ASSESSING THE CHANGES TO THE GUT MICROBIOME FOLLOWING RADIATION THERAPY IN A BLADDER CANCER CONTEXT

MATTHEW STENDEL

Department of Surgical and Interventional Science

McGill University

Montreal QC, Canada

December 2023

A thesis submitted to McGill University in partial fulfillment of the requirement of the degree of

Master of Science

©Matthew Stendel 2023

Acknowledgements

This research would not have been possible without the support from everyone around me. I will be forever grateful for the experience I have obtained and for the opportunity I was given to live out one of my dreams. This is an experience I will never forget, and it would have never happened without all the encouragement I have received along the way.

I would first like to thank my supervisor, Dr. Wassim Kassouf, for entrusting me with this project and supporting me throughout the process. I can recall many times where I've doubted me abilities to perform certain tasks or to reach certain deadlines and you were always there to help lift my confidence in whatever I was doing. Thank you for fostering an environment where I was constantly pushed to be at my best, yet where I also felt safe.

I would also like to thank my research advisory committee chair, Dr. Ahmed Aoude, and the rest of my committee, Dr. Fabio Cury and Dr. Irah King, for their insight and feedback for my project, helping me have a better grasp on the studies I was conducting.

A huge thank you to Dr. Jose João Mansure and Dr. Eva Michaud who have mentored me throughout this project. Thank you for always being there whenever I had questions or concerns about my project, and for encouraging my growth as a scientist. Additionally, one of the main themes through my time with this team has been personal growth. Thank you both so much for supporting me through my personal growth through thick and thin. Thank you also for being

with me through the several rough patches in this growth process. It means a lot to have people in the lab environment who are so supportive and who I can look up to.

I would also like to thank the other members of the Kassouf lab who I've gotten to share this amazing experience with: Dr. Surashri Shinde-Jadhav, Sabine Fehric, Jules Ebenelian, Nour Hassan, Nada Ogbahei, and Fatima Iñigo. I am lucky and grateful to have a team that is so supportive and who I can feel safe confiding in. I will never forget the time I've spent with each and every one of you, both individually and as a team. Thank you for making this the best experience I could have possibly asked for.

Lastly, I would like to thank my friends and family. The last 2 years have been some of the most challenging throughout my life, as I have put a lot of pressure on myself, both academically and in my personal life. Despite this, I can confidently say that the last 2 years have also been some of my best, and it would not have been possible without all the support of those around me. Thank you for sticking by me through all my successes, failures, and everything in between. I would not have been able to live out this dream of mine without your support.

Statement of Integrity

I, Matthew Stendel, attest that the work submitted represents solely my own efforts. I am aware of the University rules and regulations on plagiarism and subsequent penalties.

Table of Contents

List of Abbreviations……8-9

Abstract……10-11

Résumé……12-14

Contribution of Authors……15

Chapter 1: Introduction……16-62

-1.1: Intro to Bladder Cancer……16-21

1.1.1-Epidemiology……16

1.1.2-Risk Factors……16-17

1.1.3-Molecular Alterations……18

1.1.4-Symptoms……19

1.1.5-Diagnosis……19-21

-1.2: Bladder Cancer Classification……21-33

1.2.1-Introduction……21

1.2.2-Tumor Staging/Grading……21-23

1.2.3-Non-Muscle Invasive Bladder Cancer (NMIBC)……23-24

1.2.4-Muscle-Invasive Bladder Cancer (MIBC)……25-27

1.2.5-Advanced Bladder Cancer……27-29

1.2.6-Molecular Subtypes……30-32

1.2.7-Mouse Models……32-33

-1.3: Radiation Therapy……33-47

1.3.1-Introduction……33-34

1.3.2-DNA Damage and Repair……...34-36

1.3.3-Radiation-Induced Cell Death……36-39

1.3.4-Radiation and the Immune System……..39-41

1.3.5-Radioresistance……...41-44

1.3.6-Fractionation Strategies……..44-47

-1.4: Microbiome……..48-62

1.4.1-Introduction……48

1.4.2-Functions……48-51

*1.4.3-Factors Influencing Microbiome Composition/Diversity/*Health……51-52

1.4.4-Metabolites……..53-55

1.4.5-Microbiome and Cancer…….…….55-57

1.4.6-Microbiome and Radiation Therapy……...58-60

1.4.7-Microbiome and Immunotherapy……60-62

Chapter 2: Rationale……..63

Chapter 3: Hypothesis and Objectives…….……..64

Chapter 4: Materials and Methods……65-68

-4.1-Mouse Model……..65

-4.2-*In Vivo* non-Tumor Bladder Radiation Experiment……65

-4.3-MIBC Cell Line and Cell Culture…….65-66

-4.4-*In Vivo* Tumor Model…….66

-4.5-16S rRNA Gene Sequencing……..67

-4.6-Sequence Data Processing and Functional Profile Predictions……..67

-4.7-Bioinformatic Analysis…….67-68

Chapter 5: Results…….69-85

-5.1-Impact of Bladder Radiation on the Gut Microbiome *In Vivo*……..69-73

-5.2-Sex Differences in the impact of bladder radiation on the gut microbiome…….74-80

-5.3-Impact of radiation therapy on the gut microbiome in a cold tumor model *in vivo*…….81-85

Chapter 6: Discussion……..86-97

Chapter 7: Conclusion……..98-99

References:……...100-121

List of Abbreviations

Ba/Sq	Basal/Squamous (tumor subtype)
BBN	N-butyl-N-(4-hydroxybutyl) nitrosamine
BC	Bladder Cancer
BCG	Bacille Calmette-Guérin
cGAS	Cyclic GMP-AMP Synthase
CLA	Conjugated Linoleic Acid
CT	Computed Tomography
DSB	Double-Strand DNA Breaks
DNA-PKcs	DNA Protein Kinase Catalytic Subunits
EV	Extracellular Vesicle
FACC	Facility Animal Care Committee
FMT	Fecal Microbiota Transplant
GF	Germ-Free
GLP-1	Glucagon-Like Peptide 1
Gy	Gray (Unit of Ionizing Radiation)
HDI	Human Development Index
HR	Homologous Recombination
ICD	Immunogenic Cell Death
ICI	Immune Checkpoint Inhibitor
IFN	Interferon
LumNS	Luminal Nonspecified (tumor subtype)
LumP	Luminal Papillary (tumor subtype)
LumU	Luminal Unstable (tumor subtype)
MIBC	Muscle Invasive Bladder Cancer
MIP-1a	Macrophage Inflammatory Protein 1a
MVDC	Methotrexate, Vinblastine, Doxorubicin, and Cisplatin

NBI	Narrow Band Imagine
NE-Like	Neuroendocrine-Like (tumor subtype)
NF-κB	Nuclear Factor-κB
NHEJ	Non-Homologous End Joining
NMIBC	Non-Muscle Invasive Bladder Cancer
RC	Radical Cystectomy
RI-MUHC	Research Institute of the McGill University Health Centre
ROS	Reactive Oxygen Species
RT	Radiation Therapy
SCFA	Short-Chain Fatty Acid
SOP	Standard Operating Procedure
SSB	Single-Strand DNA Breaks
TMT	Trimodal Therapy
TNF	Tumor Necrosis Factor
Treg	Regulatory T-Cell
TURBT	Transurethral Resection of the Bladder Tumor
UC	Urothelial Carcinoma
UPPL	UPPL1540 (bladder tumor model)

Abstract

According to the Canadian Cancer Society, bladder cancer (BC) is projected to be the 5th most common cancer in Canada in 2023, with a projected 13,400 total cases amongst Canadians. Of those cases, 30% are muscle-invasive (MIBC), where the standard of care, radical cystectomy, involves the complete removal of the bladder. This results in reduction in quality of life, characterized by urinary and sexual impairment. Additionally, as bladder cancer is more prevalent among older individuals, many MIBC patients will not be able to undergo surgery. Because of this, there is a need for effective bladder sparing alternatives. Radiation therapy (RT) has emerged as a viable approach in appropriately selected patients, but about 25-30% of patients do not respond and will need a salvage cystectomy. Additionally, RT is likely to induce its own adverse events, thus also reducing quality of life. Various factors are thus being studied on how to improve response to radiation while minimizing adverse events. One such factor being studied is the gut microbiome, which is implicated in radiation-induced toxicity as well as response to various combination therapies commonly used with RT. Although the effects of RT on the microbiome have been heavily studied, this relationship is still unknown in a bladder cancer context.

To study this relationship, we performed several *in vivo* experiments in which RT was administered to the mouse. These experiments included a non-tumor model where 6x6Gy radiation was administered to the bladder, and a UPPL1540 (UPPL) cold bladder tumor model where 2x5Gy radiation was administered to the tumor on the right flank. After RT was

administered, stool samples were collected from all mice, and were sequenced using 16S rRNA gene sequencing. Using this sequence data, bioinformatic analysis was performed to acquire information on the composition, diversity, and functional pathways of the microbiota.

From these experiments, we found several bacteria whose relative abundance was significantly adjusted post-RT in both mouse models. Additionally, RT-induced changes to the gut microbiota were found to be both sex-dependent, and time-dependent, where different time points showed different microbial signatures. Whether these changes induce a more beneficial or harmful microbiota is still unknown, as increases and decreases were observed in both beneficial and harmful bacteria; the role of many affected features in bladder cancer is still unknown. Microbial diversity was mostly unchanged by radiation. RT was also found to also adjust the abundance of various functional pathways in the gut microbiome, most notably the *Proteasome* pathway, which was upregulated in both experiments.

A better understanding of the effects of bladder cancer RT on the gut microbiota would allow for microbiota-centric interventions to reduce radiation-induced toxicity and improve the effectiveness of combination therapies. However, although these results allow for a better understanding of these effects, the clinical implications are still unknown, and thus further research needs to be conducted to assess the clinical relevance of these changes before any interventions can be implemented.

Résumé

Selon la Société canadienne du cancer, le cancer de la vessie (CV) devrait être le cinquième cancer le plus fréquent au Canada en 2023, avec un total de 13 400 cas projetés parmi les Canadiens. Parmi ces cas, 30 % sont des cancers à invasion musculaire (CIMV), pour lesquels la norme de soins, la cystectomie radicale, implique la résection complète de la vessie. Il en résulte une réduction de la qualité de vie, caractérisée par des troubles urinaires et sexuels. En outre, le CV étant plus fréquent chez les personnes âgées, de nombreux patients atteints de MIBC ne pourront pas subir d'intervention chirurgicale. C'est pourquoi il est nécessaire de trouver des solutions efficaces pour préserver la vessie. La radiothérapie (RT) s'est imposée comme une approche viable chez les patients convenablement sélectionnés, mais environ 25 à 30 % des patients ne réagissent pas et doivent subir une cystectomie de sauvetage. En outre, la radiothérapie est susceptible d'induire ses propres effets indésirables, réduisant ainsi la qualité de vie. Différents facteurs sont donc étudiés pour améliorer la réponse à la radiothérapie tout en minimisant les effets indésirables. L'un de ces facteurs est le microbiome intestinal, qui est impliqué dans la toxicité induite par les radiations ainsi que dans la réponse à diverses thérapies combinées couramment utilisées avec la RT. Bien que les effets de la radiothérapie sur le microbiome aient été largement étudiés, cette relation est encore inconnue dans le contexte du CV.

Pour étudier cette relation, nous avons réalisé plusieurs expérimentations *in vivo* au cours desquelles la RT a été administrée à la souris. Ces expérimentations comprenaient un modèle non

tumoral dans lequel un rayonnement de 6x6Gy a été administré à la vessie, et un modèle de tumeur vésicale froide UPPL1540 (UPPL) dans lequel un rayonnement de 2x5Gy a été administré à la tumeur sur le flanc droit. Après l'administration de la RT, des échantillons de selles ont été prélevés sur toutes les souris et ont été séquencés à l'aide du séquençage du gène de l'ARNr 16S. À partir de ces données de séquence, une analyse bioinformatique a été réalisée afin d'obtenir des informations sur la composition, la diversité et les voies fonctionnelles du microbiote.

Ces expérimentations nous ont permis de découvrir plusieurs bactéries dont l'abondance relative a été ajustée de manière significative après la RT dans les deux modèles de souris. En outre, les modifications du microbiote intestinal induites par la RT se sont révélées dépendantes du sexe et du temps, des signatures microbiennes différentes étant observées à différents points dans le temps. On ne sait toujours pas si ces changements induisent un microbiote plus bénéfique ou plus nocif, car des augmentations et des diminutions ont été observées à la fois dans les bactéries bénéfiques et dans les bactéries nocives; le rôle de nombreuses caractéristiques affectées dans le cancer de la vessie est encore inconnu. La diversité microbienne n'a pratiquement pas été modifiée par les radiations. On a également constaté que la RT modifiait l'abondance de diverses voies fonctionnelles dans le microbiome intestinal, en particulier la voie du protéasome, qui a été régulée à la hausse dans les deux expérimentations.

Une meilleure compréhension des effets de la RT du CV sur le microbiote intestinal permettrait des interventions centrées sur le microbiote afin de réduire la toxicité induite par les radiations et

d'améliorer l'efficacité des thérapies combinées. Cependant, bien que ces résultats permettent de mieux comprendre ces effets, les implications cliniques sont encore inconnues, et des recherches supplémentaires doivent donc être menées pour évaluer la pertinence clinique de ces changements avant que toute intervention puisse être mise en œuvre.

Contributions of Authors

This project was given to me by Dr. Wassim Kassouf. The non-tumor experiment was designed and performed by myself under the guidance of Dr. Eva Michaud, Dr. Jose João Mansure, Dr. Wassim Kassouf, and Dr. Fabio Cury. The UPPL tumor model experiment was designed and performed by Sabina Fehric and Dr. Eva Michaud. All DNA extraction and sequencing was performed by the McGill Genome Centre. All data processing and analysis was performed by myself under the guidance of Dr. Eva Michaud and Dr. Jose João Mansure.

Chapter 1: Introduction

1.1: Intro to Bladder Cancer

1.1.1: Epidemiology

According to the Canadian Cancer Society, bladder cancer (BC) is projected to be the 5th most common cancer in Canada in 2023, with a projected 13,400 total cases amongst Canadians. It is more common in men, accounting for over 10,000 of these projected cases [1]. BC is most prevalent in Europe and North America, while being least prevalent in Latin America, Sub-Saharan Africa, and South East Asia. Interestingly, although countries with a higher Human Development Index (HDI) tend to have increased incidences of BC, they have reduced mortality to incidence ratio, indicating higher HDI is associated with greater survival [2].

1.1.2: Risk Factors

BC is known to have several biological risk factors, such as gender, age, and race. Men are 4 times more likely than women to both develop and die from BC [3,4]. In addition to gender, age also plays a role in BC risk. In the USA, 90% of BC diagnoses were in patients over 55, while 80% of diagnoses were in those over 65 [2,5]. There are also correlations between race and BC diagnosis and outcome. Previous studies have shown that white individuals get diagnosed more than black individuals [5], however, black individuals have significantly lower survival rates, even when accounting for differences in treatment strategies [6].

In addition to biological factors, many behavioral factors also influence risk of developing BC, with smoking accounting for 50-65% of all BC cases [4]. In fact, BC is the second most common cancer associated with tobacco exposure, only behind lung cancer [5]. Particularly, in both sexes, current smokers are ~4 times as likely to develop BC as never smokers, and former smokers are ~2 times as likely [2,7]. Tobacco contains various carcinogens including beta-naphthylamine and polycyclic aromatic hydrocarbons that can promote cancer via DNA adduct formation. Interestingly, patients with abnormal detoxification enzymes have increased tobacco-induced risk of BC [4].

Occupational exposure to various carcinogens associated with paint, dye, metal, petroleum, and rubber products, account for about 20% of all BC cases [4, 8, 9]. Additionally, exposure to arsenic in drinking water at high concentrations (300-500μg/L) has been shown to increase BC risk, and lower concentrations show a synergistic effect with smoking in elevating BC risk [9]. Schistosomiasis is another known risk factor of BC. Interestingly, while 90% of BC cases are urothelial carcinoma, squamous cell carcinoma is among the most common cancers in regions of Africa and the Middle East inhabited by *Schistosoma*. In these BC patients, the average age of incidence is only 40-49, compared to the 70-79 in the rest of the world [4].

While BC is not a hereditary condition, several hereditary factors can increase BC risk, such as Cowden Syndrome [10] and Lynch Syndrome [11], which are germline mutations in the PTEN gene and mixed-match repair genes, respectively.

1.1.3: Molecular Alterations

Many factors and mutations can give rise to BC. One of the most prominent factors in muscle-invasive bladder cancer (MIBC) is cell cycle regulation, where 89% of MIBC cases have mutations in a cell cycle gene, including TP53, RB1, CDKN1A, CDKN2A, CCND1, CNE1, or MDM2. Interestingly, these mutations were usually mutually exclusive, suggesting that multiple cell cycle mutations may impair MIBC growth instead of promoting it. The most common of these genes to be mutated is the tumor suppressor TP53, which occurs in 48% of cases [12, 13]. Its mutation is associated with genome doubling events. In addition to TP53, MDM2 is frequently mutated in MIBC, via amplification or overexpression, accounting for 25% of total cases [12]. MDM2 is an E3 ubiquitin ligase responsible for deleting the tumor suppressor, p53, hence its overexpression can result in tumorigenesis [14]. Another common class of mutations in BC is mutations of TERT promoters, which is present in ~62.5% of BC cases [15]. TERT encodes telomerase reverse transcriptase, part of the telomerase complex that protects the ends of telomeres from shortening when a cell divides. By regulating their telomerase levels, cells are able to control their lifespans. However, overexpressing telomerase allows tumors to divide indefinitely without losing DNA [15]. Additionally, the RTK/RAS/PI3K pathway has mutations in 71% of BC cases. Commonly mutated genes in this pathway include FGFR3, ERBB2, and PIK3CA, which are implicated in angiogenesis and tumor growth [12]. Interestingly though, FGFR3 mutations are not as common in MIBC and are associated with lower grade disease and better outcomes [13].

1.1.4: Symptoms

The most common BC symptom is blood in the urine (hematuria). Additional symptoms include changes in urinary habits, including more frequent urination, pain during urination, and difficulties with urination. More advanced BC can lead to inability to urinate, lower back pain, and appetite/weight loss, among other factors [16]. All of these symptoms are more frequent in other conditions more so than BC, thus BC could be mistaken for another condition.

1.1.5: Diagnosis

When a patient is initially suspected of having BC, they are evaluated using computed tomography (CT) imaging of the upper urinary tract, and cystoscopy, which involves inserting a camera through the urethra to observe the bladder. It is recommended for patients with gross hematuria, or over the age of 35 with microscopic hematuria [17]. Another common method of BC diagnosis is urine cytology, analyzing cells in the urine. Although urine cytology has high specificity in BC detection, it is generally not recommended due to its low sensitivity [18]. Urine cytology is a useful diagnosis tool nonetheless, as it has high sensitivity in high grade tumors and carcinoma *in situ*, and is also useful for surveillance in previously diagnosed patients [17]. In cases where an abnormal lesion is found, transurethral resection of the bladder tumor (TURBT) can be performed, in which the tumor is removed, and the surrounding muscle is sampled to assess depth. This allows for proper diagnosis, staging, and grading of BC [17, 18].

Although the current diagnosis techniques are effective, they still have limitations, and thus novel diagnosis techniques are being developed to address them. For example, new imaging

techniques are being developed that can detect residual tumors that a regular cystoscopy would miss, such as photodynamic diagnosis and fluorescence cystoscopy [19]. Photodynamic diagnosis (PDD) involves intravesical instillation of a photosensitizer, which induces accumulation of porphyrins in tumor cells that can convert blue light into red light. This technique has shown to have significantly higher sensitivity than standard white-light cystoscopy, but not specificity [20]. Laser-induced fluorescence (LIF) cystoscopy uses a particular light emission pattern without any photosensitizers, which will behave differently in tumors when compared to normal tissues [21]. Other novel techniques used to aid BC visualization include optical biopsy and narrow band imaging (NBI). Optical biopsy uses specific wavelengths of light to capture real-time dynamic images of the bladder to survey for tumors [19]. NBI uses filtered white light absorbed by hemoglobin to penetrate the urothelial surface and better visualize the bladder [20].

Additionally, several urine-based non-invasive techniques have been developed for screening BC, since the current non-invasive standard, cytology, shows poor performance in diagnosing lower-grade tumors. These tests focus on analyzing certain biomarkers in the urine to diagnose and monitor BC. Protein-based biomarkers include Nuclear mitotic apparatus protein (NMP22 BC) and Complement factor H-related protein (BTA). These proteins are assayed using either ELISA or point-of-care tests [19]. Several genomic biomarkers are used to diagnose BC, such as TRAP assay, which tests for excess telomerase based on the amplification of telomeric repeat sequences [22]. Other analyses look at DNA mutations in exfoliated cells, or mRNA in urine, with most assays looking at several mutations/mRNA sequences at once to diagnose BC [19]. In

addition to traditional genomic assays, DNA methylation-based assays have been trending as possible novel diagnostic techniques, including the utMeMa [23] and Bladder EpiCheck [24] assays. In particular, the utMeMa test has shown to be effective for detecting early stage BC [23], while the Bladder EpiCheck assay has shown to be effective for detecting high risk non-muscle invasive bladder cancer (NMIBC) [24]. Lastly, extracellular vesicle (EV) detection has shown to be promising for BC detection. Particularly, a double nano filtration technique has been developed to capture and isolate BC-associated vesicles [25]. Although there are many ways to diagnose BC, staging and classification may be even more important, as that is what ultimately determines how the cancer will manifest, and how it can be treated.

1.2: Bladder Cancer Classification

1.2.1: Introduction

BC is first classified by the cell of origin, where over 90% of BC cases originate from the urothelium. Other types of BC are much less prevalent, including squamous cell carcinoma (SCC) and adenocarcinoma, which are more common in *Schistosoma*-endemic regions and developing countries, respectively [26]. Once the histological variant of the tumor is determined, the tumor can then be staged and graded.

1.2.2: Tumor Staging/Grading

Like various other cancers, urothelial carcinoma (UC) follows the TNM staging system, where T refers to the size and extent of the primary tumor, N refers to the number of surrounding lymph nodes with cancer, and M refers to the tumor's metastasis. Each letter is followed by a number,

indicating the magnitude of that parameter [27]. More broadly, UC can be classified in 1 of 5 possible stages, ranging from Stage 0 to Stage 4, with Stage 0 referring to papillary tumors, Stage 4 referring to metastasis, and Stages 1-3 referring to different levels of tissue invasion (Fig. 1) [27, 28]. In urothelial carcinoma, Stage 0-1 are considered non-muscle invasive (NMIBC) whereas Stages 2+ are considered muscle-invasive (MIBC), as the tumor has reached the muscle layer. About 70-80% of urothelial carcinomas are considered non-muscle-invasive whereas the rest are muscle-invasive [13, 28].

In addition to staging, tumors are also given a grade ranging from 1-4, based on its levels of differentiation and resemblance to the origin cell. Higher grade tumors have lower differentiation and lose their resemblance to the origin cell more than lower grade tumors. They are also more likely to be aggressive than lower grade tumors [29]. Additionally, high- and low-grade tumors are associated with different mutation profiles. Low grade bladder tumors typically have mutations in *FGFR3, PIK3CA*, and *KDM6A*, whereas high grade tumors typically have mutations in tumor suppressor genes like *TP53* and *RB1* [30]. Although staging and grading of BC is important to better understand the cancer, treatments are usually chosen based on whether the cancer is NMIBC or MIBC, and if there is metastasis involved.

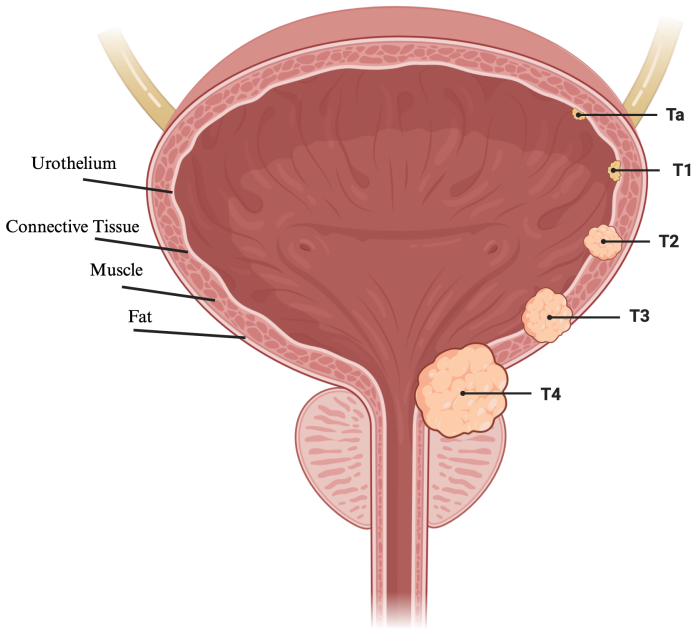

Figure 1. The different stages of urothelial carcinoma. Different stages (Ta, T1, T2, T3, T4) classified based on levels of tissue invasion. Figure adapted from BioRender.

