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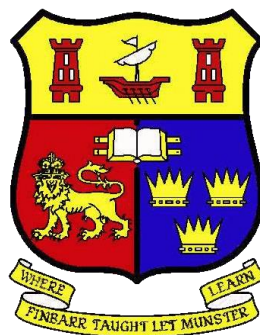
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Cancer Research @UCC



Bacteria and bacterial-produced proteins as biomarker technology for Precision Oncology

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for the degree of:

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Declaration

I hereby declare that I am the sole author of this thesis.

I authorise University College Cork to lend and photocopy this thesis to other institutions or individuals for the purpose of scholarly research.

Ciarán Devoy

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I dedicate this thesis to my son Liam Devoy and my father Paddy Devoy.

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THESIS Abstract

Biomarkers play a crucial role in cancer research, particularly in the context of precision oncology. A wide range of strategies exist to detect disease associated endogenous genes, proteins or activity. This thesis focuses on exploiting bacteria or bacterial-produced proteins in biomarker contexts. Chapter 2 describes the development of a detection system of biomarker activity, produced from bacteria; Chapter 3 describes development of a platform for deployment of biomarker detectors, produced from bacteria; while Chapter 4 examines the factors contributing to the success or failure of three clinical microbiome wide association studies (MWAS), in the context of patient accrual and the resources and systems required for set-up by measuring set-up time. Chapter 5 compares preliminary sequencing results of the MWAS in Chapter 4 to the current published literature.

Chapter 1 This literature review focusses in particular on cancer-associated bacteria. Bacterial inhabitants of the body have the potential to play a role in various stages of cancer initiation, progression, and treatment. These bacteria may be distal to the primary tumour, such as gut microbiota, or local to the tissue, before or after tumour growth. The Gut Microbiota (GM) can be involved in various stages of tumour progression. It may suppress carcinogenesis or promote it through the release of carcinogenic metabolites, sustenance of pro-inflammatory environments and/or the promotion of epigenetic changes in our genome. The GM can also mediate metastasis and reoccurrence through interactions with the immune system and can affect genome instability where host DNA damage has occurred. Furthermore, the GM composition has been recently found to influence chemotherapy, radiotherapy, and immunotherapy. Bacteria have also been found to reside in normal and malignant breast tissue. The conditions of the tumour microenvironment (TME) - low O₂, leaky vasculature and immune suppression - are supportive of tumour-selective bacterial growth, and this innate ability could enable their use as delivery agents for various therapeutics or as diagnostics.

Breast cancer is well studied in this context and this chapter uses Triple Negative Breast Cancer (TNBC) by way of an example indication. The chapter aims to provide an in-depth examination of the current knowledge on bacterial relevance to TNBC and potential uses while examining some of the remaining unanswered questions regarding mechanisms underpinning observed effects.

Chapter 2 describes methods for creating a bacterially produced synthetic protein platform for disease detection. Protease activity can serve as a highly specific biomarker for application in health, biotech and beyond. The aim of this study was to develop a protease cleavable synthetic protein platform to detect protease activity in a rapid cell-free setting. The protease sensor is modular, with orthogonal peptide tags at the N and C terminal ends, which can be uncoupled via a protease responsive module located in between. The sensor design allows for several different readouts of cleavage signal.

A protein ‘backbone’ (Green fluorescent protein (GFP)) was designed *in silico* to have both a C-terminal Flag-tag and N-Terminal 6x histidine tag (HIS) for antibody detection. A protease cleavage site, which can be adapted for any known protease cleavage sequence, enables the uncoupling of the peptide tags. Three different proteases - Tobacco Etch Virus (TEV), the main protease from coronavirus SARS-COV-2 (Mpro) and Matrix Metalloproteinase 9 (MMP9) - a cancer-selective human protease - were examined. A sandwich Enzyme-Linked Immunosorbent Assay (ELISA) was developed based on antibodies against the HIS and Flag tags. As an alternative readout, a C-terminal quencher peptide separable by protease cleavage from the GFP was also included. Purified proteins were deployed in cell-free cleavage assays with their respective protease. Western blots, fluorescence assays and immunoassay were performed on samples.

Following the design, build and validation of protein constructs, specific protease cleavage was initially demonstrated by Western blot. The novel ELISA proved to afford highly sensitive detection of protease activity in all cases. By way of alternative readout, activation of fluorescence signal upon protease cleavage was also demonstrated but did not match the sensitivity provided by the ELISA method.

This platform, comprising a protease-responsive synthetic protein device and accompanying readout, is suitable for future deployment in a rapid, low-cost, lateral flow setting. The modular protein device can readily accommodate any desired protease-response module (target protease cleavage site). This study validates the concept with three disparate proteases and applications – human infectious disease, cancer and agricultural crop infection.

Chapter 3 provides a proof of concept for the development of a bacterially produced urine directed synthetic biomarker. The convenience of urine specimen testing and its non-invasive nature make it an ideal patient sample for detecting biomarkers associated with disease states. Renal filtration of proteins is selectively based on their size and charge. In silico aided protein design facilitates the engineering of novel synthetic proteins with tailored characteristics, such as size, charge, stability, and detectability. In this study, we describe the design, build and test of a novel synthetic protein reporter with the ability, when delivered in vivo, to pass through the renal barrier and be detected in the urine.

