PF12631 and K03650, which encode for MnmE/TrmE family tRNA-modifying GTPases, in both CRC-confirmed and HIV-helminth co-infected groups suggests a possible convergence of translational stress and GTPase-mediated signaling in these conditions. Given that both GTPase activity and tRNA modification pathways are increasingly recognized as critical modulators of tumorigenesis and immune response, these findings may point to shared molecular vulnerabilities or early biomarkers of malignant transformation under chronic infectious stress.

Similarly, the RNB domain-containing exoribonuclease PF00773 was significantly associated with HIV infection (adj p = 0.00015). Huntzinger *et al*., 2023 identified HELZ2, a novel member of the RNB exoribonuclease family, which contributes to both viral genome clearance and host RNA turnover during antiviral responses, potentially

influencing the accumulation of oncogenic mRNAs [63]. Moreover, RNA-binding proteins and their counteracting exoribonucleases play a critical role in regulating RNA stability and splicing, with viral proteins frequently subverting these networks, leading to oncogenic consequences [64]. The presence of the PilT N-terminus PIN domain (PF13638) in CRC samples is implicated in apoptosis regulation, and its dysregulation promotes oncogenesis by impairing programmed cell death and enabling malignant cell survival [65].

Our findings suggest that virome-driven translational disruption could be an oncogenic vulnerability axis in chronically infected individuals, warranting further functional studies. Metabolic pathway genes such as K00647 (3-oxoacyl-[acyl-carrier-protein] reductase) exhibited contrasting expression patterns: suppressed in CRC but elevated in HIV-infected groups. This may imply that HIV-associated immune dysregulation modulates microbial fatty acid biosynthesis [66], potentially mitigating CRC-associated metabolic suppression or fostering alternative dysbiotic states, consistent with emerging evidence that viral-encoded auxiliary metabolic genes influence host microbial metabolism and inflammation. However, interpretation of these results is tempered by intraoperative stool collection, which may transiently disrupt microbial gene expression profiles, highlighting the need for future community-based sampling. Notably, the HIV and helminth co-infected group showed marked upregulation of uridine phosphorylase (K00757), an enzyme linked to suppression of anti-tumor immunity and facilitation of metastasis [67]. Its role in 5-fluorouracil activation also implicates it in chemotherapy response, underscoring the clinical relevance of virome-associated metabolic shifts. These findings align with meta-analyses demonstrating infection-driven cancer risks and amplify concerns about immunocompromised populations in high-burden settings [68].

Network-based analyses revealed that viral taxa such as *Myoviridae*, *CrAss-like viruses*, and unclassified *Caudovirales* serve as ecological hubs within the co-infected gut virome. The connectivity of lesser-known viruses implies that uncharacterized components may modulate community dynamics and host interactions, resonating with recent discoveries about virome complexity in disease [69]. This is consistent with the findings by Chen *et al*., 2023 who reported that the gut viral composition of CRC patients was drastically altered compared with that of healthy individuals, as evidenced by shifts in certain *Siphoviridae* and *Myoviridae* viruses and an enrichment of *Microviridae* further suggesting the use of these virome as novel targets for CRC treatment [70]. Thus, the modular organization of viral communities under co-infection stresses indicates a restructuring that may impact microbial homeostasis, mucosal immunity, and inflammation, all critical factors in CRC development. Collectively, our results indicate that HIV and helminth co-infections induce gut virome remodeling mirroring CRC-associated dysbiosis, not just through taxonomic shifts but via altered functional gene expression and virome-host interactions. The enrichment of transposase, reverse transcriptase, and metabolic gene domains suggests that the virome contributes to genomic instability and immune modulation, thereby fostering tumor-promoting microenvironments. These insights position the gut virome as both a biomarker and potential mediator of infection-linked cancer risk, opening avenues for functional biomarker discovery and targeted intervention strategies in high-risk populations.

5. Challenges and limitations

Characterizing the gut virome in the context of HIV-helminth co-infection is essential for understanding potential microbial mechanisms contributing to colorectal carcinogenesis in endemic African settings. This study applied shotgun metagenomic sequencing to enable broad, non-invasive profiling of viral communities and their functional pathways across clinical groups. However, several limitations warrant consideration.

Methodological constraints of metagenomics

Shotgun metagenomics captures total viral DNA irrespective of transcriptional activity and therefore cannot distinguish between active and latent viral elements. As a result, it is not possible to determine which identified viral genes are actively expressed or directly involved in inflammation, immune modulation, or oncogenic processes [71]. While metagenomic profiling has demonstrated utility in CRC biomarker discovery [72], its reliance on existing reference databases, largely derived from Western populations, may limit detection of novel or region-specific viral taxa that are underrepresented in African cohorts.

DNA extraction methodologies may also introduce compositional bias through differential lysis efficiency, potentially influencing observed viral abundance patterns [73]. Furthermore, translating metagenomic signals into successful viral or host cultivation remains challenging, as genomic data alone do not reliably predict growth requirements, metabolic dependencies, or ecological interactions within the gut ecosystem. Importantly, the present study focused exclusively on DNA viruses due to practical constraints related to RNA stability in field-collected stool samples. During intermittent sample collection between March 2020 and May 2021, immediate RNA preservation or flash freezing was not consistently feasible. Given the susceptibility of RNA to degradation under suboptimal cold-chain conditions [74], RNA viruses were not captured and may represent an underexplored component of CRC-associated virome dynamics.

Cohort size, statistical considerations, and site effects

The overall cohort size, particularly within the CRC group, was modest. Although significant differences were observed in diversity and functional enrichment analyses, limited sample size reduces power to detect subtle biological effects and increases susceptibility to inter-individual variability. Accordingly, these findings should be interpreted as hypothesis-generating rather than confirmatory. Given the high dimensionality of metagenomic datasets, multiple testing introduces a risk of false positives; differential abundance analyses were therefore conducted using count-based modelling with false discovery rate correction to mitigate type I error, although residual uncertainty cannot be entirely excluded. Validation in larger, independent cohorts will be required. CRC samples were collected from two hospitals within the same metropolitan region using standardized protocols; however, formal batch correction was not applied. While sample processing procedures were harmonized, subtle site-related influences cannot be completely excluded. In addition, the CRC sample size was insufficient to support reliable stage-stratified analyses. Inclusion of staging data under these conditions would have risked over-interpretation, and future adequately powered studies should incorporate stage-specific assessments.

Clinical and environmental confounders

Virome composition is shaped by environmental exposure, host immunity, and clinical management. All HIV-infected participants were receiving antiretroviral therapy (ART), reflecting real-world practice and reducing within-group heterogeneity. However, this limits the ability to disentangle the independent contribution of HIV infection from ART-associated virome modulation. Additional environmental and clinical covariates were not comprehensively available for multivariable adjustment and may contribute to residual variability across groups.

Statistical modelling approach

Differential abundance testing was performed using DESeq2, a negative binomial framework widely adopted for overdispersed count data, including DNA-derived metagenomic stool datasets [75,77]. Although originally developed for RNA-seq analyses, DESeq2 models dispersion directly from the data and incorporates size factor normalisation to account for library depth variation. Benchmarking studies in microbiome and metatranscriptome research demonstrate that DESeq2 maintains robust control of Type I error while providing a reasonable balance between sensitivity and specificity in moderate sample size settings [79]. While compositional methods represent valid alternatives, no single analytical approach is universally optimal, and the selection of DESeq2 was based on dataset structure, dispersion characteristics, and methodological consistency with prior related work [78].

6. Conclusion

High-throughput shotgun metagenomic sequencing has enabled comprehensive characterization of the gut virome, revealing distinct functional signatures associated with helminth and HIV-helminth co-infections that parallel changes observed in CRC. Previously observed alterations in the gut virome signatures, such as *Siphoviridae* and *Myoviridae* in CRC, together with our findings in the HIV-helminth co-infected gut virome, indicate a potential virome-level link between CRC and HIV-helminth co-infection. Notably, upregulation of GTP-binding domains (PF10396 and PF12631) and K03650 in helminth-infected individuals, and the shared enrichment of tRNA modification enzymes, GTP-binding proteins, and replication initiator proteins in both HIV-helminth co-infected and CRC groups highlight virome-mediated metabolic and genomic remodeling. These molecular alterations suggest that chronic co-infection may prime the gut environment for oncogenic signaling and genomic instability, increasing CRC susceptibility.

Our findings underscore that virome functional dynamics play a critical role in shaping disease risk in immunocompromised and parasitized hosts. This work supports the concept of the gut virome as an active intermediary in infection-driven carcinogenesis and proposes candidate virome-derived biomarkers for early cancer risk assessment. Given the high burden of HIV and helminth co-infections in many regions, integrating virome functionality into cancer risk frameworks is imperative. Future longitudinal and multi-omics studies, including mucosal sampling where possible, are needed to validate these mechanistic links and to guide targeted interventions in vulnerable populations.

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CHAPTER FIVE

Identification of specific CRC-associated methylated genes in HIV-helminth co-infected individuals as potential biomarkers for screening susceptibility to cancer development

In Chapter 4, we explored the gut virome landscape in HIV-helminth co-infected individuals, revealing shifts that may contribute to colorectal cancer (CRC) risk. While these microbial and viral signatures offer important insights, their translation into practical screening tools requires biomarkers that are both reliable and feasible for population-level use. DNA methylation analysis of stool-derived host DNA has emerged as one such approach. In this chapter, we strategically focus on vimentin methylation, a well-established, clinically validated CRC biomarker that can be detected in stool samples. This choice is informed by its demonstrated utility in diverse populations and its suitability for non-invasive screening, particularly in low-resource settings. Both HIV and helminth infections may influence methylation patterns, raising the possibility that HIV-helminth co-infection could alter the detectability or prevalence of vimentin methylation in stool DNA. This chapter investigates whether HIV-helminth co-infection impacts two key CRC screening markers, fecal occult blood and methylated vimentin, with CRC patient samples serving as positive controls. Through this approach, we aim to determine the utility of combining established molecular and biochemical markers to refine CRC risk assessment in populations with high HIV-helminth co-infection burdens.

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Vimentin Methylation as a Potential Screening Biomarker for Colorectal Cancer in HIV-Helminth Co-Infected Individuals

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Abstract

Colonoscopy remains the gold standard for colorectal cancer (CRC) screening, but its invasiveness, cost, and limited availability in resource-constrained settings pose major barriers. Stool-based methylated DNA biomarkers, such as vimentin, offer sensitive, non-invasive alternatives. Given the high burden of HIV and helminth co-infections in sub-Saharan Africa and their potential contribution to cancer susceptibility, this study investigated whether stool-derived vimentin methylation could detect early oncogenic changes in these high-risk groups. In this retrospective cross-sectional study, archived stool samples from 62 South African adults were stratified into five groups: uninfected controls, HIV-infected only, helminth-infected only, HIV-helminth co-infected, and CRC-confirmed patients. DNA was extracted, bisulfite-converted, and analyzed for vimentin methylation using a high-resolution melt assay. Fecal occult blood testing (FOBT) was also performed. Vimentin methylation differed significantly across groups ($p < 0.0001$). CRC cases showed 90% methylation, confirming its role as a CRC biomarker. Interestingly, vimentin methylation frequencies were also observed in HIV-only (92.9%, $p < 0.0001$ vs. controls), helminth-only (93.3%, $p < 0.0001$), and HIV-helminth co-infected (77.9%, $p < 0.0001$) individuals without diagnosed cancer, compared to 10% in controls. Methylation levels in infected groups were not significantly different from CRC patients (all $p > 0.05$), suggesting infection-induced epigenetic changes of comparable magnitude to malignancy. To support these results, DNMT1–RG108 molecular docking (PDB 4WXX, Maestro 2025-3) demonstrated stable binding (GlideScore −6.285 kcal/mol; ΔG_bind −49.61 kcal/mol) via hydrogen bonding with Glu1266 and Asn1578 and π–π stacking with Phe1145, providing a mechanistic explanation for infection-driven vimentin methylation. No significant differences were found between infected groups. FOBT was positive in 83.3% of CRC cases, with only sporadic positives in infected groups. These findings provide novel evidence that chronic HIV and helminth infections are associated with vimentin promoter methylation at levels indistinguishable from CRC. This supports the hypothesis that persistent infection-driven

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inflammation promotes early epigenetic reprogramming toward oncogenesis. In high-burden African settings, stool-based methylation assays could serve as early diagnostic tools to identify at-risk individuals long before clinical disease manifests, enabling targeted surveillance and prevention.

Keywords: HIV-helminth coinfection; fecal occult blood; stool vimentin methylation; stool DNA biomarker; early detection; colorectal cancer risk

1. Introduction

Several helminth species, especially schistosomes, have been implicated in the initiation and progression of cancer. The association between schistosomiasis and cancer was first described in the early 1900s, with *Schistosoma haematobium* strongly linked to bladder cancer and Schistosoma mansoni to hepatic carcinoma [1]. More recent studies have expanded this view, showing that additional helminths, including blood flukes (*Schistosoma haematobium*, *Schistosoma japonicum*, *Schistosoma mansoni*) and liver flukes (*Clonorchis sinensis*, *Opisthorchis viverrini*), contribute to human cancers [2]. However, current evidence supports but also complicates this association. Experimental studies indicate that Schistosoma and Trichuris infections promote CRC progression, whereas Taenia species may exert a protective role [3]. Schistosoma japonicum induces immunosuppressive responses that drive CRC and worsen outcomes. Interestingly, other parasites such as Eimeria granulosa can trigger anti-cancer immune activity at early stages of infection, but this effect diminishes as the infection advances, shifting immunity toward a Th2-dominant profile that promotes tumor growth and metastasis [3,4]. It is important to note that the protective effects of helminth infections may be context dependent. For example, in co-infections with Helicobacter pylori, helminths can skew immunity toward Th2 and regulatory pathways, mitigating the pro-inflammatory damage induced by *H. pylori* and thereby reducing the risk of gastric disease complications, including cancer [5]. However, in the setting of HIV, which is already well known for its immunosuppressive effects and modulation of genetic pathways that increase susceptibility to cancer and infections, including helminths [6], the dynamics shift dramatically. In this context, some helminths, particularly the intestinal groups, not only promote a cancer-favoring immunosuppressive microenvironment but also contribute to direct mechanical damage to the colon. Moreover, through the release of secretory molecules, helminths further modulate host immunity and tissue integrity, ultimately supporting carcinogenesis [7].

Further cancer-promoting mechanisms shared by helminth infections and the tumor microenvironment (TME) involve shifts in immune cell metabolism. Helminths modulate the metabolic programming of infiltrating immune cells, particularly macrophages. For example, pattern recognition receptor activation, such as toll-like receptor 4 (TLR4) signaling by lipopolysaccharide, enhances hypoxia-inducible factor 1-α (HIF1-α) expression in M1 macrophages, driving glycolysis for rapid energy acquisition. In contrast, helminth infections promote interleukin-4 (IL-4) production and polarization toward M2 macrophages. IL-4, a hallmark of type 2 immune responses, enhances oxidative phosphorylation (OXPHOS) and mitochondrial respiration in M2 macrophages surrounding parasites. This metabolic reprogramming, with reduced glycolysis, decreases the availability of intermediates required for nucleotide synthesis and limits proliferative capacity in macrophages. Notably, parallels can be drawn between helminth-infected tissue and the TME: in both contexts, M1/Th1 phenotypes are suppressed, impairing the energy-intensive demands of infiltrating immune cells and creating an immunosuppressive niche

favorable for disease progression. This convergence highlights how helminth infections may reinforce tumor-associated metabolic programs, further promoting cancer initiation and progression [7].

Helminth infections contribute to genetic instability, a hallmark of CRC. Jusakul et al. [8] showed that in fluke-associated cholangiocarcinoma (CCA), widespread epigenetic disruption drives spontaneous 5-methylcytosine deamination and CpG > TpG transitions, whereas in fluke-negative CCA, intrinsic mutations occur first, followed by methylation changes. Integrative clustering revealed four CCA subgroups: fluke-positive tumors enriched in ERBB2 amplifications and TP53 mutations, and fluke-negative tumors marked by either high copy-number alterations with PD-1/PD-L2 expression or epigenetic mutations (IDH1/2, BAP1) with FGFR/PRKA rearrangements. Whole-genome analysis also identified FGFR2 3′ UTR deletions as a driver of FGFR2 upregulation, alongside noncoding promoter mutations altering H3K27me3 sites. Distinct DNA hypermethylation patterns further suggest divergent mutational pathways between clusters [8]. These findings highlight how helminth infections not only remodel immunity and metabolism but also fuel genetic and epigenetic instability, processes central to CRC initiation and progression. Another helminth-associated pro-cancer mechanism, similar to HIV immune suppression, includes the increased expression of immune checkpoints such as PD-1. This limits the cancer-immune surveillance.

CRC harbors oncogenic mutations that serve as potential biomarkers and treatment targets. Amid the global burden of cancer, which claimed approximately 10 million lives worldwide in 2022 [9], efforts in therapeutic research are increasingly focused on targeted therapies. However, access to these advanced treatments remains determined by socioeconomic disparities, particularly pronounced in developing regions. In South Africa, communities are starkly divided into rural, township, and suburban areas, with rural populations often comprising the most economically disadvantaged. Healthcare resources, including state-of-the-art facilities, are predominantly concentrated in suburban private hospitals, accessible to a minority with greater financial means [10]. Helminth infections are highly prevalent among people living in rural and low-resource peri-urban areas, where access to costly CRC screening methods such as colonoscopy is limited. Identifying predictive CRC biomarkers in these populations could enable the establishment of targeted screening programs for individuals with helminth infections, either alone or in combination with HIV co-infection [11,12].

Dysregulated methylated events were shown to be potentially effective, non-invasive, and easily accessible screening markers of CRC. In 2004, a stool DNA panel containing APC, TP53, KRAS, and BAT26 could detect CRC at 52% sensitivity and 94% specificity. The stool DNA panel detected 16 of 31 invasive cancers, and the fecal occult blood test (FOBT) detected 4 of 31 cancers with a sensitivity of 12.9%. There was a difference of 13 cancers that were not detected by FOBT but picked up by the stool DNA panel [13]. The FOBT has been used routinely over the years as a diagnostic marker for CRC [14]. The effectiveness of using a multitarget stool sample was further studied by this approach to a commercial fecal immunochemical test (FIT) in average-risk CRC patients. The DNA test showed higher sensitivity for detecting CRC (92.3%) and advanced precancerous lesions (42.4%), exceeding FIT by about 20 percentage points. [15]. Similarly, another study reported that stool DNA–Fecal Occult Blood Testing (sDNA-FOBT) exhibited significantly higher sensitivity for the detection of CRC and precancerous lesions compared with conventional FOBT (61.70% vs. 51.06%, $p < 0.01$). The authors concluded that sDNA-FOBT represents a promising non-invasive strategy for CRC screening. However, they also emphasized the necessity of validating these results in studies with larger cohorts to ensure broader applicability [16].

Vimentin methylation detected in the stool of CRC patients has been shown to have a specificity of 100%, a sensitivity of 60%, and a positive predictive value of 100% [17]. Vimentin methylation assay is now a validated test known as the ColoGuard assay, used for the early detection of CRC. Epi proColon® 2.0 CE is another blood-based, FDA-approved test for screening of CRC [18]. These methods have been shown to be more cost-effective than the current widespread use of colonoscopy, especially in less privileged areas such as sub-Saharan Africa. The use of stool-derived DNA-based assays allows collection of stool from patients at the comfort of their own home, which will later be dropped off at the nearest health facility for transport to relevant testing sites. Studies comparing cost effectiveness between the colonoscopy and methylation studies have confirmed the cost effectiveness of the latter. For instance, a study evaluating CRC screening strategies in Alaska Native populations compared multitarget stool DNA (MT-sDNA) testing, colonoscopy, and FIT. All three approaches reduced healthcare costs and improved quality-adjusted life years (QALYs) relative to no screening. Among the strategies, MT-sDNA provided the greatest gain in QALYs. Using a Markov model, the analysis demonstrated that MT-sDNA screening was cost-effective compared with both colonoscopy and FIT across a broad range of adherence scenarios [19]. A study by Rui et al. (2025) found that all noninvasive CRC screening methods they investigated decreased cancer incidence and mortality compared with no screening. Under perfect adherence, colonoscopy every 10 years was the most cost-effective, but in real-world settings, every-3-year multitarget stool RNA testing (mt-sRNA) was preferred. Cost, test performance, and adherence were key determinants. Thus, this indicated that noninvasive methods can be cost-effective alongside all guideline-recommended strategies [20]. Similarly, another study showed that a minimum Centers for Medicare & Medicaid Services' thresholds (CMS-minimum) blood-based test reduced cancer burden but was less effective than multitarget stool DNA testing, FIT, or colonoscopy, and would only rival FIT if participation rates were markedly higher. Together, these findings reinforce the value of DNA-based stool assays as cost-effective, noninvasive strategies, particularly when adherence to traditional screening methods is suboptimal [21]. Therefore, this study used vimentin methylation and FOBT to detect potential CRC risk in HIV and helminth infected individuals. According to our knowledge, no study has looked at CRC predictive molecular markers in HIV alone and HIV-helminths co-infected patients in Sub-Saharan Africa. Thus, the overarching goal of this study is to assess the association of helminth infections with oncogenesis concerning immunomodulation, particularly in HIV-helminth co-infected individuals.

2. Materials and Methods

2.1. Study Design and Setting

This was a retrospective cross-sectional sub-study nested within a larger ethically approved project (BE351/19). Additional approval for this sub-study was granted by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC/00005458/2023). The main study was conducted in a peri-urban area south of Durban, KwaZulu-Natal, South Africa, between March 2020 and May 2021. A detailed account of the study design and setting has been published [22].

2.2. Study Population and Sampling

This sub-study utilized stored stool samples collected from consenting participants enrolled in the main study. During the collection process, unpreserved stool samples were aliquoted immediately after collection and stored at −80 °C until DNA extraction. The interval between collection and analysis ranged from approximately 2 to 3 years. Although stool without preservative may be more prone to DNA degradation at room

temperature, prior studies have shown that nucleic acids remain stable when promptly frozen and maintained at −80 °C. Sixty-two (62) participants were purposively selected based on their infection status and the availability of complete datasets for subsequent analyses. Participants were stratified into five groups: HIV-negative and helminth-negative uninfected controls (n = 10), CRC-positive controls (n = 10), HIV-infected only (n = 14), helminth-infected only (n = 15), and HIV-helminth co-infected (n = 13). HIV infection was confirmed using standard serological assays in line with national guidelines, while helminth infections were identified through stool microscopy and antigen-based tests, as described in our related publication [23] and unpublished data. Ambiguous or discordant cases were excluded from downstream analyses. These combined diagnostic and stratification approaches ensured accurate classification and minimized bias from mixed or uncertain infections. The CRC positive controls were confirmed cancer patients due for surgery. Control reactions for methylation-specific PCR were prepared using the EpiTect control DNA set (QIAGEN, Hilden, Germany) and used to verify primer specificity. A 50% methylated control was generated by mixing equal volumes of 100% methylated and 0% methylated bisulfite-converted DNA.