1.2.3: Non-Muscle Invasive Bladder Cancer (NMIBC)

Around 70% of localized bladder tumors are considered NMIBC, however, NMIBC encompasses a wide array of cancers, and can thus be further separated into low, intermediate, and high-risk cancer. The 5-year recurrence-free survival rates for low, intermediate, and high-risk NMIBC is 43%, 33%, and 23% respectively. While these rates imply that recurrence is likely for all NMIBC, progression is much less likely, as the 5-year progression-free survival rates are 93%, 74%, and 54% respectively [31]. Nevertheless, 21-45% of high-risk NMIBC cases will progress to MIBC [31, 32]. Because of this, measures to treat MIBC are still important despite having a high survival rate. To treat NMIBC, a transurethral resection of the bladder tumor is first performed (TURBT), allowing for both treatment and classification of the cancer [32]. In cases where the first TURBT is incomplete and the cancer is considered high-risk,

repeated TURBTs can be performed [33]. Afterwards, regular cystoscopies will be needed to monitor the bladder. For low-risk cancer patients, follow-ups will occur until 1-5 years post-treatment, however, intermediate and high risk patients will need regular cystoscopies for the rest of their lives [32].

After TURBT is performed and the tumor is characterized, intravesical treatments are then administered, typically in the form of chemotherapy. In low risk patients, only one instillation is typically performed, but in intermediate and high risk cases, multiple are necessary. Some of the most common chemotherapies used include mytomycin C and epirubicin [33, 34]. Mitomycin C acts as a DNA alkylating agent, thus inhibiting DNA synthesis in tumor cells [35]. Mitomycin C also leads to reduced quality of life, due to irritative urinary symptoms, pain, fatigue, and insomnia, especially in older patients [32]. Epirubicin binds to tumor DNA, thus inhibiting DNA replication, transcription, and DNA repair. Adverse events are observed in 16-25% of patients, though they are typically mild and transient [36].

Although many chemotherapy options exist, several meta analyses suggest Bacille Calmette-Guérin (BCG) to be a better adjuvant treatment post-TURBT than chemotherapy [34, 37]. BCG involves the use of a live, attenuated strain of *Mycobacterium bovis*. Despite being originally used to treat Tuberculosis, BCG has become the most commonly used immunotherapy for NMIBC, and is the standard of care for high and intermediate risk NMIBC [31, 38]. It is known that BCG induces both innate and Th1 adaptive immune responses, though it is still unclear if BCG gets internalized into the badder [38].

1.2.4: Muscle Invasive Bladder Cancer (MIBC)

While about 70% of localized bladder tumors are non-muscle invasive, the other 30% are considered muscle-invasive tumors [13]. The standard treatment for MIBC includes neoadjuvant chemotherapy, followed by radical cystectomy (RC), pelvic lymph node dissections, and urinary tract diversion [31]. The urinary tract diversion can come as an incontinent ileal conduit, an orthotopic neobladder, or a continent cutaneous diversion. The most common chemotherapeutic agent used is cisplatin, a platinum-based drug which acts by cross-linking purine based in tumor DNA, thus causing damage and preventing its repair, eventually leading to apoptosis [39]. While cisplatin with RC is known to improve 5-year survival compared to cystectomy alone, there is no such evidence yet for other drugs such as carboplatin [40]. Additionally, about 50% of patients are considered cisplatin-ineligible due to age or disease-related risk comorbidities, and must thus receive different treatment [41]. RC is also known to have a high complication rate, often leading to adverse events that are mainly gastrointestinal, infectious, wound-related, and genitourinary in nature [42].

As MIBC is more prevalent among older individuals, in which the complications often outweigh the benefits of RC, many patients will instead opt for a bladder sparing approach [41]. One such approach is to perform a partial cystectomy, removing only part of the bladder instead of the whole bladder. In carefully selected patients, partial cystectomy has similar survival rates to RC, while still maintaining sexual and urinary functions [43].

Another commonly used approach is maximal TURBT, where in the case of MIBC, resection should occur down to the detrusor muscle if feasible [44]. However, if the tumor is residing past the superficial muscle layer, TURBT cannot be performed feasibly [45]. Several studies have shown that given specific patient selection, TURBT as a monotherapy can result in similar survival rates to radical cystectomy. Radiation therapy (RT) is also used to treat MIBC, though it has shown to result in lower overall survival than RC [44, 45], and over 50% of RT-treated patients will advance to metastatic disease [45]. Additionally, RT-treated patients may need a salvage cystectomy, resulting in unwanted complications from removing the bladder in patients that were selected for bladder sparing treatment [46]. Despite its role as a neoadjuvant treatment, chemotherapy is seldomly used as a bladder-sparing monotherapy [44]. However, if neoadjuvant chemotherapy leads to a complete response, patients will often refuse radical cystectomy. This leads to a 64% overall survival and a 64% relapse rate, with 38% of relapses being muscle-invasive disease. In addition to its role as a neoadjuvant treatment to RC, chemotherapy has also been used in combination with radiation, which has a greater loco regional disease-free survival than radiation alone [45].

While TURBT, chemotherapy, and radiation have all been used as bladder sparing approaches against MIBC, these three treatments work best when combined. This is known as trimodal therapy (TMT) and consists of maximal TURBT followed by concurrent chemotherapy and radiation [41, 44, 45]. The combination of chemotherapy and RT serves two purposes. Particularly, it will sensitize the tumor to RT, making the radiation more effective. Additionally, it can aid in controlling the tumor and preventing metastases. Several chemotherapeutic drugs can

be used for implementation into TMT. These include cisplatin, 5-fluorouracil + mytomycin C, and gemcitabine, all of which have shown to improve outcome when compared to RT alone [45]. With the proper patient selection, TMT has similar oncologic outcomes to RC, and is thus the gold standard for bladder sparing treatments for MIBC [41, 44, 45].

1.2.5: Advanced Bladder Cancer

Advanced BC is considered the worst possible prognosis, and can be divided into locally advanced, and metastatic BC [41, 47, 48]. Locally advanced BC is staged at T3b/4a and N1-3 and is characterized by spreading of cancer cells to regional lymph nodes, while metastatic cancer is defined by spreading of the cancer to distant regions of the body [46]. Advanced BC is generally considered incurable, with metastatic disease in particular having an 5 year overall survival as low as 15% [49].

Despite being the worst possible prognosis, there are multiple options for managing advanced BC. One such solution is RC, which has been shown to be an effective treatment against locally advanced BC. This is because when performing radical cystectomy, lymph node removal is also performed, meaning that the tumor that has advanced into the lymph nodes would be removed [47, 48]. For metastatic disease, one of the main approaches is cisplatin-based chemotherapy, which remains the first-line option against inoperable advanced BC [48]. Historically, this would be the combination of methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC), however, due to inducing various toxicities, other combinations are used instead as first-line treatment, including dose-dense MVAC, and gemcitabine + cisplatin (GC) [41, 49].

Although cisplatin is the first-line treatment for advanced BC, about half of patients are cisplatin-ineligible, and thus other treatment options must be considered. One such option is the use of combination chemotherapy with gemcitabine + carboplatin (GCa) [41, 49]. Another possible treatment option is adjuvant RT, particularly for locally advanced disease. Several trials have compared treatments for locally advanced BC (cystectomy, or cystectomy + chemotherapy) with or without adjuvant radiation, where the addition of RT significantly improved survival and local-regional control of the cancer [50]. Immunotherapy has also shown to be an effective treatment option in advanced BC, particularly regarding immune checkpoint inhibitors (ICIs), antibodies that bind to immune checkpoint proteins such as PD-1/PD-L1 and CTLA-4 which would otherwise inhibit the anti-tumor immune response (Fig. 2). Binding to these proteins would allow T cells to maintain their anti-tumor functions without inhibition [49, 51]. Due to inducing durable anti-tumor responses, as well as promising overall survival and response rates, two ICIs have been approved by the US Food and Drug Administration (FDA) as first-line treatment for cisplatin ineligible advanced BC patients, and five ICIs are approved for advanced BC patients who progressed after chemotherapy [41, 49].

Although immunotherapy is a promising approach for advanced BC, there exist subtypes such as the luminal I subtype which is associated with non-response to immunotherapy. This subtype is notable for frequently expressing mutations in FGFR [51]. Although FGFR mutations are more prevalent in non-muscle invasive disease, they are found in up to 21% of advanced BC cases [41, 49]. Due to FGFR-mutated tumors typically responding worse to immunotherapy than other

tumors, FGFR inhibitors have found a niche in advanced BC treatment. Erdafitinib in particular has shown promising results in patients with metastatic BC with FGFR alterations who did not respond to chemotherapy [51] and has thus been approved as a second-line treatment option [41, 49].

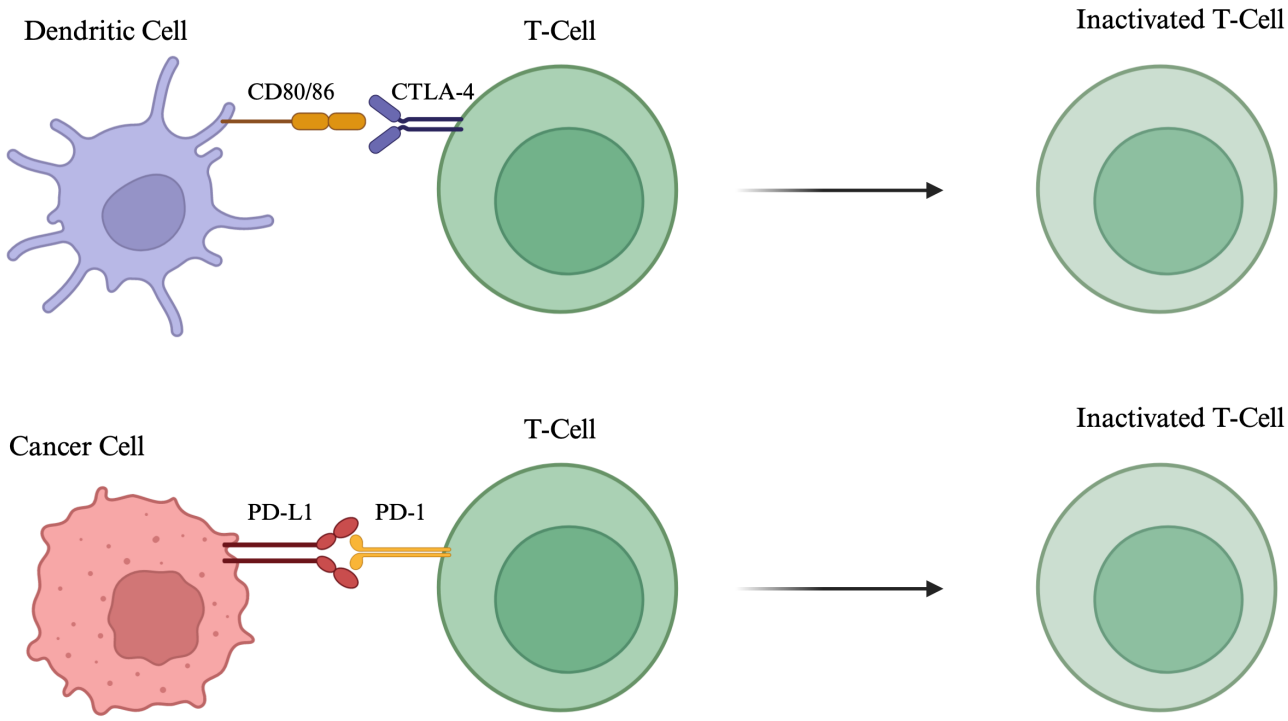

Figure 2. Immune checkpoint protein mechanisms. Mechanisms of T-Cell inactivation by CTLA-4 and by PD1/PD-L1. Figure adapted from BioRender.

1.2.6: Molecular Subtypes

Due to the heterogenous nature of BC, a one-size-fits-all approach is not recommended for BC treatment, each tumor has different gene expression signatures among various other factors that can influence how it affects the patient, and how it can be treated.

For NMIBC, tumors are classified into 3 different classes based on their gene expression patterns, based on the UROMOL study [52]. Class 1 tumors are associated with increased expression of early cell cycle genes, and generally have good prognosis, whereas class 2 tumors are associated with increased expression of late cell cycle genes. Class 2 tumors are more aggressive, and generally have poorer prognosis than Class 1 tumors. Class 1 and 2 tumors also have increased uroplakin expression, which is associated with luminal cells. Class 3 is a basal subtype with increased KRT5, KRT15, and long non-coding RNA expression, and is also associated with good prognosis [52, 53]. NMIBC can also be divided into 3 subtypes based on proteomic analysis (NMIBC proteomic subtypes, or NPSs). NPS1 is the highest-risk and is associated with increased inflammation-associated proteins. NPS2 is medium-risk, and is associated with an infiltrated, mesenchymal profile. NPS3 is the lowest risk and can resemble both Class 1 and Class 3 tumors from the UROMOL study [53, 54].

MIBC is divided into six commonly accepted molecular subtypes: Luminal papillary (LumP), luminal nonspecified (LumNS), luminal unstable (LumU), stroma-rich, basal/squamous (Ba/Sq), and neuroendocrine-like (NE-like). One way which these subtypes differ is in mRNA

differentiation signatures. All luminal subtypes overexpress urothelial differentiation signatures, whereas Ba/Sq and NE-like tumors show basal and neuroendocrine differentiation, respectively. Stroma-rich tumors show intermediate urothelial differentiation but were instead classified mainly via stromal infiltration signatures. Additionally, different subtypes are associated with up regulation of different genes, making them potentially susceptible to certain treatments. For example, LumP tumors have increased FGFR3 mutation and activation, and thus are candidates for FGFR inhibitor therapy. Meanwhile, Ba/Sq and NE-like tumors, which both have the lowest overall survival rates, show increased mutations in RB1 and TP53 [55].

Despite having poor prognosis among BC subtypes, Ba/Sq and NE-like tumors are also the most responsive to neoadjuvant chemotherapy, though only those with epithelial basal tumors, and not those with mesenchymal tumors, will respond to the treatment. Luminal tumors have the highest survival rates after treatment with neoadjuvant chemotherapy. Despite this, luminal tumors tend to not respond to neoadjuvant chemotherapy, but instead, they already have high survival rates with cystectomy alone [12, 56]. Similar trends can be observed regarding response to immunotherapy. Basal tumors tend to have increased immune infiltration and PD-L1 expression when compared to their luminal counterparts and are considered immunologically “hot” tumors. They are thus more likely to respond to ICIs [57, 58]. However, due to various factors such as TGFβ expression, basal tumors tend to only respond moderately to ICI. If those factors are cleared, as part of a combination therapy with ICI, the ICI may treat the tumor more successfully [55]. In contrast, luminal tumors are immunologically “cold”, in that they tend to have lower amounts of immune infiltration and PD-L1 expression. They are thus less likely to respond to

immunotherapy [57, 58]. In order to study these subtypes and how they react to various treatments, many mouse models for MIBC have been developed.

1.2.7: Mouse Models

Due to the heterogeneity of BC, many mouse models have been developed, as to study the disease in many different contexts. These mouse models can broadly be divided into three categories: Xenograft, allograft, and autochthonous [59].

Xenograft models are models where a tumor from a different organism is transplanted into the mouse. The main method these are developed in is via patient-derived xenografts (PDX), where cancer cells from the patient are transplanted into immunocompromised mice. These cells are injected into the mouse either subcutaneously or orthotopically, though orthotropic models are preferred due to remaining in the bladder environment. Xenograft models are not without their faults though, as it is a lengthy and expensive process, and cannot be used in immunotherapy experiments due to the need for immunocompromised mice. Additionally, the murine tumor may not perfectly reflect the patient-derived tumor, as aggressive phenotypes are more likely to be successfully transplanted [59].

Allograft models are models where a tumor from one mouse is transplanted into another. Unlike in xenografts, allografts allow for the use of immunocompetent mice, as the transplanted tumor will not be rejected by the host mouse. Commonly used allograft cell lines include MBT-2, MB49 [59], and UPPL [57]. The MBT-2 cell line is derived from C3H/He mice, by

administering N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide. This induces tumors with epithelial and basal characteristics [59, 60]. The MB49 cell line is derived from C57Bl/6 mice, by administering 7,12-dimethylbenz[a]anthracene. Both MBT-2 and MB49 tumors express PD-L1 and basal markers, with CD44 being upregulated in MB49 tumors specifically. Unlike MBT-2 though, MB49 has mesenchymal characteristics, including epithelial mesenchymal transition (EMT) signalling and fibrosis markers [59]. The UPPL cell line is also derived from C57Bl/6 mice, through conditional knockouts of TRP53 and PTEN (Upk3a- Cre^{ERT2}; $Trp53^{L/L}$; $Pten^{L/L}$; $Rosa26^{LSL\text{-}Luc}$). This cell line models human luminal muscle-invasive tumors and is considered immunologically “cold” [57].

While xenograft and allograft models aim to transplant cancer cells into a mouse, autochthonous models aim to induce BC into the mouse itself. The main method to induce BC in mice is via exposure to N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) in drinking water. This forms high-grade, basal MIBC tumors in mice after 20 weeks [61].

1.3: Radiation Therapy

1.3.1: Introduction

Radiation therapy is one of the most widely used treatments for cancer, in which ionizing radiation is used to kill the tumor. It involves firing high-energy beams of X-rays or gamma rays, which can pass through tissue, break chemical bonds, and remove electrons from atoms, thus ionizing them [62]. Traditionally, RT has been known to control tumor cells directly, via inducing DNA damage, disturbing tumor cell structure and function via organelle damage, and causing

various forms of cell death [63]. In recent years though, more emphasis has been placed on the non-targeted effects on the tumor, which occur outside the radiation field and are known as the abscopal effect [64]. Although RT has proven to be an effective anti-tumor strategy, often times, radiation will not completely kill the cancer allowing for potential recurrence. Additionally, many cancers are radioresistant. For these reasons, additional modalities are often required in combination with RT [62]. Additionally, there is potential for normal tissue in the radiation field to also be damaged [63].

1.3.2: DNA Damage and Repair

As discussed previously, the main targeted effect of RT towards the tumor is induction of DNA damage, however, RT also induces DNA damage in non-tumor cells from the surrounding tissue. RT can induce DNA damage either by direct interactions in which energy from the radiation directly interacts with DNA, or by indirect effects, such as where radiation removes electrons from atoms producing free radicals [62, 63]. DNA damage can occur as base modifications, single-strand breaks (SSB), and double-strand breaks (DSB), among various mechanisms [62].

Base modifications occur when radiation induces distortions in the DNA double-helix structure. They do not play a huge role in radiation-induced DNA damage as they are easily repaired by either base excision repair or nucleotide excision repair. In these repair methods, the damaged base/nucleotide is excised, and is then repaired using endonucleases and an undamaged template strand [62].

SSBs occur from nicks in the sugar-phosphate backbone in one strand, and are usually accompanied by a single nucleotide loss. They are repaired in a process involving poly(ADP-ribose) polymerase (PARP), polynucleotide kinase (PNK), DNA polymerase, and DNA ligase. This process involved filling in the gap created by the SSB using DNA polymerase followed by re-ligation of the nick using DNA ligase [62].

The most toxic form of DNA damage to both cancerous and non-cancerous cells are DSBs, which involve nicks in the phosphodiester backbones of both DNA strands. This can lead to loss and re-arrangement of DNA during replication and mitosis, hence why DSBs are so toxic. The two main mechanisms in which DSBs are repaired are non-homologous end joining (NHEJ) and homologous recombination (HR) [62, 66]. In NHEJ, the heterodimer Ku70-Ku80 is bound to the DNA ends followed by recruitment of DNA protein kinase catalytic subunits (DNA-PKcs). This will result in trimming incompatible ends via nucleases, followed by ligating the two ends together using a DNA ligation complex [62]. In HR, DSBs are subjected to a 5' resection, leading to 3' single-stranded overhangs, thus preventing the recruitment of Ku70-Ku80 and thus initiation of NHEJ. The 3' overhangs are then coated by Replication Protein A followed by RAD51, resulting in a RAD51-DNA nucleofilament. The nucleofilament searches for a homologous sequence elsewhere in the genome and performs strand invasion leading to establishment of a DNA replication fork. Due to requiring a homologous sequence, HR is a more precise and less error-prone form of DNA repair [62, 66].

In MIBC, very few DNA repair gene mutations are found, however, they are nonetheless important predictors of treatment outcome. Particularly, *ERCC2* is an important part of the nucleotide excision repair pathway, however, it is mutated in 12-15% of MIBC cases. Interestingly though, MIBC patients with *ERCC2* mutations show increased sensitivity to cisplatin. In addition to *ERCC2*, other DNA repair genes show increased sensitivity to treatments in MIBC. Patients harboring mutations in DNA repair genes *ATM, FANCC*, and/or *RB1* were found to be more likely to have no residual muscle-invasive tumor post-cystectomy. Meanwhile, patients harboring mutations in DNA repair genes *ATM, ERCC2, FANCD2, PALB2, BRCA1,* and/or *BRCA2* showed increased recurrence-free survival after perioperative chemotherpy [67].

1.3.3: Radiation-Induced Cell Death

Radiation can induce various forms of cell death in tumors, with the main two mechanisms of RT-induced cell death being mitotic catastrophe and apoptosis [62]. Mitotic catastrophe refers to the triggering of mitotic arrest followed by controlled cell death due to dysfunctional mitosis and is characterized by giant cells with aberrant spindle, de-condensed chromatin, and multiple micronuclei [62, 65]. RT can induce mitotic catastrophe via upregulation of various cell cycle proteins, particularly cyclin B1 and CDK-1 [62].

Apoptosis is a programmed cell death such that the cell's interior contents do not leak into the surrounding environment [65], and consists of cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and the resultant formation of apoptotic bodies where the plasma membrane remains intact [62]. Apoptosis is initiated via 3 different pathways: Intrinsic,

extrinsic, and ceramide, all of which serve to activate the initiator caspases, caspase 8 and/or 9. All of these pathways will lead to activation of executioner caspases (3, 6, and 7), which will initiate a cascade of events leading to DNA fragmentation, protein destruction and crosslinking, expression of ligands for phagocytic cells, and production of apoptotic bodies [68].

Unlike apoptosis, necrosis is an uncontrolled cell death, typically induced by external injury such as hypoxia or inflammation [65]. Although necrosis is not as common in radiation-induced cell death as apoptosis, it still occurs, and is associated with extreme changes in pH, energy loss, and ion imbalance within the cell and its microenvironment after irradiation [62, 68]. This process typically leads to an increase in inflammatory compounds like nuclear factor-κB (NF-κB), leading to plasma membrane rupture, and leakage of cellular contents into the environment, leading to a cascade of inflammation and tissue damage in the surrounding area [68]. Although RT generally favours apoptosis over necrosis, high dosage (30Gy) is associated with increased necrosis [62].

Although apoptosis and necrosis have distinct characteristics, there exist mechanisms of cell death that share properties with each. In particular, necroptosis, like necrosis, involves leakage of cell contents into the environment, however, unlike necrosis, it is highly controlled. Necroptosis is initiated by tumor necrosis factor receptor 1 (TNFR1), which leads to a series of events controlled by receptor interacting proteins 1 and 3 (RIP1/3), eventually leading to membrane permeabilization [62, 68]. Necroptosis has recently been discovered as a form of RT-induced cell death in several cancers including thyroid cancer and adrenocortical carcinoma [62].

Senescence describes cells that, although viable, undergo permanent cell cycle arrest. These cells cannot divide, and are thus not at risk of becoming cancerous. Radiation-induced senescence is typically induced via DNA damage and activation of the p53 and pRb pathways to block the cell cycle, though other factors such as oxidative stress may be implicated [62, 65]. Although senescence is a tumor-suppressive condition, it is also harmful to the surrounding environment. Particularly, senescence leads to formation of a senescence-associated secretory phenotype which could promote inflammation and tumor progression [62].

Autophagy describes the process where cellular components, including organelles, are sequestered into lysosomes and degraded, in order to recycle amino and fatty acids. This is normally a pro-survival process; however, excessive autophagy leads to cell death [62, 65, 68, 69]. The primary regulatory pathway for autophagy in cancer is the PI3K-Akt-mTOR pathway, which negatively regulates autophagy [62, 69]. RT has previously been found to induce autophagy, though the mechanisms are not well studied. It is believed that RT is involved in reducing mTOR autophosphorylation and promoting ER stress, thus leading to autophagic cell death [68, 69]. Studies have also been performed linking induction of autophagy as a way to radiosensitize tumor cells, though it has been shown both to promote radiosensitivity and radioresistance in different cell types [62].

In addition to the traditional forms of cell death, RT is also responsible for inducing immunogenic cell death (ICD), which occurs via recruitment of immune cells to kill the tumor,

also inducing immune memory. RT is able to induce antitumoral immunity by promoting dendritic cell and CD8+ T cell functionality [70]. ICD is orchestrated by three separate arms that are required for immune priming and activation, all of which have been shown to be activated by radiation: 1. Cell surface translocation of calreticulin. 2. Extracellular release of HMGB1. 3. Extracellular release of ATP [70-72]. Calreticulin acts as an “eat-me” signal for dendritic cells. It has an inhibitory counterpart, CD47, which acts as a “do-not-eat-me” signal. HMGB1, when released, acts as a danger-associated molecular pattern (DAMP) which promotes recruitment of T cells. ATP release leads to activation of immune cells via the P2XR7 purinergic receptor pathway [70-72]. Although radiation is implicated in cell death, the effect of radiation on anti-tumoral immunity can be seen as a double-edged sword, as it has been shown to induce both immune activation and immune suppression in cancer [71].