The protein reporter was designed in silico with the murine major urinary protein (MUP) as the main body of the synthetic construct with additional C-terminal Flag and N-terminal 6x histidine tags incorporated for antibody detection. The protein was modelled to ensure that its characteristics were maintained despite modifications. A novel sandwich ELISA for the detection of these tags was developed. Multiple DNA constructs for in vitro protein production were built, and the resulting bacterially produced proteins tested in vitro and in vivo.

Following intravenous administration to mice, the reporter was found to be consistently detectable (pg-level) in urine, unlike the control protein, which lacked characteristics enabling it to pass the renal barrier. The novel ELISA was capable of picogram level detection of target proteins in urine.

This strategy serves as a proof of concept for a synthetic protein platform for urine-based diagnostics, which could be adapted for development into a lateral flow device.

Chapter 4 investigates the determinants of success or failure in microbiome sample acquisition across three hospitals, focusing on comparative analysis. A cancer patient's collection of bacteria (microbiome) has potential to act a biomarker in precision oncology. Clinical microbiome wide association studies (MWAS) play a crucial role in such development. However, challenges in study set-up, patient recruitment, and sample acquisition remain significant factors influencing the success of such studies.

This chapter aims to analyse factors influencing patient participation, set-up efficiency, patient management systems, and their impact on patient and sample acquisition in clinical microbiome studies. Through comparative analysis, this chapter seeks to provide insights for optimising logistics and enhancing reliability in future academic clinical microbiome research.

Three MWAS Clinical Observational Studies (COS) were established in the course of this thesis work. Data from these studies, relating to three separate hospital sites, one private and two public, were compiled and analysed. Determinant factors of success were found to be the number of team members involved in the in the set-up phase and the use of dedicated research nurses. Understanding and addressing factors influencing patient and sample acquisition are crucial for the success of clinical microbiome studies. Through comparative analysis, this chapter provides insights into optimising logistics and enhancing reliability in future research endeavours.

Chapter 5 This study aims to analyse faecal samples from cancer patients undergoing various treatments to identify biomarkers for better patient selection and treatment strategies, and to investigate the impact of therapy type on GM composition. Samples from two sites were analysed. At Site B, 11 participants with early-stage breast and gastrointestinal cancers provided stool samples before, after, and six months post-treatment. At Site C, 13 cancer patients receiving ICIs, with or without additional treatments, provided stool samples at three time points. Stool samples were frozen, processed in batches, and microbial DNA extracted using a standardised kit. The 16S rRNA gene was amplified and sequenced, and bioinformatic analysis evaluated alpha diversity, Shannon Diversity Index, and differential abundance across samples.

Preliminary sequencing of 39 patient samples revealed the relative abundance of the top 20 bacterial genera, with *Blautia* being the most prevalent genus. Notable changes in genus representation were observed across different cancer types and treatment stages. Alpha diversity, measured by the Shannon Diversity Index, varied with treatment. Differential abundance analysis indicated significant shifts in bacterial genera, highlighting the dynamic nature of the GM, consistent with previous GM association studies.

Although the low patient number at this early stage was not expected to yield statistically significant comparisons, the analyses performed in this pilot study validate the process and demonstrate the capability to achieve the long-term aim of elucidating potential correlations between patient GM characteristics and disease or treatment outcomes.

List of Abbreviations

A280 nm	Absorbance at 280 nanometers
AA	Amino Acid
ACV	Acyclovir
ANOVA	Analysis of variance
API	Active pharmaceutical ingredient
ASV	Amplicon sequence variant
ATMP	Advanced therapy medicinal product
AUC	Area under the curve
AuNPs	Gold Nanoparticles
BC	Breast Cancer
BCA	Bicinchoninic acid assay
BCG	Bacillus Calmette-Guerin
BDEPT	Bacteria-directed enzyme prodrug therapy
β -G	β -glucuronidase

bp	Base pair
BRET	Bioluminescence Resonance Energy Transfer
CAPOX	Capecitabine and Oxaliplatin
Cas	CRISPR-associated Protein
CD	Cytosine deaminase
cfDNA	Cell-free DNA
CFP	Cyan Fluorescent Protein
CFU	Colony-forming unit
CHMP	Committee for Medicinal Products for Human Use
CHR	Chromosome
Cm	Chloramphenicol
cm	Centimetre
COS	Clinical Observational Studies
CPP	Cyclophosphamide

CRC	Colorectal Cancer
CREC	Clinical Research Ethics Committee
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRO	Contract research organisation
CRT	Chemoradiotherapy
CTLA4	Cytotoxic T Lymphocyte Antigen 4
Da	Dalton
DNA	Deoxyribonucleic acid
DPIA	Data Protection Impact Assessment
EB	Elution buffer
EBI	European Bioinformatics Institute
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
Em	Erythromycin

EMA	European Medicines Agency
EV	Extracellular vesicle
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FMT	Faecal Microbiome Transplant
FPLC	Fast Protein Liquid Chromatography
FRET	Förster Resonance Energy Transfer
FTE	Full Time Equivalent
GBM	Glomerular Basement Membrane
GCV	Ganciclovir
GFB	Glomerular filtration barrier
GFP	Green Fluorescent Protein
GI	Gastrointestinal
GIT	Gastrointestinal tract