2.3. Experimental Procedures

DNA Extraction from Stool Samples

DNA was extracted from stool samples using the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Irvine, CA, USA), optimized for microbiome and metagenome analyses. Approximately 200 mg of stool was added to a ZR BashingBead™ Lysis Tube, followed by the addition of 750 µL ZymoBIOMICS™ lysis solution. The tube was capped tightly and subjected to mechanical lysis using an Omni Bead Ruptor 4 Bead Mill homogenizer (Lasec, Johannesburg, South Africa) operated under optimized speed and duration settings suitable for 2 mL tube holders. Following lysis, tubes were centrifuged at 10,000× *g* for 1 min to pellet the debris. Up to 400 µL of the resulting supernatant was transferred to a Zymo-Spin™ III-F filter placed in a collection tube and centrifuged at 8000× *g* for 1 min before the filter was discarded.

A volume of 1200 µL of ZymoBIOMICS™ DNA binding buffer was added to the filtrate and mixed thoroughly, and 800 µL of the mixture was applied to a Zymo-Spin™ IICR column in a collection tube before centrifuging at 10,000× *g* for 1 min. This step was repeated to ensure maximum DNA capture. The bound DNA was then washed by sequential addition of 400 µL of ZymoBIOMICS™ DNA wash buffer 1 and two washes with ZymoBIOMICS™ DNA wash buffer 2 (700 µL followed by 200 µL), with centrifugation at 10,000× *g* for 1 min after each wash. The spin column was then transferred to a clean 1.5 mL microcentrifuge tube, and DNA was eluted by adding 100 µL of ZymoBIOMICS™ DNase/RNase-free water directly to the column matrix. After incubating for 1 min, the column was centrifuged at 10,000× *g* for 1 min to collect the purified DNA. To remove potential PCR inhibitors, 600 µL of ZymoBIOMICS™ HRC prep solution was added to a Zymo-Spin™ III-HRC filter in a fresh collection tube and centrifuged at 8000× *g* for 3 min. The previously eluted DNA was then passed through the prepared HRC Filter by centrifugation at exactly 16,000× *g* for 3 min. The resulting DNA was free of inhibitors and suitable for PCR and other downstream applications.

Bisulfite Conversion of Genomic DNA

Bisulfite modification of the eluted DNA was performed using the EpiJET Bisulfite Conversion Kit (Thermo Fisher Scientific, Waltham, MA, USA). Prior to use, the wash buffer, desulfonation buffer, and modification reagent were prepared according to the manufacturer's instructions. For each reaction, 20 µL of 400 ng genomic DNA was transferred to a PCR tube. A total of 120 µL of freshly prepared modification reagent was added to

each sample, mixed by pipetting, and centrifuged briefly. Bisulfite conversion was carried out using protocol A on the manufacturer's instruction manual. The protocol consists of incubation at 98 °C for 10 min followed by 60 °C for 150 min in a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Following conversion, 400 µL of binding buffer was added to a DNA purification microcolumn placed in a collection tube. The converted DNA sample was then added to the column and mixed thoroughly before centrifugation at 12,000 rpm for 30 s, and the flow-through discarded. The column was washed with 200 µL of wash buffer, centrifuged again, and treated with 200 µL of desulfonation buffer for 20 min at room temperature. After another centrifugation at 12,000 rpm for 30 s, two additional washes with 200 µL of wash buffer were performed, with the final wash centrifuged for 60 s. Purified DNA was eluted by adding 10 µL of elution buffer directly to the column, followed by centrifugation at 12,000 rpm for 60 s. We did not amplify the converted DNA but instead stored it at −20 °C until further use.

Amplifying bisulfite-converted DNA extracted from stool samples presents several technical challenges that can compromise the accurate detection of methylated genes. Firstly, bisulfite treatment chemically modifies unmethylated cytosines to uracil, a process known to cause substantial DNA fragmentation and degradation [24,25]. When combined with the naturally fragmented and low-quality DNA typically recovered from stool, due to enzymatic digestion, bacterial overgrowth, and environmental exposure, the resulting DNA may be too degraded for successful amplification [26]. In addition, bisulfite conversion requires multiple purification steps, each of which contributes to significant DNA loss, particularly problematic in samples with limited input material [27]. Finally, primer design for bisulfite-converted DNA is complicated due to the non-complementary nature of converted strands and the loss of cytosine-guanine pairing. Without carefully optimized, short amplicon primers, amplification is often inefficient or fails altogether [28]. An in-house optimization was performed using previously established primer sets, as shown in Table 1.

Table 1. Primers and probes for bisulfite-converted DNA.

Gene	Primer/Probe	Primer Sequences	References
Vimentin	Forward	5′TCGTTTCGAGGTTTTCGCGTTAGAGAC-3′	[17]
	Reverse	5′CGACTAAAACTCGACCG ACTCGCGA-3′	
ACTB	Forward	GAAAGGGTGTAGTTTTGGGAGGTTAG	[29]
	Reverse	AATAACCCAAATAAATAACCCACTACCTC	

High-Resolution Melting Analysis

High-Resolution Melting (HRM) analysis enhances post-PCR analysis by enabling precise genotyping and mutation scanning through the detection of subtle DNA sequence variations. It shortens analysis time by auto-calling genotypes, excluding no-template controls, and reducing subjectivity via automated clustering of unknown variants. The platform also supports streamlined data visualization and multi-target analysis on a single plate, improving workflow efficiency.

High-resolution melt analysis was performed using the MeltDoctor™ HRM Reagents (Thermo Fisher Scientific, Waltham, MA, USA) on a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA), in combination with the high-resolution Melt Software v3.0 for data analysis. Each HRM reaction was carried out in a final volume of 10 µL using the MeltDoctor™ HRM Master Mix, with 10 ng of bisulfite-converted template DNA as input. To validate HRM performance and ensure accurate methylation discrimination, we included the EpiTect PCR Control DNA Set (Qiagen, Hilden, Germany)

in each experiment. This human DNA control set contains both bisulfite-converted methylated and unmethylated DNA, as well as unconverted unmethylated DNA. These were used to generate standard curves and validate primer efficiency for methylation-sensitive discrimination. Reactions were assembled on ice and included 1x MeltDoctor™ HRM Master Mix, 0.2 µM each of forward and reverse primers specific for the target region, and DNA template. Negative controls (no-template controls) and positive controls from the EpiTect DNA set were included in all runs. Thermal cycling parameters, including denaturation, annealing/extension, and HRM steps, were optimized. HRM analysis was performed with a continuous ramping mode selected and a ramp rate of 0.1 °C/s. Data acquisition occurred continuously throughout the melting phase. Instrument filters were optimized using the HRM-specific Filter-1 setting (Table 2).

Table 2. Thermal cycling conditions.

Step	Temperature (°C)	Time	Notes
Initial denaturation	95	10 min	Polymerase activation
Denaturation	95	15 s	
Annealing/Extension	60	60 s	Data collected during this step
Steps 2–3 were repeated		40 cycles	
HRM preconditioning	95	10 s	
HRM step	60	Ramp at 0.1 °C	Continuous ramp with data acquisition

Following amplification, the melt curves were normalized, temperature-shifted, and analyzed using the high-resolution Melt Software v3.0 (Thermo Fisher Scientific, Waltham, MA, USA), which enables genotype and methylation calling based on curve shape and melting temperature differences. All assays were run in triplicate, and only samples with consistent amplification and clear melting profiles were included in the final analysis.

Validation of methylation cutoffs

To confirm assay specificity and ensure reliable interpretation of methylation status, commercially available DNA standards, EpiTect PCR Control DNA Set (Qiagen, JHB, SA, Cat. No. 59695), representing 0%, 50%, 75%, and 100% methylation, were used. The 0% and 100% standards served as negative and positive controls, respectively, while intermediate ratios (50% and 75%) were generated by mixing defined amounts of fully methylated and unmethylated DNA as per the manufacturer's instructions. This gradient provided reference points to validate the assay's ability to detect both full and partial methylation.

Computational Structural Modeling

The molecular docking and binding-free-energy analyses were performed using the Maestro v14.5.131 and MMshare v7.1.131 (Schrödinger Suite Release 2025-3, Windows x64). The modules used, Glide in SP (Standard Precision) mode and Prime MM-GBSA re-scoring, are integral components of this release. The crystal structure of human DNA methyltransferase 1 (DNMT1; PDB ID: 4WXX) was imported into Maestro and prepared with the Protein Preparation Wizard. Chain A, which contains the catalytic domain and the co-crystallized S-adenosyl-homocysteine (SAH) cofactor, was retained, whereas chain B and non-essential ions or solvent molecules were removed. Bond orders were reassigned, hydrogens were added, and missing side chains were rebuilt. The structure was optimized with the OPLS4 force field at physiological pH 7.4 ± 0.5 and heavy-atom restraints (RMSD 0.30 Å).

RG108 (N-[4-(hydroxycarbamoyl) benzoyl] acetamide; PubChem CID 6626651), a non-nucleoside DNMT1 inhibitor, was prepared using Lig-Prep (OPLS4, Epik

pH 7.4 ± 0.5). A receptor grid was generated around the centroid of the SAH binding pocket with inner and outer boxes of 10 Å and 24 Å, respectively, encompassing residues Cys1226, Pro1225, Gly1223, Glu1266, Arg1310, and Asn1578. Docking was executed with Glide SP (enhanced sampling and post-minimization), followed by Glide XP refinement of the five best SP poses. Binding-free energy (ΔG_bind) was estimated with Prime MM-GBSA (OPLS4/VSGB 2.0).

Fecal Occult Blood Test

The fecal occult blood test (FOBT) was performed on stool samples from the same cohort of 62 participants using the OnSite FOB-Hi Rapid Test-Cassette (CTK Biotech, Poway, CA, USA) according to the manufacturer's instructions. This test is a lateral flow immunoassay designed to qualitatively detect human hemoglobin in stool samples with a sensitivity threshold of 25 ng/mL. A small amount of stool sample was transferred into the sample collection tube containing the extraction buffer. After shaking the tube to ensure homogenization of the stool sample, 3 drops of the diluted specimen were added to the sample well of the test cassette. Results were interpreted visually within 5–10 min, following the appearance of pink to red-colored lines with a control line (C) indicating a valid test function and a test line (T) indicating the presence of occult blood. A test was considered positive if both C and T lines appeared, regardless of T line intensity, negative if only the C line appeared, and invalid if the C line was absent.

Statistical analysis

The correlation between infected and uninfected VIM methylation status was analyzed using the Chi-squared (χ^2) test and Fisher's exact test. A p-value of <0.05 was considered statistically significant. GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA) statistical software package was used for analysis.

3. Results

3.1. Assessment of Vimentin Methylation Across CRC and Infection-Related Groups

Figure 1 below represents a melt curve analysis of standard and reference genes showing distinct peaks corresponding to 0%, 50%, 75%, and 100% methylation levels, prepared from mixtures of fully methylated and unmethylated control DNA. During optimization, the reference gene (ACTB) consistently aligned with the 0% methylated standard, confirming assay specificity. These standards were therefore used to calibrate the HRM assay. The clear separation of curves confirms the assay's ability to discriminate between different methylation levels, validating the experimental conditions.

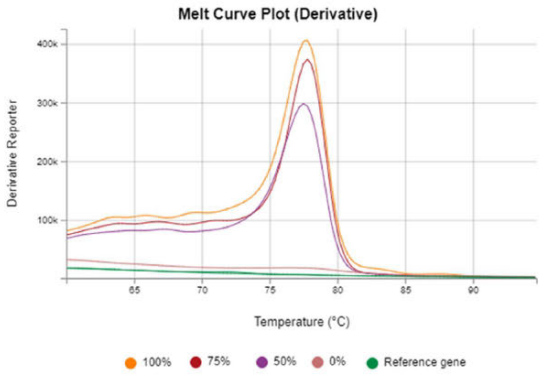


Figure 1. DNA standards Melt Curve plot.

Validation of the assay using methylated DNA standards at 0%, 50%, 75%, and 100%. The primers were methylation-specific, targeting CpG sites within the region of interest. The reference gene (ACTB), not subject to methylation at these sites, served as a control and aligned with the 0% methylated standard.

Vimentin methylation was consistent across all infected groups, with 90% methylation observed in the CRC-confirmed group, supporting its established role as a sensitive stool-based biomarker for CRC. Notably, methylation frequencies were also observed in infected groups: 92.9% in HIV-infected only, 93.3% in helminth-infected only, and 77.9% in HIV-helminth co-infected individuals, compared to only 10% in HIV- and helminth-uninfected controls (Figure 2).

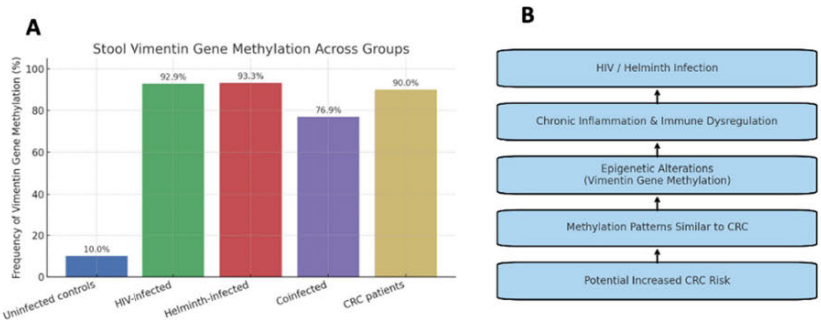


Figure 2. Correlation of Vimentin Methylation Patterns with Colorectal Cancer and Chronic Infections. (**A**) Stool vimentin methylation was observed in HIV-, helminth-, and co-infected individuals, at frequencies comparable to colorectal cancer patients. Although further studies are needed to confirm this, it is important to note that these observations suggest that chronic infection-associated methylation signatures might overlap with those previously described in colorectal cancer, posing challenges for cancer screening in regions endemic for infection. (**B**) This infographic summarizes how chronic infections like HIV and helminths cause inflammation and immune disruption, leading to vimentin gene methylation patterns similar to those in colorectal cancer patients. It highlights the potential link between infection-driven epigenetic changes and cancer risk, emphasizing the need for combined infection and cancer prevention efforts.

This distribution revealed significant variation in methylation patterns across uninfected, singly infected, co-infected, and CRC patient groups ($p < 0.0001$). While the uninfected controls exhibited markedly lower methylation compared to CRC patients ($p = 0.0011$), methylation frequencies in CRC patients were not significantly different from those in HIV-infected, helminth-infected, or co-infected individuals. These findings suggest that infection status may drive methylation changes of comparable magnitude to those seen in malignancy. When compared to uninfected controls, significantly higher vimentin methylation frequencies were observed in all infected groups, HIV-infected ($p < 0.0001$), helminth-infected ($p < 0.0001$), and coinfected individuals ($p < 0.0001$). In contrast, no significant differences in methylation levels were detected between the infected groups themselves (HIV versus helminth, coinfected versus HIV, and coinfected versus helminth) (Table 3). This indicates that while infection, whether by HIV, helminths, or both, drives elevated vimentin methylation, the type of infection does not appear to substantially influence the extent of methylation once it has occurred.

Table 3. Frequency of stool vimentin gene methylation in HIV and helminth uninfected, single-infected, and coinfected individuals, and colorectal cancer patients.

Group, (n)	Methylation, n (%)	Non-Methylation, n (%)	*p* Value	Odds Ratio (95% CI)
Uninfected controls (n = 10)	1 (10)	9 (90)		
HIV-infected (n = 14)	13 (92.9)	1 (7.1)		
Helminth-infected (n = 15)	14 (93.3)	1 (6.7)	<0.0001 *	
Coinfection (n = 13)	10 (76.9)	3 (20)		
CRC confirmed patients (n = 10)	9 (90)	1 (10)		
HIV and helminth uninfected, single-infected and coinfected individuals versus CRC confirmed patients (Positive Control)				
Uninfected controls versus CRC confirmed patients			0.0011	0.01 (0.00–0.23)
HIV-infected versus CRC confirmed patients			1.0000	1.44 (0.08–26.25)
Helminth-infected versus CRC confirmed patients			1.0000	1.56 (0.09–28.17)
Coinfection versus CRC confirmed patients			0.6036	0.37 (0.03–4.23)
HIV and helminth single and coinfected groups Versus Uninfected controls (Negative Control)				
HIV-infected versus Uninfected controls			<0.0001	117 (6.44–2126)
Helminth-infected versus Uninfected controls			<0.0001	126 (6.96–2281)
Coinfection versus Uninfected controls			<0.0001	30 (2.62–342.9)
Comparison between infected groups				
HIV-infected versus Helminth-infected			1.0000	0.93 (0.05–16.43)
Coinfection versus HIV-infected			0.3259	0.26 (0.02–2.85)
Coinfection versus Helminth-infected			0.3111	0.24 (0.02–2.64)

Footnote: * Denotes *p*-value was derived using the Chi-squared (χ^2) test. All other *p*-values were derived using Fisher's exact test.

The amplification of vimentin in CRC-confirmed samples is consistent with previous reports highlighting vimentin methylation as a potential early biomarker of colorectal oncogenesis [17].

3.2. Computational Docking and Binding-Energy Results

Docking confirmed that RG108 binds stably within the DNMT1 catalytic pocket that normally accommodates the SAM/SAH cofactor. In *Glide SP* mode, RG108 achieved a GlideScore of −6.285 kcal mol^{-1} with an Emodel of −59.3, indicating strong complementarity between ligand and receptor. *XP* redocking maintained the same orientation (GlideScore −5.293 kcal mol^{-1}), validating pose convergence and conformational stability. The 3D model (Figure 3) revealed that the hydroxamate and amide moieties of RG108 insert between Cys1226 and Glu1266, forming hydrogen bonds with Glu1266 (OE1, 2.4 Å) and Asn1578 (OD1, 2.8 Å). A π–π stacking interaction was observed between the benzoyl ring of RG108 and Phe1145, while Pro1225 and Gly1223 contributed additional van der Waals stabilization. Trp1170, Val1144, and Leu1247 reinforced hydrophobic contacts, and Glu1168 and Arg1574 maintained solvent-exposed hydrogen bonds at the pocket entrance.

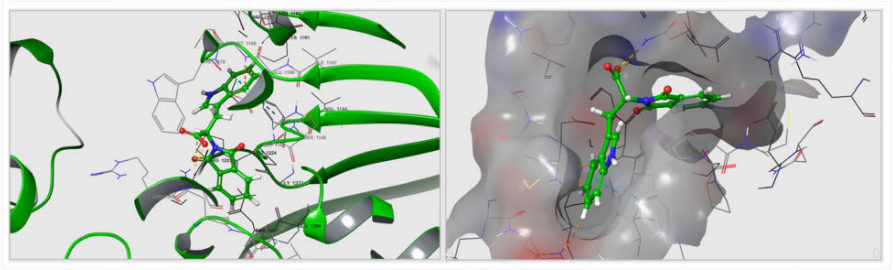


Figure 3. DNMT1–RG108 3D binding pose. Three-dimensional representation of RG108 (green sticks) bound within the DNMT1 catalytic pocket (PDB 4WXX). Hydrogen bonds (yellow dashed lines) involve Glu1266, Asn1578, and Pro1225; hydrophobic contacts include Phe1145, Trp1170, and Val1144.

The 2D interaction diagram (Figure 4) confirmed these contacts: green lines mark hydrogen bonds to Glu1266 and Asn1578; a violet line indicates π–π stacking with Phe1145; and shaded regions denote solvent exposure around Glu1168, Glu1189, and Asp1190. This configuration shows a partially buried aromatic core with a solvent-accessible hydroxamate terminus. The *Prime MM-GBSA* calculation produced a ΔG_bind of −49.61 kcal mol^{-1}, confirming a strong and energetically favorable complex. Major residue contributions were Glu1266 (−5.84), Asn1578 (−4.19), Phe1145 (−3.71), Pro1225 (−3.26), Cys1226 (−2.88), Trp1170 (−2.33), and Arg1574 (−1.95) kcal mol^{-1} (Table 4).

Table 4. Prime MM-GBSA binding-energy decomposition for the DNMT1–RG108 complex.

Residue	ΔG (kcal mol^{-1})	Interaction Type
Glu1266	−5.84	Hydrogen bond/electrostatic
Asn1578	−4.19	Hydrogen bond
Phe1145	−3.71	π–π stacking/hydrophobic
Pro1225	−3.26	Hydrophobic/backbone contact
Cys1226	−2.88	Catalytic loop stabilization
Trp1170	−2.33	Hydrophobic
Arg1574	−1.95	Polar/solvent-exposed
Total ΔG_bind	−49.61	

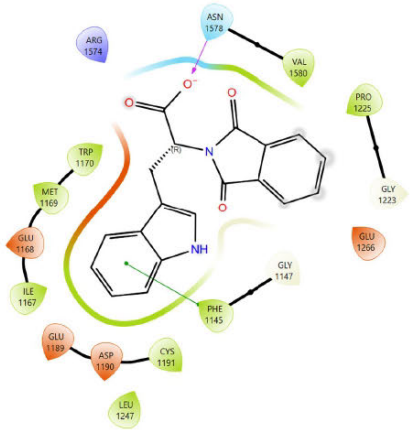


Figure 4. DNMT1–RG108 2D interaction diagram. Two-dimensional schematic generated in Maestro. Green lines show hydrogen bonds with Glu1266 and Asn1578; a violet line indicates π–π stacking with Phe1145; shaded regions depict solvent-exposed polar residues (Glu1168, Glu1189, Asp1190) surrounding the hydroxamate terminus.

Per-residue energies confirm that Glu1266, Asn1578, and Phe1145 dominate RG108 stabilization within the DNMT1 catalytic pocket (Figure 3).