1.3.4: Radiation and the Immune System

In addition to the previously mentioned ICD, radiation can activate many different components of the immune system. One example of this is upregulation of type I interferons (IFN) via the cGAS-STING pathway. Cyclic GMP-AMP synthase (cGAS) is able to recognize RT-induced cytoplasmic DNA and produce cyclic GMP (cGMP), which is then recognized by the adaptor protein STING. This then leads to IRF3/NFκB-dependent activation of type I IFN transcription [71, 72]. Radiation is also able to upregulate production of other proinflammatory cytokines, including interferon-γ (IFN-γ), interleukin 1-β, and tumor necrosis factor α (TNF-α) [72], as well as increasing class I MHC expression. Class I MHC molecules present intracellular antigens to the cell surface, where they are recognized by CD8+ T cells [71]. Radiation is also able to

increase expression of T cell co-stimulatory molecules CD80 and CD86, further promoting T cell recruitment to the tumor environment. Another immune capability of radiation is its ability to promote recruitment of CD8+ T cells via activation of chemokines such as CXCL9, CXCL10 and CXCL16, and cell adhesion molecules such as ICAM-1 and VCAM-1 [72]. Aside from T cells, radiation can also promote NK-cell mediated tumor clearance by increasing the expression of NKG2D receptor stress ligands [71, 72].

In addition to their effects on the local tumor, radiation can also induce a systemic anti-tumoral immune response, otherwise known as the abscopal effect. This is because local antigen presenting cells can migrate, thus being activating distant T cells which can then clear distant tumors. Although the abscopal effect is being increasingly reported, the overall effect is low, due to the tumors' inherent immunosuppressive qualities [73], or by an immunosuppressive phenotype induced by radiation itself.

Although radiation can sensitize tumors to host immunity in a variety of ways, it also down-regulates anti-tumoral immunity using various mechanisms. Particularly, radiation promotes attraction of various immunosuppressive cells to the tumor environment, including M2 tumor-associated macrophages, myeloid-derived suppressor cells, regulatory T cells (Tregs), and pro-tumor neutrophils, as well as inducing production of various immunosuppressive cytokines such as TGF-β and IL-10 [71, 72, 74]. Additionally, radiation can increase expression of PD-L1 on the surface of tumor cells and immunosuppressive myeloid cells [72]. One way in which PD-L1 expression can occur is via production of IFN-γ, via CD8+ T cells. IFN-γ is able to induce PD-

L1 production via the JAK-STAT-IRF pathway [71, 74, 75]. Other mechanisms in which radiation upregulated PD-L1 expression also exist. In particular, type I interferons can also promote PD-L1 expression, suggesting the cGAS–STING pathway may also induce PD-L1 expression. EGFR signalling is able to upregulate PD-L1 expression via the IL-6/JAK/STAT pathway, and DNA damage promotes its expression via ATM/ATR/Chk1 kinase activation [71]. Evidence also suggests that radiation can induce expression of TIGIT, a co-inhibitory receptor expressed on CD8+ T cells, NK cells, Tregs, and T follicular helper cell. It is associated with CD8+ T cell dysfunction [72].

1.3.5: Radioresistance

While radiation can promote an immune-resistant phenotype in tumor cells, tumors can also be resistant to radiation itself. The 6 R's of radiobiology define the six main factors influencing the response to RT. These include repair, re-assortment, repopulation, reoxygenation, intrinsic radiosensitivity, and most recently, re-activation of the immune response [72]. One of the main factors affecting a tumor's radiosensitivity is its ability to repair DNA DSBs. As double-stranded DNA breaks are the primary way that RT kills tumors, tumors with highly efficient DNA repair will have increased radioresistance. One way to induce radiosensitivity is thus to target DNA repair [62].

Another way in which tumors exhibit radioresistance is via adhesion to the extracellular matrix. This is mediated by integrins, which, due to adhesion, allow the tumors to better interact with their environment. Particularly, β_1 integrins have been found to be associated with radio

resistance due to interactions with various factors including JNK, Akt, p130Cas, and paxillin [62, 76].

Tumors are also able to exhibit radioresistance via the immune system. Particularly, the balance between pro- and anti-inflammatory signals can determine a tumor's susceptibility to RT, in which a pro-inflammatory environment is more conducive to successful treatment [62, 75]. This balance can be influenced by numerous factors, including radiation dose, tissue type, and the intrinsic characteristics of tumor cells [76].

The molecular oxygen status of the tumor environment is another factor that influences radioresistance. As radiation can induce DSBs via reactive oxygen species (ROS), hypoxic tumor environments that cannot produce as much ROS will not lead to efficient formation of DNA breaks, thus leading to radioresistance. Additionally, killing of normoxic cells frees up oxygen to be delivered to the tumor environment, leading to oxidative stress and thus HIF-1 activation, which modulates the expression of over 100 genes involved in the regulation of proliferation, apoptosis, and angiogenesis, independently promoting radioresistance. [62, 76].

The EGFR-PI3K-Akt pathway is another way in which tumors can exhibit radioresistance. Specifically, radiation induces EGFR signalling of PI3K and Akt, the latter of which forms a complex with DNA-PKcs and stimulates their kinase activity, leading to increased repairing of RT-induced DSBs, thus conferring radioresistance. Blocking the EGFR-PI3K-Akt pathway is thus a promising strategy for increasing radiosensitivity in tumors [62].

Radiation is also known to promote stemness in tumors, leading to increased tumor self-renewal. Particularly, the Hedgehog signalling pathway is known to promote cancer stem cell renewal in many types of cancer [62]. Radiation increases expression of Gli1, a Hedgehog effector transcription factor, thus leading to increased Hedgehog expression and radioresistance. Using cyclopamine to block the Hedgehog pathway was shown to enhance tumor radiosensitivity [77].

Lastly, it has been found that exposure to lower-dose radiation is known to confer protection from later, higher-dose radiation. Several factors are involved in inducing “radioadaptive resistance”, including mitochondrial signalling, RT-induced cytosolic cyclin D1 accumulation, NF-κB pathways, as well as promotion of apoptosis inhibitors and antioxidant defence mechanisms, are among the factors implicated [62]. Methods of radioresistance have been summarized in Figure 3.

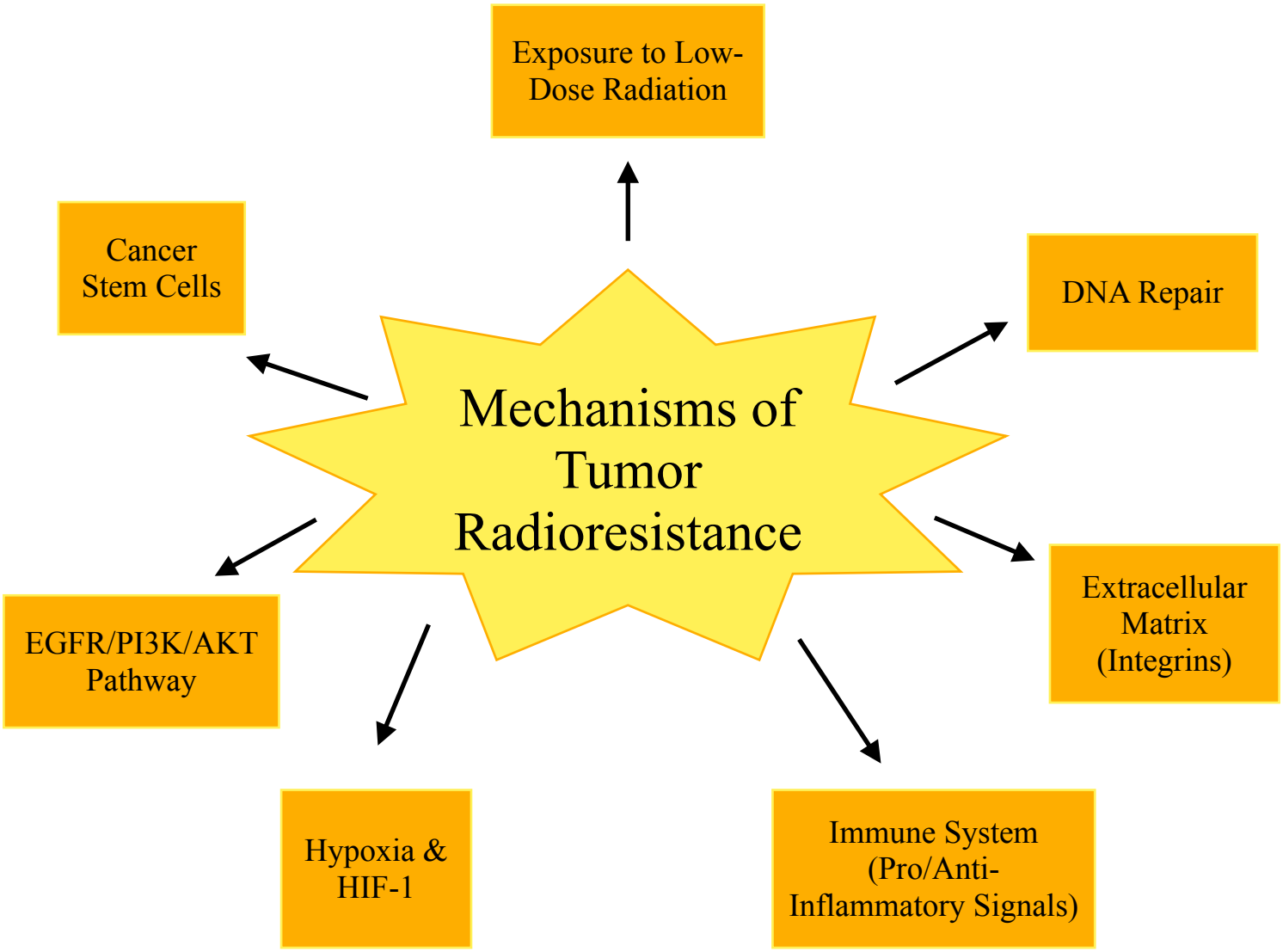

Figure 3. Mechanisms of Tumor Radioresistance. Summary of section 1.3.5, detailing different mechanisms in which tumors can exhibit radioresistance.

1.3.6: Fractionation Strategies

While the 6Rs of radiobiology describe the six main factors influencing the response to RT, they also explain our growing understanding of how RT functions. Originally, there were 4Rs: Repair, redistribution, reoxygenation, and repopulation. Eventually a 5th and 6th R was added as our understanding of RT furthered. This increased understanding in the functionality of RT led to different strategies being developed to enhance its effectiveness, such as the use of different fractionation regimes. Fractionation is important in RT as it takes advantage of the differences

between cancerous and non-cancerous cells to give the most effective treatment possible with minimal damage to normal tissue. For example, since normal cells traditionally have better DNA repair than tumor cells, fractionation will allow the normal cells to repair the RT-induced DNA damage, while said damage will accumulate further in tumor cells [78].

Conventional RT regimens consist of ~1.8-2.5Gy dose per fraction, 5 days per week, for ~4-8 weeks [79-81]. There also exists hyper- and hypo-fractionated RT regimens. Hyperfractionated RT refers to smaller doses (0.5-1.8Gy) with more fractions, often multiple per day, whereas hypofractionated RT refers to large doses (3-20Gy) with fewer fractions. Different regimens have shown differing levels to effectiveness, depending on the cancer being treated [79]. In BC, a conventional regimen of 64Gy total, over 32 fractions (2Gy/fraction) is used, though another regimen is also commonly used, consisting of 55Gy total over 20 fractions (2.75Gy/fraction). The 55Gy regimen has shown to be non-inferior to the 64Gy regimen in toxicity, and superior regarding invasive locoregional control [82]. In TMT, various radiation regimens are used, though the most popular is 64Gy to the tumor bed with partial irradiation (~54Gy) going to the whole bladder. Pelvic lymph nodes may also be irradiated as part of TMT, though some studies have shown that this may not be necessary [83].

Hypofractionated RT can be further divided into ablative and subablative doses, which are used in regards to stereotactic ablative radiotherapy (SABR) and stereotactic body radiation therapy (SBRT). Ablative regimens consist of 1-5 fractions, with the goal of reaching 90%+ local tumor control. Examples of ablative regimens include 34Gy x1, 18Gy x3, and 10Gy x5. Sub-ablative

regimens are also commonly used in order to accommodate radiation tolerance of at-risk organs. These regimens typically consist of 1-5 fractions, and doses of 5-10 Gy/fraction [80]. Ablative regimens have been shown to induce an immune response characterized by increased class-I MHC expression, with different proteins being expressed with MHC-I in irradiated or non-irradiated cells [81]. Despite this, ablative regimens are not as good as sub-ablative regimens at inducing antitumoral immunity. In preclinical studies combining radiation with anti-CTLA-4 in a breast cancer model, an ablative 20Gy x1 regimen failed to improve the efficacy of the immunotherapy while also failing to induce an abscopal effect. Meanwhile, combining anti-CTLA-4 with sub-ablative regimens of 8Gy x 3 or 6Gy x 5 showed increased anti-tumor response, in both the irradiated and non-irradiated field [84]. In BC, sub-ablative regimens can be used for patients not suitable for curative treatment. In those cases, 7Gy x 3 has been shown to provide equivalent symptomatic improvement to 3.5Gy x 10 with equivalent treatment efficacy and no increase in toxicity. A sub-ablative regimen of 6Gy x 5-6 has resulted in 70% tumor control in elderly patients with locally advanced BC, with moderate acute and low late-stage toxicities [85].

Additionally, although both ablative and sub-ablative regimens can induce ICD, negative feedback pathways exist to limit its effectiveness in ablative, but not sub-ablative regimens. Normally, when radiation induces accumulation of cytosolic DNA, it is recognized by the cGAS-STING pathway, leading to increased IFN-β secretion by tumor cells, leading to increased CD8+ T cell priming. High enough single doses of radiation (above 12-18Gy) induce expression of the exonuclease Trex1, which degrades cytosolic DNA, rendering it unable to activate the cGAS-

STING pathway to promote antitumoral immunity [86]. Ablative doses have also been shown to increase levels of tumor-promoting macrophages [80].

While ablative doses of radiation have shown immunosuppressive effects compared to their sub-ablative counterparts, conventional and hyperfractionated/low-dose radiation regimens also show immunosuppressive properties. Particularly, when compared to 8Gy x3, a conventional 2Gy x 18 regimen led to an increased myeloid response, including increases in myeloid derived suppressor cells (MDSCs) and an increased ratio of M2/M1 tumor-associated macrophages, thus promoting an immunosuppressive phenotype. The 2Gy x 18 also increased PD-L1 expression when compared to the 8Gy x3 regimen, and it was less effective than the sub-ablative regimen when combined with anti-TIGIT + anti-PD-L1 [87]. Additionally, macrophages exposed to low-dose radiation (0.1-0.5Gy) exhibit immunosuppressive properties, showing reduced IL-1β and increased TGF-β [72]. Despite this, low-dose radiation has also shown to have immunogenic properties. A 2Gy radiation dose can induce production of nitric oxide buy tumor-associated macrophages, as well as releasing pro-inflammatory cytokines, leading to repopulation of the tumor environment by immunogenic cells [80]. Although sub-ablative regimens show the most promise when combined with immunotherapy, the effects of radiation regimens on treatment efficacy and combination with immunotherapy are complex, and there is no consensus on an optimal RT dose-fractionation regimen.

1.4: Microbiome

1.4.1: Introduction

The microbiota refers to the collective of microorganisms populating a specific area, whereas the microbiome refers to their collective genome [88]. The microbiota consists of bacteria, archaea, fungi, protozoa, and viruses [88-91]. Although archaea, fungi, protozoa, and viruses have not been studied much, bacteria have been extensively studied [89]. The human microbiota contains ~10^{14} bacterial cells, outnumbering our own cells 10:1. Additionally, the microbiota is known to colonize every surface of our body that is exposed to the external environment, though most of the microbiota is located in the gastrointestinal tract, where it is commonly referred to as the gut microbiota [89, 90].

The human gut microbiota is estimated to consist of over 35,000 different bacterial species [88, 89], with most of them being obligate anaerobes [89]. The two most prevalent phyla in a healthy gut microbiota are *Firmicutes* and *Bacteroidetes*, followed by *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia*. Yet, the gut microbiota is not homogenous, as there are differences in diversity and abundance of bacteria in different parts of the gastrointestinal tract. These range from 10^{1} per gram of contents in the esophagus and stomach to 10^{12} per gram of contents in the colon and distal gut [88, 89].

1.4.2: Functions

The physiological effects of the gut microbiota lie primarily in its symbiotic relationship with the mucosa. Particularly, the microbiota can aid the host via, immunoregulatory, protective, and

metabolic functions. The microbiota plays various roles in immunoregulation and can regulate both local and systemic immunity. This can be seen from studies involving germ-free (GF) mice, which have abnormal numbers of certain immune cells, deficits in local/systemic lymphoid structures, poorly formed spleens and lymph nodes, hypoplastic Peyer's patches, and decreased number of mature isolated lymphoid follicles [89]. The microbiota is able to regulate production of various CD4+ T cell subtypes with various mechanisms. For example, although GF mice tend towards a Th2 cytokine response, *Bacteroides fragilis* polysaccharide A (PSA) can produce IFN-γ, thus promoting a Th1 response instead [92]. Other mechanisms exist to promote an anti-inflammatory phenotype in the gut, such as Treg induction via short-chain fatty acids (SCFAs) [88], and conditioning of intestinal epithelial cells to promote Th2-cell and Treg induction via various microbial isolates from bacteria such as *Escherichia coli* and *Lactobacillus* [89]. The microbiota can also trigger a Th17 response, such as with various *Bacteroidetes* being implicated in induction of Th17 cells in the small intestinal lamina propria [93].

Aside from its role in immunoregulation, the gut microbiota also serves a role in protecting the host from various factors, including infection from pathogens, and abnormal growth of the microbiota itself. This primarily happens by inducing the production of antimicrobial proteins in Paneth cells via a pattern-recognition receptor-mediated mechanism. These antimicrobials include defensins, cathelicidins, and C-type lectins, among others [88, 89]. Certain bacteria, including *Lactobacillus*, are able to produce antimicrobial products against many different kinds of bacteria, including both Gram-positive and -negative species [89]. One example of this is their production of lactic acid, which can disrupt the outer membrane of Gram-negative pathogens,

thus rendering them susceptible to host lysozyme [94]. The microbiota is also able to regulate bacterial growth by inducing immunoglobulin expression. Gram-negative bacteria, such as *Bacteroides* can activate dendritic cells, which induce plasma cells in the intestinal mucosa to produce secretory IgA, in turn, restricting microbial translocation from the intestinal lumen to circulation [89].

The gut microbiota can also protect the host by maintaining gut barrier structural integrity. This is accomplished by maintaining cell-to-cell junctions and promoting epithelial cell repair following injury. Various bacteria have additional ways of maintaining epithelial structural integrity, such as *Lactobacillus rhamnosus GG*, which produces soluble proteins p40 and p75, leading to decrease in cytokine-induced apoptosis in epithelial cells [95]. Additionally, *Bacteroides thetaiotaomicron* can express sprr2A, which aids in maintaining desmosomes at the epithelial villus. The microbiota can also promote epithelial repair, such as via TLR2 signalling stimulated by microbial peptidoglycan. In addition to its role in maintaining gastrointestinal structural integrity, the gut microbiota can also aid in its development. For example, the microbiota is capable of modulating mucosal glycosylation patterns. These glycosylation patterns act as attachment sites for the microbiota to the epithelial cell surface, allowing them to perform their other functions. Another way that the microbiota can aid in the gastrointestinal structural development is via production of angiogenin-3 via Paneth cell signaling, which aids in developing the intestinal vasculature, and therefore nutrient intake [88, 89].

In addition to nutrient intake, the microbiota also plays a role in nutrient melabolism. Many bacteria are able to break down indigestible fibres to produce SCFAs, an important host energy source. The microbiota has also been implicated in inducing lipid and protein metabolism, as well as vitamin and bile acid synthesis, breakdown of polyphenols into various active compounds, and metabolism of various xenobiotics and drugs [88, 89, 96].

1.4.3: Factors Influencing Microbiome Composition/Diversity/Health

The microbiota is a dynamic system that varies greatly between individuals, and as such, its composition can be influenced by many factors. Firstly, the microbiota is influenced by various prenatal and birth-related factors. Maternal diet, obesity, smoking, and antibiotic use during pregnancy can influence the infant's microbiome [97]. Mode of delivery is also known to influence the infant microbiota [88, 98, 99]. Particularly, vaginal delivery has been associated with colonization by *Lactobacillus* and *Prevotella* [88, 98], as well as certain *Bacteroides* species [97]. Cesarean-section delivery is associated with colonization by *Streptococcus, Corynebacterium,* and *Propionibacterium* [88, 98], and decreased bacterial richness and diversity in the gut [97]. The microbiota matures quickly upon aging though, as after 3 years, a child's microbiota will begin to resemble the adult microbiota [88, 98].

In addition to age and prenatal factors, diet is also known to influence the gut microbial composition. This first becomes apparent regarding the infant diet, particularly, breast-fed and formula-fed infants have different microbial profiles. Particularly, breast-fed infants have increased abundance in *Lactobacillus* and *Bifidobacterium* due to containing certain bioactive

human milk oligosaccharides easily digested by those bacteria. Meanwhile, formula-fed infants have increased *Enterococcus, Enterobacteria, Bacteroides, Clostiridia*, and *Streptococcus* [88, 97, 98]. Regarding adult diets, those rich in insoluble carbohydrates, such as dietary fibre, lead to an increase in bacteria capable of digesting said carbohydrates. These include various members of the *Firmicutes* phylum, including *Ruminococcus bromii, Roseburia* and *Eubacterium rectale*. A high-fat, Western diet is known to increase abundance of *Bacteroidetes* while decreasing abundance of *Firmicutes* and reducing bacterial diversity [88, 98, 100, 101].

Another factor influencing microbial composition is antibiotic use. Although antibiotics are generally used to target pathogenic bacteria, they also destroy non-pathogenic bacteria as well. Use of broad-spectrum antibiotics have been found to reduce bacterial diversity and abundance of beneficial bacteria, while increasing abundance of opportunistic pathogens [98, 102]. In most individuals though, the microbiota will recover to its original state, however, in some cases, effects will persist [88, 102]. Additionally, antibiotic use in early life can confer changes to the microbiota, as it can disrupt the microbiota, leading to a loss of health-promoting bacteria, and reduced expression of antibacterial agents and immunoglobulin G. These factors can either be transient or can persist [97], and are also involved in development of inflammatory bowel disease. There is also evidence suggesting antibiotic use early in life can also lead to obesity later in life due to changes in the microbiota [98]. Lastly, antibiotic use can lead to development of resistance genes in the microbiota that can be transferred via horizontal gene transfer, potentially leading to the transfer of antibiotic resistance from the microbiota to a pathogen [88, 97, 102].

1.4.4: Metabolites

Although the microbiota can perform its functions via direct interactions with the host, microbial metabolites also play a major role in various biological processes. These metabolites are typically small molecules derived from the breakdown of indigestible food by the microbiota, which can exert a variety of biological functions on the host [103, 104]. Normally, the gut microbiota is limited spatially, as microbes are located only in the gastrointestinal lumen, where they can interact with local epithelial and immune cells. To bypass their spatial limitations and expand the scope of their interactions, bacteria in the microbiota release metabolites, such that they can interact with the host and other microbes from a larger distance [105].

The most researched metabolites in the microbiome are SCFAs [106, 107], fatty acids with less than six carbons produced by metabolism of dietary fibres in the gut, and include acetate, butyrate, and propionate, among others. Importantly, SCFAs are able to be carried by host circulation such that they can reach distal sites to induce broad-range effects [104-107]. They play significant role in metabolism, including lipid biosynthesis, gluconeogenesis [105], and conversion of ammonia into ammonium [103]. SCFAs are also known to promote pathogen clearance while also inducing an anti-inflammatory environment via up regulating the T_H2 and Treg response [104-106]. They are also implicated in modulating gut motility [105] and upregulating tight junction production, thus maintaining structural integrity in the gut and increasing resistance to inflammation and drug-induced injury [103]. In addition to their role in gut structural integrity, SCFAs are implicated in maintaining blood-brain barrier integrity, thus preventing gut metabolites from entering the brain [102, 108].