GM	Gut Microbiome
GO	Graphene Oxide
GRAVY	Grand average of hydropathicity
h	Hour
HIS	Histidine
HPRA	Health Products Regulatory Authority
HRB	Health Research Board
HRP	Horseradish peroxidase
HSV-1	Herpes simplex virus type 1
HTS	High-throughput sequencing
ICI	Immune Checkpoint Inhibitor
IDT	Integrated DNA Technologies
IPTG	Isopropyl β -D-1-thiogalactopyranoside
irAEs	Immune-Related Adverse Events

IV	Intravenous
kg	Kilogram
kV	Kilovolt
l	Litre
LARC	Locally Advanced Rectal Cancer
LB	Lysogeny Broth
LC	Lipocalin
LC	Liquid Crystal
LFA	Lateral Flow Assay
LPS	Lipopolysaccharide
M	Molar
m	Metre
MAB	Monoclonal Antibody
MDT	Multidisciplinary Team

mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
mm	Millimetre
MMP9	Matrix Metalloproteinase 9
MPR	Major Pathological Response
Mpro	Main Protease from Coronavirus SARS-CoV-2
MPS	Mononuclear phagocyte system
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MTA	Material Transfer Agreement
MUP	Major Urinary Protein
MUPt	Tagged major urinary protein

MWAS	Microbiome-Wide Association Studies
MWCO	Molecular Weight Cut-Off
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
nCRT	Neoadjuvant Chemoradiotherapy
NDA	New drug application
NEB	New England Biolabs
ng	Nanogram
NGS	Next-generation sequencing
nM	Nanomolar
NSLC	Non-Small Cell Lung Cancer
NTR	Nitroreductase
O.D	Optical Density
OC	Oesophageal Cancer

OD ₅₉₀	Optical density at 590 nanometres
OD ₆₀₀	Optical density at 600 nanometres
OI	Optical imaging
OTU	Operational Taxonomic Unit
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline with Tween 20
PBS-TS	PBS plus 0.1% tween 20 and 0.05% saponin from quillaja bark
PCA	Principal Component Analysis
pCCA	Perihilar Cholangiocarcinoma
PCR	Polymerase chain reaction
PD-1	Programmed Death-1
PD-L1	Programmed Death-Ligand 1
PET	Positron emission tomography

PFS	Progression Free Survival
PI	Principal Investigator
PIL	Patient Information Letter
PKS	polyketide synthase
PM	Patient Management
pmol	Picomole
PVDF	Polyvinylidene fluoride
QDs	Quantum Dots
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
R&D	Research and development
RBS	Ribosome Binding Site
RCOA	Renal clearable optical agents
RNA	Ribonucleic acid

Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal RNA
SCFA	Short-Chain Fatty Acid
SDAU	Statistics, Data & Analysis Unit
SDI	Shannon Diversity Index
SDS	Sodium dodecyl sulfate
Sec	Second
SERS	Surface-Enhanced Raman Scattering
SME	Small and medium-sized enterprise
SNP	Single nucleotide polymorphism
SPF	Specific-pathogen-free
SpR	Specialist Registrar
SPR	Surface Plasmon Resonance

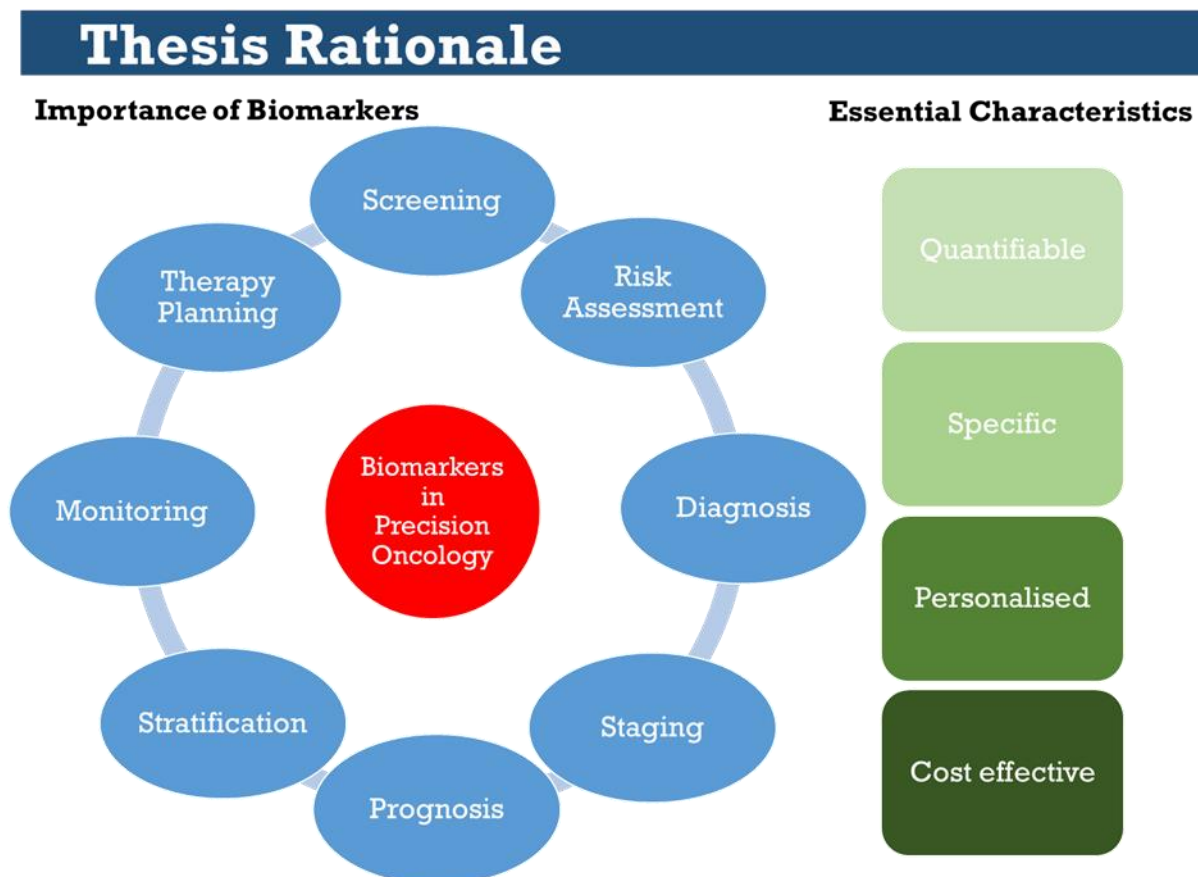
SSWHG	South and South-West Hospital Group
SWAT	Study Within A Trial
TBS	Tris-buffered saline
TEAE	Treatment-emergent adverse event
TEV	Tobacco Etch Virus
TK	Thymidine kinase
TKI	Tyrosine Kinase Inhibitors
TTO	Technology transfer office
UCC CTG:	University College Cork Cancer Trials Group
VEGF	Vascular endothelial growth factor
WGS	Whole-genome sequencing
µg	Microgram
µl	Microlitre
µM	Micromolar