3.3. Assessment of Fecal Occult Blood Test Across CRC and Infection-Related Groups

Among the CRC confirmed group, 10 out of 12 samples (83.3%) tested positive for occult blood. In contrast, only 1 sample each from the HIV-only infected group (1/13; 7.7%) and the HIV-helminth co-infected group (1/12; 8.3%) showed a positive FOBT result. All other participants, including those in the helminth-only group (0/15) and HIV and helminth-uninfected controls (0/10), tested negative. Integrating methylation profiling with FOBT enhances the accuracy of CRC screening by compensating for FOBT's limited sensitivity in early lesions. In our cohort, cases with positive FOBT results, particularly those with HIV or HIV/helminth co-infection, also showed altered vimentin methylation, suggesting a potential complementary diagnostic value between the two approaches.

4. Discussion

This study aimed to investigate the association between HIV-helminth co-infections and predictive biomarkers of CRC, focusing on the fecal occult blood test and vimentin methylated DNA marker. Given the high burden of infectious diseases in sub-Saharan Africa and their potential role in chronic inflammation and cancer susceptibility [30], we sought to explore whether individuals with HIV infection only, helminth infection only, or HIV-helminth co-infected individuals exhibit molecular signatures that may reflect susceptibility to CRC development. Understanding such associations is critical for improving non-invasive, accessible CRC screening strategies tailored to high-risk populations in resource-limited settings.

4.1. Vimentin Methylation

Methylation analysis of the vimentin gene in stool samples showed a marked difference between HIV and helminth uninfected controls and all infection groups, supporting our hypothesis that chronic infectious co-states may elevate CRC risk through epige-

netic mechanisms. In HIV-helminth uninfected controls, vimentin methylation was rarely detected (1/10, 10%), in contrast to the CRC-confirmed group (9/10, 90%). Inflammation-associated infectious states mirrored this elevation: HIV-only (13/14, 92.9%), helminth-only (14/15, 93.3%), and HIV-helminth co-infected individuals (10/13, 76.9%). Vimentin methylation is a well-established stool-based biomarker for CRC, with high specificity in distinguishing cancerous lesions from healthy tissue [17,31]. Moreover, infectious agents are increasingly implicated in oncogenic epigenetic modulation. Infection-driven DNA methylation changes are recognized as mechanisms through which pathogens may promote tumorigenesis [32].

The present study findings demonstrate that vimentin gene methylation is not exclusive to CRC but is also significantly elevated in individuals with HIV or helminth infections, irrespective of coinfection status. The comparable methylation levels between infected groups and CRC patients suggest that chronic infection-driven inflammation may induce epigenetic alterations of a magnitude similar to those associated with malignancy. This aligns with evidence linking persistent immune activation and inflammatory signaling to aberrant DNA methylation patterns, which may facilitate tumorigenic pathways [33]. Importantly, in this case, the absence of differences between HIV-only, helminth-only, and HIV-helminth coinfected groups indicates that the presence of infection, rather than its specific etiology, is the dominant driver of this methylation change. Dysregulated DNA methylation is associated with HIV infection, with the potential for developing genetic therapies targeting methylated HIV viral genes [34]. Such infection-associated epigenetic modulation could represent an early carcinogenic event, particularly in populations with high burdens of infectious diseases. These findings show the potential of vimentin methylation as both a biomarker of CRC and a marker of infection-related epigenetic reprogramming, with implications for screening strategies in endemic regions.

4.2. Mechanistic and Translational Insights

The computational modeling provides a structural and energetic context linking DNMT1 inhibition by RG108 to vimentin (VIM) promoter hypermethylation observed experimentally. DNMT1 is essential for maintenance methylation during DNA replication; chronic activation causes aberrant CpG methylation of tumor-suppressor genes, including VIM. Docking and MM-GBSA analyses reveal that RG108 occupies the SAM/SAH-binding channel, establishing strong hydrogen bonds with Glu1266 and Asn1578, and π–π stacking with Phe1145, key residues that govern methyl-transfer catalysis. These contacts obstruct the correct alignment of the methyl donor and acceptor, effectively blocking enzymatic turnover. The top SP pose (GlideScore -6.285 kcal mol^{-1}; Emodel -59.3) and MM-GBSA energy (ΔG_bind -49.61 kcal mol^{-1}) agree with reported affinities for validated DNMT1 inhibitors [35,36]. Electrostatic stabilization dominated by Glu1266, followed by Asn1578 and Phe1145, underscores the role of polar and aromatic interactions in ligand affinity [35].

The structural overlap between RG108 and SAH indicates competitive inhibition, particularly relevant under inflammatory conditions with elevated S-adenosylmethionine (SAM) levels [36,37]. These mechanistic insights extend the biological observations from stool-based methylation analyses. In chronic infections such as HIV and helminthiasis, oxidative and cytokine stress upregulate DNMT1, promoting aberrant VIM methylation. By binding to the catalytic loop (Glu1266-Asn1578-Pro1225), RG108 may suppress infection-driven epigenetic silencing and restore tumor-suppressor gene expression [36,38]. The favorable MM-GBSA profile and stable hydrogen-bond/π–π network confirm RG108's demethylating potential and provide a structural rationale for DNMT1 inhibition in infection-associated colorectal carcinogenesis.

Overall, this computational component strengthens the translational narrative by linking experimental methylation data with a molecular-mechanistic explanation. The integration of in silico docking, MM-GBSA energetics, and experimental methylation profiles establishes RG108 as a mechanistically plausible DNMT1 inhibitor capable of reversing infection-induced VIM silencing and supporting the design of next-generation DNMT1-targeted therapeutics.

With further evaluation of potential markers for CRC susceptibility, the FOBT demonstrated relatively strong diagnostic alignment with CRC, with 83.3% (10/12) in the CRC confirmed group. This supports existing evidence that FOBT, despite its simplicity, remains a useful initial screening tool for CRC, especially in low-resource settings where more advanced molecular testing may be unavailable. However, the detection of positive FOBT results in one participant in the HIV-infected only group and another in the HIV-helminth co-infection group raises concerns about the specificity of FOBT in populations burdened with chronic infections. The detection of blood in these cases may be correlated with previous studies that demonstrated a significantly higher false-positive rate associated with infections [39] or altered mucosal integrity [40]. While FOBT remains cost-effective and non-invasive, its lack of molecular specificity [41] emphasizes the need to complement it with additional validated biomarkers to improve diagnostic accuracy. These findings suggest that combining FOBT with methylation markers, or integrating them into a multimodal screening algorithm, may enhance early CRC detection, particularly in high-risk populations with co-infections.

5. Challenges and Limitations

It is important to note that genetic variability plays an important role in methylation studies. Thus, genetic and epigenetic variability in African populations may influence assay performance. African populations have the greatest genomic diversity globally [42], including polymorphisms that may occur within primer binding sites, potentially affecting amplification efficiency or specificity. In their study on genetic variability in helminth infections, Papaiakovou et al. [43] investigated single-nucleotide variants within nuclear repeat regions, with a specific focus on primer binding sites, to assess their impact on the performance of diagnostic qPCR assays. The authors found that genetic variation within these target regions can significantly influence qPCR assay sensitivity and specificity. Such variability may lead to reduced amplification efficiency or complete failure of detection, thereby increasing the risk of false-negative results. These findings underscore the importance of continuously monitoring genetic diversity in diagnostic targets, particularly in endemic regions, to ensure the reliability and accuracy of qPCR-based diagnostics [43].

Additionally, while the frequency of methylation in infection groups approached that of CRC cases, it is important to consider that its elevation in non-cancer infectious disease cohorts likely reflects background epithelial turnover, chronic inflammation, or infection-associated epigenetic remodeling hence future studies need to also consider the use of blood-based analysis, as indicated in a study by Gopal et al., 2023 to ascertain which route would be best for use as a susceptibility marker [44]. Stool-derived DNA, while advantageous due to its non-invasive collection and acceptability in low-resource settings, can be more variable in quality and prone to degradation over time [45]. Furthermore, sample-to-sample variability, particularly in stool-derived DNA, which contains a complex mixture of human, microbial, dietary, and inhibitory substances, should be taken into consideration when performing this kind of assay. Variability in epithelial cell shedding, the degree of microbial contamination, and PCR inhibitors can all affect DNA quality and downstream assay performance [15].

Optimization of stool-derived DNA methylation markers remains a challenge, and this can be further compounded by variable sample quality [46]. In this study, archived stool samples collected several years prior were utilized. Stool samples are inherently more prone to variability: they contain mixed DNA populations (host, microbial, dietary) and are technically more challenging to preserve than blood or plasma. Studies demonstrate that stool DNA integrity and microbial community composition significantly deteriorate if not frozen within 15 min of defecation, aliquoted immediately, and stored at −80 °C, with degradation increasing with daily freeze–thaw cycles [47]. While some microbiome data remain stable in domestic freezers at −20 °C for up to six months [48], high-sensitivity assays like methylation-specific PCR are likely affected by even small losses in DNA integrity.

This study did not assay IL-6, TNF-α, or similar inflammatory mediators in the same stool samples used for vimentin methylation profiling. Although related work in the same cohort has demonstrated elevated Th1/Th17 cytokine expression in HIV-helminth co-infection [49], the causal relationship between chronic inflammation and methylation remains hypothetical and warrants further targeted investigation. Lastly, we acknowledge that the group sizes in our study are relatively small, which limits the statistical power to detect subtle differences in methylation frequency. However, we used appropriate statistical methods for small samples (Fisher's exact test) and reported exact *p*-values, effect sizes (odds ratio), and 95% confidence intervals as evident in Table 3. We therefore recommend validation in larger, independent cohorts to confirm these findings.

6. Future Directions

Helminth infections are well known to induce a dominant Th2 immune profile characterized by elevated secretion of IL-4, IL-5, IL-10, IL-13, and TGF-β, accompanied by the activation of alternatively activated macrophages (M2 phenotype) and regulatory T cells. These immune signatures create an anti-inflammatory, tissue-repairing milieu that mirrors tumor-promoting environments, favoring immune suppression, angiogenesis, and epithelial proliferation [7]. Chronic exposure to Th2 cytokines such as IL-4 and IL-13 has been shown to enhance cellular proliferation, modulate DNA methyltransferase activity, and promote tumorigenesis through persistent epigenetic reprogramming [50]. To strengthen causal inference, future studies should integrate cytokine profiling with DNA methylation analyses to assess whether specific inflammatory pathways are correlated with methylation at cancer-related loci such as the VIM promoter. Correlative and mediation analyses between Th2 cytokine concentrations and stool-based methylation markers could delineate the contribution of immune dysregulation to epigenetic alterations that precede tumor initiation. Given the ethical limitations of obtaining colon biopsies from asymptomatic HIV- or helminth-infected individuals, future research should prioritize non-invasive sampling approaches. These include fecal cytokine profiling [51], which offers a promising strategy to assess intestinal immune activity alongside stool-based methylation and microbial analyses. Combining these complementary non-invasive biomarkers may enable longitudinal monitoring of infection-associated inflammation and its epigenetic consequences, providing an opportunity to identify early molecular changes that precede CRC development.

Moreover, integrated multi-omics studies that simultaneously assess the gut microbiome, well established for its role in cancer initiation and progression, yet notably altered in the presence of helminth infection [52], together with host cytokine networks and DNA methylation patterns, could elucidate how infectious co-states reconfigure the intestinal microenvironment and contribute to carcinogenesis. Experimental validation in cell-based and animal models should investigate whether parasite-induced cytokines or helminth excretory/secretory molecules (E-S products) drive DNMT expression or direct methylation

of oncogenic targets [53]. Such mechanistic insights may open translational avenues for early detection and intervention, potentially reversing or halting precancerous epigenetic changes following infection clearance or immune modulation. Finally, contextualizing these investigations within Sub-Saharan African settings is essential. In this region, HIV and helminth infections remain prevalent, and access to invasive diagnostic modalities is limited. Validating stool-based DNA methylation and fecal cytokine markers in these populations could enable low-cost, community-based screening for individuals at elevated CRC risk. Ultimately, this integrated approach may inform precision prevention strategies aimed at reducing the infection-associated cancer burden in Africa.

7. Conclusions

This study provides the first evidence linking HIV-helminth co-infections to molecular signatures consistent with increased CRC susceptibility in a Sub-Saharan African context. We demonstrated that stool-based vimentin promoter methylation, a well-established CRC biomarker, was markedly elevated not only in CRC-confirmed patients but also in individuals with HIV infection, helminth infection, and HIV-helminth co-infection, compared to uninfected controls. These findings strongly support our hypothesis that chronic infectious co-states may drive epigenetic remodeling, mimic oncogenic methylation patterns, and potentially predispose to malignancy. However, the clinical utility of infection-associated methylation as a screening tool remains underexplored in the absence of longitudinal follow-up linking these changes to actual cancer development. Thus, this study provides a foundation for future large-scale, long-term studies to validate these observations and establish translational relevance. The FOBT showed substantial concordance with CRC diagnosis, attesting to its ongoing relevance as a low-cost screening tool in resource-limited settings. However, positive FOBT results in the 2 individuals in the non-cancer infection groups highlight the risk of false positives in populations with chronic inflammatory or mucosal pathology, reinforcing the need for molecular adjuncts to enhance specificity. Overall, our findings suggest that integrating stool-based DNA methylation assays might improve early CRC detection in high-risk, infection-burdened populations. Longitudinal studies are now warranted to determine whether infection-associated methylation changes precede neoplastic transformation, thereby offering predictive value for targeted prevention strategies, Figure 5.

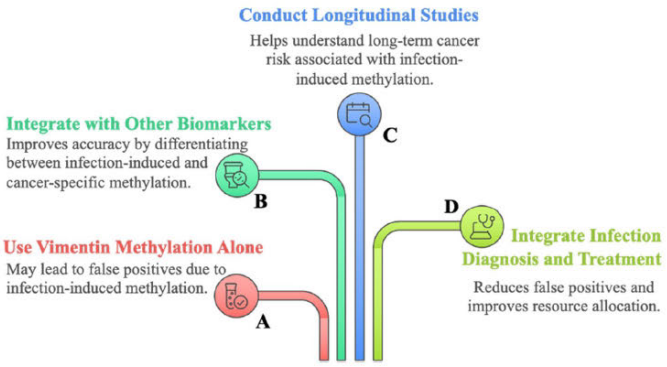


Figure 5. Concluding Figure. Conceptual framework for CRC screening in populations with high HIV and helminth prevalence based on vimentin methylation status. The schematic illustrates different

approaches for interpreting stool-based vimentin methylation results. (**A**) Using vimentin methylation alone may result in false positives due to infection-induced methylation in HIV- and helminth-infected individuals. (**B**) Combining vimentin methylation with other CRC-specific biomarkers enhances screening accuracy by distinguishing infection-induced epigenetic changes from malignant alterations. (**C**) Integration with infection diagnosis and treatment helps reduce false positives, ensuring appropriate resource allocation. (**D**) Conducting longitudinal studies in infected populations allows assessment of long-term CRC risk associated with infection-driven methylation, guiding targeted surveillance strategies. This framework emphasizes the need for a multifaceted approach to early CRC detection in high-risk, infection-burdened regions.

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Abbreviations

The following abbreviations are used in this manuscript:

CRC	Colorectal cancer
CpG	Cytosine phosphate guanine dinucleotide
DNA	Deoxyribonucleic acid
HRM	High-resolution melt
HIV	Human immunodeficiency virus
qPCR	Quantitative polymerase chain reaction

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CHAPTER SIX

Intersecting Pathways in Gut Virome, DNA Methylation, and Metabolism in Infection-Associated CRC Risk

While the assessment of vimentin and FOBT as screening markers provided valuable insights, they only represent part of the biological picture. Colorectal cancer risk in high-burden settings may be influenced by a more complex interaction between chronic infections, viral activity, epigenetic regulation, and cancer-related metabolic pathways. Emerging evidence highlights the gut virome as an active participant in disease processes, capable of altering host gene expression through methylation changes and modulating cellular metabolism to promote tumor development. In populations affected by HIV and helminth infections, these viral influences intersect with persistent inflammation, immune regulation, and tissue remodeling, potentially creating molecular signatures similar to those seen in CRC. In this chapter, we expand our investigation to explore these connections, examining whether virome-driven epigenetic and metabolic changes in HIV-helminth co-infected individuals reflect the patterns observed in CRC patients.

Introduction

Epigenetic modifications play a pivotal role in the onset and progression of disease [1]. The immune evasion strategies employed by helminth infections are well established, and while certain helminths have been linked to cancer, the molecular pathways connecting these infections to carcinogenesis remain poorly understood. Dysregulated DNA methylation has been observed throughout the life cycle of some parasitic infections, such as the nematode Trichinella spiralis [2], and is a recognised driver of CRC initiation [3]. In sub-Saharan Africa, where HIV-helminth co-infections remain prevalent, the potential cancer risk warrants further investigation, particularly given that HIV itself is known to induce aberrant DNA methylation [4]. In addition to epigenetic changes, CRC tumours reprogram metabolic pathways to adapt to hypoxic conditions and to develop resistance to therapy [5]. Similarly, metabolic reprogramming is a hallmark of HIV infection [6,7]. Helminth infections also exploit host metabolic processes to promote their own survival, which further assists in modulating immune responses to facilitate immune evasion [8,9]. Therefore, in this study, we assessed gut virome DNA methylation and cancer metabolism-related gene expression across defined clinical groups. Our goal was to identify molecular signatures that could serve as novel biomarkers of CRC risk in this unique South African cohort.

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This paper aligns with the journal's mission to showcase scientific findings in the African continent. This chapter underscores the importance of integrating microvirome, epigenetic, and metabolic data to inform targeted surveillance and prevention strategies in underrepresented regions.

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Virome remodeling rewires epigenetic and metabolic pathways linked to infection-associated colorectal cancer risk*

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ABSTRACT

Background: Colorectal cancer (CRC) incidence is rising in sub-Saharan Africa, coinciding with the high prevalence of immune-modulating infections such as HIV and helminths. The gut virome, a critical yet understudied component of the microbiome, may influence oncogenic processes through epigenetic and metabolic alterations. However, the interplay between gut viral communities, HIV-helminth co-infection, and CRC risk remains poorly characterized in African populations. This study aimed to investigate gut virome-associated epigenetic and metabolic signatures linked to CRC susceptibility among South African adults, with a focus on HIV and helminth co-infection dynamics.

Methods: Untargeted shotgun metagenomic sequencing was performed on stool DNA samples from 62 adults stratified into five groups: uninfected controls (n = 10), HIV-only (n = 14), helminth-only (n = 15), HIV-helminth co-infected (n = 13), and CRC-confirmed patients (n = 10). Bioinformatic analyses were used to identify differentially abundant viral genes and to functionally annotate epigenetic and metabolic pathways associated with infection status and CRC occurrence.

Results and discussion: Adenine-specific DNA methylase (COG2189) emerged as one of the most significantly enriched epigenetic markers across all infected and CRC groups, CRC (7.0 ± 1.26, q = 2.98e-06), helminth-only (7.1 ± 1.16, q = 1.30e-07), HIV-only (6.2 ± 1.21, q = 1.28e-05), and co-infected 6.5 ± 1.21, q = 6.11e-06), suggesting a shared viral epigenomic mechanism potentially contributing to tumorigenesis. Additionally, diverse metabolism-related genes were differentially abundant, particularly those linked to butyrate metabolism, oxidative stress response, and polyamine biosynthesis, metabolic pathways known to influence tumor initiation,

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immune evasion, and disease progression. These findings indicate that the gut virome may play an intermediary role in modulating host epigenetic and metabolic landscapes in infection-driven CRC risk.
Conclusion: This study identifies novel gut virome-associated epigenetic and metabolic functional signatures that may serve as early, non-invasive biomarkers of CRC susceptibility in HIV- and helminth-endemic populations. Integrating such molecular indicators into cancer surveillance and prevention frameworks could enhance early detection strategies and precision cancer care in underrepresented, high-infection burden African regions.

1. Introduction

Colorectal cancer (CRC) can take years to develop. In the initial stages, benign growth (polyps) on the epithelial layer lining the colon or the rectum can gradually become malignant and continue to grow without restriction. This is accompanied by mutational burden facilitated by genetic instability and DNA damage, which are the primary drivers of CRC development (Deschoolmeester et al., 2010). Over 20% of CRC patients usually present with an advanced stage of the disease due to a lack of early diagnosis, which is complicated by asymptomatic disease. Helminth infection may contribute to cancer development by inducing CRC-related mutations (Brindley et al., 2015; Scholte et al., 2018). Epigenetic regulation, particularly DNA methylation, is essential for maintaining genomic stability, directing cell fate decisions, and modulating immune responses (Liotti et al., 2022; Fritz et al., 2022). Studies suggest that people living with HIV (PLWHIV) display accelerated epigenetic aging, as evidenced by increased DNA methylation age, when compared to HIV-negative individuals. This epigenetic age acceleration may underlie their increased susceptibility to age-associated comorbidities and oncogenesis (Nelson et al., 2017). Moreover, epigenetic modifications induced by HIV infection have been increasingly recognized, with DNA methylation emerging as both a promising biomarker and a potential epi-therapeutic target (Arumugam et al., 2021).

Infection-driven CRC is characterized by epigenomic perturbations that play a key role in reshaping the intestinal microenvironment toward malignancy through chronic inflammation and microbial exposure. Aberrant DNA methylation, including both classical 5-methylcytosine (5 mC) and emerging markers such as N6-methyladenine (6 mA), has been implicated in oncogenesis and may serve as both a biomarker and a mechanistic link between infection and tumorigenesis (Eijk et al., 2012). The gut virome, comprising both bacteriophages and eukaryotic viruses, has been increasingly recognized as a potent modulator of host epigenetics. Viral elements can encode or influence methylation machinery, thereby altering host gene expression and immune responses (Nishijima et al., 2022; Yang et al., 2021). These interactions are dynamic and context-dependent, shaped by host genetics, co-infections, environmental exposures, and microbial community structure (Zuo et al., 2020).

On their own, evidence shows that helminth infections can induce epigenetic changes that help the parasite evade host immunity (Bohnacker et al., 2024; Banihashemian and Mirmajlessi, 2025), while simultaneously rewiring host metabolism in ways that sustain an immunosuppressive microenvironment and enable long-term parasite persistence (Kokova et al., 2021). In parallel, HIV induces metabolic reprogramming that can persist despite effective antiretroviral therapy, locking the immune system into a dysregulated state (Deme et al., 2022). Cancer cells, notorious for hijacking metabolic pathways to fuel growth and evade immune attack, may exploit these infection-driven shifts. The convergence of helminth- and HIV-mediated metabolic alterations may therefore create a permissive metabolic landscape that could accelerate oncogenesis in co-infected individuals.