Another class of widely researched microbial metabolites are bile acids. Bile acids originate in the liver as products of cholesterol oxidation [104-106] and are then stored in the gallbladder and released into the duodenum [104, 105]. While most of the bile acid pool is reabsorbed into the liver, ~5-10% reach the colon, where they can be transformed by the microbiota from primary into secondary bile acids [104-106]. Secondary bile acids can lead to reduced emulsification of hydrophobic fat droplets, leading to maldigestion of lipids and malabsorption of fat-soluble vitamins [103], and have also been implicated increased triglyceride levels [109]. However, bile acids also increase glucose tolerance and serum glucagon-like peptide-1 (GLP-1) in mice [105], and are also implicated in protection against HFD-induced metabolic injury by increasing energy expenditure [109]. Their role in immunoregulation is disputed as they have both pro- and anti-inflammatory characteristics [105]. Bile acids also play a role in gut integrity, as some bile acids are agonists of the farnesoid X receptor (FXR) can stabilize the gut-vascular barrier, thus preventing microbes from entering the bloodstream [103]. Like SCFAs, secondary bile acids have also been implicated in maintaining blood-brain barrier integrity [105, 108].

Aside from SCFAs and bile acids, tryptophan derivatives have also been heavily researched. These include tryptamine, skatole, indole/indole derivatives [105], serotonin, and kynurenine [106]. These metabolites mostly act as ligands for aryl hydrocarbon receptor, which has a wide variety of biological effects [104-106]. Regarding their role in metabolism have been implicated in increasing glucose tolerance and serum glucagon-like peptide-1 (GLP-1) in mice [104, 106].

Tryptophan derivatives have also been found to upregulate Treg differentiation, IL-22 production [107], and tight junction production [103].

Other metabolites can also be produced by the microbiota, including peptidoglycan fragments [104], various gases [105], and siderophores [106].

1.4.5: Microbiome and Cancer

In addition to various other diseases, the microbiome has recently been found to be a major player in influencing cancer development, through both tumor promoting and suppressing effects. It is well known that cancer patients have altered microbiota, both in the tumor environment, and in distant areas, such as the gut microbiome for non-gastrointestinal cancers [110]. This relationship was also found to be causative. Particularly, the first bacteria in the microbiota with a known mechanism of inducing cancer in humans is *Helicobacter pylori*, which secretes the toxin CagA. CagA can then induce aberrant ERK signalling or bind to PAR1 proteins to induce tumor development [111]. Various other bacteria can promote tumor development via their toxins, which can be seen on Table 1 [112-116].

Another way in which the microbiota can indirectly promote tumorigenesis is by inducing chronic inflammation. *F. nucleatum* has been associated with upregulation of TNF-α, β-catenin, and NF-κB, as well as shifting macrophage phenotypes to the immunosuppressive M2 form, thus allowing tumor growth by inhibiting antitumor immunity. Other mechanisms exist in which the microbiota can promote inflammation, thus leading to tumor development. For example,

microbial toxins can reduce mucosal surface integrity, allowing foreign antigens to more easily enter and generate an inflammatory response. Additionally, defects in the inflammasome complex can lead to microbe-induce tumorigenesis. Dysbiosis-induced Th17 immune responses have also been linked to pro-tumor effects [115].

While the microbiota is implicated in promoting cancer development, it has also been implicated in suppressing it. One strategy the microbiota uses to suppress cancer development is by binding to carcinogens, thus blocking their activity. This is especially seen in probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* [117]. Microbes can also produce metabolites associated with cancer reduction, such as SCFAs and conjugated linoleic acid (CLA). SCFAs such as butyrate are implicated in promoting apoptosis, improving intestinal barrier function, and suppressing inflammation [118]. Butyrate and propionate have also been found to inhibit host histone deacetylase function, which has shown anti-tumor effects in both colorectal cancer and lymphoma [113]. CLA can increase expression of the peroxisome proliferator-activated gamma receptor, which can modulate lipid metabolism, apoptosis, and anti-tumoral immunity. Certain bacteria are also involved in regulation of the cellular detoxification process by upregulating enzymes such as catalase, superoxide dismutase, and GST. This leads to reduction in free radical activity [118]. The most well-known way in which the microbiota suppresses tumor development is by promoting anti-tumoral immune responses. This can be best shown by experiments involving GF mice or antibiotics, which have reduced ability to clear tumors. Several *Bifidobacterium* species have been shown to strengthen dendritic cell function, which can then recruit cytotoxic T cells to the tumor environment. Additionally, the microbiota has been

associated with increased CD8+ T cell antitumoral response, as seen by the fact that GF mice do not respond to anti-CTLA-4, but they do respond once *Bacteroides* is added [114]. Fecal microbiota transplant (FMT) experiments have confirmed this effect as well [112]. In addition to promoting a CD8+ T cell response, the microbiota has been found to promote the CD4+ response as well, such as by mediating the balance between Th17 and Treg cells [115]. Additionally, LPS can induce activation of toll-like receptor 4, leading to an anti-tumor response [112, 113]. Although the microbiota has anti-cancer mechanisms on its own, it also plays a role in response to various anticancer therapies.

Although the gut microbiota has been heavily linked to various cancer types, only one clinical study has linked it with bladder cancer. In that study, *Prevotella* and *Clostridium* cluster XI, as well as the SCFA, butyrate, were negatively associated with bladder cancer, while LPS was positively associated with bladder cancer [119]. More studies exist relating bladder cancer with the urinary microbiota, though there is no consensus on specific impactful bacteria [120].

Microbe	Toxin	Effect on Host
Helicobacter pylori	CagA	Interacts with host E-cadherin and induce β-catenin signaling, leading to increased cell proliferation and tumorigenic potential. Can also induce aberrant ERK signalling or bind to PAR1 proteins to induce tumor development.
Escherichia coli	Colibactin and Cytolethal Distending Toxin	DNase activity
Shigella flexeri	Inositol Phosphate Phosphatase D and Cysteine Protease-Like Virulence Gene A	Induces host p53 degradation
Fusobacterium nucleatum	*Fusobacterium nucleatum* Effector Adhesin A	Interacts with host E-cadherin and induce β-catenin signaling, leading to increased cell proliferation and tumorigenic potential.
Bacteroides fragilis	*Bacteroides fragilis* Metalloproteinase Toxin	Interacts with host E-cadherin and induce β-catenin signaling, leading to increased cell proliferation and tumorigenic potential.
Fusobacterium nucleatum	Fap2	Binds to the NK-Cell inhibitory receptor, TIGIT, thus blocking the NK-Cell-mediated anti-tumor response

Table 1. Several microbial toxins and their pro-tumor effects on the host.

1.4.6: Microbiome and Radiation Therapy

Potentially the most important reason why microbiome studies are so prevalent in current cancer research is due to their bidirectional relationship with anticancer therapies; the microbiome can influence the efficacy of the therapy, meanwhile, the therapy induces changes in microbiome composition. This can be seen with RT, in which RT induces changes to the microbiota, and the microbiota in turn affects RT outcome. RT generally reduces overall richness and diversity of the microbiota while increasing abundance of opportunistic pathogens and decreasing abundance of beneficial bacteria. Particularly, RT increases relative abundance of the genera *Proteobacteria* and *Fusobacteria* while decreasing the Firmicutes:Bacteroidetes ratio [121-123]. Well-known probiotic bacteria such as *Lactobacillus* and *Bifidobacterium*, as well as the known prominent and beneficial bacteria, *Faecalibacterium prausnitzii*, all showed decreased abundance [121, 122]. Interestingly, although RT generally increases proportion of opportunistic pathogens and decreases proportion of beneficial bacteria, it is also known to increase the relative abundance of *Akkermansia muciniphila*, a known beneficial species that promotes response to ICIs [124-127].

While RT is known to affect the microbial profile, this relationship is bidirectional, as the microbiota can also affect the efficacy and toxicities of RT. Although experiments regarding the role of the microbiota in response to RT are scarce, one bladder cancer study in mice showed that *Bacteroides acidifaciens* is associated with response to RT while *Parabacteroides* is associated with non-response [128]. Additionally, it is believed that the microbiota can influence the immunomodulatory properties of an RT-induced abscopal effect [124-126]. Evidence of this relationship does exist though. Radiation has been shown to induce bacterial translocation to

mesenteric lymph nodes leading to a stronger anti-tumor response [124]. Additionally, addition of vancomycin has been shown to improve response to RT, leading to CD8+ T cells priming and IFN-γ production. This may occur due to the increase in immunogenic bacteria like *Akkermansia muciniphila* [124-127]. Microbial metabolites have also been implicated in promoting response to RT, such as SCFAs, potentially due to their role in DNA repair [128, 129]. In bladder cancer, it was found that the SCFAs acetate, butyrate, and propionate all promote response to radiation synergistically [128].

Although the microbiota likely affects RT efficacy, the impact of the microbiome on RT-induced toxicities has been explored much more. Generally, increases in opportunistic pathogens such as *Proteobacteria* and *Fusobacteria* are increased in patients with RT-induced gastrointestinal toxicities, while diversity and abundance of certain beneficial bacteria are reduced [124, 130]. However, changes in the most abundant bacteria in the microbiota remain controversial, as some studies report an increase in *Firmicutes* and decrease in *Bacteroidetes* in patients with RT-induced toxicities, while other studies report the opposite [124-127, 130, 131]. Interestingly though, germ-free (GF) mice experience less toxicities than conventionally raised mice [126]. Additionally, some mice given high doses of total body irradiation were able to survive a normal lifespan. These mice, termed “elite survivors” had increased *Lachnospiraceae* and *Enterococcaceae*. When feces of elite survivors were transplanted in GF or conventionally raised mice, RT-induced toxicity was reduced, suggesting a causative relationship of the microbiota on RT-induced toxicities [126]. Ways the microbiota can protect against RT-induced damage include activation of TLR-mediated immunooregulatory pathways via LPS and flagellin [132, 133],

SCFA-mediated immunoregulation via G-protein couple receptors and histone deacetylase, as well as promoting mucin secretion and increasing abundance of tight junction proteins [132].

1.4.7: Microbiome and Immunotherapy

Due to its role in immune regulation, the role of the microbiome in immunotherapy has been studied greatly. Particularly, the microbiome has shown to have a role in modulating efficacy of adoptive T cell transfer, TLR agonists, and ICIs. Various bacteria in the *Clostridium* class and *Bacteroidetes* phylum are associated with better response to CD19 chimeric antigen receptor (CAR) T cell therapy, whereas antibiotic use targeting anaerobes are generally associated with worse overall survival [134].

Certain bacteria, including *Ruminococcus* and *Alistipes shahii* were shown to improve CpG-oligodeoxynucleotide (CpG-ODN) efficacy, which would induce a strong TNF/IL-12 mediated anti-tumor response. *Lactobacillus fermentum* was shown to negatively affect CpG-ODN [135].

The microbiota has also shown to be a major player in regulating response to ICIs such as anti-CTLA-4 and anti-PD-1/PD-L1. GF or antibiotic-treated mice have shown to respond poorly to anti-CTLA-4 treatment, and response to treatment was restored following gavage with *Bacteroides thetaiotaomicron* or *Bacteroides fragilis*, which induces dendritic cell maturation in tumors and a Th1 response in draining lymph nodes [136-139]. Additionally, it was shown that *B, thetaiotaomicron* or *B. fragilis*, and *Burkholderia cepacia* all promoted response to anti-CTLA-4 [136]. *Bacteroidetes* are also associated with reduction in immune-related adverse

events, whereas *Firmicutes* such as *F. prausnitzii* are increased [137]. Interestingly, anti-CTLA-4 can also influence gut microbial composition, as it enriches *Firmicutes* and *Fusobacteria* in the stool while reducing abundance of *Bacteroidetes*. The inverse effect was shown in the small intestine. *B. fragilis* did not show significant changes, though [136]. The microbiota has also been shown to regulate response to PD-1/PD-L1 blockade. Like with CTLA-4, antibiotic-treated patients resisted PD-1 blockade. In a study on MIBC patients treated with neoadjuvant anti-PD-1 prior to RC, 15% of patients taking antibiotics achieved a complete response, as opposed to 50% of patients not taking antibiotics [140].

When regarding specific bacteria, a study by Routy et. al showed that various bacteria in the *Firmicutes* phylum, as well as the genera, *Akkermansia* and *Alistipes* are overrepresented in responders to anti-PD-1 [141]. Other studies show different findings though, as Matson et. al found eight bacteria overrepresented in responders: *Enterococcus faecium, Collinsella aerofaciens, Bifidobacterium adolescentis, Klebsiella pneumoniae, Veillonella parvula, Parabacteroides merdae, Lactobacillus sp.,* and *Bifidobacterium longum*. Two bacteria are underrepresented in responders: *Ruminococcus obeum* and *Roseburia intestinalis* [142]. These results suggest a complex relationship between the gut microbiota and PD-1 blockade. Additionally, it has been found that FMT has shown to improve response to anti-PD-1 in PD-1–refractory melanoma patients, though this was not always the case. *Bifidobacterium longum, Colinsella aerofaciens,* and *F. prausnitzii* were all shown to be associated with clinical benefit from FMT [143].

Immune checkpoint inhibitors can also induce immune-related adverse events, which are also associated with the microbiota. One study showed that *Clostridium hathewayi, Ruminococcus torques, Bacteroides massiliensis, Paraprevotella clara, Parabacteroides distasonis* and *Megamonas* are associated with fewer adverse events, whereas *Bifidobacterium dentium, Rothia mucilaginosa* and *Gemella haemolysans* were associated with increased adverse events. Additionally, adverse events in specific cancer types, as well as specific types of adverse events, were each associated with a different microbial profile [144].

Chapter 2: Rationale

Radiation therapy (RT) serves as an appealing bladder sparing treatment in muscle-invasive bladder cancer (MIBC), however, it does pose several challenges. Firstly, due to its close proximity to the gut, bladder radiation therapy is responsible for various gastrointestinal toxicities [145], where ~60-80% of pelvic or abdominal cancer patients who receive RT experience some symptoms of acute bowel toxicity [146]. Secondly, there are a significant number of patients that do not respond to RT. When combining with TURBT and chemotherapy as part of TMT, ~25-30% do not respond, and will need a salvage cystectomy [45]. As such, combining RT with immunotherapy has emerged as a promising alternative. Because combining RT with immunotherapy is still novel, many questions remain unanswered, including how to implement treatment sequencing, dose/fractionation regimen, RT site, and immune-related adverse events [147]. As previously mentioned, the microbiome is implicated in all of the above factors: It plays a role in modulating radiation-induced intestinal toxicities [124-127, 130-133], as well as response to RT [124-129] and immunotherapy [134-137, 141-143]. The microbiome is thus an important tool in understanding how to improve the efficacy of RT. Various studies exist regarding the effects of RT on the gut microbiome exist in several cancers and in various animal studies. These studies generally show an increase in harmful bacteria and a decrease in beneficial bacteria [121, 122]. However, this relationship has not been sufficiently studied in BC. Understanding the impact of RT on the gut microbiome in a BC context will allow for greater understanding of cancer therapies and will allow for the implementation of various strategies to induce the microbiome to a state most conducive for bladder preservation treatment.

Chapter 3: Hypothesis and Objectives

We hypothesize that in the context of bladder cancer treatment, radiation therapy will induce changes in the gut microbiota.

Aim 1) Evaluate the impact of bladder radiation on the gut microbiome *in vivo*.

Aim 2) Evaluate the impact of radiation therapy on the gut microbiome in a poorly immunogenic tumor model *in vivo*.

Chapter 4: Materials and Methods

4.1: Mouse model

Male and female C57Bl/6 mice were obtained from Charles River Laboratories, Inc, and held in the animal research facility at the Research Institute of the McGill University Health Centre (RI-MUHC). (AUP #7585) The facility animal care committee (FACC) approved protocol follows the standard operating procedures (SOPs) put in place.

4.2: *In Vivo* Non-Tumor Bladder Radiation Experiment

Mice were either untreated or were given 3 fractions per week of 6Gy image-guided radiation specifically to the bladder, for 2 weeks, to mirror a commonly used fractionation schedule in the clinic. Stool samples were collected from all mice at the start of the experiment (before RT), and after 1, 2, and 4 weeks post-RT. In all experiments, RT was given using a X-RAD SmART Irradiator Pxi 225cx (Precision X-Ray). The X-RAD is calibrated annually in accordance with guidelines from the American Association of Physicists in Medicine, task group 61. CT Scans were taken of the mice to assist in RT planning. SmART-ATP (SmART Scientific Solutions) was used to specify the location and angle of the beam.

4.3: MIBC Cell Line and Cell Culture

For cell line experiments, a UPPL1540 syngeneic bladder cancer cell line was used. This cell line was gifted by Dr. William Kim (University of North Carolina). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Wisent) supplemented with 10% fecal bovine serum (FBS,

Wisent), and were incubated at 37˚C with 5% CO_2. Cells were passaged at ~70-80% confluency. Passages were performed by detaching cells with 0.25% trypsin (Wisent). Cells were passaged 2-3 times before injection in mice. Cell counting was performed using a Vi-cell-XR viability analyzer (Beckman Coulter).

4.4: *In Vivo* Tumor Model

Male C57Bl/6 mice were obtained from Charles River Laboratories, Inc, and held in the animal research facility at the Research Institute of the McGill University Health Centre (RI-MUHC). The facility animal care committee (FACC) approved protocol follows the standard operating procedures (SOPs) put in place. To model bladder cancer in mice, $5x10^6$ UPPL1540 cells were injected subcutaneously in the right flank of the mice. The mice were monitored regularly for tumor growth until tumors reached 0.1-0.15cm^3. Tumor volume was calculated using length and width values measure using a caliper, and using an ellipsoidal volume formula (estimated tumor volume = $4/3*(3.14159)*(Length/2)*(Width/2)^2$) [148]. Mice were then monitored every two days, and were either untreated or were given 2 fractions of 5Gy RT, 24 hours apart, in accordance with the protocols from previous experiments from our group on subcutaneous bladder tumor models [148]. Stool samples were collected from all mice at the start of the experiment (tumor volume 0.1-0.15cm^3), midpoint (tumor volume 0.6-0.8cm^3), and primary endpoint (tumor volume 1.5-2.0cm^3).

4.5: 16S rRNA Gene Sequencing

DNA extraction from stool samples and sequencing was performed by the McGill Genome Centre. DNA samples were extracted using the Uneasy Powersoil Pro kit (Qiagen, cat. #47016). Each sample was resuspended by adding 5mL of 1X phosphate-buffered saline (PBS)/25% glycerol, vortexed. ~50mg of sample was transferred in a PowerBead Pro tube. An incubation of 70˚C for 10 minutes was then added. Extraction was performed as recommended by the manufacturer. Polymerase Chain Reaction was performed using primers for the v3-v4 variable region of the 16S rRNA gene, after which gene data was sequenced using Illumina sequencing.

4.6: Sequence Data Processing and Functional Profile Predictions

Sequence data was processed using QIIME2-2022.8 and q2studio-2022.8.0. Firstly, sequence data was imported into QIIME2 [149] by creating a manifest folder. Once the sequences were imported, they were denoised using the DADA2 plugin, creating a frequency table and a denoised sequence file. By using a gg-99 V3-V4 classifier (scikit-learn 0.24.1), sequences were compared to the Greengenes database, and OTUs were assigned based on 99% sequence identity. PICRUSt2 was used to predict functional profiles of the microbiota.

4.7: Bioinformatic Analysis

Sequence data was imported to MicrobiomeAnalyst. Low count filter and low variance filter were set to 0%, data scaling was set to ‘do not scale my data’, and data transformation was set to ‘relative log expression (RLE)’. Relative abundance and diversity calculations, as well as PCoA plots were performed using MicrobiomeAnalyst [150]. Further analysis on relative abundance,

and relative abundance graphing, was performed using GraphPad Prism 10.0.2. Relative abundance of functional profile data was performed using PICRUSt2 [151], MicrobiomeAnalyst [150], and the R package, ggpicrust2 [152], using the statistical anlalysis tool, edgeR.

Chapter 5: Results

5.1: Impact of bladder radiation on the gut microbiome *in vivo*

Bladder radiation does not significantly affect gut microbial diversity *in vivo*

The gut microbiome is known to play a major role in RT efficacy, however, its relationship with RT is still unknown in bladder cancer. We thus assessed the effects of bladder radiation on the gut microbiome. In this experiment, 20 male and 20 female non-tumor bearing C57Bl/6 mice were randomized into untreated and RT-treated groups. Mice were given 3 fractions of 6Gy bladder radiation per week for 2 weeks (6x6Gy total). Stool samples were collected at the start of the experiment, as well as 1, 2, and 4 weeks afterwards, followed by 16S rRNA gene sequencing. From this sequence data, we analyzed bacterial diversity and composition of the stool samples. Mice in the RT arm did not differ significantly in terms of α-diversity, diversity of bacteria within one individual sample, (Fig. 4A) or β-diversity, variability of the microbiota between different samples/groups (Fig. 4B-G) over time compared to mice in the control arm. These results suggest that bladder radiation does not play a significant role in shaping overall gut microbial composition and diversity.

Bladder radiation affects relative abundance of specific gut bacteria

Although microbial diversity was not affected by the radiation, microbial diversity looks at the microbiota as a whole, and thus analyzing microbial diversity may overlook changes in relative abundance to specific microbial features. When comparing relative abundance at week 1 to baseline, RT mice showed significant ($p<0.05$) increase in the species *Akkermansia muciniphila*

and *Bifidobacterium pseudolongum* compared to control mice (Fig. 5A-B). RT mice showed significant decrease in the genus *rc4_4* (Fig. 5C). At week 2, RT mice showed significant increase in the order *Erysipelotrichales*, and species *Bifidobacterium animalis* (Fig. 5D-E), and decrease in the genus *rc4_4* compared to control mice (Fig. 5C). At week 4, mice showed significant increase in the family *S24_7* and decrease in the class *Mollicutes*, and order *Anaeroplasmatales* (Fig. 5F-H). These results suggest that bladder radiation induces differing short-term and long-term changes in the relative abundance of specific microbial features in the gut.

Bladder radiation increases Proteasome pathway expression in the gut microbiota

Although changes in specific features were observed, the presence of these bacteria may not equate to changes in the functional pathways that exert biological effects on the host. We thus used PICRUSt2 to predict the functional profile of the gut microbiome using the 16S rRNA gene sequence data. Functional prediction showed an increase in the KEGG pathway *Proteasome* at weeks 1, 2, and 4 in RT-treated mice (Fig. 6). These results suggest that RT promotes proteasomal function in the gut microbiota.

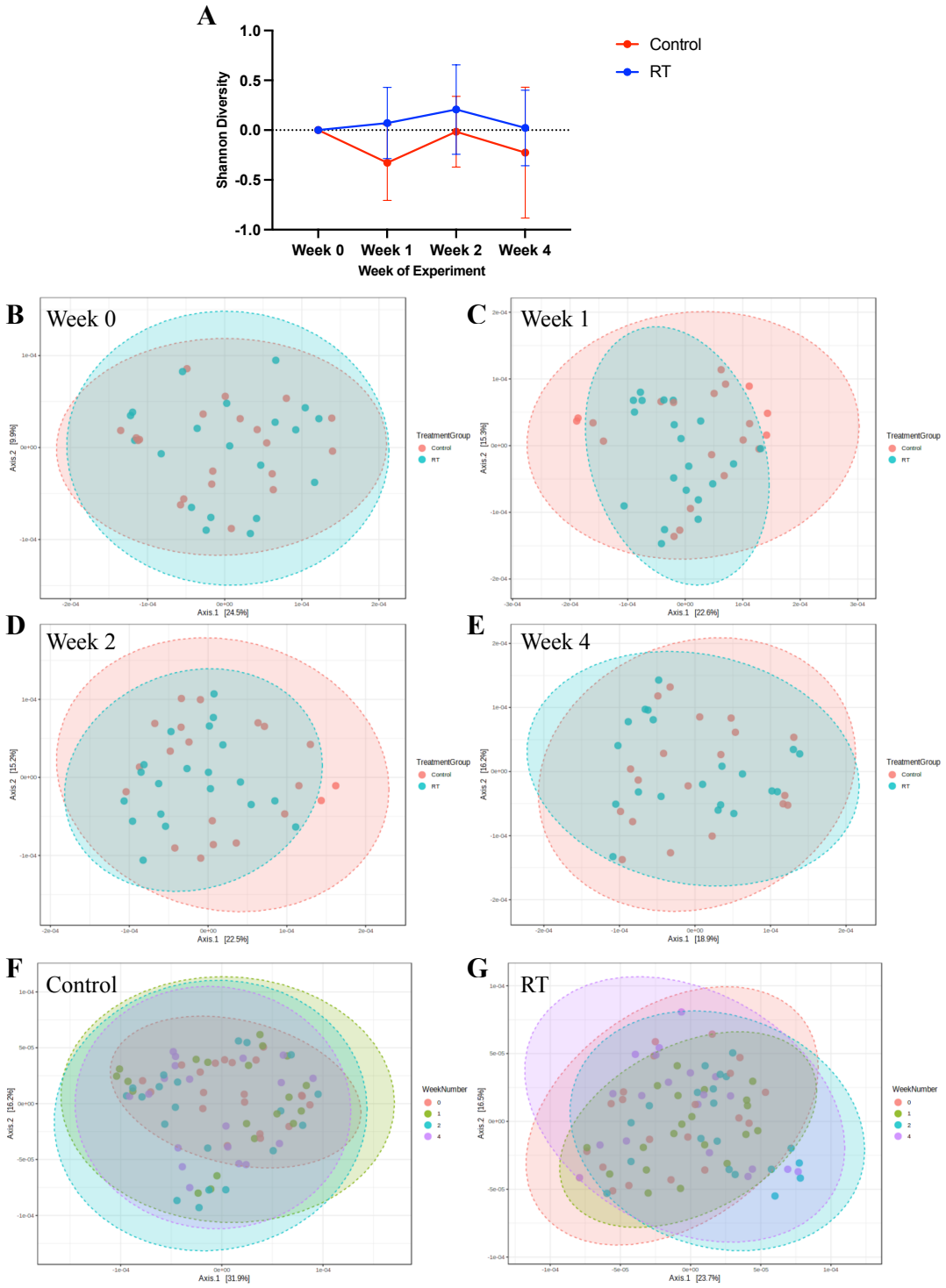

Figure 4. Changes in alpha and beta diversity of the microbiome of mice over time with vs without bladder radiation treatment. (A) Change in alpha diversity of the microbiota at each time-point compared to baseline (week 0). Alpha diversity measured using Shannon index. (B-E) Principal coordinate analysis (PCoA) of the microbiota of control compared to RT-treated mice at (B) baseline, (C) week 1, (D) week 2, and (E) week 4. (F-G) PCoA of the microbiota at different time-points in (F) control and (G) RT-treated mice. In all cases, PCoA used to measure beta diversity based on Bray-Curtis dissimilarity. Statistical analyses performed using ANOVA: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Error bars represent 95% confidence intervals.