°C	Degrees Celsius
16S rRNA	16S Ribosomal RNA
5-FC	5-fluorocytosine
5-FU	5-Fluorouracil

INTRODUCTION

Early detection and diagnosis of many diseases can lead to earlier medical interventions with more favourable outcomes for patients. Biomarkers play a crucial role in cancer research, particularly in the context of precision oncology. There is a raft of endogenous biomarkers from cell free nucleic acids, proteins, and lipids to a multitude of metabolites which can be collected from biofluids including blood, urine, and exhaled breath (Pinsky et al., 2017, Duffy et al., 2010, Zhao and Sanyal, 2022, Butler et al., 2020, Qiu et al., 2023, Fuchs et al., 2010). There exists a number of synthetic biomarker reporting systems, including biologically inert materials such as certain dyes or fluorophores to measure normal bodily function or aspects of a disease state (Cheng and Pu, 2021, Rizk et al., 2018).

This thesis focuses on exploiting bacteria or bacterial-produced proteins in biomarker contexts, and this chapter focusses in particular on cancer associated bacteria.



Biomarker Detection technology: Synthetic biomarker systems have an advantage by their very nature of having a low background signal. They can be activity based, for example a tumour specific protease activatable reporter whereby a synthetic peptide substrate is usually attached to an inert transporter molecule which upon cleavage by its specific protease will release reporters into the urine. Carriers include polyethylene glycol PEG, iron oxide nanoparticles or precious metals like gold and silver (Huang et al., 2022a, Loynachan et al., 2019, Kwong et al., 2013). Synthetic protein design and technology is a rapidly evolving field (Walker et al., 2021). As the technology progresses, opportunities increase for exploitation of designer proteins in detecting targets of interest (Flores Bueso and Tangney, 2017). More traditional proteins (antibodies) are already commonplace as part of rapid ‘in the field’ tests, such as lateral flow tests used for the detection of biomarkers including microbial antigens (e.g. Covid-19 antigen tests) (Candel et al., 2020) or hormones (pregnancy tests) (Leuvering et al., 1980).

Two examples of exploitation of bacterially produced synthetic proteins are studied in this thesis (Chapter 2, 3). The first is utilising a synthetic protein itself as a detection system of biomarker activity, specifically, protease activity. Proteases can act as highly specific indicators of the activity of multiple biological processes and can therefore serve as useful as biomarkers for a wide range of targets of value in health, food and other key sectors of global social and economic importance. Matrix Metalloproteinase 9 (MMP9) is an example of a cancer selective human protease. Currently, protease activity can be detected using laboratory instruments using a number of methods, reviewed elsewhere (Ong and Yang, 2017, Chung and Lin, 2020). Briefly, these include mass spectrometry based assays (Ritorto et al., 2014, Yan et al., 2011); colourimetry based chemistry (Lou et al., 2010, Guarise et al., 2006); fluorescent protein based techniques (Nicholls et al., 2011, Callahan et al., 2010, Heim et al., 1994, Zhang, 2004, Stawarski et al., 2014); bioluminescence based enzymes (Wigdal et al., 2008, Yao et al., 2007); quantum dots (QDs) (Medintz et al., 2006); nanoparticles (Laromaine et al., 2007, Loynachan et al., 2019, Jin et al., 2012, Song et al., 2013, Berdichevsky et al., 2003). More recent developments involve using liquid crystal (LC) based protease assays which can allow for naked eye detection of protease activity (Jannat and Yang, 2018). Transcription based assays have also been described and offer extremely high sensitivity (Fink and Jerala, 2022, Liu et al., 2021e). In terms of existing diagnostic kits, these can be divided into two broad categories: nucleic acid based molecular diagnosis and antigen/antibody based immunoassays. Nucleic acid quantification methods include quantitative reverse transcription

polymerase chain reaction (qRT PCR), loop mediated isothermal amplification (LAMP) based assays, clustered regularly interspaced short palindromic repeats (CRISPR) associated protein (Cas) system, and RNA sequencing, with qRT-PCR currently deemed the gold standard for COVID-19 detection.