Building on these insights, this study focused on characterizing methylation-associated genes within the gut virome, specifically COGs related to DNA methyltransferases, across distinct clinical groups: HIV-only, helminth-only, HIV-helminth co-infected, and CRC-confirmed groups. We hypothesized that chronic infection leads to shared and unique epigenetic and metabolic disruptions that have the potential to contribute to CRC risk. Our findings highlight how viral and bacterial components may collaborate to reshape the host epigenetic landscape, promoting genomic instability and immune evasion. Beyond cancer susceptibility, variations in gut virome composition and associated methylation pathways have been linked to a broad range of conditions, including obesity, diabetes, and autoimmune disorders (Yang et al., 2021; Cochetel et al., 2025).

2. Study methods

2.1. Study design and population

This cohort of study patients consisted of 62 South African adults across five clinical groups: uninfected controls (n = 10), HIV-only (n = 14), helminth-only (n = 15), and HIV-helminth co-infected (n = 13). Full details of the recruitment and study demographics are published elsewhere (Mpaka-Mbatha et al., 2023). Stool samples from CRC-confirmed patients (n = 10) were collected 24 h preoperatively, before bowel preparation and antibiotics were administered by a qualified surgeon from the Department of Surgery, University of KwaZulu-Natal, South Africa.

2.2. DNA extraction

Stool-derived genomic DNA was extracted from 200 mg of stool sample using the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Irvine, CA, USA; Cat. No. D4300) according to the manufacturer's instructions. Mechanical lysis was performed using the Omni Bead Ruptor 4 prior to extraction to ensure sample homogeneity. DNA purification was completed using Zymo-Spin™ columns and humic removal columns. Quantification was performed with the Qubit™ 4 Fluorometer using the Qubit™ dsDNA HS Assay Kit (Thermo Fischer Scientific, Waltham, MA, USA).

2.3. Next generation sequencing

Libraries were constructed using the Illumina® DNA Prep Kit (Illumina Inc., San Diego, CA, USA, Cat. No. 20060060) according to the manufacturer's instructions. A limited-cycle PCR was conducted to amplify the library while incorporating sample-specific dual indexes. Magnetic bead-based purification was then used to eliminate excess reagents and perform size selection of the DNA fragments. Sequencing was carried out on the Illumina NextSeq 2000 platform with 2 × 150 bp paired-end reads. Quality control was performed using TrimGalore, and metagenomic analysis was conducted using the SqueezeMeta pipeline (v1.6.2) in coassembly mode:

SqueezeMeta.pl -m coassembly -p PROJECT -s Samplelist. txt -f TRIMMED -t 32.

Open reading frames (ORFs) were predicted with Prodigal, and functional annotation was performed via DIAMOND-based searches against the COG database. Furthermore, taxonomic classification of contigs was performed by SqueezeMeta, utilising a combination of ORF queries using DIAMOND, marker genes, and the authors' published script to combine results. Contigs identified as viral in origin by SqueezeMeta were extracted for downstream analyses:

viruses = subsetTax(PROJECT, 'superkingdom', tax = 'Viruses', rescale_copy_number = F).

Downstream analysis focused on COG functional categories, with differentially abundant functions identified using the R (v4.4.2) package SQMtools (v1.6.2) (Puente-Sánchez et al., 2020) and DESeq2 (Love et al., 2014). After defining sample groups COG abundances were extracted as follows:

rownames(metadata) = colnames(viruses$functions$COG$abund).

A DESeq2 object was formed as follows:

dds = DESeqDataSetFromMatrix(countData = viruses$functions$COG $abund, colData = metadata, design = ~ condition).

Rows summing to less than 10 were removed. Differential abundance comparisons were performed with contrasts and repeated for all comparisons, e.g.:

results = results (dds, contrast = c("condition","Controls","CRC"))

Statistical significance was determined using adjusted p-values with Benjamini-Hochberg correction with a threshold of 0.05.

3. Results

3.1. Putative viral sequences identified in stool metagenomes

From the assembled metagenomic contigs, a subset showed high similarity to known viral genomes (BLAST identity >90%, alignment >1 kb), including sequences related to BK023550.1, BK036789.1, and BK047185.1. These contigs are referred to as the viral elements throughout the study, reflecting inferred viral content from total metagenomic data. Their functional potential was predicted based on sequence homology, providing insight into virus-associated metabolic/epigenetic and host-interaction pathways within the gut ecosystem (Supplementary Table 1). The differential expression patterns of abundant genes are visualized in Fig. 1, which presents a clustered heatmap of significantly modulated COG metabolic pathways across the infection groups. The heatmap highlights distinct metabolic signatures, illustrating the divergent microbial responses associated with HIV and helminth infections. Together, these findings suggest that HIV and helminth infections induce distinct viral elements metabolic adaptations, with HIV infections favouring lipid metabolic activity, while helminth infections are associated with broader suppression of viral elements metabolic pathways.

3.2. Functional classification of differentially abundant functions

3.2.1. Differentially abundant functions annotated by COG categories

A majority of the differentially abundant functions were commonly detected across all infected groups and CRC cases when compared to HIV and helminth uninfected healthy controls. Notably, several genes with uncharacterized functions, such as ENOG410ZGRA, showed distinct patterns between infection groups. ENOG410ZGRA was underrepresented in the HIV-only group (-19.9 ± 4.53, $q = 0.00036$) but enriched in the HIV-helminth co-infected group (19.2 ± 4.57, $q = 0.00086$), suggesting infection-specific regulatory dynamics. Similarly, ENOG4112065 was significantly abundant in both the HIV-helminth co-infection group (24.6 ± 3.57, $q = 8.94 \times 10^{-10}$) and CRC samples (23.9 ± 3.74, $q = 1.86 \times 10^{-8}$). Interestingly, ENOG41122JG was consistently enriched in the helminth-only (18.7 ± 4.25, $q = 0.00041$) and CRC

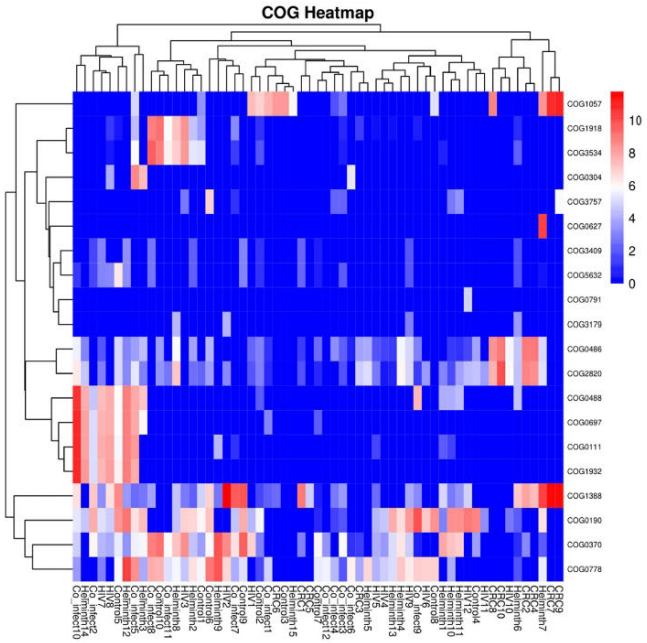


Fig. 1. Differential expression heatmap of functionally annotated microbial genes (COGs) across infection groups. The heatmap displays the normalized expression levels of selected COGs significantly altered between helminth-only, HIV-only, HIV-helminth co-infected, HIV and helminth uninfected controls, and CRC-confirmed groups. Hierarchical clustering reveals distinct metabolic profiles associated with each group. Color intensity represents Z-score normalized abundance values (red = high expression; blue = low expression). Rows represent viral elements; columns represent infection groups. Only statistically significant COGs (adjusted $p < 0.05$) are shown.

groups (18.5 ± 4.65, q = 0.0018), suggesting possible functional overlap or shared immunometabolic disruptions across parasitic infection and CRC states (Fig. 2), as further detailed in Table 1.

3.2.1.1. Specific infection-associated metabolic reprogramming as a potential driver of CRC risk. Comparative analysis between infected groups revealed that COG1932, encoding phosphoserine aminotransferase (PSAT1), an enzyme central to amino acid and coenzyme metabolism, was consistently under-represented in HIV-helminth co-infection (−15.3 ± 4.80, q = 0.02044) and HIV-only comparisons (−19.0 ± 4.79, q = 0.00139) but enriched in helminth only group (21.1 ± 4.60, q = 0.00024), Fig. 2. This pattern suggests a helminth-specific metabolic modulation, potentially associated with immune evasion strategies or nutrient competition within the host gut microenvironment, and highlights broader disruptions in host-microbial metabolic crosstalk in helminth-dominated infections. Interestingly, PSAT1 activity appears enriched in helminth-only infections relative to HIV-associated groups, indicating that helminth infection may stimulate serine biosynthesis, possibly through anti-inflammatory pathways that modulate host metabolic demand. Supporting this, previous studies have reported multiple serine proteases in parasitic helminths, implicated in parasite development, nutrient acquisition, tissue and cell invasion, anticoagulation, and immune evasion. The presence of these enzymes provides a mechanistic basis for the observed microbial PSAT1-like upregulation, suggesting that helminth-associated metabolic adaptations enhance serine biosynthesis and one-carbon metabolism (Yang et al., 2015; Cummings et al., 2022).

The phosphoglycerate dehydrogenase (COG0111) involved in amino acid and coenzyme metabolism was under-represented in HIV-helminth co-infection (−15.7 ± 3.64, q = 0.00052), HIV only (−19.4 ± 3.62, q = 4.66e-06), and CRC confirmed (−16.9 ± 3.78, q = 0.000317) cases. Similarly, 5,10-methylene-tetrahydrofolate dehydrogenase, involved in coenzyme metabolism (COG0190), was modulated in HIV-helminth co-infection (−5.7 ± 1.29, q = 0.00040), HIV only (−6.8 ± 1.29, q = 6.56e-06), helminth only (−6.0 ± 1.25, q = 9.34e-05), and CRC confirmed (−5.5 ± 1.34, q = 0.00122) cases. The predicted GTPase with regulatory functions (COG0486) was consistently modulated in both helminth-only (3.5 ± 1.33, q = 0.0244) and helminth-HIV coinfected individuals (5.0 ± 1.20, q = 0.00092). Other genes dysregulated in the HIV-only group included exoribonuclease R (COG0557) (4.5 ± 1.11, q = 0.01030), which was also detected in helminth-only (−4.4 ± 1.06, q = 0.00010) and CRC-confirmed (−3.9 ± 1.56, q = 0.01055) samples. There was a common under-representation of permeases of the drug/metabolite transporter superfamily (COG0697) involved in amino acid and carbohydrate metabolism in HIV-helminth co-infection (−15.5 ± 4.43, q = 0.00964) and HIV infected individuals (−18.9 ± 4.41, q = 0.00046).

In contrast, comparison of all infected groups to the CRC-confirmed cohort revealed enriched COG0304 in both HIV-only and HIV-helminth co-infected samples. COG0304 encodes 3-oxoacyl-(acyl-carrier-protein) synthase, a key enzyme in fatty acid elongation essential for microbial membrane integrity and secondary metabolite production. This enrichment may reflect infection-driven adaptations of the viral elements in the context of chronic immune activation, particularly in HIV infection, where lipid metabolism is critical for immune regulation and microbial persistence. These findings collectively highlight how different infections distinctly modulate host metabolic circuits,

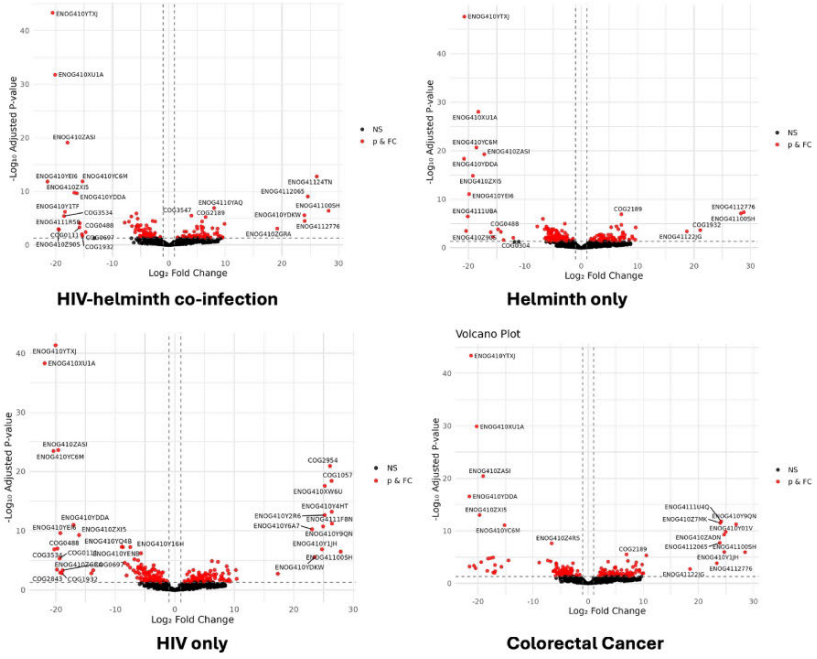


Fig. 2. Volcano Plot depicting differentially abundant functions between uninfected healthy controls, all the infected groups, and the CRC positive controls. The viral elements analysis was functionally categorized using clusters of orthologous groups (COG) pathways. The volcano plot displays gene distribution based on $\log_2$ fold change and adjusted p-values, indicating significantly enriched or down-abundant genes across conditions.

Table 1
Metabolic pathways observed in infected groups directly linked to cancer.

Gene Name	Functional Category	Cancer Association	References
Phosphoglycerate dehydrogenase	Amino acid/ Coenzyme metabolism	PHGDH is often amplified in cancers such as breast cancer and melanoma, contributing to serine biosynthesis and supporting rapid cell proliferation.	(Liu et al., 2013; Kuzuoglu-Ozturk, 2023)
5,10-methylene-THF dehydrogenase (MTHFD2)	Coenzyme metabolism	MTHFD2 is consistently enriched in various cancers, promoting tumor growth and metastasis, making it a potential therapeutic target.	(Pardo-Lorente and Sdelci, 2024; Ramos et al., 2024)
ABC transporter ATPase components	General function prediction	ABC transporters are linked to cancer hallmarks, including proliferation, metastasis, and drug resistance, by facilitating the efflux of chemotherapeutic agents.	Muriithi et al. (2020)
Nitroreductase (NTR)	Energy production and conversion	NTRs are overexpressed in various cancers, including colorectal, breast, and liver cancers, and are being explored for targeted cancer therapies.	Shang et al. (2024)
N-acetylmuramoyl-L-alanine amidase	Cell envelope biogenesis	This enzyme has been studied for its potential to induce cytotoxicity in cancer cells when combined with other agents, suggesting a role in cancer therapy.	Fuentes-Baile et al. (2020)
Phosphoserine aminotransferase (PSAT1)	Amino acid/ Coenzyme metabolism	PSAT1 is overexpressed in several cancers, including colorectal and breast cancer, supporting tumor cell proliferation and survival.	Yang et al. (2023)
Uridine phosphorylase (UPP1)	Nucleotide transport and metabolism	UPP1 supports metastasis in cancers such as breast cancer and is considered a potential prognostic factor in oral squamous cell carcinoma.	(Yang et al., 2023; Nwosu et al., 2023)

potentially mitigating or amplifying CRC risk. While metabolic adaptations can support viral persistence and immune evasion, they may also create a microenvironment conducive to neoplastic transformation, including enhanced lymphocyte infiltration, immune exhaustion, and redox imbalance (Ren et al., 2022). Significant abundance of Adenine-specific DNA methylase (COG2189) was shared by CRC (7.0 ± 1.26, q = 2.98e-06), helminth-only (7.1 ± 1.16, q = 1.30e-07), and co-infected groups (6.5 ± 1.21, q = 6.11e-06). This enzyme catalyzes the methylation of adenine residues (N6-methyladenine, 6 mA), a modification increasingly recognized for its regulatory role in microbial gene expression and genome stability. The consistent enrichment across helminth-only, HIV-helminth co-infected, and CRC groups suggests a shared adaptive or stress response within the gut microenvironment, potentially driven by chronic inflammation or altered immune signaling. Adenine methylation serves as a mechanism for phase variation, DNA repair, and protection against restriction enzymes needed for survival under immune pressure. Its overrepresentation in the infected groups may therefore indicate enhanced microbial resilience and persistence in response to host immunity. Similarly, in CRC, elevated 6 mA methylation has been linked to shifts in microbial virulence and to epigenetic crosstalk that can alter host gene regulation and chromatin dynamics (Chen et al., 2023). The parallel enrichment of COG2189 in both infection and cancer states points to a possible convergence of microbial adaptation and tumor-promoting conditions. It is plausible that infection-induced microbial methylation may reprogram the gut epigenetic landscape, creating an environment that favors both microbial persistence and malignant transformation. This observation indicates a potential mechanistic bridge between chronic infection and CRC risk, mediated through epigenetic modulation at the viral elements-host interface.

Furthermore, Table 1 outlines the COG viral elements-associated pathways that show potential links to cancer-related processes. These pathways were identified through functional profiling of viral genes enriched in the CRC group and compared to other infection states. The table highlights pathways associated with genome maintenance and metabolic processes. Of note, among these are genes involved in DNA repair, protein metabolism, and host immune evasion, potentially highlighting the role of the viral elements in modulating tumorigenic pathways.

When comparing all infected groups to the CRC group, both the HIV-helminth co-infection and HIV-only groups had the uncharacterized, ENG410ZZ4I (4.6 ± 1.13, q = 0.0058) and (5.1 ± 1.13, q = 0.00042), respectively. COG0304 (3-oxoacyl-(acyl-carrier-protein) synthase) (31.5 ± 4.78, q = 1.60e-08 and 31.3 ± 4.78, q = 1.23e-08, respectively) in common. Additionally, ENOG4112065 was shared between the HIV-only group (−18.1 ± 3.61, q = 4.19e-05) and the helminth-only group (−18.1 ± 3.44, q = 2.73e-05), Fig. 3A. Of interest, when comparing infected groups against each other, there were genes that were also shared amongst groups. For instance, COG1932 was also downregulated in Helminth only vs HIV only (−40.2 ± 4.33, q = 9.76e-18) and HIV-helminth co-infection (−36.4 ± 4.34, q = 1.96e-14), Fig. 3B.

Gut viral elements-related differentially abundant genes that were significantly expressed in infected groups are tabulated in Table 2. These findings indicate gut viral elements in HIV-helminth co-infection and their potential utility as a source of candidate biomarkers or mechanistic targets in infection-associated disease progression.

These shared alterations suggest convergent functional pressures, particularly affecting metabolic pathways. A central feature of the infection-altered gut viral signature landscape observed in this study was the structured reprogramming of viral elements' metabolic gene expression, reflecting dynamic shifts in the core biosynthetic and energy-producing pathways (Fig. 4).

Furthermore, differential pathway enrichment analysis was performed to quantify coordinated metabolic activity across infection and CRC groups using gene set variation analysis (GSVA; Table 3). Overall, modest but biologically relevant alterations were observed in one-carbon metabolism, amino acid pathways, and transporter activity. In infection states relative to controls, the one-carbon metabolism by folate pathway showed mild down-abundance ($log_2FC = -0.23$; adj. P = 0.23), while glycine, threonine, and serine metabolism displayed a trend toward enrichment ($log_2FC = +0.15$; adj. P = 0.35). ATP transporter activity remained largely unchanged ($log_2FC = -0.03$; adj. P = 0.83), suggesting limited energy transport alteration during infection. Although none of these pathways reached false discovery rate (FDR) significance, their consistent directionality points to a subtle remodeling of one-carbon and amino acid metabolism under infection conditions.

In the helminth-infected group, similar low-amplitude but coherent alterations were observed. The glycine, threonine, and serine metabolism pathway showed modest enrichment ($log_2FC = +0.19$; adj. P = 0.38), implying slight upregulation of one-carbon substrate utilization and amino acid turnover during helminth infection. This pathway contributes to folate-mediated methylation and nucleotide synthesis, processes that may reflect adaptive host metabolic adjustments to chronic infection. In contrast, ATP transporter activity ($log_2FC = -0.14$; adj. P = 0.38) and one-carbon metabolism by folate ($log_2FC = -0.05$; adj. P = 0.65) were under-represented, consistent with reduced energy transport and partial suppression of folate-dependent reactions. While these differences were not statistically significant, the pattern suggests a shift toward an energy-conserving phenotype and altered amino acid flux.