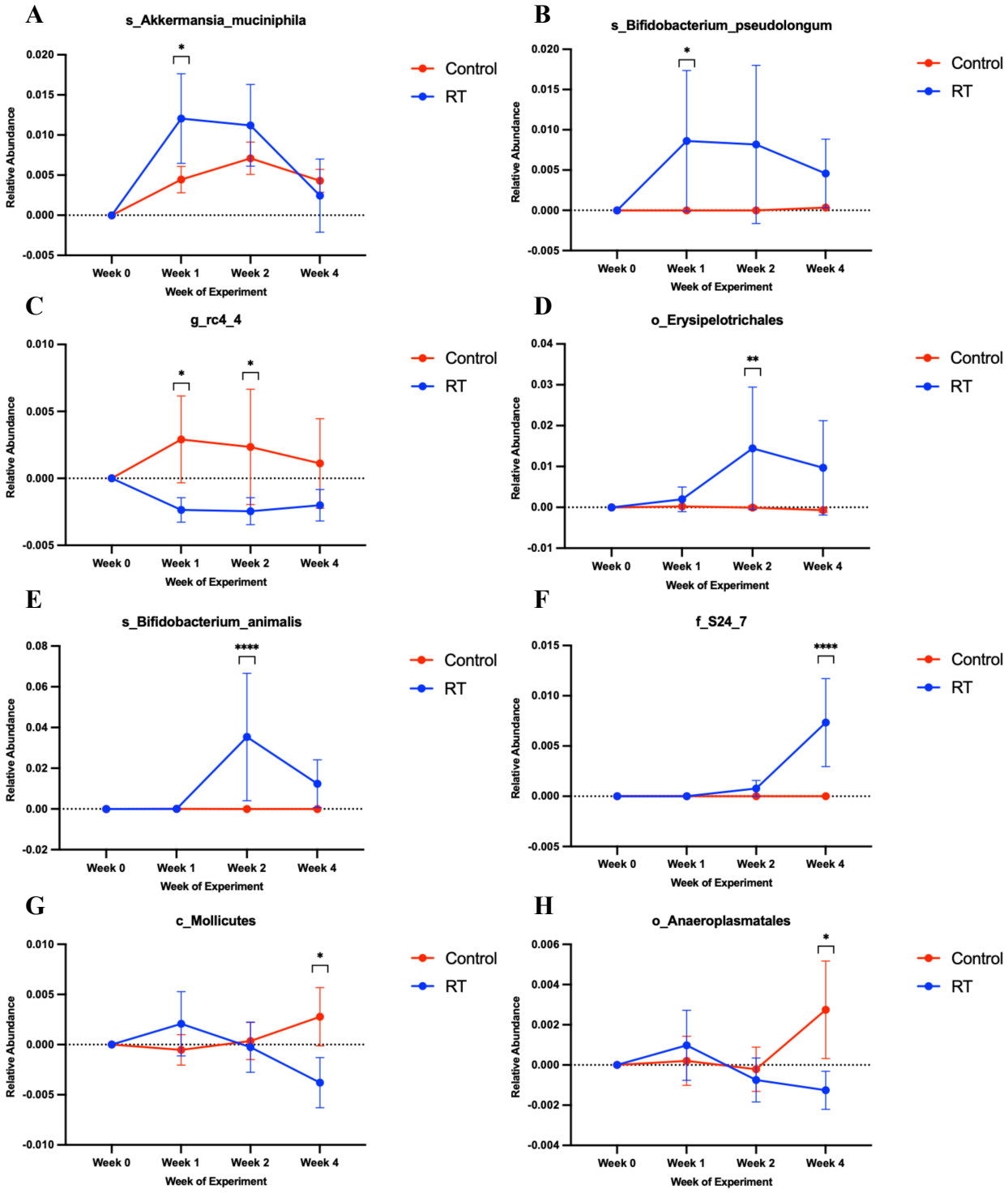

Figure 5. Changes in relative abundance of specific taxa in mice over time with vs without bladder radiation treatment. Change in relative abundance of the species (A) *Akkermansia muciniphila* and (B) *Bifidobacterium pseudolongum*, the genus (C) *rc4_4*, the order (D) *Erysipelotrichales*, the species (E) *Bifidobacterium animalis*, the family (F) *S24_7*, the class (G) *Mollicutes*, and the order (H) *Anaeroplasmatales* at each time-point compared to baseline (week 0). Statistical analyses performed using ANOVA: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Error bars represent 95% confidence intervals.

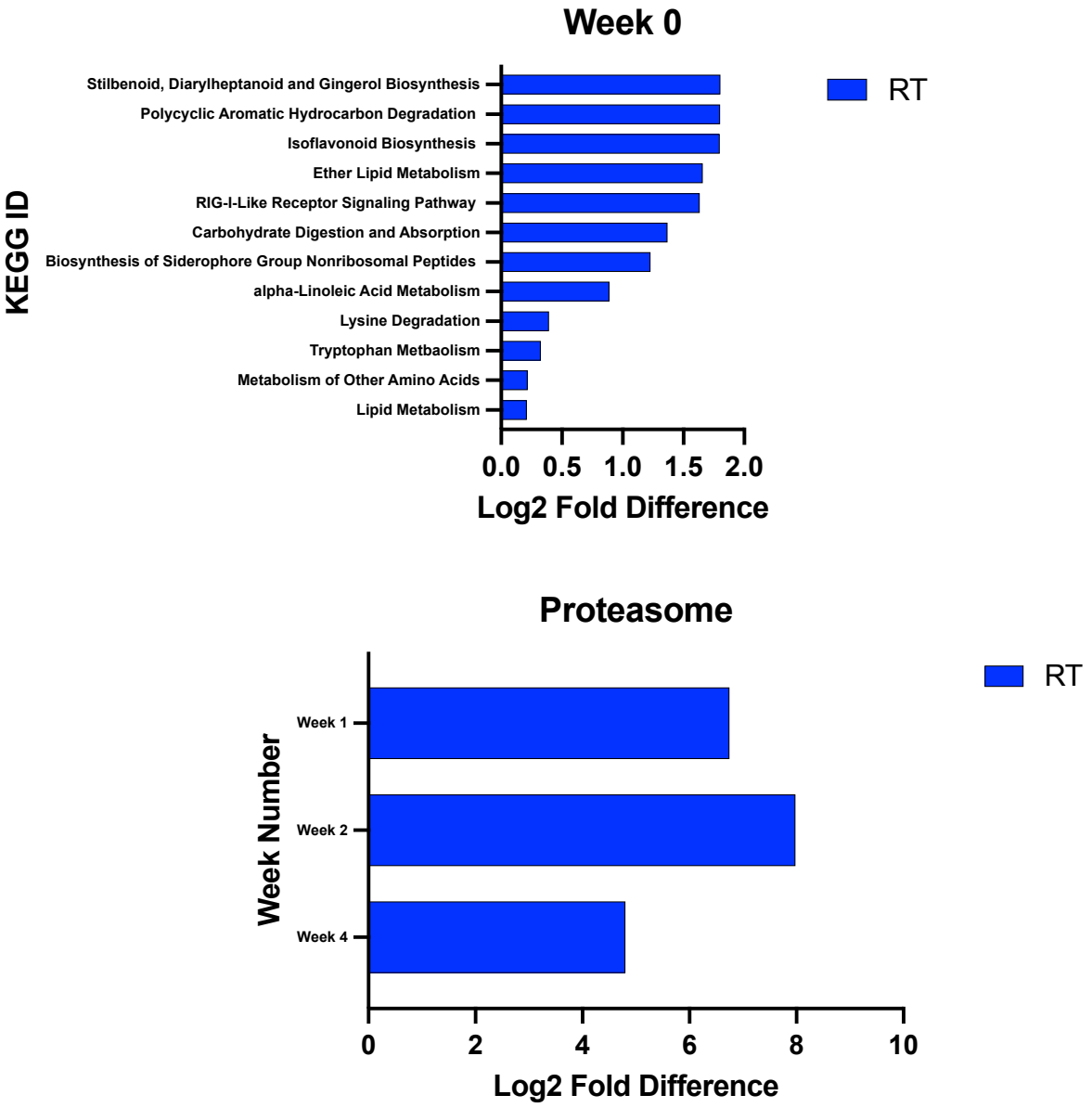

Figure 6. Differences in predicted functional pathways in mice with vs without bladder radiation treatment. Log2 fold difference of predicted functional pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database between control and RT mice at (A) week 0, and (B) in the Proteasome pathway at weeks 1, 2, and 4. Functional prediction performed using PICRUSt2. All features shown are statistically significant ($p < 0.05$) using edgeR statistical analysis.

5.2: Sex differences in the impact of bladder radiation on the gut microbiome

Bladder radiation decreases alpha diversity in female mice, does not affect beta diversity

Sex differences within both the human and murine microbiome have been studied extensively [153], ranging from differences in composition among sexes to the effect of sex on the relationship between the microbiome and BC [154]. For this reason, we decided to further divide our analysis by comparing sex differences with bladder radiation. Bacterial diversity and composition were analyzed once again using the same mice from the previous analysis. Regarding α-diversity analysis, there were no significant differences between any of the groups (male/control, female/control, male/RT, female/RT) in either sex (Fig. 7A-B).

Regarding β-diversity analysis, there was clear separation between male and female mice, however, no clustering occurred between untreated and RT-treated mice. This clustering pattern occurred at all time points (Fig. 8A-D). Additionally, no clustering occurred between samples at different time points among all four groups (male/control, female/control, male/RT, female/RT) (Fig. 8E-H). Overall, these results suggest a potential role of bladder radiation in altering gut microbial diversity among females without inducing striking changes in overall microbial composition. These results also suggest sex plays a larger role in the overall gut microbiota composition than treatment with bladder RT.

Bladder radiation changes relative of abundance of certain features differently in male vs female mice

When analyzing sex-specific changes in gut microbial composition, the only features to show significant changes in RT compared to control is an increase in the genus *Bifidobacterium* in females at week 4 (Fig. 9A). Orders *Burkholderiales* and *Coriobacterales* showed greater relative abundance in RT-treated males than RT-treated females at week 1 (Fig. 9B-C), whereas at week 4, the genus *Bifidobacterium*, and species *Bacteroides uniformis* showed greater relative abundance in RT-treated females than RT-treated males (Fig 9A, D). These results suggest that bladder radiation leads to different changes in specific microbial features in males when compared to females, and that these differences change over time.

Bladder radiation changes relative of abundance of certain functional pathways differently in male vs female mice

In terms of sex-specific functional differences between control and RT-treated mice, males showed increases in the pathways *Proteasome* at weeks 1 and 2, and no differences at week 4 (Fig. 10A-B). In females, functional pathway differences that were not observed at baseline include increases in the pathways *Proteasome* at week 2, and *Endocytosis, Flavonoid biosynthesis,* and *Proteasome* at week 4 (Fig. 10C-E). When comparing male vs female mice post-RT, week 1 showed an increase in the pathways *Proteasome, Renin-angiotensin system, Bladder cancer, Betalain biosynthesis, Indole alkaloid biosynthesis,* and *Fluorobenzoate degradation* in males. No differences between RT-treated males and females at week 2. At week 4, the pathways *Valine, leucine and isoleucine degradation* and *Glycosphingolipid biosynthesis* -

globo and isoglobo series were overexpressed in males, whereas the pathways *Dioxin degradation*, *Lysine degradation, RIG-I-like receptor signaling pathway, Colorectal cancer, Small cell lung cancer,* and *Stilbenoid, diarylheptanoid and gingerol biosynthesis,* were over expressed in females (Fig. 11). These results suggest that RT changes the functional signature of the gut microbiota differently in males and females, and that these changes will differ over time.

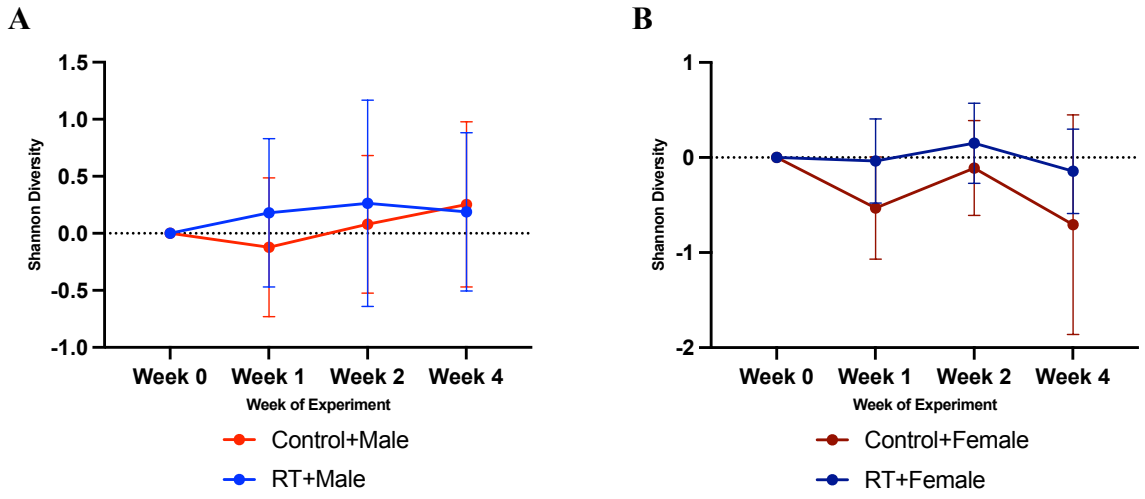

Figure 7. Sex differences in the changes in alpha diversity of the microbiome of mice over time with vs without bladder radiation treatment. Change in alpha diversity of the microbiota at each time-point compared to baseline (week 0). Alpha diversity measured using Shannon index in (A) males and (B) females. Statistical analyses performed using ANOVA: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Error bars represent 95% confidence intervals.

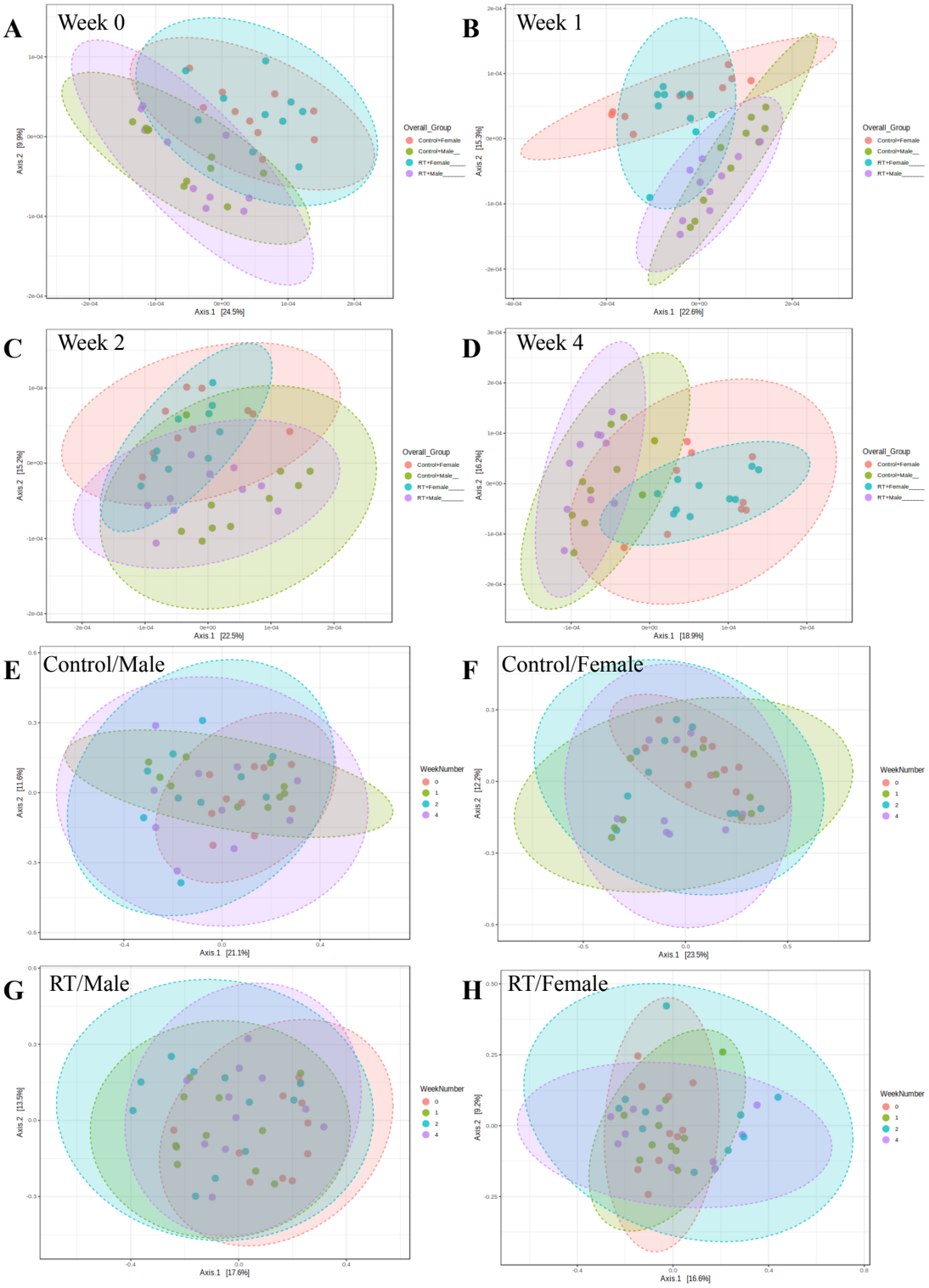

Figure 8. Sex differences in the changes in beta diversity of the microbiome of mice over time with vs without bladder radiation treatment. (A-D) Principal coordinate analysis (PCoA) of the microbiota of control/male, control/female, RT/male, and RT/female mice at (A) baseline, (B) week 1, (C) week 2, and (D) week 4. (E-H) PCoA of the microbiota at different time-points in (E) control/male, (F) control/female, (G) RT/male, and (H) RT/female mice. In all cases, PCoA used to measure beta diversity based on Bray-Curtis dissimilarity.

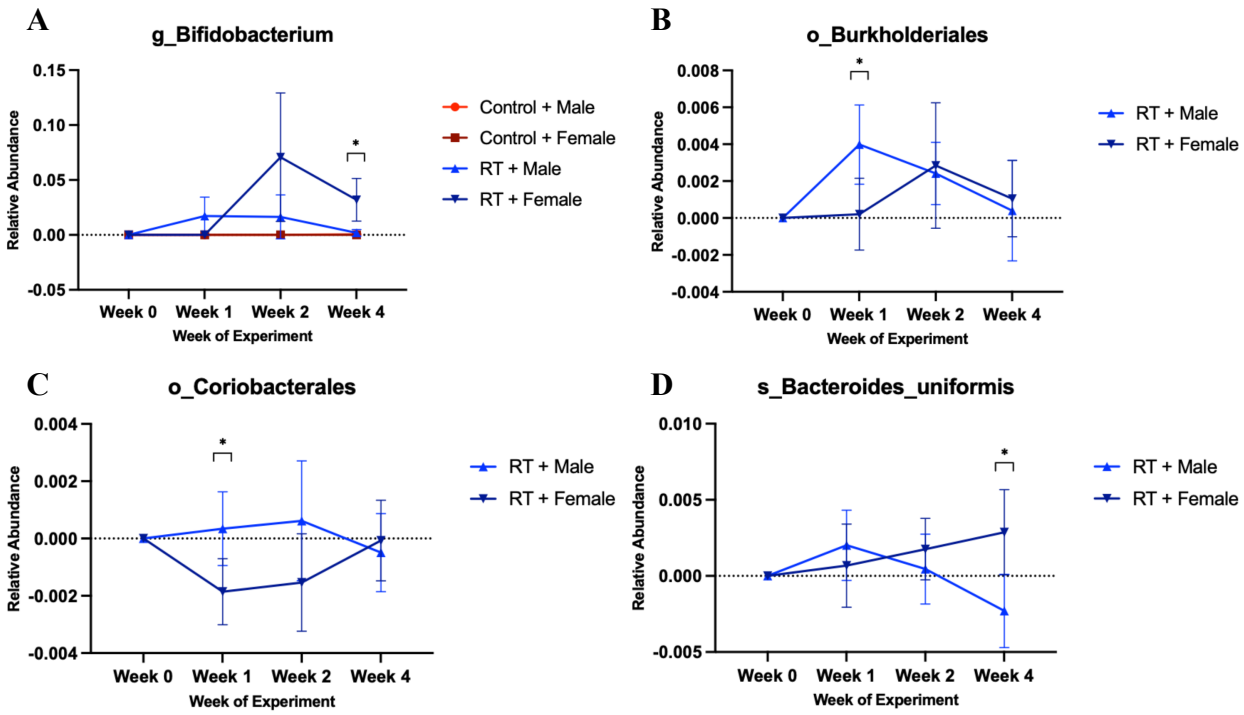

Figure 9. Sex differences in the changes in relative abundance of specific taxa in mice over time with vs without bladder radiation treatment. Change in relative abundance of the genus (A) *Bifidobacterium*, the orders (B) *Burkholderiales* and (C) *Coriobacterales*, as well as the species (D) *Bacteroides uniformis* at each time-point compared to baseline (week 0). Statistical analyses performed using ANOVA: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Error bars represent 95% confidence intervals.

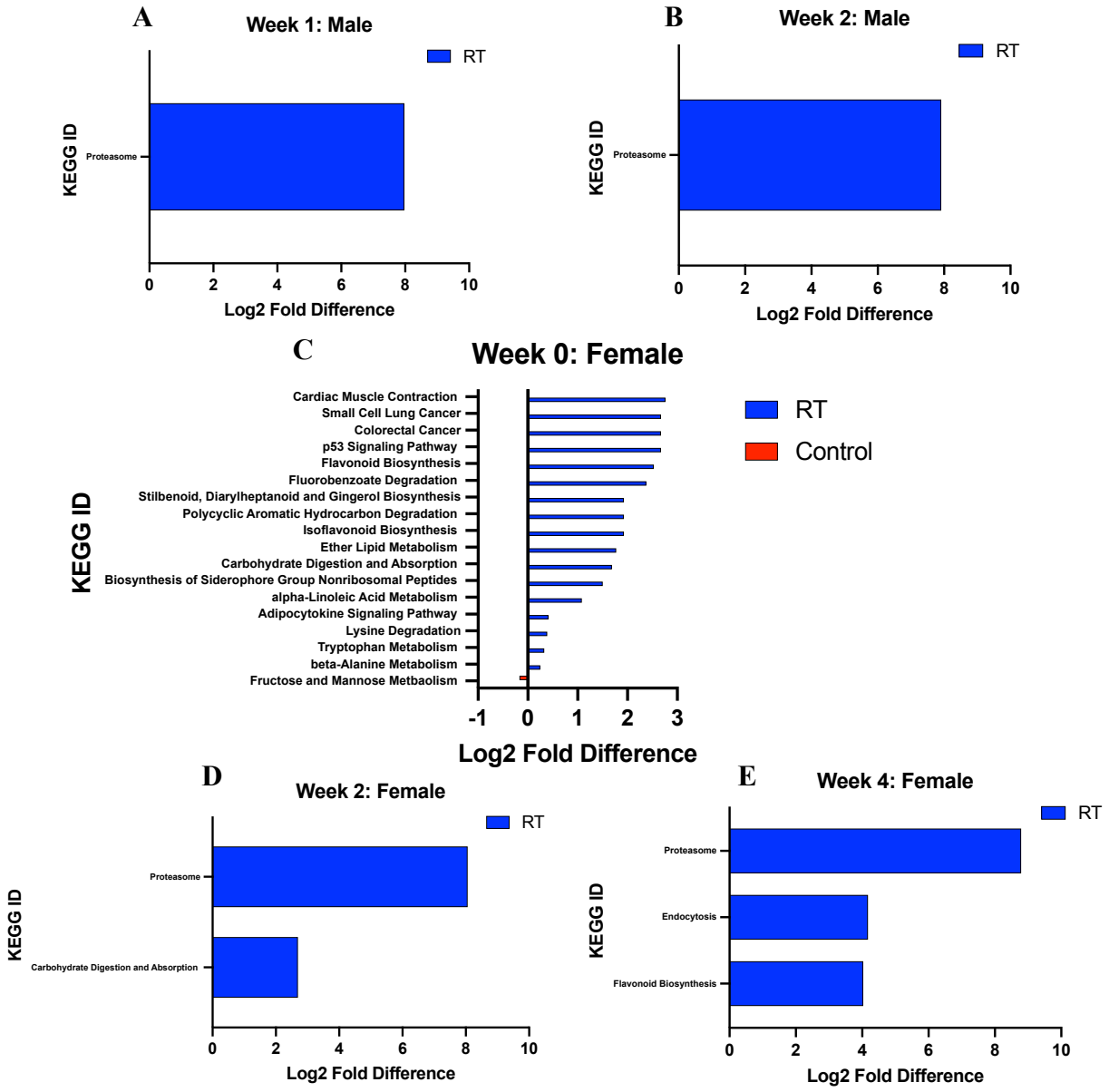

Figure 10. Differences in predicted functional pathways in male and female mice with vs without bladder radiation treatment. Log2 fold difference of predicted functional pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database between male control and RT mice at weeks (A) 1, and (B) 2, as well as female control and RT mice at weeks (C) 0, (D) 2, and (E) 4. Functional prediction performed using PICRUSt2. All features shown are statistically significant ($p < 0.05$) using edgeR statistical analysis.