Sample types: Protein based protease detectors could potentially be applied to any biological liquid sample, such as blood, saliva or urine for detection of protease activity to reveal cancer biomarkers. Urine serves as an ideal biological sample due to its non-invasive nature and ease of collection, allowing for unlimited repeated sampling, including in the at home setting. Chapter 3 describes development of a protein platform for deployment of biomarker detectors, involving producing a stable, detectable protein that safely transports itself to urine when made available systemically in the body. While less glamorous, stool also presents a relatively accessible, non-invasive sample, and of key value for bacterial detection.

Chapter 4 investigates the factors influencing the success of clinical microbiome wide association studies (MWAS) in understanding the relationship between gut microbial communities and cancer outcomes. Focusing on patient participation, study setup (SU) efficiency, and patient management (PM) across three hospitals, the study aims to provide insights for optimizing logistics and enhancing reliability in clinical microbiome research. Through comparative analysis of data from three MWAS clinical observational studies, the chapter identifies key determinants such as team hierarchy, patient recruitment and retention strategies, and the impact of clinical personnel involvement. Expectedly, the study finds that dedicated research nurses significantly enhance sample acquisition rates, and larger SU teams correlate with longer setup times. By addressing these factors, this chapter aims to improve future clinical microbiome studies, offering a systematic analysis of influences on patient and sample acquisition within an Irish context.

The final chapter focuses on analysing faecal samples from cancer patients undergoing various treatments to identify biomarkers for improved patient selection and treatment strategies, as well as investigating the impact of therapy type on gut microbiome (GM) composition. Samples from 11 participants with early-stage breast and gastrointestinal cancers at Site B and 13 cancer patients receiving immune checkpoint inhibitors at Site C were collected at multiple time points, frozen, processed, and analysed for microbial DNA. Significant changes in genus representation were

observed between patient sampling timepoints across different cancer types and treatment stages. Despite the low patient number, the analyses validate the process and demonstrate the potential to elucidate correlations between patient GM factors and disease or treatment outcomes.

1 CHAPTER 1:

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1.1 THE HUMAN MICROBIOME & CANCER

Humans are a symbiont of human and microbial cells, with a ratio of 1.3-2.5 bacterial: human cells (Sender et al., 2016). Although the majority of these microbes reside within our gut, distinctive collections of microbes are also found in most body parts (possibly including the brain). These distinctive microbial signatures, are known as *the microbiome* (Whipps et al., 1988, Ursell et al., 2012). The term microbiome, as Whipps and co-workers originally postulated, includes not only the community of the microorganisms but also their “theatre of activity”. These ecosystems, created by a multitude of microbes that may include bacteria, archaea, fungi, yeast, viruses, are site specific. The microbiome of each body part has distinctive characteristics regarding population dynamics and diversity of microbial species and these site specific diversity and dynamics can be regarded as a health indicator. For example, high diversity in the gut microbiome is generally linked to good health. Our microbiomes represent a virtual organ that perform essential body functions that maintain our homeostasis, such as metabolising nutrients, maintaining the integrity of the mucosal barriers, developing a healthy immune system, modulating a healthy neuronal development (including regulating our moods) and defending us against pathogens (Jandhyala et al., 2015, Appleton, 2018).

1.2 DISTAL (GUT) MICROBIOME AND CANCER

1.2.1 GM Composition & Dysbiosis

Microbes start colonising our body as early as in the 2nd trimester of foetal development, where low levels of microbial signals can be detected in the foetal gut, skin, placenta, and lung tissue (Mishra et al., 2021). However, the first major colonisation event in early life happens at birth, where the mode of delivery determines the neonate microbiome composition to resemble either a vaginal or skin microbiome. After this, our microbiomes are shaped by external factors such as diet, lifestyle, and environmental biodiversity and as such, our microbiome composition varies with age. In early neonatal life, breastfeeding enables the vertical transmission (mother to infant) of bacteria. Thus, neonates exhibit a microbiome composition resembling their mother’s milk. The adult type of GM composition starts appearing at ages 3-5 years (Rodríguez et al., 2015). At this age, 90% of all species of the adult GM would have already colonised the gut.

Overall, a diverse GM is a healthy and robust GM, well able to perform the multiple tasks that define our health status (see Figure 1). On average, the adult GM is composed of approximately 300-500 species, comprised in 12 bacterial phyla and one Archaeal taxon, with the majority of species belonging to the Bacteroidetes and Firmicutes phyla and a smaller proportion to the Proteo and Actinobacteria phyla. However, GM composition differs between individuals and starting in mid-to-late adulthood (40-50 years age) it increasingly diverges towards a microbiome that is unique to each individual (Thursby and Juge, 2017, Wilmanski et al., 2021). Compositional uniqueness is more accentuated among the elderly (>65 years old), since at this life stage, microbiome diversity can decrease significantly. In this age group, uniqueness has been positively associated with a healthy status (Claesson et al., 2012, Wilmanski et al., 2021).