When CRC samples were compared with HIV-helminth co-infected samples, a distinct metabolic bias emerged. One-carbon metabolism by folate was enriched ($log_2FC = +0.30$; adj. P = 0.054), supporting the notion of enhanced folate-dependent methylation and nucleotide biosynthesis, hallmarks of tumorigenic metabolic reprogramming. Conversely, ATP transporter ($log_2FC = -0.23$; adj. P = 0.11) and glycine, serine, and threonine metabolism ($log_2FC = -0.09$; adj. P =

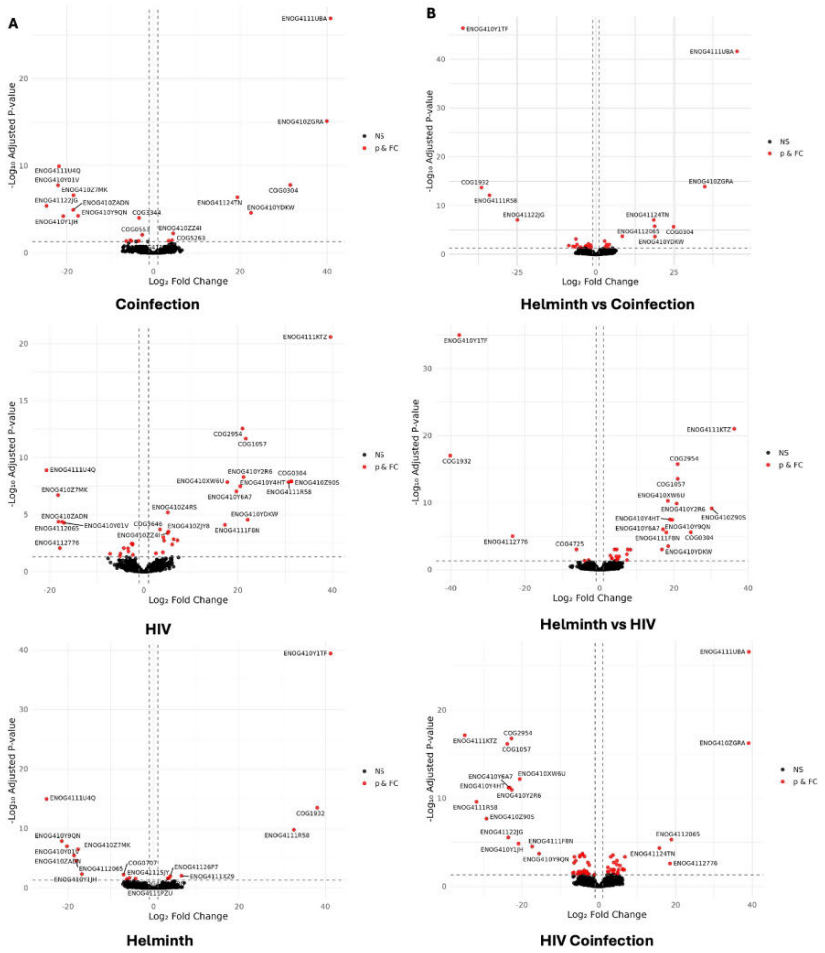


Fig. 3. Differentially abundant microbial functional categories across infection and CRC groups. (A) Volcano plots showing differentially abundant protein function categories between CRC-positive controls and all infected groups combined, identifying broad functional and evolutionary relationships associated with CRC. (B) Volcano plots comparing all infected groups (HIV-only, helminth-only, and HIV–helminth co-infected) against each other to reveal infection-specific functional signatures. Functional annotation and clustering were performed using Clusters of Orthologous Groups (COG) classification. Each point represents a distinct COG function, plotted by $\log_2$ fold change (x-axis) and -$\log_{10}$ adjusted p-value (y-axis). Red points denote significantly differentially abundant functions (FDR <0.05). Functions on the right indicate enrichment, while those on the left indicate reduced abundance relative to the reference group. Together, the analyses highlight distinct microbial functional remodeling associated with infection states and CRC pathogenesis.

0.49) were under-represented, implying reduced energy and amino acid homeostasis. Together, these findings suggest that HIV-helminth co-infection may foster a pro-tumorigenic metabolic environment characterized by increased one-carbon flux and suppressed energy balance.

Comparisons between CRC and helminth-only groups revealed comparable but subtler shifts. One-carbon metabolism by folate was modestly elevated in CRC ($\log_2$FC = +0.13; adj. P = 0.33), suggesting gradual metabolic reorientation toward nucleotide synthesis and methylation support. In contrast, glycine, serine, and threonine metabolism ($\log_2$FC = −0.12; adj. P = 0.33) and ATP transporter activity ($\log_2$FC = −0.12; adj. P = 0.33) were under-represented, reflecting reduced amino acid turnover and energy transport. Although not statistically significant, these coordinated shifts align with emerging evidence of metabolic reprogramming during CRC development, particularly in helminth-exposed individuals.

Finally, CRC versus HIV comparisons showed more pronounced divergence. Glycine, serine, and threonine metabolism was significantly under-represented ($\log_2$FC = −0.3282; adj. P = 0.032), consistent with reduced one-carbon substrate cycling and potential depletion of methyl donors required for DNA methylation. Down-abundance of ATP

Table 2
Significantly expressed metabolic genes in infected groups.

COG ID	Gene Name/Annotation	Functional Category	Group(s)	mean ± 95% CI	q-Value
0111	Phosphoglycerate dehydrogenase	Amino acid/Coenzyme metabolism	HIV only, Helminth, Co-infection	−19.4 ± 3.62 −12.0 ± 3.50 −15.8 ± 3.64	4.66e-06 0.00897 0.00052
0190	5,10-methylene-THF dehydrogenase	Coenzyme metabolism	HIV only, Helminth, Co-infection	−6.8 ± 1.29 −6.1 ± 1.25 −5.7 ± 1.29	6.56e-06 9.34e-05 0.00040
2189	Adenine-specific DNA methylase	Mediates N6-methyladenine modification known to regulate microbial gene expression and virulence.	HIV only, Helminth, Co-infection	6.2 ± 1.21 7.1 ± 1.16 6.5 ± 1.21	1.28e-05 1.30e-07 6.11e-06
0304	3-oxoacyl-(acyl-carrier-protein) synthase	Secondary metabolites biosynthesis, transport and catabolism/Lipid metabolism	HIV	31.2 ± 4.78	1.23e-08
0370	Fe^{2+} transport system protein B	Inorganic ion transport/metabolism	HIV only, Helminth	−6.0 ± 1.32 −5.4 ± 1.27	0.00017 0.00089
0486	Predicted GTPase	General function prediction	HIV only, Co-infection	4.24 ± 1.19 5.0 ± 1.20	0.005137 0.00091
0488	ATPase components of ABC transporters with duplicated ATPase domains	General function prediction	HIV only, Helminth	−19.8 ± 3.27 −14.7 ± 3.15	9.89e-08 0.00016
0697	Permeases of the drug/metabolite transporter (DMT) superfamily	General function prediction only/Amino acid transport and metabolism/Carbohydrate transport and metabolism	HIV Co-infection	−15.5 ± 4.43 −18.9 ± 4.42	0.00964 0.00046
0778	Nitroreductase	Energy production and conversion	HIV, Helminth	−5.7 ± 1.27 −5.2 ± 1.23	0.00027 0.00074
1057	Nicotinic acid mononucleotide adenylyltransferase	Coenzyme metabolism	HIV	26.4 ± 2.80	2.16e-12
1388	FOG: LysM repeat	Cell envelope biogenesis, outer membrane	HIV, Co-infection	7.2 ± 1.42 6.1 ± 1.42	1.73e-05 0.00058
1932	Phosphoserine aminotransferase	Amino acid transport and metabolism/Coenzyme metabolism	HIV Helminth Co-infection	−19.0 ± 4.79 21.1 ± 4.60 −15.5 ± 4.80	0.00139 0.00024 0.02044
2820	Uridine phosphorylase	Nucleotide transport and metabolism	Co-infection	5.0 ± 1.12	0.00035
3534	Alpha-L-arabinofuranosidase	Carbohydrate transport and metabolism	HIV, Helminth, Co-infection	−20.3 ± 3.39 −14.2 ± 3.27 −18.5 ± 3.39	1.36e-07 0.00052 4.05e-06
3969	Predicted phosphoadenosine phosphosulfate sulfotransferase	General function prediction only	Helminth, Co-infection	6.2 ± 1.23 5.7 ± 1.29	4.02e-05 0.00033
ENOG410XU3I	No name	Function unknown	HIV, Helminth	−6.2 ± 1.53 −5.1 ± 1.47	0.00096 0.00821
ENOG410XQ2T	type iii restriction	Defense mechanisms	HIV, Helminth	6.0 ± 1.42 7.1 ± 1.37	0.00058 1.92e-05
ENOG410XNW6	Phage protein	Function unknown	HIV	6.7 ± 1.53	0.00039
ENOG410XRH6	Integrase	Replication, recombination and repair	Co-infection	−6.5 ± −4.49	0.00030
ENOG410XUQ2	YjcQ protein	Function Unknown	HIV	−5.3 ± 1.29	0.00086
ENOG410ZGRA	No name	Function unknown	HIV, Co-infection	−19.9 ± 4.53 19.2 ± 4.57	0.00036 0.00086
ENOG410Y1TF	No name	Function unknown	HIV, Co-infection	−13.8 ± 3.20	0.00045 6.39e-07

(*continued on next page*)

Table 2 (*continued*)

COG ID	Gene Name/Annotation	Functional Category	Group(s)	mean ± 95% CI	q-Value
				−18.4 ± 3.15	
ENOG410Y1ZG	No name	Function unknown	HIV	−4.0 ± 0.96	0.00078
ENOG410YB2E	No name	Function unknown	Co-infection	9.9 ± 2.08	0.00012
ENOG410YDR6	No name	Function unknown	HIV, Helminth	−6.2 ± 1.39	0.00024
				−5.8 ± 1.33	0.00055
ENOG410YK3D	No Name	Function Unknown	HIV, Co-infection	−6.2 ± 1.19	9.71e-06
				−5.5 ± 1.19	0.00018
ENOG410YPT3	No name	Function Unknown	HIV, Helminth, Co-infection	−6.17 ± 1.22	0.00022
				−5.7 ± 1.27	4.18e-05
ENOG410YUBV	No name	Function Unknown	HIV	−7.0 ± 1.62	0.00029
ENOG410Z7Y1	No name	Function unknown	HIV	10.3 ± 2.37	0.00046
ENOG410ZHCN	No name	Functional Unknown	HIV, Helminth, Co-infection	−4.4 ± 1.06	0.00042
				−3.5 ± 1.01	0.00086
				−3.9 ± 1.06	0.00897
ENOG4111FT5	fibronectin type III domain protein	Function Unknown	HIV, Helminth, Co-infection	8.9 ± 4.30	0.00433
				9.7 ± 1.96	0.00044
				8.6 ± 2.06	6.83e-05
ENOG4111PAV	Phage-related protein	Function unknown	HIV, Helminth	3.1 ± 0.72	0.00089
				2.4 ± 0.68	0.00042
ENOG4111R20	No name	Function unknown	HIV	−6.2 ± 1.33	0.00851
ENOG4111W9D	No name	Function Unknown	Co-infection	−4.0 ± 0.86	0.00013
ENOG4111WWI	No name	Function unknown	HIV	−2.7 ± 0.66	0.00021
ENOG4112065	No name	Function unknown	Co-infection	24.6 ± 3.57	0.00078
ENOG4112923	Caudovirus prohead protease	Function unknown	HIV, Helminth, Co-infection	8.7 ± 1.85	8.94e-10
				7.4 ± 1.72	9.77e-05
				7.7 ± 1.81	0.00061
ENOG411297B	Dj domain protein	Posttranslational modification, protein turnover, chaperones	Helminth, Co-infection	−5.3 ± 1.19	0.00071
				−5.6 ± 1.24	0.00040
ENOG4111XQJ	No name	Function unknown	HIV	−5.7 ± 1.24	0.00030
					0.00016

Footnote: Where more than one p-value is given, they represent statistical significance for each group individually.

transporter and one-carbon metabolism by folate pathways further supports suppressed energy metabolism and folate turnover. Together, these data highlight HIV-associated metabolic modulation that could influence CRC pathogenesis through disturbed cellular energetics and epigenetic instability. Although statistical significance was not reached for most pathways, the coherent directionality across multiple comparisons may be an indication of biologically meaningful remodeling. These trends warrant validation in a larger cohort, where increased statistical power could confirm the subtle metabolic reprogramming observed here.

3.3. Evidence of viral DNA methylation machinery

Investigation of viral elements-associated epigenetic modulation revealed significant alterations in DNA methylation-related genes in individuals with HIV or helminth infections only, confirmed CRC, and HIV and helminth uninfected controls, Fig. 5. Most notably, COG2189, which encodes an adenine-specific DNA methylase, was consistently enriched in the CRC-confirmed (adj. p = 2.98e-06), helminth-only (adj. p = 1.30e-07), and HIV-helminth co-infected (adj. p = 6.11e-06) groups. This epigenetic pattern was further supported by the enrichment of COG0286, which encodes a Type I restriction modification (RM) system methyltransferase. Its selective expression in the HIV-helminth co-infected group (adj. p = 0.0238) indicates a potential microbial mechanism of host-epigenetic interference. Additionally, COG0190, encoding 5,10-methylene-tetrahydrofolate dehydrogenase (MTHFD2), was significantly enriched in HIV-only, helminth-only, and co-infected groups (adj. p < 0.0005).

4. Discussion

The interplay between chronic infections, particularly HIV and helminth infections, and host epigenetic reprogramming presents a critical axis for understanding CRC susceptibility. This study aimed to explore how chronic infections may influence CRC risk by examining viral elements-associated epigenetic and metabolic changes in HIV- and helminth-infected individuals. Our stratified metagenomic analysis identified distinct methylation/metabolism-associated signatures across

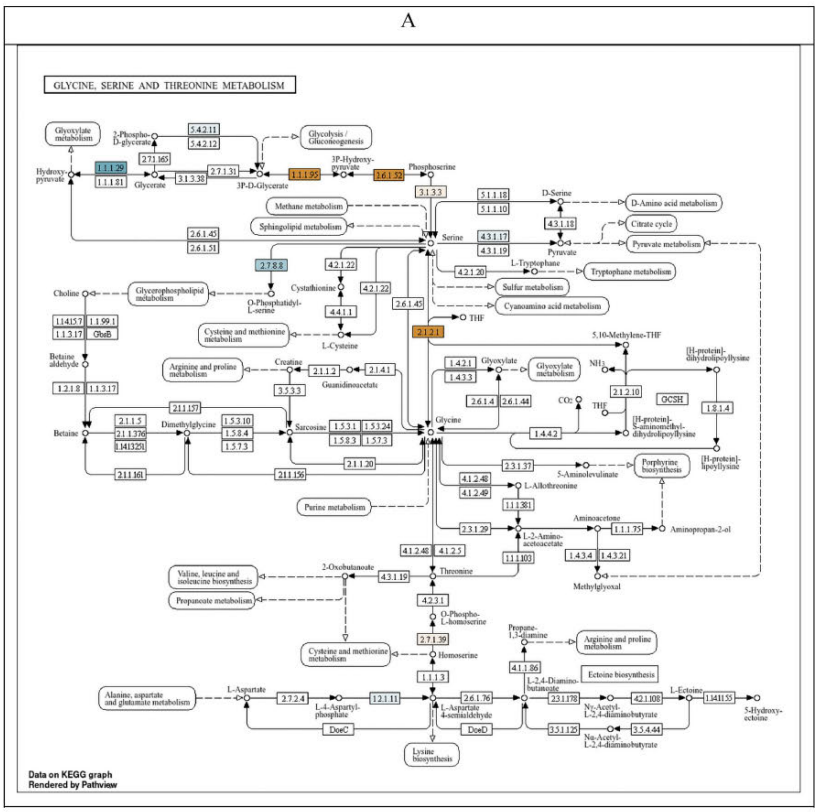


Fig. 4. Infection-induced metabolic reprogramming reveals coordinated activation of amino acid, folate, heme, and transporter pathways. (A) Serine-glycine metabolism: Infection and co-infection states show enhanced flux through the serine-glycine biosynthetic arm linking glycolytic intermediates to one-carbon units. Key enzymes, including PHGDH, PSAT1, and SHMT1/2, are enriched, increasing serine-glycine interconversion and supplying substrates for nucleotide and methyl-donor synthesis. (B) One-carbon pool by folate: Elevated activity of MTHFD2 and associated enzymes sustains the folate-mediated one-carbon cycle, generating 5,10-methylene-THF and S-adenosylmethionine (SAM). (C) Porphyrin and heme metabolism: Upregulation of ALAS1, UROD, and FECH indicates increased iron turnover and altered heme homeostasis. (D) ABC transporters: Induction of multiple ABC transporter families enhances trafficking of iron complexes, peptides, and xenobiotics.

the HIV-only, helminth-only, CRC confirmed, and HIV-helminth co-infected groups, with each exhibiting unique patterns of viral elements enzyme expression implicated in methylation dynamics. Both metabolic reprogramming and epigenetic modifications are established cancer hallmarks, enabling transformed cells to support proliferation, evade immune detection, and persist in hostile microenvironments (Xu et al., 2023). Shotgun metagenomic sequencing of stool DNA revealed a shared "onco-viral elements" between CRC patients and HIV-helminth co-infected individuals. The HIV-helminth co-infection group exhibited the most extensive methylation-related reprogramming. Both 5,10-methylene-tetrahydrofolate dehydrogenase (COG0190) and Adenine-specific DNA methylase (COG2189) were significantly enriched, along with COG0286, which encodes a Type I restriction-modification system methyltransferase. The co-expression of microbial and host-mimetic methyltransferases suggests epigenetic convergence through viral, bacterial, or horizontal gene transfer (Miyashita et al., 2002).

4.1. Epigenetic reprogramming in infection-associated CRC risk

Adenine-specific DNA methylase catalyzes the formation of N6-methyladenine (6 mA), a DNA modification increasingly recognized for its regulatory roles in eukaryotic systems, particularly in transcriptional activation, stem cell maintenance, and chromatin organization (Chen et al., 2017). Elevated 6 mA levels have been associated with genomic instability, proliferation, and oncogenic progression in multiple tumor types (Li et al., 2017), suggesting that its presence in infection may constitute a precursor to malignant transformation. Previous studies on tissue samples observed significantly higher levels of 5-methylcytosine (5 mC) compared to 6 mA. However, a substantial number of samples exhibited either upregulation or downregulation of both 5 mC and 6 mA in hepatocellular carcinoma (Zhang et al., 2022). Of interest, 6 mA has sparked interest in multiple studies investigating its role in parasitic infections (Lizarraga et al., 2020) and viral infections (Zhang

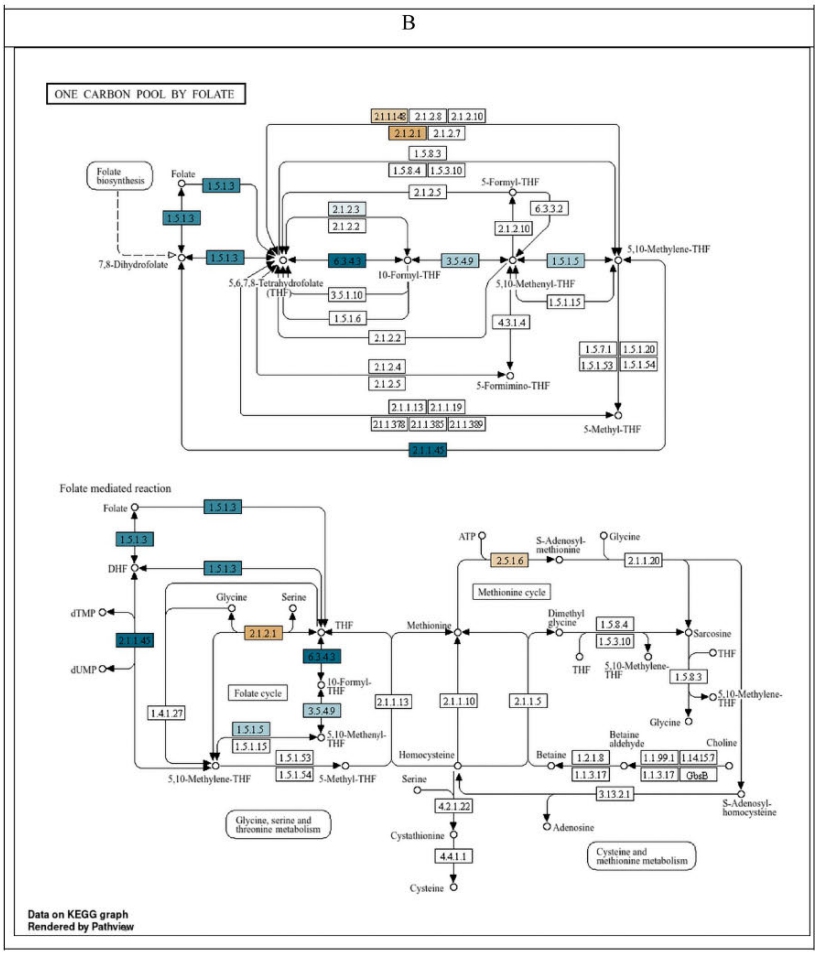


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et al., 2023). Fig. 6 presents a comparative overview of the regulatory functions of these methylation marks. It is important to note that an integrated framework of these methylation marks has been proposed for precision epigenome editing aimed at treating methylation-related diseases (Liu et al., 2025).

Although traditionally involved in bacterial defense, RM system methyltransferases have been implicated in the regulation of virulence, phase variation, and modulation of host transcriptional responses (Pflueger et al., 2018; Zemach et al., 2013). Their presence in the viral elements of HIV-helminth co-infected individuals may reflect epigenetic mimicry strategies used to evade host immune response. The Type I RM system methyltransferase makes up an important component of a multi-subunit antiviral defense complex. Type I RM systems comprise three proteins, HsdR, HsdS, and HsdM, that assemble into different configurations to control whether foreign DNA is cleaved or protected. In their pentameric arrangement, these complexes function as restriction endonucleases that recognise and cut unmethylated or improperly methylated DNA from invading bacteriophages (Fig. 7). This activity requires ATP, Mg^{2+}, and S-adenosyl-methionine (AdoMet) as cofactors (Zhang et al., 2017). The enrichment of Type I restriction modification methyltransferases (e.g., COG0286) in HIV-helminth co-infection may alter viral composition, reshape bacteriophage-host dynamics, and drive changes in viral gene exchange. Such shifts may destabilize viral elements' community structure, influence immune regulation, and sustain chronic inflammation processes that hold potential relevance for cancer risk in affected individuals.