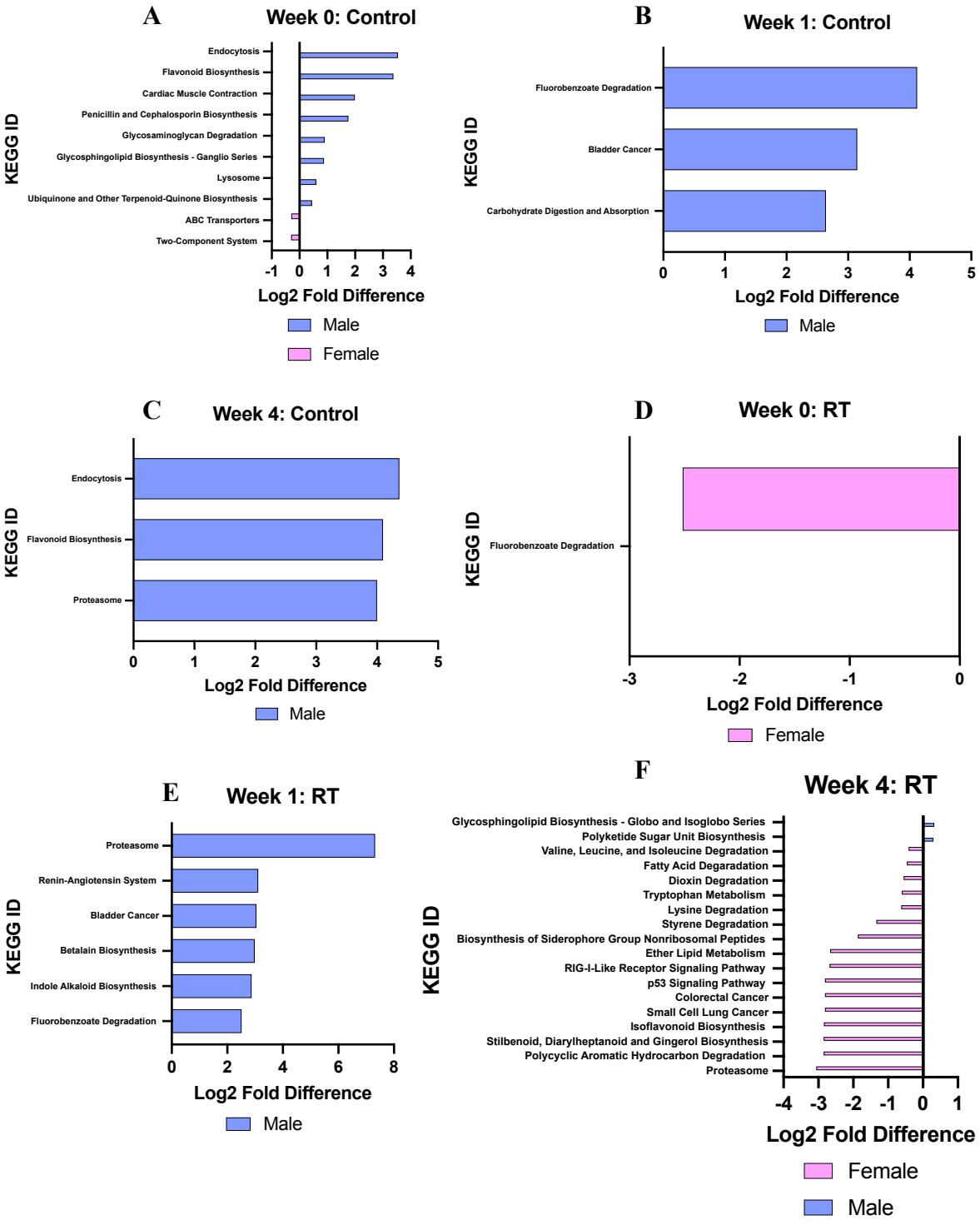

Figure 11. Sex-specific differences in predicted functional pathways in mice with and without bladder radiation treatment. Log2 fold difference of predicted functional pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database between male and female control mice at weeks (A) 0, (B), 1, and (C) 4, as well as male and female RT mice at weeks (D) 0, (E) 1, and (F) 4. Functional prediction performed using PICRUSt2. All features shown are statistically significant ($p < 0.05$) using edgeR statistical analysis.

5.3: Impact of radiation therapy on the gut microbiome in a cold tumor model *in vivo*

Radiation therapy in a cold tumor model does not significantly affect gut microbial diversity *in vivo*

Previously, we had explored the effects of bladder RT on the gut microbiome in the absence of a tumor. In order to fully understand the impact of RT on the gut microbiome in a bladder cancer context, we also need to explore this relationship while a tumor is developing. To accomplish this, mice were injected with a UPPL bladder cancer cell line in the right flank, and then randomized into untreated and RT-treated groups, which were given 2x5Gy RT to the tumor site. We once again analyzed bacterial diversity and composition of the stool samples using the collected sequences. Mice in the RT arm did not differ significantly in terms of α-diversity (Fig. 12A). In terms of β-diversity, no clustering was observed between control and RT samples at any time point (Fig. 12B-D), however, in control mice, day 0 and day 30 samples showed slight separation from each other. This clustering was not observed at day 15 (Fig. 12E). Additionally, this clustering pattern did not occur in RT mice (Fig. 12F). These results suggest that RT in an immunologically cold bladder tumor model does not significantly affect microbial composition and diversity.

Radiation therapy in a cold tumor model changes relative abundance of several microbial features and increases Proteasome pathway expression in the gut microbiota

At day 15, decreases in relative abundance in RT mice compared to control mice were observed in the genera *Family_XIII_UCG_001* and *Ruminoccus_1* (Fig. 13A-B). At day 30, increase in the genus *Staphylococcus* and decrease in the genus *Ruminoccus_1* was observed (Fig. 13B-C). No functional pathways were differentially expressed between control and RT-treated mice at days 0 and 15 post-RT. At day 30, the pathway *Proteasome* were increased in the RT-treated mice compared to controls (Fig. 14). These results suggest that RT in an immunologically cold bladder tumor model has a time-dependent effect on the abundance of several gut microbial features, as well as promoting proteasome functions in the gut microbiota.

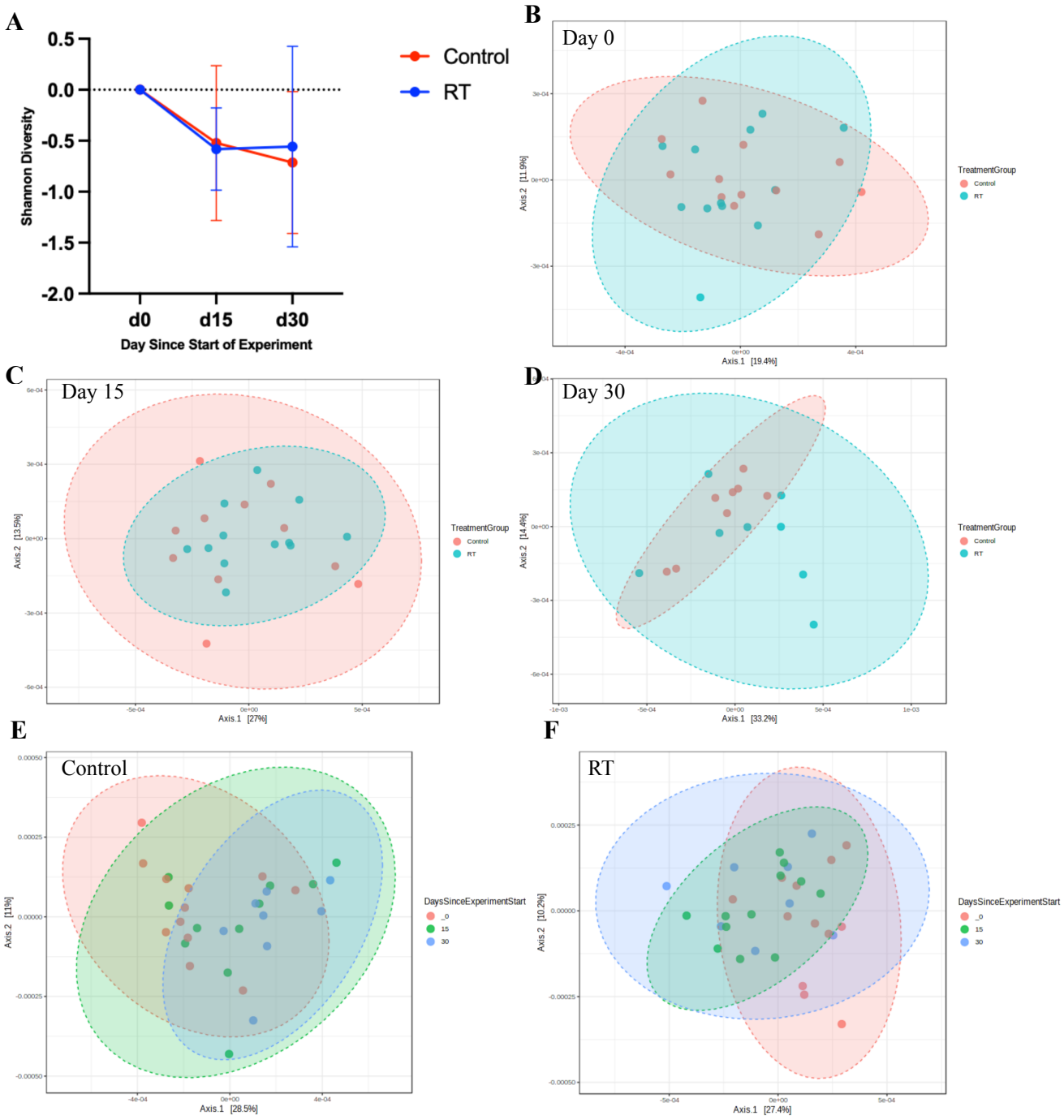

Figure 12. Changes in alpha and beta diversity of the microbiome of mice with UPPL tumors on the right flank over time given RT vs no RT. (A) Change in alpha diversity of the microbiota at each time-point compared to baseline (week 0). Alpha diversity measured using Shannon index. (B-D) Principal coordinate analysis (PCoA) of the microbiota of control compared to RT-treated mice at (B) baseline, (C) Day 15, and (D) day 30. (E-F) PCoA of the microbiota at different time-points in (E) control and (F) RT-treated mice. In all cases, PCoA used to measure beta diversity based on Bray-Curtis dissimilarity. Statistical analyses performed using ANOVA: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Error bars represent 95% confidence intervals.

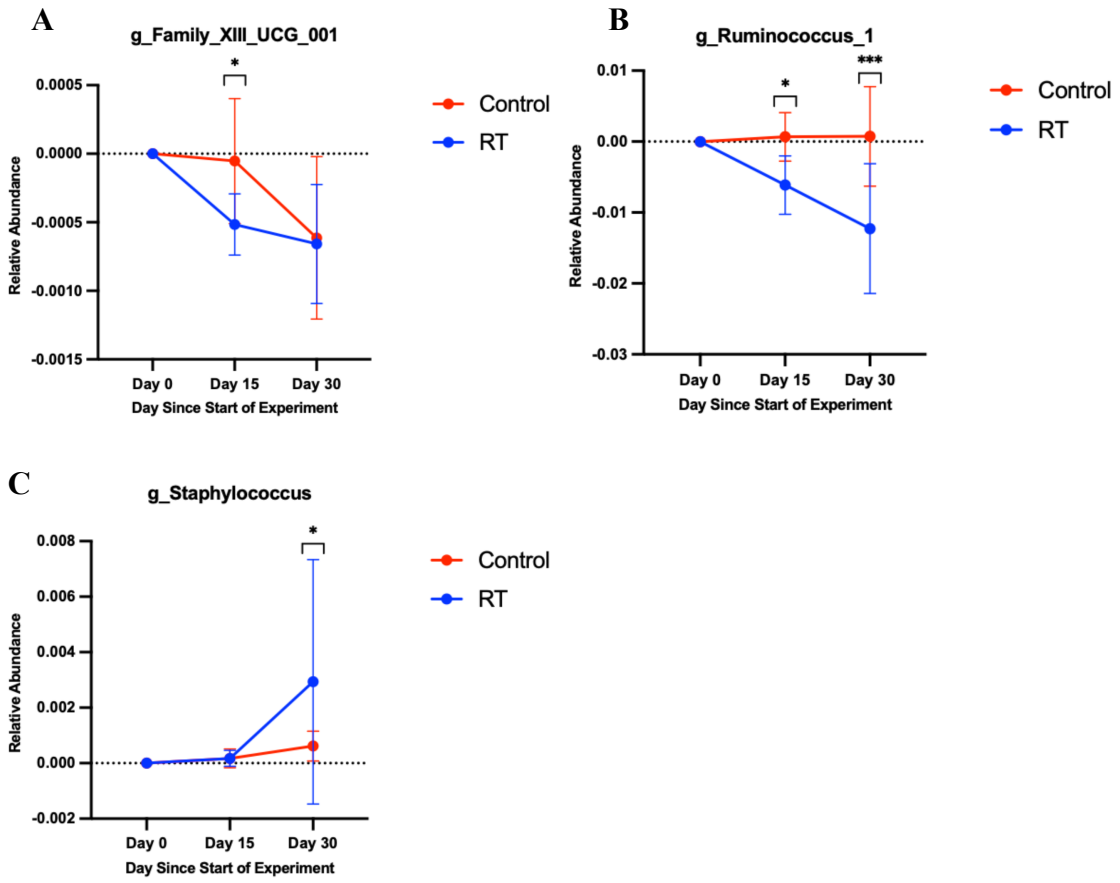

Figure 13. Changes in relative abundance of specific taxa in mice with UPPL tumors on the right flank over time given RT compared to no RT. Change in relative abundance of the genera (A) *Family_XIII_UCG_001*, (B) *Ruminococcus_1*, and (C) *Staphylococcus* at each time-point compared to baseline (week 0). Statistical analyses performed using ANOVA: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Error bars represent 95% confidence intervals.

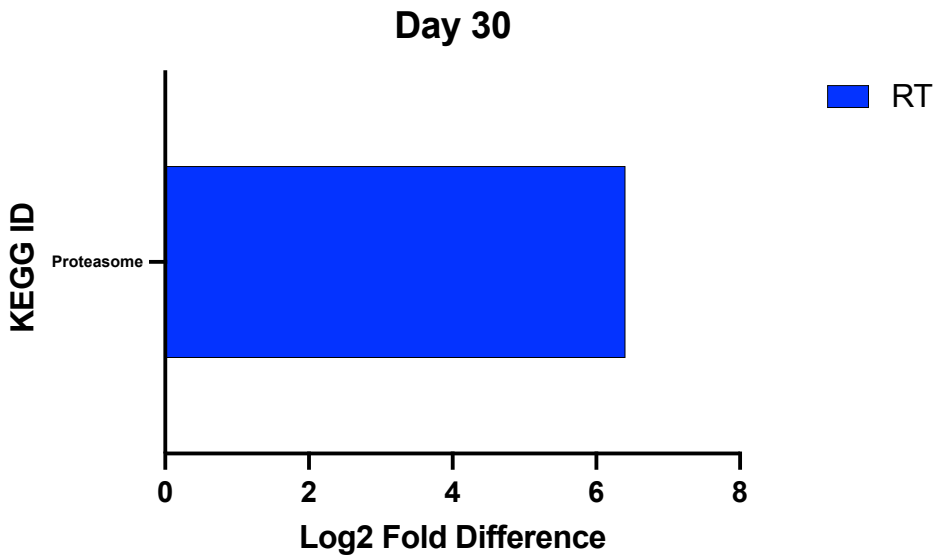

Figure 14. Differences in predicted functional pathways in mice with UPPL tumors on the right flank given RT compared to no RT. Log2 fold difference of predicted functional pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database between control and RT mice at day 30 post-RT. Functional prediction performed using PICRUSt2. All features shown are statistically significant ($p < 0.05$) using edgeR statistical analysis.

Chapter 6: Discussion

MIBC is a cancer with a low survival rate, where the standard of care involves the complete removal of the bladder, which leads to reduced quality-of-life. Because of this, there is a need to develop and optimize bladder sparing alternatives. One widely used alternative is RT, which has similar overall survival to RC. However, due to the fact that 30% of patients do not respond to RT, and that many patients will develop radiation-induced toxicities, there is a need to improve RT as a bladder sparing alternative. One approach that can be taken is to study the various downstream effects of radiation that could be influencing bladder cancer treatment. Particularly, RT is known to alter the gut microbiome, which can in turn, influence response to treatment and induce adverse events. Although the effects of radiation on the gut microbiota are well studied, information regarding bladder cancer is scarce, hence why we focused our attention at observing the effects of RT on the gut microbiome in a bladder cancer context. Our findings suggest that RT influences changes in the relative abundance of specific microbial features rather than sweeping changes to the microbiota, and that these changes differ at different time-points. Additionally, RT was found to induce changes to the functional profile of the gut microbiota, such as increasing expression of the proteasome pathway.

In our non-tumor experiments, we opted to administer a 6Gy x 6 RT fractionation regimen, which has not been previously used in murine models evaluating the effects of radiation. Nonetheless, this regimen was used due to mirroring commonly used RT regimens in BC as well as its applications in combination with immunotherapy. In MIBC patients unsuitable for

cystectomy or daily RT, this regimen was found to result in 92% tumor control and 63% 1-year overall survival, while only having a 6.5% 6-month rate of grade ≥3 late toxicities [155]. A similar regimen has also been found to promote immunogenicity when combined with anti-CTLA-4 in a breast cancer model [84]. This is pertinent to our objective, as while this RT regimen has been shown to promote response to anti-CTLA-4, the microbiome is also implicated in response to ICIs such as anti-CTLA-4 [135-139]. In order to best administer combination therapy between RT and ICIs, it is important to understand how RT will affect the microbiome, and how those effects will influence the relationship between the two treatment modalities.

When studying the effects of bladder RT on the gut microbiota, we found changes in several bacteria that have previously been implicated in response to BC. Particularly, our findings suggest that bladder radiation increases the relative abundance of *Akkermansia* and *Bifidobacterium* in the gut. While the findings regarding *Akkermansia* seem to match previous literature [124-127], the findings regarding *Bifodbacterium* are contrary to previous literature, which describes a decrease in the relative abundance of *Bifidobacterium* post-RT [121, 122]. *Akkermansia muciniphila*, the lone species in the genus *Akkermansia*, is a mucin-degrading bacteria well known for its immunogenicity and is associated with increased response to ICIs such as anti-CTLA-4 and anti-PD-L1 [124-127, 141, 156]. Although *Akkermansia* is considered an immunogenic bacteria, it also has anti-inflammatory properties. It is associated with Treg differentiation and is associated with protection from age-associated inflammation [157]. It is also associated with maintaining gut homeostasis and radioprotection via promoting intestinal stem cell development [158] and with survival in various cancers such as pancreas and bladder

[157, 159]. Like *Akkermansia*, *Bifidobacterium* is also associated with response to ICIs, including anti-PD-1 [142] and anti-PD-L1 [160], and has also been shown to improve anti-tumoral immunity by strengthening dendritic cell function, leading to the induction of an anti-tumor T cell response [114]. Additionally, *Bifidobacterium* has been shown to correlate negatively with bladder cancer [161], and has also been shown to reduce RT-induced toxicities such as diarrhea [162].

In addition to *Akkermansia* and *Bifidobacterium*, we found other bacteria associated with immunity and RT-induced toxicity that were altered by bladder radiation. Our findings show a decrease in the class *Mollicutes*, the order *Anaeroplasmatales*, and an increase in the family *S24_7*, all of which can manipulate various immune factors. The class *Mollicutes* is associated with reduction in the pro-inflammatory cytokine, macrophage inflammatory protein 1a (MIP-1a) [163]. MIP-1a is associated with protection from various cancers including melanoma [164] and nasopharyngeal cancer [165] but is also associated with worse prognosis in BC [166]. This suggests that RT-induced reduction in *Mollicutes* may be beneficial in other cancers, but is deleterious in BC. The genus *Anaeroplasma*, within the order *Anaeroplasmatales*, which is also in the class *Mollicutes*, is associated with increased TGF-ß expression [167], which itself is associated with worse prognosis in BC [168], suggesting that the relationship between *Mollicutes* and BC is complex and multi-layered. Additionally, the family *S24_7* is associated with increased PD-L1 expression [169], suggesting it may induce stronger response to anti-PD-L1.

Bladder radiation was also found to alter the abundance of bacteria associated with RT-associated toxicity, particularly through increases in the order *Erysipelotrichales* and decreases in the genus *rc4_4*. *Erysipelotrichia*, the class containing *Erysipelotrichales*, has previously been associated with protection from radiation-induced gastrointestinal injury [170] and has been negatively correlated with various cancers, including colorectal [171] and pancreatic [172]. This relationship is controversial though, as it has also been positively associated with colorectal cancer [173] and non-response to a combination of chemotherapy and anti-PD-L1 [174]. Meanwhile, *rc4_4* has previously been associated with low-grade (grade 2) radiation enteritis [175]. Put together, these results suggest that bladder radiation may induce changes in the gut microbiota that reduce gastrointestinal toxicity, however, it also induces both pro- and anti-immunogenic bacteria, and thus the effects on response to immunotherapy may be hard to discern.

In addition to analyzing the role of bladder radiation on the gut microbiota, we also explored the role sex plays in this relationship. We found the impact of sex pertinent to our study due to the impact that sex plays in all facets of this project. Firstly, sex differences exist in the diagnosis and prognosis of BC. Although BC is more common in males than females, females have a lower 5-year overall survival than males in both localized and locally advanced BC [176]. This relationship extends to response to radiation, where females with MIBC have a lower relative survival than males after treatment with TMT [177]. The gut microbiome is also influenced by the sex of the host. In mice, microbial diversity and relative abundance of specific bacteria

differed between males and females. This finding can be translated to humans as well, as females tend to have higher alpha diversity and a reduced abundance of the phylum *Bacteroidetes* [153].

When analyzing the effect of bladder radiation on alpha diversity in male and female mice, we observed several sex differences in how bladder radiation affected specific bacteria. Particularly, RT-treated males showed higher relative abundance of orders *Coriobacterales* and *Burkholderiales* after week 1 when compared to RT-treated females, though by week 4, RT-treated females had higher relative abundance of genus *Bifidobacterium* and species *Bacteroides uniformis* than males. Previous studies in the gut microbiota have shown *Coriobacteriaceae*, a family within the order *Coriobacterales* to be enriched in patients with colorectal cancer [173] and is associated with resistance to neoadjuvant chemoradiation in locally advanced rectal cancer [178]. Interestingly though, a study on the urinary microbiota shows that *Coriobacterales* is reduced in female patients with BC compared to female healthy controls, but not in males [179]. A similarly complex relationship between bacteria and cancer can be observed with *Burkholderiales*. *Burkholderia cepacia*, a species within the order *Burkholderiales*, has been found to promote response to anti-CTLA-4 [136], however various bacteria within the order *Burkholderiales* have been associated with various cancers [180-182], and *Burkholderiales* was found to be enriched in neoplastic bladder tissue compared to non-neoplastic tissue [179]. The role of *Bacteroides uniformis* in cancer is controversial, as its abundance has been shown to be increases in colorectal cancer [183] while decreased in metastatic colorectal cancer vs non-metastatic disease [184]. Additionally, there is no information on its role in BC. However, *Bacteroides uniformis* has also been shown to promote response to chemotherapy + anti-PD-L1

in various solid tumors [174]. Taken together with *Bifidobacterium*, results from this experiment show that RT promotes bacteria in both males and females that are associated with both beneficial and harmful effects on the host. Due to the scarcity in studies on the gut microbiota in bladder cancer, the effects that these features may have are purely speculative, and thus further research needs to be conducted to better understand the relationship between sex and the gut microbiota following bladder radiation.