A healthy microbiome safeguards host microbiome homeostasis. Here, different microbe populations regulate the abundance of neighbouring commensal or pathogenic bacteria by occupying a niche and adjusting the niche environment. Bacteria compete for nutrients, release bacteriocins (peptides which are toxic to and inhibit/regulate the growth of similar or closely related bacterial strains) or bacterial signals (MAMPs, see 1.2) to communicate with the host in order to modulate the release of antimicrobial peptides, mucin and ultimately, immune responses (Schnupf et al., 2018). Together, these actions contribute to the formation and maintenance of a healthy GIT mucosal “firewall”, which by segregating the GM from host cells, prevents microbial translocation and adverse immune priming events (Ge et al., 2021, Mezouar et al., 2018). Overall, a healthy GM safeguards host microbiome homeostasis by modulating immune tolerance against gut commensals and eliciting pertinent immune responses (Belkaid and Hand, 2014). These GM immune cell interactions are essential for the proper development of the gut associated lymphoid tissues (GALT), which is the largest mass of lymphoid tissue in the body (more in 1.2) (Claud and Walker, 2008).

The loss of beneficial microbes, expansion of pathobionts (commensals that at higher densities can cause harm), and/or the overall loss of microbial diversity can alter the GM composition in a way in which the abovementioned self regulation and host microbiome homeostasis functions are impaired. This altered and impaired GM composition is known as dysbiosis (Petersen and Round, 2014). GM Dysbiosis has been found to induce tumorigenesis, mainly through its interactions with

the immune system, where it has been found to prime and sustain systemic chronic inflammation and/or immunosuppression (see Figure 1 and section 1.3) (Ge et al., 2021).