4.1.1. Biological implications of 6 mA methylation

In the current study, the persistent enrichment of COG2189, which encodes an adenine-specific DNA methylase, in both the infection-only

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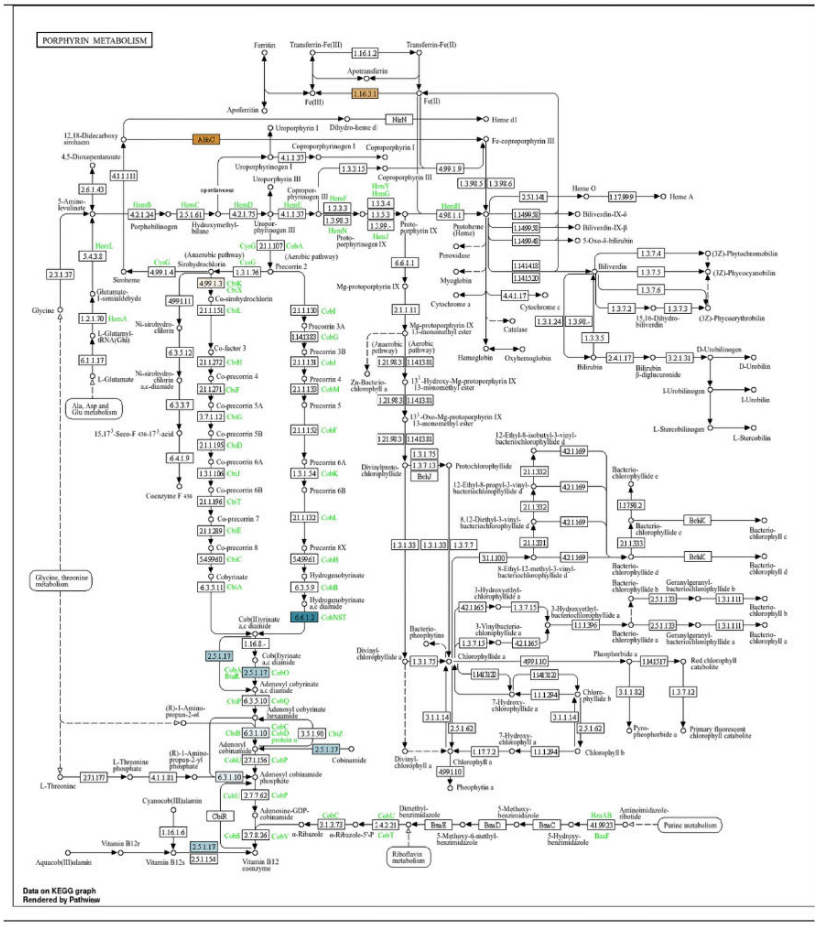


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and CRC-confirmed groups highlights the potential of microbial 6 mA-modifying enzymes to contribute to host epigenetic reprogramming. This trend suggests a shared molecular axis between chronic infection and early oncogenic transformation, possibly mediated by viral elements-encoded methylation machinery. Once integrated or expressed within the host intestinal environment, these enzymes may disrupt normal methylation homeostasis and reinforce transcriptional programs that favour proliferation, immune suppression, or epithelial-to-mesenchymal transition (Xie et al., 2020). N6-methyladenine (6 mA) methylation represents a significant expansion of the classical DNA methylation paradigm, which is traditionally dominated by 5-methylcytosine (5 mC). While 5 mC is well established in gene silencing and heterochromatin formation, emerging research in eukaryotic systems has revealed that 6 mA serves distinct, often activating, roles in gene regulation. Notably, 6 mA is frequently enriched in heterochromatic regions, particularly within histone H3K9me3-marked domains, where it appears to counterbalance repression and support the selective transcriptional activation (Liu et al., 2025).

In developmental and disease contexts, 6 mA exhibits dynamic and context-specific regulation. Its enrichment in stem-like populations has been linked to the maintenance of tumor-initiating cell phenotypes, facilitating the expression of genes involved in self-renewal, metabolic adaptation, and resistance to apoptosis (Xiao et al., 2017). This epigenetic landscape not only supports sustained tumor growth but also contributes to immune evasion by modulating the expression of immune-related genes, including major histocompatibility complex (MHC) molecules and co-stimulatory ligands (Luan et al., 2024). The functional significance of 6 mA is further underscored by its regulation

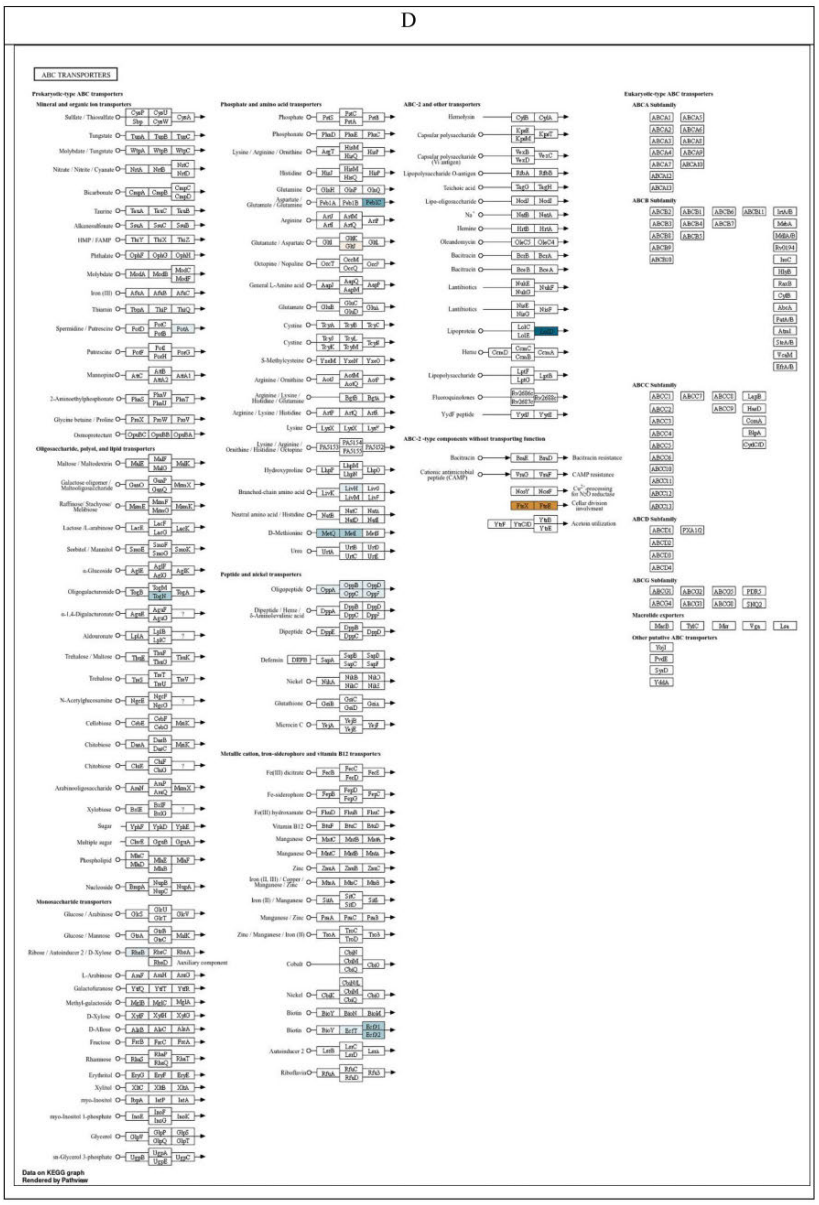


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via demethylases, such as ALKBH1, which actively remove this mark. Aberrant expression of ALKBH1 has been reported in glioblastoma, gastric cancer, and other malignancies, where it contributes to epigenomic instability, therapeutic resistance, and cellular invasiveness (Koh et al., 2018). Its dysregulation reinforces the view that 6 mA is not merely a microbial vestige but a malleable epigenetic switch with

Table 3
Differential pathway activity across comparison groups.

Comparison	Pathway	log_2FC	adj.P.Val	Direction	Interpretation
Control vs Co-infected	One-carbon by folate metabolism	−0.23	0.23	Under-represented	Mild suppression during infection
Control vs Co-infected	Glycine, threonine, and serine metabolism	+0.15	0.35	Enriched	Slight amino acid activation
Control vs Co-infected	ATP transporter activity	−0.03	0.83	No change	Unaltered energy transport
CRC vs Co-infected	One-carbon metabolism by folate	0.304	0.054	Enriched	Increased folate-dependent methylation
CRC vs Co-infected	ATP transporter	−0.229	0.110	Under-represented	Reduced energy transport
CRC vs Co-infected	Glycine, serine and threonine metabolism	−0.087	0.493	Under-represented	Lower amino acid flux
CRC vs Helminth	One-carbon metabolism by folate	0.1311	0.3305	Enriched	Trend toward higher folate activity
CRC vs Helminth	Glycine, serine and threonine metabolism	−0.1219	0.3305	Under-represented	Reduced amino acid turnover
CRC vs Helminth	ATP transporter	−0.1182	0.3305	Under-represented	Lower energy metabolism
CRC vs HIV	Glycine, serine and threonine metabolism	−0.3282	0.0320	Under-represented	Significant suppression of one-carbon substrates
CRC vs HIV	ATP transporter	−0.2044	0.1642	Under-represented	Reduced energy-related transport
CRC vs HIV	One-carbon metabolism by folate	−0.0988	0.4377	Under-represented	Minimal folate pathway change

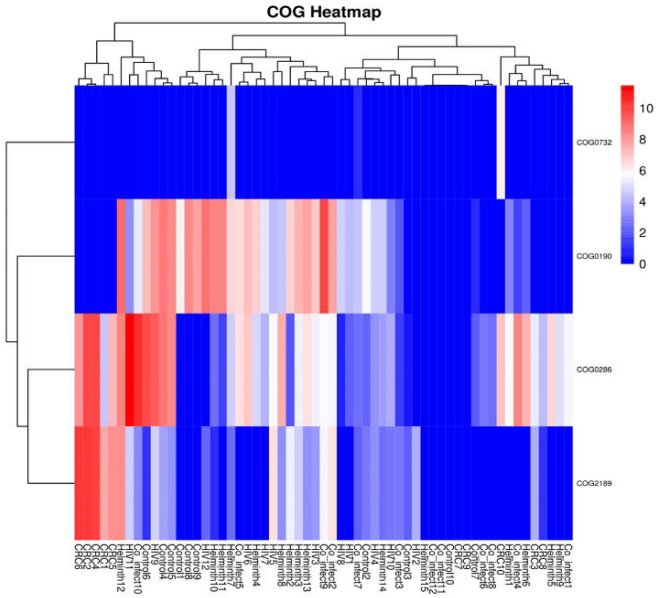


Fig. 5. Heatmap illustrating Virome-Derived Epigenetic Enzymes Differentially Abundant Across Clinical Groups. The heatmap illustrates the clustering of COG gene expression profiles across HIV and helminth uninfected controls, HIV only, Helminth only, HIV-helminth co-infected, and CRC confirmed groups. Rows represent specific COG categories (COG0732, COG0190, COG0286, COG2189), while columns corresponding to individual samples are indicated on the x-axis. The color scale represents the expression levels, with red indicating higher expression, blue indicating lower expression, and white representing intermediate levels. Hierarchical clustering dendrograms are shown for both samples (top) and COG categories (left), highlighting patterns of similarity in gene expression across samples and functional groups.

oncogenic potential in mammalian systems, including humans.

Moreover, the identification of infection-specific 6 mA methylation signals suggests that such epigenetic changes may occur before the emergence of genetic mutations, positioning them as early and potentially reversible events in tumorigenesis. From a translational perspective, this raises the possibility of exploiting microbial 6 mA methylation patterns as non-invasive biomarkers in populations with elevated infection and CRC risk. Collectively, these findings support a conceptual shift in our understanding of methylation dynamics, from a static host-centered model to a bidirectional host-microbe epigenetic interface. Viral signatures and their enzymatic repertoire may play a critical role in shaping the epigenome under chronic inflammatory conditions, with implications for both cancer initiation and progression. Future studies should explore the therapeutic targeting of microbial methyltransferases or the restoration of host 6 mA demethylation capacity as avenues for modulating this axis in CRC prevention and care.

4.1.2. Virulence-associated type I restriction modification systems

Supporting this, COG0286 (Type I restriction-modification methyltransferase) and COG0190 (Type II site-specific DNA methyltransferase) were also differentially abundant. These genes are known components of bacterial restriction-modification systems, which not only protect against phage infection but also modulate transcription and epigenetic landscape (Takahashi et al., 2025). Helminth infections were also associated with site-specific methylation patterns, with shared CpG sites detected across all individuals at both the nestling and fledged juvenile

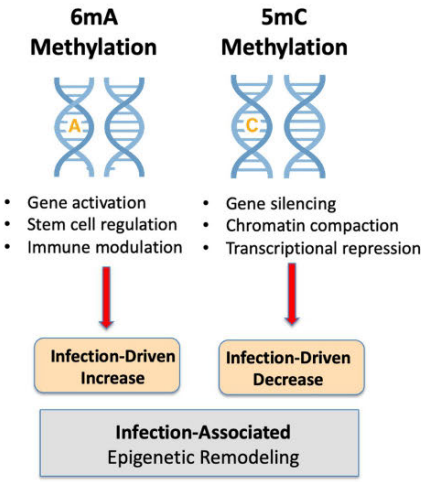


Fig. 6. Comparison between N6-methyladenine (6 mA) and 5-methylcytosine (5 mC) in the context of infection-associated epigenetic remodeling. While 6 mA is linked to gene activation, stem cell regulation, and immune modulation (with infection-driven increases), 5 mC is typically associated with gene silencing and chromatin compaction, and may be reduced in chronic infection contexts. Together, their imbalance reflects a shift toward a tumor-permissive epigenetic state.

stages. These CpG sites were located in gene promoters, transcription start sites, exons, first introns, other introns, or in intergenic regions (Lundregan et al., 2022). The expression of these methyltransferases suggests a functional role of the gut viral signature's epigenetic machinery in mediating infection-driven molecular changes that may promote colorectal oncogenesis.

4.1.3. *MTHFD2 and the metabolic-epigenetic interface in cancer*

In the HIV-only cohort, there was a selective enrichment of COG0190, which encodes 5,10-methylene-tetrahydrofolate dehydrogenase (MTHFD2), a central enzyme in the one-carbon metabolism pathway. MTHFD2 is instrumental in the biosynthesis of S-adenosylmethionine (SAM), a universal methyl donor required for DNA and histone methylation. Its abundance suggests an enhanced methylation potential within host cells, which could predispose the epigenome to aberrant hypermethylation of tumor suppressor loci (Corley et al., 2021; Oriol-Tordera et al., 2020). This mechanism is consistent with reports of epigenetic silencing of antiviral genes in HIV latency and the emergence of chronic inflammation-associated methylation signatures that affect immune pathways and cognitive function (Corley et al., 2016).

Key metabolic genes, COG1932 (phosphoserine aminotransferase), COG0111 (phosphoglycerate dehydrogenase), and COG0190 (5,10-methylene-tetrahydrofolate dehydrogenase), were consistently enriched across infected groups. These enzymes participate in the serine-glycine-one-carbon pathway, crucial for nucleotide biosynthesis and redox homeostasis, and are frequently co-opted during tumorigenesis (Nong et al., 2023; Jack et al., 2001). Additionally, COG0304, which encodes 3-oxoacyl-(acyl-carrier-protein) synthase involved in fatty acid synthesis, was abundant in HIV-infected individuals, supporting lipid biosynthesis, a key component of cancer metabolic rewiring. Interestingly, COG0486, a predicted regulatory GTPase, was also enriched, suggesting potential changes in microbial and viral signaling that may influence host cell cycle control or apoptosis. Infection-specific modulations were observed as well: in HIV-only participants, genes such as COG0557 (exoribonuclease R) and COG0697 (a drug/metabolite transporter family member) were uniquely dysregulated, possibly reflecting virus-induced adaptations in microbial stress response and nutrient transport (Manna et al., 2014). These functional changes reinforce the idea that infection-driven microbiome alterations may support a pro-tumorigenic microenvironment.

4.2. *Metabolic reprogramming in infection-associated CRC risk*

The differential abundance of viral elements involved in one-carbon metabolism (COG0190: MTHFD2-like), serine biosynthesis (COG0111: PHGDH-like; COG1932: PSAT1-like), fatty acid elongation (COG0304), and iron transport (COG0370) indicates a coordinated shift in viral

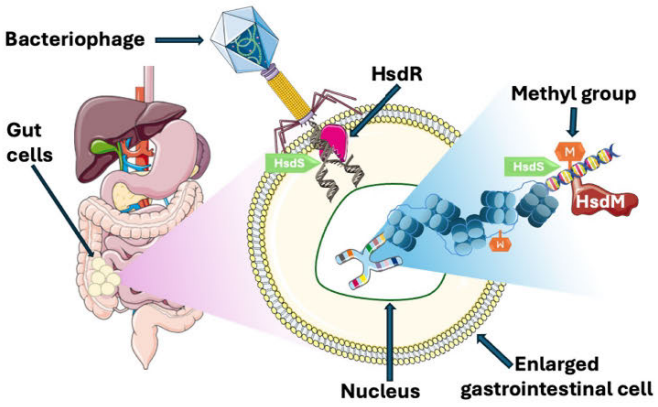


Fig. 7. Type I restriction modification (RM) enzyme function within the gut viral signatures and its potential significance in HIV-helminth co-infection. Within the gut virome, bacteriophages infect bacterial hosts, triggering defense mechanisms such as the Type I R-M system. The specificity subunit (HsdS) recognises defined DNA sequences, guiding the methyltransferase subunit (HsdM) to methylate host bacterial DNA and protect it from cleavage. Incoming phage DNA lacking the host-specific methylation pattern is targeted for restriction-mediated degradation (HsdR).

elements' function. These pathways are well-established components of colorectal tumor metabolism, facilitating nucleotide synthesis, redox balance, immune evasion, and cellular proliferation (Nong et al., 2023; Zhang et al., 2018). Importantly, these metabolic signatures were not limited to patients with CRC. They were consistently detected in HIV-only and co-infected individuals, indicating that chronic infection may precondition the gut microenvironment by establishing a metabolic state similar to that of neoplastic transformation. In contrast, helminth-only individuals exhibited selective underrepresentation of anabolic enzymes, such as PSAT1, suggesting a distinct metabolic suppression profile, indicating that infection type may dictate divergent microbial responses.

The functional convergence of these infection-specific shifts with known CRC hallmarks suggests that chronic infections may induce or mimic early metabolic remodeling in the absence of overt neoplasia. This is consistent with the finding that metabolic reprogramming in cancer often begins at the adenoma stage, driven by transcriptional regulators such as MYC (Satoh et al., 2017). In this context, infection-triggered microbial shifts in methyl donor production and redox metabolism may act synergistically with inflammation to shape a tumor-permissive microenvironment. Furthermore, studies on the role of the microbiome in CRC have highlighted how bacteria, such as *Fusobacterium nucleatum*, promote tumor progression through metabolic and immune signaling (Kostic et al., 2013). The patterns observed in this study align with these models, suggesting the idea that microbial metabolism may serve as both a marker and mechanism of CRC risk in chronically infected populations. Finally, the broader potential implications of these findings are supported by the identification of distinct metabolic signatures in colorectal tumors with prognostic relevance (Shang et al., 2021). The detection of similar signatures in the infection-only groups suggests that gut microbial metabolism may function as a biosensor of early disease risk and a target for preventive intervention.

4.2.1. One-carbon metabolism and methyl donor flux

One-carbon metabolism emerged as a potential critical axis of metabolic reprogramming across all infection groups in this study, primarily via the enrichment of COG0190, which encodes MTHFD2. This enzyme functions at the core of the folate cycle, catalyzing the interconversion of methylene-THF to formyl-THF while generating NADPH, an essential reducing equivalent for oxidative stress buffering and biosynthetic activity. In cancer biology, MTHFD2 overexpression is strongly associated with enhanced de novo nucleotide synthesis, epigenetic flexibility, and redox homeostasis, enabling tumor cells to survive under proliferative and inflammatory pressures (Zhang and Wang, 2024; Maddocks et al., 2016). Its infection-specific enrichment in this dataset, notably in HIV-only, helminth-only, and co-infected individuals, may mirror a pre-neoplastic shift in microbial metabolism that converges mechanistically with pathways commonly dysregulated in CRC.

Elevated SAM production by MTHFD2, in tandem with infection-induced inflammation, may foster methylation drift, a precursor to epigenetic instability, gene silencing, and oncogene activation, which are key hallmarks of tumorigenesis (Mentch and Locasale, 2015; Mentch et al., 2015; Ser et al., 2016; Zhang et al., 2021). The relevance of this viral elements metabolic remodeling is further emphasized by the parallels with viral infections, such as SARS-CoV-2, which hijacks host one-carbon metabolism to support viral replication (Qiu et al., 2014). This suggests that the abundance of MTHFD2 is not exclusive to tumor contexts but reflects a broader mechanism of infection-induced metabolic exploitation in host cells. Collectively, the infection-induced abundance of MTHFD2 suggests a microbial contribution to the host epigenetic and metabolic vulnerability. In this context, microbial one-carbon metabolism may serve as both a mechanistic link and an early biosensor for CRC risk in high-exposure populations.

4.2.2. Amino acid biosynthesis: serine-glycine axis and tumor metabolism

The relationship between amino acid biosynthesis, particularly the serine-glycine axis, and tumor metabolism has gained prominence in understanding infection-associated CRC risk. This axis is central to the serine-glycine-one-carbon (SGOC) network, which links glycolytic intermediates to the synthesis of amino acids, nucleotides, glutathione, and methyl donors. These metabolic processes are essential for redox homeostasis, epigenetic maintenance, and rapid cell proliferation, which are hallmarks of cancer progression (Wang et al., 2019; Ananieva, 2015). In this study, the abundance of COG0111 (phosphoglycerate dehydrogenase, PHGDH-like) and COG1932 (phosphoserine aminotransferase, PSAT1-like) was observed in HIV-infected and co-infected individuals, indicating potential activation of the serine biosynthesis pathway. PHGDH catalyzes the oxidation of 3-phosphoglycerate to 3-phosphohydroxypyruvate, whereas PSAT1 converts this intermediate into phosphoserine, a precursor of serine and subsequently glycine. This pathway contributes to the folate cycle, glutathione maintenance, and production of SAM, a universal methyl donor for DNA and histone methylation. Additionally, microbial homologs of methylenetetrahydrofolate dehydrogenase (MTHFD2, COG0190) contribute to the mitochondrial folate-dependent one-carbon metabolism pathway. This enzyme plays a crucial role in producing SAM (Valle-Casuso et al., 2019) (Fig. 8). Elevated levels of MTHFD2 have been widely observed in various cancers, where it contributes to tumor growth, maintenance of stem cell-like properties, and resistance to chemotherapy. Due to its role in driving tumor progression and association with poor clinical outcomes, MTHFD2 represents a promising target for anticancer therapies. Our findings suggest a mechanistic link between infection-induced viral elements and metabolic reprogramming.

Thus, activation of the serine-glycine biosynthesis pathway in helminth individuals may reflect a multi-layered metabolic adaptation that simultaneously supports persistent infection and primes the host for oncogenic transformation. Its suppression in helminth-only cases further underscores the infection-specific metabolic trajectories. Thus, the SGOC axis serves as both a mechanistic bridge between infection and CRC risk and a potential intervention point for early cancer prevention in high-risk populations. The SGOC axis emerges as a promising potential therapeutic target. Modulating PHGDH or PSAT1 activity could impair tumor-supportive metabolic fluxes while restoring immune functionality. As Wang et al. (2019) suggested, targeting enzymes in this pathway could simultaneously limit infection-driven immune exhaustion and tumor proliferation.