While studying the effects of bladder radiation on the microbiota does give insight into effects that may potentially be observed in the clinic, also exploring this relationship with a developing tumor will give additional clinical insight. For this tumor model, we decided to use a UPPL bladder cancer cell line. One of the main strategies being researched to overcome radioresistance in MIBC is to combine RT with immunotherapies such as ICIs. Given that immunologically cold tumors have lower response rates to ICIs [57], and the microbiota has a known role in response to ICI [139-144], it makes sense to study the microbiota in an cold tumor model where it will be most impactful in the relationship between RT and antitumor immunity.

Interestingly, from our findings, the effects of tumor RT and the effects of bladder radiation on the murine gut microbiota differed in terms of the changes in specific features. This can be explained by a variety of factors that differed between the UPPL and non-tumor experiments, such as differences in the treatment regimen used, site of radiation, and the presence of a tumor. In particular, the specific features differentially expressed between control and RT-treated mice with UPPL tumors include decreases in the genera *Family_XIII_UCG_001* and *Ruminococcus_1*,

and increases in the genus *Staphylococcus*. Currently, no data regarding *Family_XIII_UCG_001* exists in BC, however, studies regarding this bacteria do exist in other cancers. Particularly, a reduction of *Family_XIII_UCG_001* show is observed in colorectal cancer [185], and an increase in the family of *Family_XIII_UCG_001*, *Anaerovoracaceae*, is seen in N0 stage esophageal squamous cell carcinoma when compared to higher stages [186]. The relationship between *Ruminococcus* in the gut microbiota and cancer vary between different cancer types, as it is causatively related to castration resistant prostate cancer [187], and upregulated in lung cancer [188] and cholangiocarcinoma [189], but is downregulated in colorectal cancer [185]. Additionally, urinary microbiota studies indicate a decrease in *Ruminococcus-1* in male NMIBC patients compared to healthy controls [190]. On the contrary, *Staphylococcus* is enriched in the bladder mucosal microbiome of BC patients when compared to healthy controls [191], though it may also be associated with increased peripheral lymphocyte count [192]. Overall, these RT-induced changes in the gut microbiota suggests that RT in a cold tumor model may induce a pro-tumorigenic microbiota with potential for increased immunogenicity.

One interesting observation found in both the non tumor and UPPL findings is that RT-induced changes to the microbiota are time dependent, and each time point has its own microbial signature, rather than specific features increasing or decreasing in relative abundance at a steady rate. This can be explained via the process in which RT alters the gut microbiota. Administration of RT leads to the elimination of a certain proportion of the gut microbiota. The microbes most resistant to this dysbiotic event will have increased abundance relative to the rest of the microbiota despite not increasing in abundance themselves. Eventually, the microbiota will

repopulate, causing the overall abundance of the microbiota to reach its previous amount. Microbial features affected by RT at earlier time points are defined by their ability to resist RT-induced dysbiosis, whereas features affected at later time points refer to bacteria most or least capable of repopulating the microbiota post-RT.

In addition to analyzing RT-induced changes in microbial composition, diversity, and relative abundance, we also explored RT-induced changes to functional pathway expression in the microbiome. These pathways allow for further understanding of how the microbiome changes, and the physiological effects that these changes will have. Due to sequencing only the 16S rRNA gene and not the rest of the genomic information from the stool, functional pathways cannot be determined with 100% certainty. Instead, a functional prediction software, such as PICRUSt2, needs to be used.

In both the non-tumor and UPPL models, as well as in both males and females, RT was found to upregulate expression of the *Proteasome* pathway. This has implications in BC, as the proteasome subunit genes PSDM2 and PSDM8 have previously been shown to be upregulated in BC while also being associated with worse prognosis [193]. This suggests a potential role for proteasome inhibitors as a combination with radiation to treat BC. Proteasome inhibitors have previously been explored heavily in myeloma, though previous findings in solid tumors have not been promising [194].

In addition to the *Proteasome* pathway, RT was also found to upregulate expression of the *Endocytosis* and *Flavonoid biosynthesis* pathways in female mice. Although aberrant endocytosis is known to occur in various cancers, the relationship between cancer and endocytosis is complex. There are various endocytosis-regulating proteins that are associated with cancer when up- or down-regulated. In BC, these include Dab2, Mdm2, RAB27, KAI1, and MTSS1 [195, 196]. Increase in the *Endocytosis* pathway can thus have pro- or anti-tumorigenic effects on the host. Increase in the *Flavonoid biosynthesis* pathway is likely an artifact from the mice's feeding, in which bacteria involved in synthesizing the flavonoids in the mice's food were more resistant to RT-induced dysbiosis.

In addition to comparing control and RT mice for each sex, male and female mice were also compared to each other, where differences in several functional pathways were observed. For example, at week 1, females showed reduction of the *Renin Angiotensin System* pathway compared to males. Several parts of this system are upregualted in BC, and inhibiting the renin-angiotensin system has shown promise in NMIBC in improving 3-year and 5-year survival [197]. Interestingly, females also showed reduction of the *Bladder Cancer* pathway compared to males at week 1, suggesting males harbor a more pro-tumorigenic microbiome during RT. At week 4, females showed increases in several pathways, including *Fatty Acid Degradation, Ether Lipid Metabolism*, and *p53 Signalling Pathway*, all of which have implications in cancer. Particularly, fatty acid metabolism is increased in BC [198]. Ether lipids have increased abundance in various cancers. Additionally, it was found that inhibiting the enzyme alkylglyceronephosphate synthase, one of the key steps in ether lipid synthesis, it associated with increased survival in breast cancer

[199]. Meanwhile, p53 is a tumor suppressor that is frequently mutated in many cancers, including BC. Upregulation of the p53 signalling pathway could act as a double-edged sword in BC, as it can provide a tumor-suppressive effect when not mutated but could lead to increased tumorigenesis when mutated [200]. *Tryptophan metabolism* was also increased in females post-RT compared to males at week 4, suggesting an increase in tryptophan derivative production, which serves various roles in metabolism, immunoregulation, and tight junction protection [100-104]. Another pathway of interest is *RIG-I-like receptor signaling pathway*, which was once again increased in females post-RT compared to males at week 4. This pathway is known to induce radiosensitivity in various cancers, including BC [201], and the use of RIG-I agonists is an immunotherapy strategy known to work against several cancer types [202]. Lastly, females also have increases in several cancer-associated KEGG pathways post-RT compared to males at week 4, including colorectal and small cell lung cancer, suggesting a pro-tumorigenic microbiome. Overall, the combination of pro-tumor and anti-tumor pathways that are differentially expressed between sexes suggests that the relationship between RT, sex, the microbiome, and bladder cancer is not straightforward, and needs further research to investigate which pathways are the drivers of tumorigenesis and tumor suppression in a BC radiation context.

Although this project produced various interesting findings, there are also several limitations with this project. Firstly, the housing of the mice in both the non-tumor and UPPL experiments may have skewed the results. In the non-tumor experiment, mice were caged according to their sex and treatment group. Potential exchanges of microbiome components between mice may

amplify the observed changes to the microbiota; effects observed in one mouse may be transferred to others. In the UPPL experiment, the opposite phenomenon is observed. As mice were not caged according to their treatment group, potential exchanges in microbiome components between mice of different groups during feeding may dampen any potential findings instead. Additionally, in the UPPL model, the short term effects of RT on the gut microbiome were not studied. In the non-tumor experiment, samples were collected before, during, and after the radiation regimen, and it was found that RT induces different short- and long-term effects on the gut microbiota. However, the soonest stool sample collection post-RT happens after 2 weeks, and thus the shorter-term effects are not being observed.

Another limitation in this experiment regards the use of 16S rRNA gene sequencing. While whole genome sequencing can capture a complete picture of the microbiome, it is also expensive and not convenient to use and is only necessary if higher resolution is needed [203, 204]. By using 16S sequencing, certain components of the gut microbiome such as fungi cannot be studied, and functional analysis necessitates the use of functional prediction software instead of using the sequence data directly. This limitation will be accounted for as more methods for microbiome sequencing get developed. One such method that shows promise is 2bRad-M, which uses restriction sites to assist in sequencing, and can sequence ~1% of the metagenome while still allowing for high resolution analysis and functional prediction [205].

One future direction that can be implemented is the use of an orthotopic tumor model. While the subcutaneous UPPL model can be used to study systemic effects of RT on the gut microbiota,

using an orthotopic model allows for the study of both bladder irradiation and the effects of the tumor, and is thus the most clinically relevant model. An experiment using BBN and a 8Gy x 3 bladder radiation regimen is currently ongoing to address this future direction. Additionally, different radiation regimens can be tested in the future to determine which regimen induces the most favourable changes in the microbiome to prevent toxicities and aid in combination therapy.

Another future direction that can be implemented is the analysis of the urinary microbiome. Although urine was thought to be sterile until recently, most microbiome studies in BC involve studying the urinary microbiome rather than the gut. Currently, there is no standard method of collecting urinary microbiome samples, though, improvements in sampling methods could lead to urinary microbiome studies being easier to perform while still being able to obtain enough of the microbiota to feasibly study. Lastly, although the studies described previously were all performed in mice, studying the gut microbiome from human MIBC patients through the use of human stool samples is both feasible and improves upon our understanding of how radiation would affect the microbiome in a clinical setting.

Chapter 7: Conclusion

As expected, radiation did induce changes in the murine gut microbiome in a BC context. These changes were found to be dependent on both sex and the amount of time elapsed after treatment, and were also found to change when implementing the UPPL cold bladder tumor model. When regarding the time-dependent changes in the microbiota, abundance of specific microbial features were not linearly dependent on time elapsed. Rather, each time point produced a unique microbial signature, suggesting that the bacteria most resistant to RT-induced dysbiosis and the bacteria most efficiently able to repopulate the microbiota are different. While various changes to specific bacteria were observed, alpha and beta diversity remained unchanged after RT. Changes were also observed in the functional pathways of the gut microbiome post-RT. Notably, the *Proteasome* pathway was upregulated after radiation in both the non-tumor and UPPL models, including in both male and female mice in the non-tumor model.

In previous studies examining the role of radiation on the gut microbiota, radiation generally increased the abundance of deleterious features while decreasing the abundance of beneficial features. This would imply that in order to best administer combination therapies, it may be beneficial to administer antibiotics and/or wait for the microbiota to recover post-RT such that the gut microbiota will be more responsive to combination therapy. However, this study does not necessarily support findings from previous studies, as pro- and anti-tumorigenic bacteria were both upregulated and downregulated after bladder radiation. Although these results allow for a better understanding of the effects of radiation on the gut microbiota in a bladder cancer context,

the clinical implications are still unknown, and further research needs to be conducted to assess the clinical relevance of these changes.

References

1. Canadian Cancer Society / Société canadienne du. Canadian Cancer Statistics. *Canadian Cancer Society* https://cancer.ca/en/research/cancer-statistics/canadian-cancer-statistics.
2. Cumberbatch, M. G. K. *et al.* Epidemiology of Bladder Cancer: A Systematic Review and Contemporary Update of Risk Factors in 2018. *European Urology* **74**, 784–795 (2018).
3. Sung, H. *et al.* Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA A Cancer J Clin* **71**, 209–249 (2021).
4. Saginala, K. *et al.* Epidemiology of Bladder Cancer. *Medical Sciences* **8**, 15 (2020).
5. Mossanen, M. The Epidemiology of Bladder Cancer. *Hematology/Oncology Clinics of North America* **35**, 445–455 (2021).
6. Wang, Y., Chang, Q. & Li, Y. Racial differences in Urinary Bladder Cancer in the United States. *Sci Rep* **8**, 12521 (2018).
7. Freedman, N. D. Association Between Smoking and Risk of Bladder Cancer Among Men and Women. *JAMA* **306**, 737 (2011).
8. Burger, M. *et al.* Epidemiology and Risk Factors of Urothelial Bladder Cancer. *European Urology* **63**, 234–241 (2013).
9. Letašiová, S. *et al.* Bladder cancer, a review of the environmental risk factors. *Environ Health* **11**, S11 (2012).
10. Riegert-Johnson, D. L. *et al.* Cancer and Lhermitte-Duclos disease are common in Cowden syndrome patients. *Hered Cancer Clin Pract* **8**, 6 (2010).

11. van der Post, R. S. *et al.* Risk of urothelial bladder cancer in Lynch syndrome is increased, in particular among MSH2 mutation carriers. *J Med Genet* **47**, 464–470 (2010).
12. Robertson, A. G. *et al.* Comprehensive molecular characterization of muscle invasive bladder cancer. *Cell* **171**, 540-556.e25 (2017).
13. Chandrasekar, T., Erlich, A. & Zlotta, A. R. Molecular Characterization of Bladder Cancer. *Curr Urol Rep* **19**, 107 (2018).
14. Iwakuma, T. & Lozano, G. MDM2, an introduction. *Mol Cancer Res* **1**, 993–1000 (2003).
15. Wan, S. *et al.* The role of telomerase reverse transcriptase (TERT) promoter mutations in prognosis in bladder cancer. *Bioengineered* **12**, 1495–1504.
16. American Cancer Society. Bladder Cancer Signs and Symptoms. *American Cancer Society* https://www.cancer.org/cancer/types/bladder-cancer/detection-diagnosis-staging/signs-and-symptoms.html.
17. DeGEORGE, K. C., Holt, H. R. & Hodges, S. C. Bladder Cancer: Diagnosis and Treatment. *afp* **96**, 507–514 (2017).
18. Sharma, S., Ksheersagar, P. & Sharma, P. Diagnosis and Treatment of Bladder Cancer. *afp* **80**, 717–723 (2009).
19. Hu, X., Li, G. & Wu, S. Advances in Diagnosis and Therapy for Bladder Cancer. *Cancers (Basel)* **14**, 3181 (2022).
20. Russo, G. I. *et al.* Performance of Narrow Band Imaging (NBI) and Photodynamic Diagnosis (PDD) Fluorescence Imaging Compared to White Light Cystoscopy (WLC) in Detecting Non-Muscle Invasive Bladder Cancer: A Systematic Review and Lesion-Level Diagnostic Meta-Analysis. *Cancers (Basel)* **13**, 4378 (2021).

21. Bochynek, K., Aebisher, D., Gasiorek, M., Cieślar, G. & Kawczyk-Krupka, A. Evaluation of autofluorescence and photodynamic diagnosis in assessment of bladder lesions. *Photodiagnosis and Photodynamic Therapy* **30**, 101719 (2020).

22. Sanchini, M. A. *et al.* Relevance of Urine Telomerase in the Diagnosis of Bladder Cancer. *JAMA* **294**, 2052–2056 (2005).

23. Chen, X. *et al.* Urine DNA methylation assay enables early detection and recurrence monitoring for bladder cancer. *Journal of Clinical Investigation* **130**, 6278–6289 (2020).

24. Mancini, M., Righetto, M., Zumerle, S., Montopoli, M. & Zattoni, F. The Bladder EpiCheck Test as a Non-Invasive Tool Based on the Identification of DNA Methylation in Bladder Cancer Cells in the Urine: A Review of Published Evidence. *Int J Mol Sci* **21**, 6542 (2020).

25. Liang, L.-G. *et al.* An integrated double-filtration microfluidic device for isolation, enrichment and quantification of urinary extracellular vesicles for detection of bladder cancer. *Sci Rep* **7**, 46224 (2017).

26. Felsenstein, K. M. & Theodorescu, D. Precision medicine for urothelial bladder cancer: update on tumour genomics and immunotherapy. *Nat Rev Urol* **15**, 92–111 (2018).

27. Cancer Staging - NCI. *National Cancer Institute* https://www.cancer.gov/about-cancer/diagnosis-staging/staging (2015).

28. Berdik, C. Unlocking bladder cancer. *Nature* **551**, S34–S35 (2017).

29. Tumor Grade - NCI. *National Cancer Institute* https://www.cancer.gov/about-cancer/diagnosis-staging/diagnosis/tumor-grade (2013).

30. Damrauer, J. S. *et al.* Intrinsic subtypes of high-grade bladder cancer reflect the hallmarks of breast cancer biology. *Proc Natl Acad Sci U S A* **111**, 3110–3115 (2014).

31. Lenis, A. T., Lec, P. M., Chamie, K. & Mshs, M. Bladder Cancer: A Review. *JAMA* **324**, 1980 (2020).

32. McConkey, R. W. & Dowling, M. Supportive Care Needs of Patients on Surveillance and Treatment for Non-Muscle-Invasive Bladder Cancer. *Seminars in Oncology Nursing* **37**, 151105 (2021).

33. Chang, S. S. *et al.* Diagnosis and Treatment of Non-Muscle Invasive Bladder Cancer: AUA/ SUO Guideline. *Journal of Urology* **196**, 1021–1029 (2016).

34. Non-muscle-invasive Bladder Cancer - Introduction - Uroweb. *Uroweb - European Association of Urology* https://uroweb.org/guidelines/non-muscle-invasive-bladder-cancer.

35. Schmidt, S. *et al.* Intravesical Bacillus Calmette-Guérin versus mitomycin C for Ta and T1 bladder cancer. *Cochrane Database of Systematic Reviews* (2020) doi:10.1002/14651858.CD011935.pub2.

36. Onrust, S. V., Wiseman, L. R. & Goa, K. L. Epirubicin: A Review of its Intravesical Use in Superficial Bladder Cancer. *Drugs & Aging* **15**, 307–333 (1999).

37. Packiam, V. T., Johnson, S. C. & Steinberg, G. D. Non–muscle-invasive bladder cancer: Intravesical treatments beyond Bacille Calmette-Guérin. *Cancer* **123**, 390–400 (2017).

38. Pettenati, C. & Ingersoll, M. A. Mechanisms of BCG immunotherapy and its outlook for bladder cancer. *Nat Rev Urol* **15**, 615–625 (2018).

39. Dasari, S. & Tchounwou, P. B. Cisplatin in cancer therapy: molecular mechanisms of action. *Eur J Pharmacol* **740**, 364–378 (2014).

40. Chang, S. S. *et al.* Treatment of Non-Metastatic Muscle-Invasive Bladder Cancer: Aua/ Asco/Astro/Suo Guideline. *J Urol* **198**, 552–559 (2017).

41. Patel, V. G., Oh, W. K. & Galsky, M. D. Treatment of muscle-invasive and advanced bladder cancer in 2020. *CA: A Cancer Journal for Clinicians* **70**, 404–423 (2020).

42. Shabsigh, A. *et al.* Defining Early Morbidity of Radical Cystectomy for Patients with Bladder Cancer Using a Standardized Reporting Methodology. *European Urology* **55**, 164–176 (2009).

43. Capitanio, U. *et al.* Partial Cystectomy Does Not Undermine Cancer Control in Appropriately Selected Patients With Urothelial Carcinoma of the Bladder: A Population-based Matched Analysist. *Urology* **74**, 858–864 (2009).

44. Hamad, J., McCloskey, H., Milowsky, M. I., Royce, T. & Smith, A. Bladder preservation in muscle-invasive bladder cancer: a comprehensive review. *Int Braz J Urol* **46**, 169–184 (2020).

45. Tholomier, C., Souhami, L. & Kassouf, W. Bladder-sparing protocols in the treatment of muscle-invasive bladder cancer. *Transl Androl Urol* **9**, 2920–2937 (2020).

46. Cooke, P. W. *et al.* Long–Term Risk of Salvage Cystectomy after Radiotherapy for Muscle–Invasive Bladder Cancer. *European Urology* **38**, 279–286 (2000).

47. Khochikar, M. V. Treatment of locally advanced and metastatic bladder cancer. *Indian J Urol* **24**, 84–94 (2008).

48. Abufaraj, M. *et al.* Management of muscle invasive, locally advanced and metastatic urothelial carcinoma of the bladder: a literature review with emphasis on the role of surgery. *Translational Andrology and Urology* **5**, 73544–73744 (2016).

49. Nadal, R. & Bellmunt, J. Management of metastatic bladder cancer. *Cancer Treatment Reviews* **76**, 10–21 (2019).

50. Baumann, B. C., Zaghloul, M. S., Sargos, P. & Murthy, V. Adjuvant and Neoadjuvant Radiation Therapy for Locally Advanced Bladder Cancer. *Clinical Oncology* **33**, 391–399 (2021).

51. Loriot, Y. *et al.* Erdafitinib in Locally Advanced or Metastatic Urothelial Carcinoma. *N Engl J Med* **381**, 338–348 (2019).

52. Hedegaard, J. *et al.* Comprehensive Transcriptional Analysis of Early-Stage Urothelial Carcinoma. *Cancer Cell* **30**, 27–42 (2016).

53. Tran, L., Xiao, J.-F., Agarwal, N., Duex, J. E. & Theodorescu, D. Advances in bladder cancer biology and therapy. *Nat Rev Cancer* **21**, 104–121 (2021).

54. Stroggilos, R. *et al.* Proteome-based classification of Nonmuscle Invasive Bladder Cancer. *International Journal of Cancer* **146**, 281–294 (2020).

55. Kamoun, A. *et al.* A Consensus Molecular Classification of Muscle-invasive Bladder Cancer. *Eur Urol* **77**, 420–433 (2020).

56. McConkey, D. J. & Choi, W. Molecular Subtypes of Bladder Cancer. *Curr Oncol Rep* **20**, 77 (2018).

57. Saito, R. *et al.* Molecular subtype-specific immunocompetent models of high-grade urothelial carcinoma reveal differential neoantigen expression and response to immunotherapy. *Cancer Res* **78**, 3954–3968 (2018).

58. Goutas, D. *et al.* Immunohistochemical Study of Bladder Cancer Molecular Subtypes and Their Association with PD-L1 Expression. *Cancers (Basel)* **15**, 188 (2022).

59. Ruan, J.-L. *et al.* Mouse Models of Muscle-invasive Bladder Cancer: Key Considerations for Clinical Translation Based on Molecular Subtypes. *European Urology Oncology* **2**, 239–247 (2019).

60. Seo, H. K. *et al.* The establishment of a growth-controllable orthotopic bladder cancer model through the down-regulation of c-myc expression. *Oncotarget* **8**, 50500–50509 (2016).

61. Fantini, D. & Meeks, J. J. The BBN model: a mouse bladder cancer model featuring basal-subtype gene expression and MLL3/MLL4 genetic disruption. *Oncoscience* **5**, 172–173 (2018).

62. Kim, B. M. *et al.* Therapeutic Implications for Overcoming Radiation Resistance in Cancer Therapy. *Int J Mol Sci* **16**, 26880–26913 (2015).

63. Wang, J., Wang, H. & Qian, H. Biological effects of radiation on cancer cells. *Mil Med Res* **5**, 20 (2018).

64. Suek, N., Campesato, L. F., Merghoub, T. & Khalil, D. N. Targeted APC Activation in Cancer Immunotherapy to Enhance the Abscopal Effect. *Front Immunol* **10**, 604 (2019).

65. D'Arcy, M. S. Cell death: a review of the major forms of apoptosis, necrosis and autophagy. *Cell Biol Int* **43**, 582–592 (2019).

66. Trenner, A. & Sartori, A. A. Harnessing DNA Double-Strand Break Repair for Cancer Treatment. *Front Oncol* **9**, 1388 (2019).

67. Mouw, K. W. DNA Repair Pathway Alterations in Bladder Cancer. *Cancers (Basel)* **9**, 28 (2017).

68. Sia, J., Szmyd, R., Hau, E. & Gee, H. E. Molecular Mechanisms of Radiation-Induced Cancer Cell Death: A Primer. *Front Cell Dev Biol* **8**, 41 (2020).

69. Tam, S. Y., Wu, V. W. C. & Law, H. K. W. Influence of autophagy on the efficacy of radiotherapy. *Radiat Oncol* **12**, 57 (2017).

70. Golden, E. B. & Apetoh, L. Radiotherapy and immunogenic cell death. *Semin Radiat Oncol* **25**, 11–17 (2015).

71. Sato, H., Okonogi, N. & Nakano, T. Rationale of combination of anti-PD-1/PD-L1 antibody therapy and radiotherapy for cancer treatment. *Int J Clin Oncol* **25**, 801–809 (2020).

72. Boustani, J., Grapin, M., Laurent, P.-A., Apetoh, L. & Mirjolet, C. The 6th R of Radiobiology: Reactivation of Anti-Tumor Immune Response. *Cancers (Basel)* **11**, 860 (2019).

73. Liu, Y. *et al.* Abscopal effect of radiotherapy combined with immune checkpoint inhibitors. *J Hematol Oncol* **11**, 104 (2018).

74. Shevtsov, M., Sato, H., Multhoff, G. & Shibata, A. Novel Approaches to Improve the Efficacy of Immuno-Radiotherapy. *Front Oncol* **9**, 156 (2019).

75. Dovedi, S. J. *et al.* Acquired resistance to fractionated radiotherapy can be overcome by concurrent PD-L1 blockade. *Cancer Res* **74**, 5458–5468 (2014).