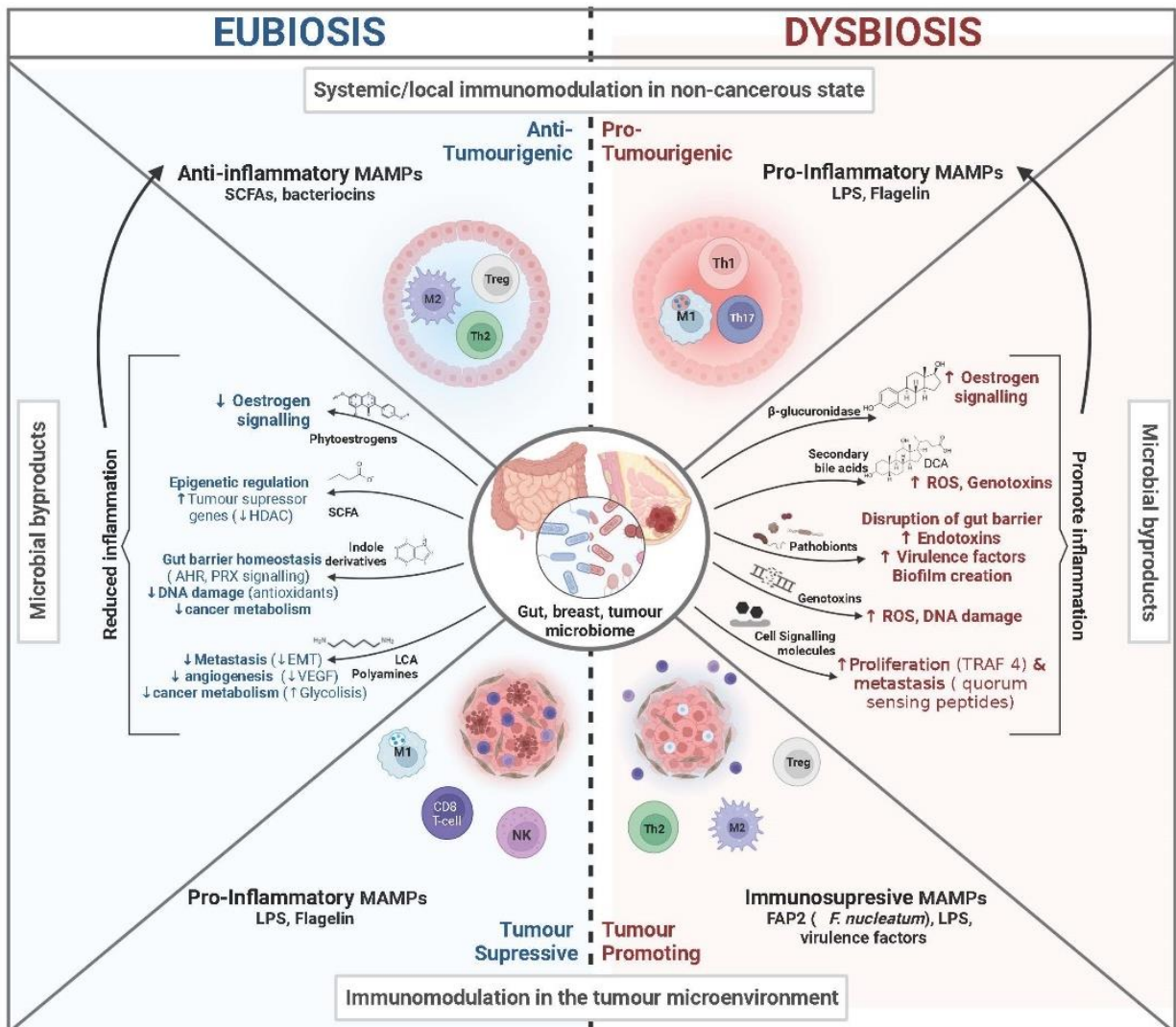


Figure 1. The microbiome influence in Cancer. In Eubiotic states the microbiome promotes our health status and prevents oncogenesis and tumour progression by influencing the immune system, promoting gut barrier integrity and influencing cell signalling. Conversely, during dysbiosis there is a loss of barrier integrity that can lead to potentially harmful bacterial translocation, chronic inflammation in distal sites and the production of cancer promoting bacterial by products.

1.2.2 The GM and Immunity

The microbiome promotes the development and maintenance of the GIT mucosa and associated lymphoid tissues by producing microbial motifs (antigens) known as microbe associated molecular patterns (MAMPs) (e.g., lipopolysaccharide (LPS), short chain fatty acids (SFCA), and peptidoglycans) (Toor et al., 2019, Kumar et al., 2011, Janeway, 1989). MAMPs are recognised by pattern recognition receptors (PRRs) in Antigen Presenting Cells (APCs)). The GM uses MAMPs to communicate, trains and supports the maturation of the innate immune system, in order to: **(i)** modulate tolerance by discerning self from non self (Medzhitov and Janeway, 1997); **(ii)** ensure homeostatic levels of innate immune cells (e.g. macrophages and dendritic cells) (Gorjifard and Goldszmid, 2016); **(iii)** bridge the innate and adaptative immune systems, through the production of co stimulatory signals that induce an adaptive immune system response (Li and Wu, 2021, Amarante-Mendes et al., 2018). See Figure 2.

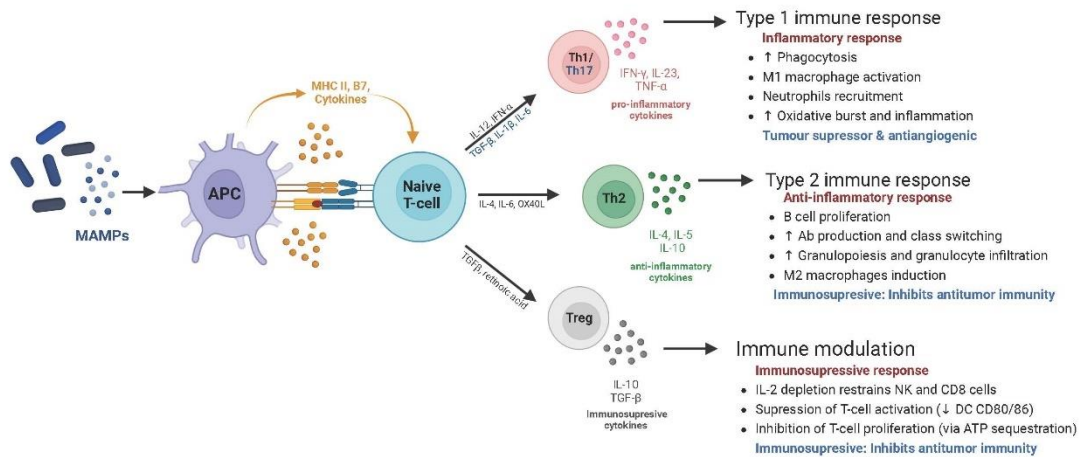


Figure 2. The microbiome influence on the host immune system. The microbiome modulates the immune system through MAMP production. These are sensed by antigen presenting cells (APCs),

which process them to be presented to CD4⁺ naïve T-cells to induce either immune tolerance or an immune response.

1.2.3 GM influence in cancer via Chronic Inflammation

Chronic inflammation contributes to tumorigenesis at all stages of oncogenesis, progression and dissemination, by promoting genomic instability and epigenetic modifications, inducing proliferation, strengthening anti-apoptotic pathways, and stimulating angiogenesis and metastasis (Mantovani, 2005, Gonzalez et al., 2018). Approximately 90% of all cancers are a product of somatic mutations caused by environmental or life style factors (e.g. diet and infection) that lead to some form of chronic inflammation (Singh et al., 2019). Among these, up to 20% of all cancers are preceded by chronic inflammation at the cancer site (Francescone et al., 2014).