4.2.3. Lipid biosynthesis and membrane remodeling

A distinct feature of the infection-altered metabolic landscape in this study was the enrichment of COG0304, which encodes 3-oxoacyl-ACP synthase, particularly in HIV-positive individuals. This enzyme is a key catalyst in the fatty acid elongation cycle, driving de novo lipid biosynthesis, which fuels membrane remodeling, vesicle formation, and proliferation. In both viral infections and cancer, fatty acid synthesis is frequently reprogrammed to accommodate the increased membrane turnover and signaling needs of rapidly replicating cells (He et al., 2018; Lorizate et al., 2013; Waheed and Freed, 2010). In the context of HIV infection, this metabolic shift suggests that the host lipid machinery is repurposed to support viral replication while sustaining inflammatory and regenerative responses within the gut microenvironment. Elevated lipid biosynthesis not only provides the structural lipids necessary for viral envelope assembly but also contributes to bioenergetic flexibility and immune modulation in the host (Chan et al., 2010; Heaton and Randall, 2011). Such changes may synergize with broader tumorigenic processes, as metabolic autonomy through lipid production is a hallmark of both infections and neoplastic transformation.

These findings are consistent with evidence from other viral systems. For instance, Hepatitis C Virus (HCV), coronaviruses, and herpesviruses have been shown to hijack host fatty acid biosynthesis pathways to create replication organelles or evade immune detection (Syed et al.,

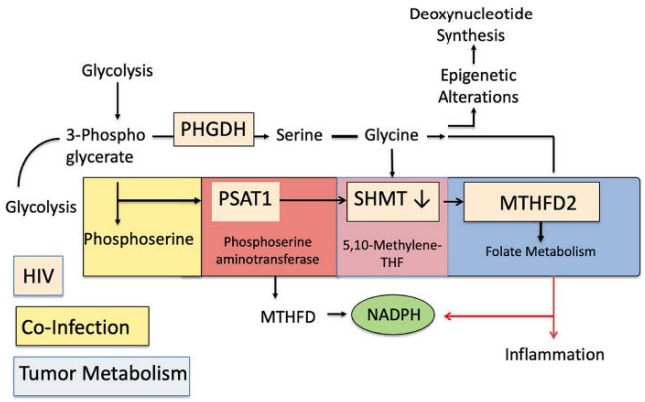


Fig. 8. The serine-glycine-one-carbon (SGOC) metabolic axis linking glycolytic intermediates to folate cycle activation, NADPH production, and epigenetic modulation. Enriched enzymes in HIV and co-infection contexts (PHGDH, PSAT1, MTHFD2) contribute to increased methyl donor synthesis and redox buffering, mirroring tumor metabolism and enhancing CRC risk.

2010; Song et al., 2024). This convergence suggests a conserved viral strategy that mimics cancer-like metabolic remodeling to maximize replication efficiency. Viruses and tumors share dependencies on elevated lipid turnover, redox buffering, and membrane-bound signaling platforms (Butler et al., 2020; Bartolacci et al., 2021). Moreover, microbial interactions may have amplified this metabolic phenotype. Altered gut viral elements configurations in HIV-positive individuals may stimulate compensatory lipid biosynthesis as part of a broader host-microbe adaptation to inflammation and oxidative stress. While these shifts are initially protective, they may inadvertently prime epithelial tissues for malignant progression by promoting cell survival and immune escape mechanisms. In summary, the abundance of lipid biosynthesis genes in HIV-infected individuals may reflect a metabolic interface between chronic viral infection and tumorigenesis, where HIV-driven reprogramming of host lipid pathways parallels oncogenic metabolic processes. This metabolic convergence suggests that inhibition of fatty acid synthesis may simultaneously impede HIV replication and mitigate infection-associated carcinogenic risk.

4.2.4. Ion transport, redox balance, and proliferation signals

Dysregulation of iron metabolism is increasingly recognized as a critical factor in the pathological processes associated with HIV infection and may contribute to the mechanisms underlying colorectal carcinogenesis. Evidence suggests that an increase in microbial iron transport genes, such as COG0370, occurs in individuals infected with HIV and helminths, indicating that these pathogens or their associated dysbiosis exploit host iron to support microbial survival. This activity may inadvertently generate a pro-oxidative environment in the gastrointestinal tract. Iron, a redox-active metal, facilitates Fenton chemistry, producing hydroxyl radicals that promote DNA damage, lipid peroxidation, and inflammation, all of which are closely linked to tumor initiation and genomic instability (Shankaran et al., 2017; Shytaj et al., 2020).

Furthermore, the upregulation of COG0486, a predicted regulatory GTPase, in samples from HIV and helminth infected individuals suggests alterations in host-microbe signaling networks that affect cell proliferation and migration. GTPases are central to cell cycle progression, cytoskeletal remodeling, and vesicle transport, and have been increasingly implicated in cancer cell invasion and chemoresistance (Gonçalves et al., 2019; Kerins and Ooi, 2018). Although the exact function of the microbial GTPase identified here remains unknown, its consistent upregulation implies a microbial strategy of mimicking or modulating host signaling to promote epithelial transformation. Additionally, research has shown that HIV-infected cells exhibit increased iron uptake and oxidative stress, partly due to reduced glutathione (GSH) levels, which weakens their antioxidant defenses and renders them more susceptible to damage and transformation (Bhaskar et al., 2015). Dysregulation of hepcidin, the central regulator of iron homeostasis, further exacerbates this vulnerability by disrupting systemic iron distribution, contributing to iron overload or anaemia, depending on the context (Cunha et al., 2015; Huibers et al., 2020). These findings align with the microbial gene expression shifts observed in this study and indicate a multi-level disturbance of redox balance.

In summary, the coordinated upregulation of microbial genes involved in iron acquisition and signal transduction during chronic infection, particularly in the setting of HIV or co-infection, may reshape the mucosal microenvironment in favour of proliferation, inflammation, and DNA instability. These mechanisms represent converging pathways that can potentiate colorectal cancer development, emphasizing the need to consider iron metabolism and oxidative stress as key mediators of infection-associated carcinogenesis.

4.2.5. Convergence on tumor-promoting metabolic states

The provided data highlight that infection-associated shifts in microbial metabolism, especially under HIV-helminth co-infection, may remodel metabolic states in ways that mirror early tumorigenesis. This is reflected in the observed upregulation of microbial genes supporting key biosynthetic processes, including SAM synthesis, amino acid and fatty acid metabolism, and iron transport. These coordinated metabolic changes suggest a structured reprogramming of the gut microbiome that may precede and facilitate the neoplastic transformation. Chronic infections, particularly those involving HIV, disrupt microbial community composition and function, leading to a dysbiotic gut environment that supports oncogenic signaling through specific metabolic routes. For example, the upregulation of COG0370, which encodes an Fe^{2+} transport system protein, was observed in the HIV- and helminth-infected groups. Iron is a key redox-active metal that, when dysregulated, promotes oxidative stress through the generation of hydroxyl radicals, damaging DNA, and promoting inflammation. Such oxidative and inflammatory environments can promote genomic instability and contribute to tumorigenesis.

A broader understanding of these shifts is captured by the concept of functional redundancy, whereby different microbial communities,

regardless of their species composition, converge functionally by expressing similar metabolic capabilities (Glymenaki et al., 2017; Villéger et al., 2019). This functional convergence implies that chronic infections can lead to distinct microbial assemblages that activate shared tumor-promoting metabolic networks, particularly those involved in redox balance and proliferation signals. The concurrent upregulation of genes such as MTHFD2 and PHGDH in this context underscores the tight link between microbial metabolism and host cellular pathways (Aarnoutse et al., 2019; Gopalakrishnan et al., 2018). This crosstalk may enhance the biosynthetic and oxidative capacity of the local microenvironment, priming tissues for neoplastic transformation even before pathological changes become histologically visible. Moreover, these infection-altered microbial gene signatures have implications beyond pathogenesis, as they offer opportunities for early detection and therapeutic modulation. Recent findings suggest that modifying the gut microbiota can influence host responses to cancer therapy (Villéger et al., 2019; Oh et al., 2021). Such interventions may be particularly beneficial for high-risk individuals exposed to chronic infections, where microbial iron transport and redox regulation act as upstream modulators of tumorigenesis. In conclusion, dysregulation of ion transport and redox-related microbial genes in individuals co-infected with HIV and helminths reveals a mechanism by which infection-induced metabolic reprogramming contributes to early events in colorectal cancer development. These findings emphasize the therapeutic value of targeting microbial metabolic functions, such as iron acquisition and oxidative stress modulation, as part of a personalized strategy for CRC prevention and intervention.

4.2.6. Differential pathway activity

Aggregate pathway analysis revealed distinct metabolic signatures associated with CRC in the context of HIV and helminth co-infection. The upregulation of the one-carbon metabolism by the folate pathway in CRC relative to the co-infection group (logFC = 0.30, adj. p = 0.054) suggests enhanced folate-dependent methylation and nucleotide biosynthesis that could facilitate tumor proliferation. This observation aligns with the known role of folate metabolism in sustaining rapid cell division and maintaining DNA methylation patterns that favour oncogenic transcriptional programs. In contrast, the downregulation of ATP transporter and glycine, serine, and threonine metabolism pathways implies suppressed mitochondrial activity and amino acid flux, potentially reflecting metabolic trade-offs during tumor adaptation in immune-altered environments. Together, these findings suggest that disruption of folate and energy metabolism may represent a critical mechanistic link between HIV-helminth co-infection and heightened CRC susceptibility through dysregulated methylation and metabolic reprogramming. When compared with helminth infection alone, CRC samples showed a consistent, though nonsignificant, elevation in folate-mediated one-carbon metabolism, reinforcing the notion that even mild shifts in this pathway may signal early tumorigenic events under chronic inflammatory or parasitic conditions. Meanwhile, the CRC versus HIV comparison revealed a significant suppression of the glycine, serine, and threonine metabolism pathway (adj. p = 0.032), suggesting that HIV infection may alter amino acid utilization and one-carbon substrate availability. Such alterations could compromise metabolic flexibility, thereby influencing tumor energetics and methylation balance in a manner distinct from helminth-driven effects.

Collectively, these results point toward an intricate metabolic interplay at the intersection of infection and tumorigenesis. They highlight the need for deeper investigation into how chronic infectious exposures modulate methylation-linked metabolism and thereby shape cancer risk in African populations, where HIV-helminth co-infection remains prevalent.

The GSVA-based pathway quantification revealed context-dependent but consistent modulation of folate-linked and amino acid pathways across infection and cancer states. The upregulation of one-carbon metabolism in CRC, contrasted with its suppression in infection, suggests adaptive reactivation of methylation and nucleotide synthesis pathways to support tumor growth. Concurrent downregulation of amino acid and ATP transporter activity indicates disrupted energy balance, likely compounding the metabolic stress induced by chronic infection. While most pathways did not reach FDR significance, these coordinated shifts provide a mechanistic framework linking infection-driven immune alterations to CRC-associated metabolic reprogramming.

Overall, this study's findings suggest that microviral elements' metabolism and epigenetics represent central axes through which chronic infections may modulate the gut environment, potentially reprogramming it to promote colorectal oncogenesis. Although causality cannot be established from this cross-sectional metagenomic study, the convergence of immunosuppressive profiles, virome network restructuring, and metabolic and epigenetic reprogramming supports a model in which chronic infection disrupts host-microbiome equilibrium in ways that increase cancer susceptibility. These insights are particularly important for African populations, where HIV-helminth co-infections are endemic and virome-related cancer research remains underexplored. Future longitudinal and mechanistic studies are essential to unravel the causal pathways linking chronic infection, virome perturbation, and CRC risk.

4.3. Clinical and translational relevance

The identification of infection-associated microbial methylation enzymes, particularly COG2189 (adenine-specific DNA methylase) and COG0286 (Type I restriction-modification methyltransferase), has substantial implications for early detection of CRC and targeted intervention. These enzymes, consistently enriched in the helminth-only, co-infected, and CRC-confirmed groups, appear to reflect a state of heightened methylation permissiveness in the gut epithelium. Their microbial origin and infection-specific enrichment suggest that they may serve as early, non-invasive biomarkers of epigenetic dysregulation and cancer risk. Stool-based metagenomic detection of methylation-related enzymes presents a practical and scalable diagnostic approach, particularly suited to low-resource regions where access to conventional screening modalities, such as colonoscopy and histopathology, remains limited (Emlet et al., 2020; Li et al., 2022). In sub-Saharan Africa, where both the infectious disease burden and CRC incidence are rising, such tools could enable earlier detection and stratified surveillance among high-risk populations (Luo et al., 2022; Sun et al., 2023).

Importantly, these microbial methyltransferases not only serve as potential biomarkers but may also act as therapeutic targets for CRC. For instance, COG2189 and its associated 6 mA activity have been linked to transcriptional activation in repressive chromatin regions, tumor stemness, and immune evasion. Similarly, the co-expression of COG0286 in co-infection states underscores the role of virome-encoded epigenetic machinery in shaping host gene expression. Targeted inhibition of these microbial methylases or modulation of downstream host pathways disrupted by 6 mA accumulation could restore epigenetic equilibrium and mitigate carcinogenic potential (Chen et al., 2022; Qin et al., 2018). Restoration of host epigenetic surveillance may also involve the reactivation or therapeutic enhancement of demethylating enzymes, such as ALKBH1, whose dysregulation in cancer models promotes 6 mA persistence and epigenomic instability (Chen et al., 2022). Integrating these microbial targets with host methylation regulators could form the basis for multi-modal epigenetic therapies.

Moreover, by coupling metagenomic signatures with host genomic and transcriptomic data, precision oncology frameworks can be developed to better characterize infection-associated CRC subtypes (Li et al., 2022). This approach supports a paradigm shift from static host-centric models to dynamic host-microbe interactions, in which chronic infection and microbial metabolism synergize to rewire the epigenome and promote malignancy (Emlet et al., 2020; Luo et al., 2023). In conclusion, the integration of viral elements associated methylation markers into

clinical practice offers an innovative strategy for detecting and disrupting infection-driven carcinogenesis. As sequencing technologies become more accessible and epidemiological surveillance expands, these findings can inform cost-effective screening programs, guide molecularly targeted therapies, and improve CRC outcomes in regions facing the overlapping burdens of infection and cancer (Zuo et al., 2022; Borin et al., 2023).

5. Challenges and limitations

All stool samples were collected before surgical intervention, bowel preparation, or antibiotic administration, minimizing external perturbations of the gut microbiota. Nonetheless, detailed perioperative metadata (e.g., diet, medications, and clinical parameters) were not systematically recorded, which restricted deeper contextual analysis. Although excluding or adjusting for perioperative conditions would have strengthened the analysis, this was not feasible due to the limited sample size and lack of standardized metadata. Nonetheless, because all CRC samples were obtained under comparable preoperative conditions, intra-group variability is likely minimal. The relatively small sample size limited the statistical power to detect subtle microbial-epigenetic associations; however, consistent patterns observed across groups highlight biologically meaningful trends that warrant further exploration. Future studies should increase cohort size, include longitudinal sampling, and apply integrated multi-omics to validate causal pathways linking infection-driven microbiome remodeling, host methylation changes, and CRC susceptibility. Standardized and well-documented sample collection protocols are also noted for future studies with a larger cohort to validate these findings. Despite these limitations, this study provides the first evidence in a Sub-Saharan African context linking HIV-helminth co-infection to gut microbial and epigenetic signatures resembling those observed in CRC.

6. Conclusion

In a setting burdened by overlapping infectious diseases and an escalating CRC burden, our integrative multi-omic approach illuminates how chronic HIV and helminth co-infections reprogram the molecular landscape toward oncogenesis. This framework enabled a comprehensive interrogation of metabolic and epigenetic dynamics in a South African cohort uniquely positioned at the intersection of infection and cancer vulnerability. These poorly characterized viral elements suggest a cryptic reservoir of oncogenic potential, supporting the emerging concept of an "onco-viral elements" in African populations, where dysbiotic viral communities may drive early carcinogenic processes. Our findings reveal a potential novel convergence of viral reprogramming, viral elements metabolism, and epigenetic dysregulation that together may foster a permissive microenvironment for CRC initiation. This study not only advances our understanding of infection-associated carcinogenesis but also emphasizes the critical need to integrate infection history and viral elements ecology into CRC risk stratification, particularly in resource-limited regions facing syndemic disease pressures. Our findings also suggest that chronic infections may enhance the host's methylation potential not only through the direct expression of viral methyltransferases but also via metabolic upregulation of the methyl donor biosynthesis machinery (Fig. 9). Together, these observations underscore the potential for both direct and indirect epigenetic modulation by the gut virome in the risk of infection-associated CRC. The integration of viral elements methyltransferase expression (e.g., COG2189 and COG0286) with metabolic reprogramming (COG0190) reveals a multi-pronged mechanism through which chronic infections may prime the host intestinal epigenome toward a pro-oncogenic state. These trends align with the broader metagenomic findings of this study, which illustrate the distinct enrichment of methylation-related genes across the infected and CRC-positive groups. Such insights are particularly important in regions such as sub-Saharan Africa, where overlapping infectious burdens are high and molecular predictors of cancer susceptibility remain poorly characterized. The convergence of microbial signaling, metabolic plasticity, and host epigenetic dysregulation strengthens the hypothesis that chronic infection is a modifiable risk factor for CRC via methylation-driven genomic instability.

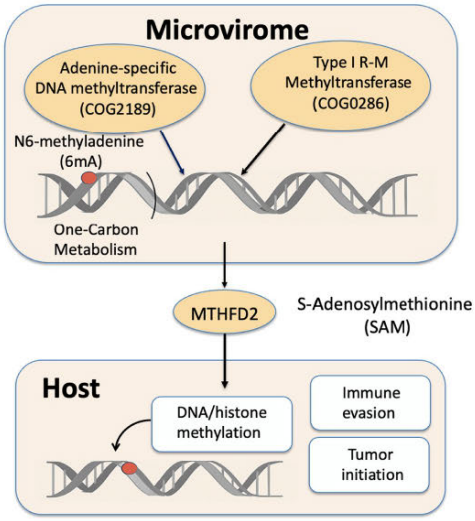


Fig. 9. Intersecting Viral elements Regulatory pathways. Viral elements-derived epigenetic enzymes (COG2189, COG0286) and one-carbon metabolism (MTHFD2) collaboratively drive host DNA and histone methylation via S-adenosylmethionine (SAM) production. These infection-associated mechanisms promote immune evasion and tumor initiation, establishing a methylation-permissive microenvironment in the gut epithelium.

CRediT authorship contribution statement

Botle Precious Damane: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Jonathan Featherston:** Writing – review & editing, Visualization, Methodology, Formal analysis. **Shakeel Kader:** Writing – review & editing, Data curation. **Mohammed Alaouna:** Writing – review & editing, Visualization. **Pragalathan Naidoo:** Writing – review & editing, Validation, Supervision. **Zodwa Dlamini:** Writing – review & editing, Validation, Supervision, Funding acquisition. **Zilungile Lynette Mkhize-Kwitshana:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Data availability statement

Data generated from this study can be accessed from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate. The study was conducted in accordance with the Declaration of Helsinki and approved by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (BREC/00005458/2023) on May 18, 2023. Informed

consent was obtained from all subjects involved in the study.

Clinical trial number

Not applicable.

Consent to publish declaration

All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Botle Precious Damane, Zodwa Dlamini and Zilungile Mkhize-Kwitshana reports financial support was provided by South African Medical Research Council and National Research Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.amolm.2026.100103.

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CHAPTER SEVEN

This thesis set out to investigate an underexplored frontier, the intersection of the gut virome, host epigenetics, cancer-related metabolism, and immune regulation in HIV-helminth co-infected individuals, within a South African population carrying some of the world's highest burdens of infection-associated cancers. While each of these domains has been studied independently, their convergence in shaping colorectal cancer (CRC) risk in high-burden settings has remained largely unexplored. Through a stepwise approach, this work mapped virome shifts linked to HIV and helminth infection, evaluated established CRC screening tools such as methylated vimentin, and integrated virome-driven methylation and metabolic gene expression profiles. Importantly, we also observed the upregulation of immune checkpoint molecules, suggesting the presence of an immunosuppressive tumour-like microenvironment in these infection states. This finding aligns with known mechanisms of immune evasion in cancer and points towards possible implications for immunotherapy responsiveness in such populations.

The findings demonstrate that infection-driven biological alterations are not peripheral, transient phenomena but central, measurable processes with the potential to be harnessed for early CRC detection and risk stratification. By combining microvirome ecology, molecular oncology, metabolic profiling, and immune regulation analyses, this study advances a more integrated model for understanding cancer risk in immunologically complex settings. Beyond its scientific novelty, the work responds directly to a pressing public health need. South Africa, like much of sub-Saharan Africa, faces the dual challenge of a rising cancer burden alongside persistent infectious diseases. The tools and insights developed here could inform the development of affordable, non-invasive, and context-appropriate screening and prevention strategies, while also opening avenues for tailored immunotherapy approaches in infection-associated cancer contexts. This chapter synthesises the study's key findings, situates them within the broader scientific and clinical landscape, and outlines their implications for research, policy, and practice. It also reflects on the challenges and limitations encountered and sets forth a vision for future investigations that build on these foundations to deliver tangible health benefits for vulnerable populations.

Synthesis

7.1 Preamble

Colorectal cancer incidence is rising in sub-Saharan Africa, a region where HIV and helminth infections remain highly prevalent and often coexist in populations burdened by poverty, poor sanitation, and limited healthcare access. The success of ART has extended the lifespan of PLWH,

but it has also unmasked an increasing burden of non-communicable diseases, notably CRC. Persistent immune activation despite ART, coupled with helminth-driven Th2-skewed responses and regulatory cell enrichment, contributes to a chronically dysregulated immune environment that may promote oncogenesis. This study supports the hypothesis that HIV-helminth co-infection is associated with features linked to CRC susceptibility, including alterations in key immune, microbial, and epigenetic pathways. We demonstrated that co-infection significantly modulated immune checkpoint expression, marked by upregulation of PD-1, PD-L2, and TIM-3, and downregulation of the co-stimulatory molecule CD28, reflecting an exhausted and immunosuppressive T cell phenotype. These patterns parallel immune escape mechanisms commonly observed in oncogenesis.