76. Barker, H. E., Paget, J. T. E., Khan, A. A. & Harrington, K. J. The Tumour Microenvironment after Radiotherapy: Mechanisms of Resistance and Recurrence. *Nat Rev Cancer* **15**, 409–425 (2015).

77. Gan, G. N. *et al.* Hedgehog signaling drives radioresistance and stroma-driven tumor repopulation in head and neck squamous cancers. *Cancer Res* **74**, 7024–7036 (2014).

78. Ghaderi, N. *et al.* A Century of Fractionated Radiotherapy: How Mathematical Oncology Can Break the Rules. *Int J Mol Sci* **23**, 1316 (2022).

79. Makinde, A. Y., Eke, I., Aryankalayil, M. J., Ahmed, M. M. & Coleman, C. N. Exploiting Gene Expression Kinetics in Conventional, Hyperfractionation, and Hypofractionation for Targeted Therapy. *Semin Radiat Oncol* **26**, 254–260 (2016).

80. Demaria, S. *et al.* Radiation dose and fraction in immunotherapy: one-size regimen does not fit all settings, so how does one choose? *J Immunother Cancer* **9**, e002038 (2021).

81. Demaria, S. & Formenti, S. C. Radiation as an immunological adjuvant: current evidence on dose and fractionation. *Front Oncol* **2**, 153 (2012).

82. Choudhury, A. *et al.* Hypofractionated radiotherapy in locally advanced bladder cancer: an individual patient data meta-analysis of the BC2001 and BCON trials. *Lancet Oncol* **22**, 246–255 (2021).

83. Premo, C., Apolo, A. B., Agarwal, P. K. & Citrin, D. Trimodality therapy in bladder cancer: Who, what and when? *Urol Clin North Am* **42**, 169–vii (2015).

84. Dewan, M. Z. *et al.* Fractionated but not single dose radiotherapy induces an immune-mediated abscopal effect when combined with anti-CTLA-4 antibody. *Clin Cancer Res* **15**, 5379–5388 (2009).

85. Portner, R. *et al.* A practical approach to bladder preservation with hypofractionated radiotherapy for localised muscle-invasive bladder cancer. *Clin Transl Radiat Oncol* **31**, 1–7 (2021).

86. Vanpouille-Box, C. *et al.* DNA exonuclease Trex1 regulates radiotherapy-induced tumour immunogenicity. *Nat Commun* **8**, 15618 (2017).

87. Grapin, M. *et al.* Optimized fractionated radiotherapy with anti-PD-L1 and anti-TIGIT: a promising new combination. *J Immunother Cancer* **7**, 160 (2019).

88. Jandhyala, S. M. *et al.* Role of the normal gut microbiota. *World J Gastroenterol* **21**, 8787–8803 (2015).

89. Sekirov, I., Russell, S. L., Antunes, L. C. M. & Finlay, B. B. Gut Microbiota in Health and Disease. *Physiological Reviews* **90**, 859–904 (2010).

90. Dzutsev, A., Goldszmid, R. S., Viaud, S., Zitvogel, L. & Trinchieri, G. The role of the microbiota in inflammation, carcinogenesis, and cancer therapy. *European Journal of Immunology* **45**, 17–31 (2015).

91. Roy, S. & Trinchieri, G. Microbiota: a key orchestrator of cancer therapy. *Nat Rev Cancer* **17**, 271–285 (2017).

92. Mazmanian, S. K., Liu, C. H., Tzianabos, A. O. & Kasper, D. L. An Immunomodulatory Molecule of Symbiotic Bacteria Directs Maturation of the Host Immune System. *Cell* **122**, 107–118 (2005).

93. Ivanov, I. I. *et al.* Specific microbiota direct the differentiation of Th17 cells in the mucosa of the small intestine. (2009).

94. Alakomi, H.-L. *et al.* Lactic Acid Permeabilizes Gram-Negative Bacteria by Disrupting the Outer Membrane. *Appl Environ Microbiol* **66**, 2001–2005 (2000).

95. Yan, F. *et al.* Colon-specific delivery of a probiotic-derived soluble protein ameliorates intestinal inflammation in mice through an EGFR-dependent mechanism. *J Clin Invest* **121**, 2242–2253 (2011).

96. Swanson, H. I. Drug Metabolism by the Host and Gut Microbiota: A Partnership or Rivalry? *Drug Metab Dispos* **43**, 1499–1504 (2015).

97. Vandenplas, Y. *et al.* Factors affecting early-life intestinal microbiota development. *Nutrition* **78**, 110812 (2020).

98. Wen, L. & Duffy, A. Factors Influencing the Gut Microbiota, Inflammation, and Type 2 Diabetes. *J Nutr* **147**, 1468S-1475S (2017).

99. Hasan, N. & Yang, H. Factors affecting the composition of the gut microbiota, and its modulation. *PeerJ* **7**, e7502 (2019).

100. Su, Q. & Liu, Q. Factors Affecting Gut Microbiome in Daily Diet. *Frontiers in Nutrition* **8**, (2021).

101. Bajinka, O., Tan, Y., Abdelhalim, K. A., Özdemir, G. & Qiu, X. Extrinsic factors influencing gut microbes, the immediate consequences and restoring eubiosis. *AMB Express* **10**, 130 (2020).

102. Modi, S. R., Collins, J. J. & Relman, D. A. Antibiotics and the gut microbiota. *J Clin Invest* **124**, 4212–4218 (2014).

103. Spivak, I., Fluhr, L. & Elinav, E. Local and systemic effects of microbiome-derived metabolites. *EMBO Reports* **23**, e55664 (2022).

104. Li, C., Liang, Y. & Qiao, Y. Messengers From the Gut: Gut Microbiota-Derived Metabolites on Host Regulation. *Frontiers in Microbiology* **13**, (2022).

105. Liu, J. *et al.* Functions of Gut Microbiota Metabolites, Current Status and Future Perspectives. *Aging Dis* **13**, 1106–1126 (2022).

106. Ma, Y., Liu, X. & Wang, J. Small molecules in the big picture of gut microbiome-host cross-talk. *eBioMedicine* **81**, 104085 (2022).

107. McCarville, J. L., Chen, G. Y., Cuevas, V. D., Troha, K. & Ayres, J. S. Microbiota Metabolites in Health and Disease. *Annu. Rev. Immunol.* **38**, 147–170 (2020).

108. Ahmed, H. *et al.* Microbiota-derived metabolites as drivers of gut–brain communication. *Gut Microbes* **14**, 2102878.

109. Agus, A., Clément, K. & Sokol, H. Gut microbiota-derived metabolites as central regulators in metabolic disorders. *Gut* **70**, 1174–1182 (2021).

110. Zong, Y. *et al.* The Interaction Between the Microbiome and Tumors. *Front Cell Infect Microbiol* **11**, 673724 (2021).

111. Hatakeyama, M. Structure and function of Helicobacter pylori CagA, the first-identified bacterial protein involved in human cancer. *Proc Jpn Acad Ser B Phys Biol Sci* **93**, 196–219 (2017).

112. Matson, V., Chervin, C. S. & Gajewski, T. F. Cancer and the Microbiome—Influence of the Commensal Microbiota on Cancer, Immune Responses, and Immunotherapy. *Gastroenterology* **160**, 600–613 (2021).

113. Vivarelli, S. *et al.* Gut Microbiota and Cancer: From Pathogenesis to Therapy. *Cancers (Basel)* **11**, 38 (2019).

114. Akbar, N., Khan, N. A., Muhammad, J. S. & Siddiqui, R. The role of gut microbiome in cancer genesis and cancer prevention. *Health Sciences Review* **2**, 100010 (2022).

115. Sadrekarimi, H. *et al.* Emerging role of human microbiome in cancer development and response to therapy: special focus on intestinal microflora. *Journal of Translational Medicine* **20**, 301 (2022).

116. Zitvogel, L. *et al.* (116) Cancer and the gut microbiota: An unexpected link. *Sci Transl Med* **7**, 271ps1 (2015).

117. Górska, A., Przystupski, D., Niemczura, M. J. & Kulbacka, J. Probiotic Bacteria: A Promising Tool in Cancer Prevention and Therapy. *Curr Microbiol* **76**, 939–949 (2019).

118. Dos Reis, S. A. *et al.* Review of the mechanisms of probiotic actions in the prevention of colorectal cancer. *Nutrition Research* **37**, 1–19 (2017).

119. Canxia He *et al.* Gut microbial composition changes in bladder cancer patients: A case-control study in Harbin, China. *Asia Pacific Journal of Clinical Nutrition* **29**, (2020).

120. Gwon, Y.-N. *et al.* Microbiome in Bladder Cancer: A Systematic Review. *Diagnostics (Basel)* **13**, 84 (2022).

121. Fernandes, A., Oliveira, A., Soares, R. & Barata, P. The Effects of Ionizing Radiation on Gut Microbiota, a Systematic Review. *Nutrients* **13**, 3025 (2021).

122. Fernandes, A., Oliveira, A., Soares, R. & Barata, P. The Effects of Ionizing Radiation on Gut Microbiota: What Can Animal Models Tell Us?—A Systematic Review. *Curr Issues Mol Biol* **45**, 3877–3910 (2023).

123. Li, Z. *et al.* New Insights into the Relationship between Gut Microbiota and Radiotherapy for Cancer. *Nutrients* **15**, 48 (2022).

124. Tonneau, M. *et al.* The role of the gut microbiome on radiation therapy efficacy and gastrointestinal complications: A systematic review. *Radiotherapy and Oncology* **156**, 1–9 (2021).

125. Li, Z. *et al.* New Insights into the Relationship between Gut Microbiota and Radiotherapy for Cancer. *Nutrients* **15**, 48 (2022).

126. Amit, U., Facciabene, A. & Ben-Josef, E. Radiation Therapy and the Microbiome; More Than a Gut Feeling. *Cancer J* **29**, 84–88 (2023).

127. Liu, J., Liu, C. & Yue, J. Radiotherapy and the gut microbiome: facts and fiction. *Radiation Oncology* **16**, 9 (2021).

128. Then, C. K., Paillas, S., Wang, X., Hampson, A. & Kiltie, A. E. Association of Bacteroides acidifaciens relative abundance with high-fibre diet-associated radiosensitisation. *BMC Biology* **18**, 102 (2020).

129. Eaton, S. E. *et al.* Exploiting dietary fibre and the gut microbiota in pelvic radiotherapy patients. *Br J Cancer* **127**, 2087–2098 (2022).

130. Yi, Y., Lu, W., Shen, L., Wu, Y. & Zhang, Z. The gut microbiota as a booster for radiotherapy: novel insights into radio-protection and radiation injury. *Exp Hematol Oncol* **12**, 48 (2023).

131. Moraitis, I., Guiu, J. & Rubert, J. Gut microbiota controlling radiation-induced enteritis and intestinal regeneration. *Trends in Endocrinology & Metabolism* **34**, 489–501 (2023).

132. Xin, J.-Y. *et al.* Potential role of gut microbiota and its metabolites in radiation-induced intestinal damage. *Ecotoxicology and Environmental Safety* **248**, 114341 (2022).

133. Fernandes, D. & Andreyev, J. The Role of the Human Gut Microbiome in Inflammatory Bowel Disease and Radiation Enteropathy. *Microorganisms* **10**, 1613 (2022).

134. Smith, M. *et al.* Gut microbiome correlates of response and toxicity following anti-CD19 CAR T cell therapy. *Nat Med* **28**, 713–723 (2022).

135. Iida, N. *et al.* Commensal Bacteria Control Cancer Response to Therapy by Modulating the Tumor Microenvironment. *Science* **342**, 967–970 (2013).

136. Vétizou, M. *et al.* Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. *Science* **350**, 1079–1084 (2015).

137. Elkrief, A., Derosa, L., Zitvogel, L., Kroemer, G. & Routy, B. The intimate relationship between gut microbiota and cancer immunotherapy. *Gut Microbes* **10**, 424–428 (2019).

138. Roy, S. & Trinchieri, G. Microbiota: a key orchestrator of cancer therapy. *Nat Rev Cancer* **17**, 271–285 (2017).

139. Fernandes, M. R., Aggarwal, P., Costa, R. G. F., Cole, A. M. & Trinchieri, G. Targeting the gut microbiota for cancer therapy. *Nat Rev Cancer* **22**, 703–722 (2022).

140. Pederzoli, F. *et al.* Is There a Detrimental Effect of Antibiotic Therapy in Patients with Muscle-invasive Bladder Cancer Treated with Neoadjuvant Pembrolizumab? *European Urology* **80**, 319–322 (2021).

141. Routy, B. *et al.* Gut microbiome influences efficacy of PD-1–based immunotherapy against epithelial tumors. *Science* **359**, 91–97 (2018).

142. Matson, V. *et al.* The commensal microbiome is associated with anti-PD-1 efficacy in metastatic melanoma patients. *Science* **359**, 104–108 (2018).

143. Davar, D. *et al.* Fecal microbiota transplant overcomes resistance to anti–PD-1 therapy in melanoma patients. *Science* **371**, 595–602 (2021).

144. Zhang, Y. *et al.* Correlation of the gut microbiome and immune-related adverse events in gastrointestinal cancer patients treated with immune checkpoint inhibitors. *Front Cell Infect Microbiol* **13**, 1099063 (2023).

145. Olcina, M. M. & Giaccia, A. J. Reducing radiation-induced gastrointestinal toxicity — the role of the PHD/HIF axis. *J Clin Invest* **126**, 3708–3715.

146. Hauer-Jensen, M., Denham, J. W. & Andreyev, H. J. N. Radiation Enteropathy – Pathogenesis, Treatment, and Prevention. *Nat Rev Gastroenterol Hepatol* **11**, 470–479 (2014).

147. Daro-Faye, M. *et al.* Combined radiotherapy and immunotherapy in urothelial bladder cancer: harnessing the full potential of the anti-tumor immune response. *World J Urol* **39**, 1331–1343 (2021).

148. Rompré-Brodeur, A. *et al.* PD-1/PD-L1 Immune Checkpoint Inhibition with Radiation in Bladder Cancer: *In Situ* and Abscopal Effects. *Molecular Cancer Therapeutics* **19**, 211–220 (2020).

149. Bolyen, E. *et al.* Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* **37**, 852–857 (2019).

150. Dhariwal, A. *et al.* MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res* **45**, W180–W188 (2017).

151. Douglas, G. M. *et al.* PICRUSt2 for prediction of metagenome functions. *Nat Biotechnol* **38**, 685–688 (2020).

152. Yang, C. *et al.* ggpicrust2: an R package for PICRUSt2 predicted functional profile analysis and visualization. *Bioinformatics* **39**, btad470 (2023).

153. Kim, Y. S., Unno, T., Kim, B.-Y. & Park, M.-S. Sex Differences in Gut Microbiota. *World J Mens Health* **38**, 48–60 (2020).

154. Heidar, N. A., Bhat, T. A., Shabir, U. & Hussein, A. A. The Urinary Microbiome and Bladder Cancer. *Life* **13**, 812 (2023).

155. Hafeez, S. *et al.* Clinical Outcomes of Image Guided Adaptive Hypofractionated Weekly Radiation Therapy for Bladder Cancer in Patients Unsuitable for Radical Treatment. *Int J Radiat Oncol Biol Phys* **98**, 115–122 (2017).

156. Zheng, Y. *et al.* Gut microbiome affects the response to anti-PD-1 immunotherapy in patients with hepatocellular carcinoma. *J Immunother Cancer* **7**, 193 (2019).

157. Martin, A., Woolbright, B. L., Umar, S., Ingersoll, M. A. & Taylor, J. A. Bladder cancer, inflammageing and microbiomes. *Nat Rev Urol* **19**, 495–509 (2022).

158. Kim, S. *et al.* Mucin degrader Akkermansia muciniphila accelerates intestinal stem cell-mediated epithelial development. *Gut Microbes* **13**, 1892441.

159. Kharofa, J. *et al.* Analysis of the fecal metagenome in long-term survivors of pancreas cancer. *Cancer* **129**, 1986–1994 (2023).

160. Sivan, A. *et al.* Commensal Bifidobacterium promotes antitumor immunity and facilitates anti–PD-L1 efficacy. *Science* **350**, 1084–1089 (2015).

161. Min, K., Kim, H. T., Lee, E. H., Park, H. & Ha, Y.-S. Bacteria for Treatment: Microbiome in Bladder Cancer. *Biomedicines* **10**, 1783 (2022).

162. Chitapanarux, I. *et al.* Randomized controlled trial of live lactobacillus acidophilus plus bifidobacterium bifidum in prophylaxis of diarrhea during radiotherapy in cervical cancer patients. *Radiat Oncol* **5**, 31 (2010).

163. Xue, F., He, Z., Zhuang, D.-Z. & Lin, F. The influence of gut microbiota on circulating inflammatory cytokines and host: A Mendelian randomization study with meta-analysis. *Life Sci* **332**, 122105 (2023).

164. Nakasone, Y. *et al.* Host-Derived MCP-1 and MIP-1α Regulate Protective Anti-Tumor Immunity to Localized and Metastatic B16 Melanoma. *The American Journal of Pathology* **180**, 365–374 (2012).

165. Yang, M.-J. *et al.* Decreased macrophage inflammatory protein (MIP)-1α and MIP-1β increase the risk of developing nasopharyngeal carcinoma. *Cancer Communications* **38**, (2018).

166. Kumari, N. *et al.* Predictive role of serum and urinary cytokines in invasion and recurrence of bladder cancer. *Tumour Biol.* **39**, 1010428317697552 (2017).

167. Khan, I. *et al.* Far infrared radiation induces changes in gut microbiota and activates GPCRs in mice. *J Adv Res* **22**, 145–152 (2019).

168. Stojnev, S. *et al.* Prognostic Impact of Canonical TGF-β Signaling in Urothelial Bladder Cancer. *Medicina (Kaunas)* **55**, 302 (2019).

169. Zhu, T. *et al.* D-galactose protects the intestine from ionizing radiation-induced injury by altering the gut microbiome. *J Radiat Res* **63**, 805–816 (2022).

170. Xu, L. *et al.* Crosstalk between the gut microbiome and clinical response in locally advanced thoracic esophageal squamous cell carcinoma during neoadjuvant camrelizumab and chemotherapy. *Annals of Translational Medicine* **10**, 325–325 (2022).

171. Sun, L. *et al.* The difference of human gut microbiome in colorectal cancer with and without metastases. *Frontiers in Oncology* **12**, (2022).

172. Half, E. *et al.* Fecal microbiome signatures of pancreatic cancer patients. *Sci Rep* **9**, 16801 (2019).

173. Chen, W., Liu, F., Ling, Z., Tong, X. & Xiang, C. Human Intestinal Lumen and Mucosa-Associated Microbiota in Patients with Colorectal Cancer. *PLoS One* **7**, e39743 (2012).

174. Wu, Z. *et al.* The gut microbiota modulates responses to anti–PD-1 and chemotherapy combination therapy and related adverse events in patients with advanced solid tumors. *Front Oncol* **12**, 887383 (2022).

175. Wang, Z. *et al.* Gut microbial dysbiosis is associated with development and progression of radiation enteritis during pelvic radiotherapy. *J Cell Mol Med* **23**, 3747–3756 (2019).

176. Nakayama, M. *et al.* Impact of sex difference on survival of bladder cancer: A population-based registry data in Japan. *International Journal of Urology* **26**, 649–654 (2019).

177. Ballas, L. K. *et al.* Disparities in male versus female oncologic outcomes following bladder preservation: A population-based cohort study. *Cancer Med* **10**, 3004–3012 (2021).

178. Yi, Y. *et al.* Gut Microbiome Components Predict Response to Neoadjuvant Chemoradiotherapy in Patients with Locally Advanced Rectal Cancer: A Prospective, Longitudinal Study. *Clinical Cancer Research* **27**, 1329–1340 (2021).

179. Pederzoli, F. *et al.* Sex-specific Alterations in the Urinary and Tissue Microbiome in Therapy-naïve Urothelial Bladder Cancer Patients. *European Urology Oncology* **3**, 784–788 (2020).

180. Sims, T. T. *et al.* Tumor Microbial Diversity and Compositional Differences among Women in Botswana with High-Grade Cervical Dysplasia and Cervical Cancer. *International journal of gynecological cancer : official journal of the International Gynecological Cancer Society* **30**, 1151 (2020).

181. Lee, C.-C. *et al.* Unraveling the connections between gut microbiota, stress, and quality of life for holistic care in newly diagnosed breast cancer patients. *Sci Rep* **13**, 17916 (2023).

182. Alomair, A. O. *et al.* Colonic Mucosal Microbiota in Colorectal Cancer: A Single-Center Metagenomic Study in Saudi Arabia. *Gastroenterol Res Pract* **2018**, 5284754 (2018).

183. Zhou, P. *et al.* Differences in tissue-associated bacteria between metastatic and non-metastatic colorectal cancer. *Frontiers in Microbiology* **14**, (2023).

184. Zhao, L., Cho, W. C. & Nicolls, M. R. Colorectal Cancer-Associated Microbiome Patterns and Signatures. *Front Genet* **12**, 787176 (2021).

185. Park, J. *et al.* Fecal Microbiota and Gut Microbe-Derived Extracellular Vesicles in Colorectal Cancer. *Front Oncol* **11**, 650026 (2021).

186. Zhang, B., Xiao, Q., Chen, H., Zhou, T. & Yin, Y. Comparison of tumor-associated and nontumor-associated esophageal mucosa microbiota in patients with esophageal squamous cell carcinoma. *Medicine (Baltimore)* **101**, e30483 (2022).

187. Liu, Y., Yang, C., Zhang, Z. & Jiang, H. Gut Microbiota Dysbiosis Accelerates Prostate Cancer Progression Through Increased LPCAT1 Expression and Enhanced DNA Repair Pathways. *Front Oncol* **11**, 679712 (2021).

188. Zheng, Y. *et al.* Specific gut microbiome signature predicts the early-stage lung cancer. *Gut Microbes* **11**, 1030–1042.

189. Zhang, T. *et al.* A Predictive Model Based on the Gut Microbiota Improves the Diagnostic Effect in Patients With Cholangiocarcinoma. *Front Cell Infect Microbiol* **11**, 751795 (2021).

190. Oresta, B. *et al.* The Microbiome of Catheter Collected Urine in Males with Bladder Cancer According to Disease Stage. *Journal of Urology* **205**, 86–93 (2021).

191. Mansour, B. *et al.* Bladder Tissue Microbiome Composition in Patients of Bladder Cancer or Benign Prostatic Hyperplasia and Related Human Beta Defensin Levels. *Biomedicines* **10**, 1758 (2022).

192. Schluter, J. *et al.* The gut microbiota is associated with immune cell dynamics in humans. *Nature* **588**, 303–307 (2020).

193. Salah Fararjeh, A., Al-Khader, A., Al-Saleem, M. & Abu Qauod, R. The Prognostic Significance of Proteasome 26S Subunit, Non-ATPase (PSMD) Genes for Bladder Urothelial Carcinoma Patients. *Cancer Inform* **20**, 11769351211067692 (2021).

194. Manasanch, E. E. & Orlowski, R. Z. Proteasome Inhibitors in Cancer Therapy. *Nat Rev Clin Oncol* **14**, 417–433 (2017).

195. Mellman, I. & Yarden, Y. Endocytosis and Cancer. *Cold Spring Harb Perspect Biol* **5**, a016949 (2013).

196. Khan, I. & Steeg, P. S. Endocytosis: a pivotal pathway for regulating metastasis. *Br J Cancer* **124**, 66–75 (2021).

197. Samara, M. *et al.* Renin-Angiotensin System Single Nucleotide Polymorphisms Are Associated with Bladder Cancer Risk. *Curr Oncol* **28**, 4702–4708 (2021).

198. Yang, Y.-Y. *et al.* Characterization of the Lipid Metabolism in Bladder Cancer to Guide Clinical Therapy. *J Oncol* **2022**, 7679652 (2022).

199. Benjamin, D. I. *et al.* Ether lipid generating enzyme AGPS alters the balance of structural and signaling lipids to fuel cancer pathogenicity. *Proc Natl Acad Sci U S A* **110**, 14912–14917 (2013).

200. Wu, G. *et al.* Significance of TP53 mutation in bladder cancer disease progression and drug selection. *PeerJ* **7**, e8261 (2019).

201. Ranoa, D. R. E. *et al.* Cancer therapies activate RIG-I-like receptor pathway through endogenous non-coding RNAs. *Oncotarget* **7**, 26496–26515 (2016).

202. Jiang, Y. *et al.* Exploiting RIG-I-like receptor pathway for cancer immunotherapy. *Journal of Hematology & Oncology* **16**, 8 (2023).

203. Qian, X.-B. *et al.* A guide to human microbiome research: study design, sample collection, and bioinformatics analysis. *Chinese Medical Journal* **133**, 1844 (2020).

204. Jarett, J. K., Kingsbury, D. D., Dahlhausen, K. E. & Ganz, H. H. Best Practices for Microbiome Study Design in Companion Animal Research. *Front Vet Sci* **8**, 644836 (2021).

205. Sun, Z. *et al.* Species-resolved sequencing of low-biomass or degraded microbiomes using 2bRAD-M. *Genome Biology* **23**, 36 (2022).