Shotgun metagenomic sequencing revealed that the gut virome in co-infected individuals resembled that of confirmed CRC cases. Both groups exhibited enrichment of viral genes involved in metabolic regulation (COG0304, COG1932, COG0111, COG0190), immune evasion, and epigenetic modulation, most notably MnmE GTPase (K03650) and adenine-specific DNA methylase (COG2189). These findings suggest that viral communities may contribute to host stress responses, metabolic reprogramming, and methylation changes, thereby fostering a microenvironment conducive to malignancy. The emergence of several uncharacterized viral genes further points to a potentially novel "onco-microvirome," reflecting a virome-driven functional landscape enriched with cancer-relevant traits. Parallel evaluation of stool-based methylation markers highlighted important context-specific limitations. While Vimentin retained high sensitivity for CRC detection, it showed low specificity in infected controls.

Collectively, this body of work highlights a complex interplay between infection, immune dysregulation, viral functional activity, and epigenetic alterations. These findings suggest an infection-associated oncogenic landscape characterised by features such as immune exhaustion, viral mimicry, and disrupted epigenetic control. While causality cannot be inferred, these insights contribute to existing theoretical frameworks and underscore the potential importance of integrating infection history, microbial ecology, and immune profiling into CRC risk stratification and screening strategies. These findings highlight urgent opportunities for context-specific biomarker discovery, early cancer surveillance, and infection-targeted prevention strategies, particularly in underrepresented and high-burden populations.

7.2 Summary of current findings

Using a retrospective observational design, we analyzed archived stool and blood samples from a well-characterized South African cohort stratified by infection status into HIV-only, helminth-only, HIV-helminth co-infected, and uninfected control groups. This approach allowed us to interrogate how chronic infections modulate immunological, viromic, and epigenetic landscapes in a population facing a high burden of infectious disease and rising CRC incidence. Our immune profiling revealed pronounced dysregulation of checkpoint and co-stimulatory molecules. Helminth-infected individuals exhibited a significant reduction in B and T lymphocyte attenuator (BTLA), a regulatory checkpoint that interfaces with HVEM to bridge critical T cell signaling pathways. This loss suggests an early disruption of immune homeostasis that may facilitate persistent inflammation or inadequate cancer surveillance. In HIV-infected and co-infected participants, CD28, a vital co-stimulatory molecule essential for T cell activation, was markedly downregulated, consistent with sustained T cell exhaustion. Additionally, PD-L2, a ligand characteristic of alternatively activated (M2) macrophages, was significantly elevated in co-infected individuals, indicating a shift toward a Th2-skewed, immunosuppressive microenvironment favorable to tumor progression. Collectively, these immune alterations reflect an environment of immune suppression and dysfunction that likely diminishes antitumor immunity, accelerating CRC development.

Shotgun metagenomic sequencing of stool DNA uncovered a distinctive “onco-microvirome” shared between CRC patients and HIV-helminth co-infected individuals. Viral taxa, including *Podoviridae*, *Myoviridae*, and CrAss-like phages, emerged as central nodes within viral ecological networks, implying pivotal roles in shaping bacterial communities linked to inflammation and oncogenesis. Functional annotation revealed consistent upregulation of genes such as the MnmE GTPase (K03650), implicated in translational regulation during cellular stress, and adenine-specific DNA methylase (COG2189), which participates in DNA replication, recombination, and repair. These viral functions indicate that chronic infection induces a virome capable of modulating host metabolic and epigenetic pathways implicated in tumorigenesis. Notably, many enriched viral taxa remain unclassified, underscoring a critical gap in virome characterization within African populations and highlighting potential novel contributors to infection-driven carcinogenesis.

Epigenetic biomarker analysis further revealed population-specific challenges. Although Vimentin methylation demonstrated 100% sensitivity in detecting CRC, its poor specificity, due to high methylation prevalence across non-CRC groups, limits its utility as a discriminative

biomarker. The fecal occult blood test performed well in CRC detection but was insufficient for risk prediction among infected individuals. These findings emphasize the necessity for tailored validation of non-invasive biomarkers in regions burdened by endemic infections.

Extending beyond these observations, our analysis highlighted consistent modulation of microbial genes involved in core metabolic processes across infected groups. Key enzymes such as COG0304 (3-oxoacyl-[acyl-carrier-protein] synthase), integral to lipid metabolism, and COG1932 (phosphoserine aminotransferase), pivotal in amino acid and coenzyme metabolism, were differentially expressed. Additional metabolic genes, including COG0111 (phosphoglycerate dehydrogenase) and COG0190 (5,10-methylene-tetrahydrofolate dehydrogenase), further reinforced a pattern of microbial metabolic reprogramming, a hallmark of the tumorigenic microenvironment. The detection of COG0486, a predicted regulatory GTPase, suggests shifts in viral regulatory pathways potentially contributing to cellular transformation. Concurrently, the upregulation of COG2189 across helminth-only, HIV-helminth co-infected, and CRC groups reflects a shared epigenetic dysregulation likely predisposing host tissues to genomic instability.

Importantly, several novel, uncharacterized viral genes were differentially expressed, particularly in co-infection contexts, suggesting the presence of unexplored molecular signatures that may mediate infection-driven immune perturbation and early oncogenic processes. These findings align with the emerging concept of an “onco-microvirome,” where a dysbiotic viral community expresses functional genes that contribute to cancer hallmarks. Chronic immune activation from HIV, combined with the immunomodulatory effects of helminth infections, appears to drive these virome-level shifts, reshaping the microbial gene pool in patterns convergent with CRC-associated signatures.

Taken together, this study supports an association between HIV-helminth co-infection and a microenvironment with features linked to colorectal tumorigenesis. By bringing together findings on immune checkpoint dysregulation, viral functional changes, microbial metabolic shifts, and epigenetic modifications, this work highlights how chronic infections may be connected to pathways involved in cancer development. While causality cannot be inferred, these findings underscore the importance of considering infection history and microbial ecology in CRC risk assessment and biomarker validation, particularly in resource-limited settings with a high infectious disease burden. Future functional studies on these microbial-host interactions will be critical for advancing early detection, risk stratification, and targeted interventions in infection-

associated cancers.

7.3 Discussion and Conclusion

This study demonstrates that chronic HIV and helminth infections, individually and combined, significantly alter the immune system and gut microbial environment in ways that mimic potential CRC development. HIV-helminth co-infected individuals exhibit immunosuppressive profiles that likely impair cancer surveillance, while the gut virome shows functional shifts with enrichment of viral taxa and genes involved in metabolism and epigenetic regulation, underscoring the microbial role in fostering a cancer-promoting microenvironment. Our immune profiling revealed a constellation of checkpoint disruptions across infection states. Notably, helminth-infected individuals exhibited significant downregulation of BTLA, a co-inhibitory receptor that binds herpesvirus entry mediator (HVEM) to regulate T cell signaling. The loss of this regulatory axis may reflect early immune dysregulation, favouring chronic inflammation or impaired tumor surveillance. In contrast, HIV-infected and co-infected individuals displayed reduced expression of CD28, a critical costimulatory molecule necessary for effective T cell activation, an alteration consistent with progressive T cell exhaustion. The co-infected group also showed significant upregulation of PD-L2, a marker associated with alternatively activated (M2) macrophages and Th2-skewed immune responses. These shifts collectively suggest an immunosuppressive and tumor-permissive environment, aligning with cancer-prone immune phenotypes observed in chronic infections.

These interconnected changes reveal how persistent infections may drive an oncogenic landscape through sustained immune activation, microvirome dysbiosis, and epigenetic remodeling, predisposing individuals, especially in endemic regions, to early oncogenesis. The overlap between infection-associated and cancer-associated signatures also highlights challenges in using conventional diagnostic tools, emphasizing the need for context-specific strategies that integrate infection status, immune function, and microbial ecology to enhance CRC prevention and early detection in resource-limited settings. Notably, the high prevalence of vimentin methylation in both CRC and infected individuals further supports the notion that HIV-helminth co-infections may predispose these individuals to an increased risk of CRC. Overall, our findings suggest that HIV and helminth infections are associated with features linked to colorectal carcinogenesis by orchestrating immune suppression and microbial functional reprogramming. Thus, while causality cannot be inferred, understanding this potential infection-associated oncogenic microenvironment is crucial for developing integrated prevention and cancer control strategies tailored to high-risk populations in endemic settings.

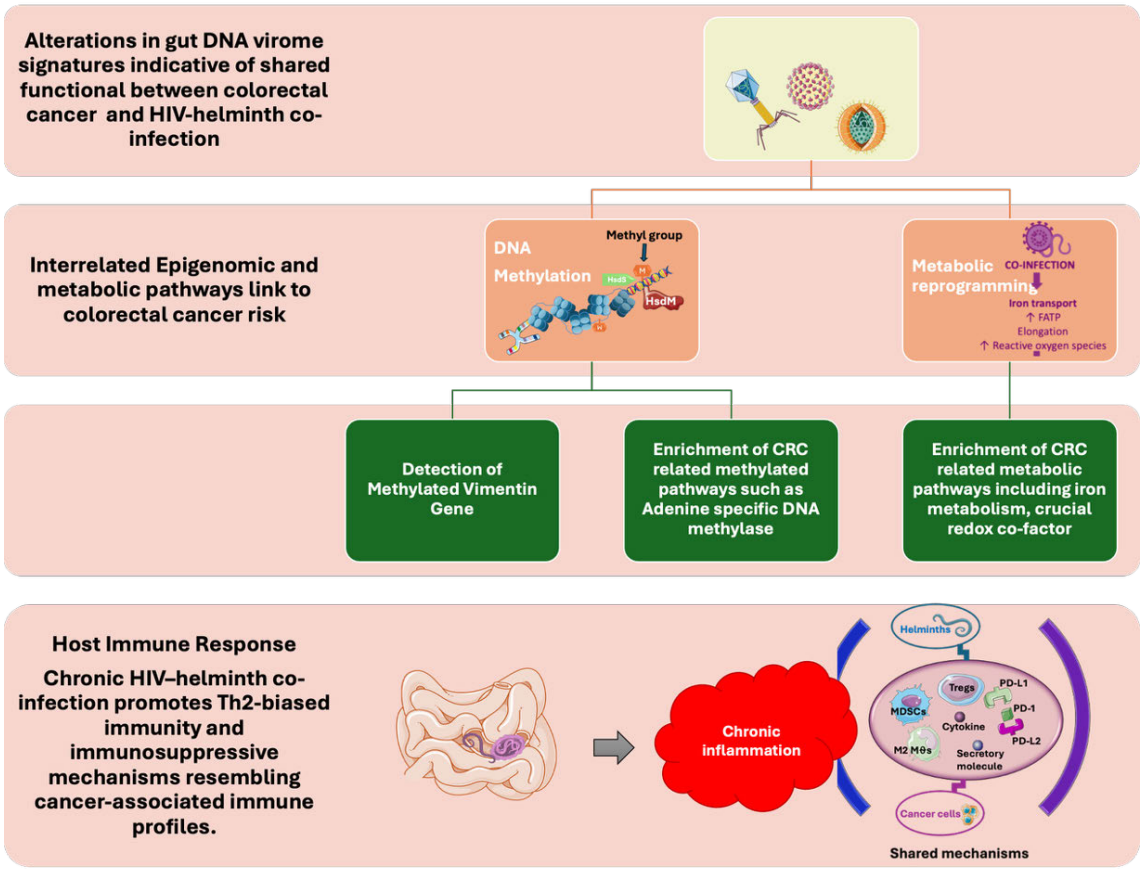


Figure 7.1: Concluding Figure

Persistent HIV-helminth co-infection may contribute to an oncogenic landscape through immune dysregulation, microvirome imbalance, altered metabolic pathways, and epigenetic remodeling, potentially increasing susceptibility to early carcinogenesis, particularly in endemic populations.

7.4 Challenges and limitations

This study faced several methodological and biological challenges that may have influenced the findings. One key limitation is the high frequency of vimentin methylation observed across both CRC and non-CRC groups, which reduces its specificity as a standalone biomarker. These findings highlight the need for more sensitive, population-adapted, multiplexed methylation assays to enhance the accuracy of fecal-based CRC screening, particularly in populations burdened by infectious diseases. In addition, the use of stool-derived DNA, while advantageous due to its non-invasive collection and relevance in low-resource settings, presents challenges related to sample quality, DNA degradation, and biomarker detection sensitivity.

In this study, archived stool samples collected several years prior were used. Stool is inherently more complex, containing a heterogeneous mix of host, microbial, and dietary DNA. It is also technically more difficult to preserve than blood or plasma. Research indicates that optimal preservation of stool DNA and microbial profiles requires freezing within 15 minutes of defecation, immediate aliquoting, and storage at -80°C. Degradation increases significantly with delayed processing or repeated freeze-thaw cycles. While some microbial data remain stable at -20°C for short periods, high-sensitivity assays like methylation-specific PCR are more likely to be compromised by even minor losses in DNA integrity. DNA extraction methods may also introduce compositional bias, potentially influencing observed viral abundance, while translating metagenomic profiles into successful viral or host cultivation remains technically challenging. For practical reasons, this study focused exclusively on DNA viruses; RNA viruses, which are more labile and could not be consistently preserved during field collection, were not captured and may represent an underexplored aspect of CRC-associated virome dynamics. Despite these constraints, the study provides preliminary insights into infection-associated microbial and viral patterns, highlighting the need for validation in larger, independent cohorts under standardized collection and storage protocols.

Further limitations of this study are the modest overall cohort size, particularly within the CRC group used as positive controls to examine potential links between HIV-helminth co-infection and CRC risk. While diversity and functional enrichment analyses revealed significant differences, the small sample size limits the ability to detect subtle biological effects and increases susceptibility to inter-individual variability. Consequently, these results should be interpreted as hypothesis-generating rather than definitive. The high dimensionality of metagenomic data also introduces the possibility of false positives; to address this, differential abundance analyses were performed using count-based modeling with false discovery rate correction, although some residual uncertainty may remain. CRC samples were obtained from two hospitals within the same metropolitan region following standardized protocols, but formal batch correction was not applied, which could contribute to unaccounted technical variation. Thus, subtle site-specific factors cannot be entirely excluded, despite harmonized sample processing procedures. The limited CRC sample size also precluded reliable stage-stratified analyses, as incorporating staging information under these conditions could risk over-interpretation; adequately powered future studies should include stage-specific assessments. Despite these constraints, the study offers valuable preliminary insights, highlighting the need for validation in larger, independent cohorts to strengthen evidence for mechanistic links between co-infection and colorectal carcinogenesis.

Several unmeasured or partially characterized factors may have influenced the findings of this study. The cross-sectional design precludes assessment of temporal dynamics, and longitudinal studies would be necessary to establish causal relationships between HIV-helminth co-infection and immune or virome changes. The unknown duration of both HIV and helminth infections complicates interpretation, as distinct immunological alterations are likely to occur across acute versus chronic phases. Similarly, the duration and timing of antiretroviral therapy were not fully documented, particularly in the context of South Africa's "test and treat" policy implemented in 2016, which may influence virome composition independently of HIV infection. Although this study evaluated selected co-stimulatory and co-inhibitory immune checkpoints, future investigations should broaden immune profiling to include additional modulatory molecules implicated in antitumor immunity. Finally, while all HIV-infected participants were on ART, reducing within-group heterogeneity, other environmental and clinical variables were not comprehensively captured, potentially contributing to residual variability in virome and immune profiles.

By combining virome dynamics, epigenetic modulation, cancer-related metabolic reprogramming, and immune checkpoint regulation within the context of HIV-helminth co-infection, this thesis has charted new scientific territory. The patterns uncovered here do not merely speak to known CRC signatures; they reveal how infection-driven biology may actively shape cancer risk and progression in vulnerable African populations. The findings of this study have practical as well as theoretical significance. They offer a scientific foundation for context-appropriate, non-invasive cancer screening strategies and point towards precision medicine approaches that could improve early detection, prevention, and even therapeutic decision-making in settings historically left at the margins of cancer research. Importantly, they emphasise the importance of conducting studies that benefit our population. The message here is that African populations are not merely recipients of global cancer knowledge but active contributors to the discovery of solutions that can benefit the world. While the work presented here opens new research avenues, it also provides a clear roadmap for addressing them. Its legacy will be measured not only by its academic contribution, but by its potential to transform the way cancer is understood, detected, and prevented in the presence of persistent infectious disease burdens. In that sense, this thesis is a potential start of a more inclusive, integrated approach to tackling cancer in Africa and beyond.

7.5. Future perspectives

This study highlights a growing imperative in global health: the need to recognize and respond to the syndemic interplay between chronic infections and non-communicable diseases such as cancer. In many LMICs, particularly in sub-Saharan Africa, the coexistence of HIV and helminth infections creates a unique immunological and microbial landscape that may potentiate CRC development. Our findings suggest that this altered landscape, marked by chronic immune activation, microbial dysbiosis, and virome reshaping, acts as a permissive environment for oncogenesis. These results indicate a broader shift in our understanding of cancer risk, moving beyond traditional genetic and lifestyle factors to encompass infection-driven immune modulation. Despite the success of ART in extending the lives of PLWH, this increased longevity has revealed a hidden burden: persistent immune dysregulation and inflammatory signaling that may predispose to malignancy. The additional presence of helminth infections, still prevalent in many resource-limited communities, adds another layer of immunological complexity that conventional cancer risk models fail to address.

Looking ahead, several critical research directions emerge. First, future studies should aim to functionally annotate novel viral and microbial genes identified in co-infected individuals. These may play active roles in immune evasion, inflammation, or oncogenic signaling. Longitudinal cohort studies that integrate cytokine profiling, metagenomics, and spatial transcriptomics will be crucial in distinguishing early causative events from downstream consequences of tumor development. Particularly, the temporal dynamics of immune checkpoint dysregulation in co-infected individuals warrant deeper investigation, as they may offer early windows for immune-modulatory interventions. In parallel, there is an urgent need to redefine CRC screening strategies for populations burdened by infectious comorbidities. While current methylation markers such as vimentin, have shown some utility, their poor specificity and sensitivity in this setting highlight the limitations of applying Eurocentric biomarker panels to African populations without local validation. Emerging epigenetic candidates, including LINE-1, MGMT, TFAP2E, RASSF2, and SFRP2 [1, 2], offer more promise and should be explored further, particularly in stool-derived formats. However, the effectiveness of these markers will depend on improvements in sample preservation protocols, including prompt freezing and minimized freeze-thaw cycles, to maintain DNA integrity.

Furthermore, the integration of virome signatures into CRC risk prediction holds transformative potential. However, the lack of functional validation remains a limitation of this study. Future work should prioritise experimental validation of identified targets, including enriched metabolic

pathways and differentially methylated genes, to confirm their biological and clinical relevance. Techniques such as digital PCR (dPCR) could be employed for sensitive and quantitative validation of candidate methylation markers in larger, independent cohorts. In addition, functional microbial markers, such as viral methyltransferases and genes involved in host-microbiome signaling, may serve as next-generation classifiers that capture both infection status and cancer susceptibility. In tandem, therapeutic trials exploring the benefits of deworming, microbiome modulation, or immune checkpoint inhibitors alongside ART could inform novel prevention strategies tailored to co-infected individuals. Ultimately, this work calls for a precision public health approach, one that accounts for the unique immune, microbial, and virome profiles of high-risk groups. By building multi-omic, locally informed cancer risk models, we can improve early detection and reduce cancer disparities in settings where dual burdens of infectious and chronic diseases converge. This study lays the foundation for such efforts and highlights the importance of bridging cancer biology with infectious disease epidemiology to advance global cancer prevention.

In particular, prioritising the integration of infection control and cancer prevention represents a critical step toward reducing the burden of infection-associated malignancies in African settings. The findings of this study note the wider public health significance of HIV and helminth co-infections, particularly in high-prevalence settings where their combined effects may shape long-term disease trajectories. Targeting these co-infections within healthcare systems could improve overall health outcomes and may help alleviate the burden of infection-related cancers. Policy priorities should include incorporating infection-informed risk stratification into existing screening programmes, strengthening community awareness to encourage early care-seeking, and expanding preventive interventions such as deworming. In parallel, improving diagnostic infrastructure for the timely identification of infections and early neoplastic changes, alongside ensuring access to appropriate anti-parasitic therapies, will be critical. Furthermore, greater alignment between infectious disease and oncology policies is needed to support a coordinated and efficient approach to prevention and care. Together, these strategies have the potential to enhance long-term health outcomes and may contribute to reducing the incidence of colorectal and other infection-associated cancers in affected populations.

References

1. Nagasaka, T., Tanaka, N., Cullings, H. M., Sun, D. S., Sasamoto, H., Uchida, T., M. Koi, N. Nishida, Y. Naomoto, C. R. Boland*, et al.* Analysis of fecal DNA methylation to detect gastrointestinal neoplasia. *Journal of the National Cancer Institute* 101 (2009): 1244-58. 10.1093/jnci/djp265.

2. Fatemi, N., Tierling, S., Es, H. A., Varkiani, M., Mojarad, E. N., Aghdaei, H. A., Walter, J., and Totonchi, M. DNA methylation biomarkers in colorectal cancer: Clinical applications for precision medicine. *International Journal of Cancer* 151 (2022): 2068-81. https://doi.org/10.1002/ijc.34186.

LIST OF APPENDICES

Ethics Approval for the main study

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

22 December 2022

Prof Z Kwitshana
School of Laboratory Medicine and Medical Sciences
College of Health Sciences
Kwitshana@mut.ac.za

Dear Prof Kwitshana

Protocol: A pilot study on helminthiasis and microbes interactions: macrobiotic control of microbiota and the effects on Human Immunodeficiency Virus and Mycobacterium tuberculosis diseases, immune responses and nutritional status: Huma and *in vitro* studies
Degree: Non-Degree
BREC Ref No: BE351/19

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 24 January 2023
Expiration of Ethical Approval: 23 January 2024

I wish to advise you that your application for Recertification received on 19 December 2022 for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 14 February 2023.

Yours sincerely

[redacted]

Ms P.Pillay
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Ethics Approval for the current study

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

18 May 2023

Ms Botle Dirontso Precious Damane (222131177)
School of Laboratory Medicine & Medical Science
Medical School

Dear Ms Damane,

Protocol reference number: BREC/00005458/2023
Project title: Assessment of helminth-HIV co-infection as a potential risk factor for colorectal cancer in South African adult population
Degree: PhD

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 18 May 2023. Please ensure that any outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from 18 May 2023. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on RIG on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2020) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 13 June 2023.

Yours sincerely,

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
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Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

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