Inflammation is a mechanism mainly orchestrated by the innate immune system to defend the body against pathogens and injuries, which can be perpetuated by exogenous stimuli (e.g. MAMPs) (Serhan et al., 2010, Abudukelimu et al., 2018). The prolonged/recurrent exposure to stimuli can lead to an uncontrolled infiltration and accumulation of immune cells, which can become polarised towards pro-inflammatory, tumour promoting cell types (Type 1/17, 2 in Figure 2). It can also lead to the release/production of damage associated molecular patterns (DAMPs), which signal a status of altered self and amplify and perpetuate immune reactions through a vicious cycle of inflammation and DAMP production (Roh and Sohn, 2018, Bianchi, 2007),

The most relevant mechanism for bacterial driven chronic inflammation is gut barrier failure (Schwabe and Jobin, 2013, Mezouar et al., 2018, Thoo et al., 2019). This prevents an inflammatory response in the intestinal mucosa and its translocation into compartments where they can elicit a systemic immune response and establish systemic immune memory against it (Macpherson and Uhr, 2004). Their anatomical separation is achieved through a multi-level gut barrier, which ensures **(i)** segregation: preventing direct contact between the GM and host tissue; and **(ii)** compartmentalisation – ensuring that responses to commensal bacteria are kept locally (Thoo et al., 2019). Disruption of segregation/compartmentalisation allows microbes or their by products to be distributed systemically to other body sites, where they can incite local and systemic

inflammatory responses. The recurrent injury to the tissue caused by these responses can consequentially prime the immune system (via DAMPs) towards chronic inflammation (Murch, 2021). In fact, a commonality across GM associated diseases is that they are a product of barrier dysfunction (Francescone et al., 2014, Stolfi et al., 2022). This has been correlated to localised digestive tract diseases, such as colon (Genua et al., 2021), liver (Yu and Schwabe, 2017) and pancreatic cancer (Akshintala et al., 2019), among others and systemic inflammatory diseases, such as metabolic (Tilg et al., 2020) and autoimmune diseases (Kinashi and Hase, 2021), and cancer in other body sites (Ge et al., 2021), including the breast (Ruo et al., 2021).

1.2.4 GM influence in cancer via GM byproducts

The GM produces a large array of small molecules during the metabolism of food and xenobiotics (compounds of nonhost origin that enter the gut with the diet, lifestyle or are produced by microbes). These can be in the form of low molecular weight metabolites, peptides, and proteins. In fact, the GM is associated with many biochemical pathways and in the synthesis of specific metabolites that are absorbed into the circulation. In this way, the GM contributes to the host biology a circulating pool of bacterially derived metabolites that can potentially exceed concentrations typically achieved by drugs (10 μ M–1 mM) (Dodd et al., 2017). Many of these byproducts play critical roles in interbacterial (between different microbial species) and host GM signalling by engaging with specific host receptors (Nicholson et al., 2012). As such, GM derived metabolites can signal distant organs in the body and facilitate a connection between the host immune and hormone system, brain (the gut brain axis), and metabolism. The beneficial or detrimental effects of these GM derived metabolites will depend on the context and the state of the host, considering that the primordial nature of the symbiotic microbiota is to ensure its host health (Agus et al., 2021). Accordingly, below are outlined some of the pathways, metabolites and/or byproducts by which the GM can exert an effect in cancer, with a focus on Triple Negative Breast Cancers (TNBC).

TNBC characteristically lack, or express at very low levels, human growth factor receptor 2 (HER2), progesterone (PR) and/or oestrogen receptors (ER) (National Cancer Institute (NCI), November 2018). In 2020, 12% -17% of the 2.3 million new breast cancer (BC) cases and over 685,000 deaths worldwide can be attributed to TNBC (Lei et al., 2021, Foulkes et al., 2010). TNBC disproportionately affects young premenopausal women with west African ancestry, particularly

African American and Ghanaian women. Other relevant risk factors include BRCA-1/2 mutations, smoking history, and obesity (Kohler et al., 2015, Nwagu et al., 2021). Generally, TNBC originates in the milk duct as ductal carcinoma and less frequently in mammary lobules as lobule carcinoma. Based on its genotype profiling and cellular origin, TNBC can be classified into four subtypes: basal subtypes 1/2, mesenchymal subtype and an androgen receptor expressing luminal subtype (Lehmann et al., 2016). TNBC is highly invasive and has no standard treatment care options for the metastatic disease stage. Therapeutic schemes for this disease are constrained to conventional cytotoxic chemotherapy as endocrine or receptor based therapies (e.g. HER2, ER and PR) are completely ineffective for TNBC (Almansour, 2022). Surgery (lumpectomy or mastectomy) followed by radiation are available for early stage patients and immune checkpoint inhibitor therapies are offered on a case by case basis (Li et al., 2019a). For TNBC patients with BRCA 1/2 mutations, the *Poly (ADP ribose) polymerase* PARP inhibitor therapy can be an option, as they respond positively to this therapeutic approach (Liu et al., 2021d).

1.2.4.1 Oestrogen regulation via β -glucuronidase

The risk for developing receptor positive breast cancers in post-menopausal women is highly associated with the levels of circulating oestrogens and the time of exposure (Russo and Russo, 2006, Fuhrman et al., 2014). Whilst oestrogen metabolism is not traditionally considered an essential factor in TNBC, it may be to a degree, as some TNBCs express alternative oestrogen receptors. In addition, different jurisdictions have different cut offs for the expression of either oestrogen receptor (ER) and/or progesterone receptor (PR). For example, in the US, TNBC is diagnosed when receptor expression is lower than 1%. On the other hand, in the European Union, TNBC is diagnosed when the expression levels are lower than 10%. Even though TNBC lacks canonical ER- α , oestrogen signalling in TNBC can also be maintained by constitutively active oestrogen related receptors (ERRs) (Treeck et al., 2020). Expression of alternative oestrogen receptors ER β and the G protein coupled oestrogen receptor 1 (GPER-1) enables some degree of oestrogen reactivity in TNBC, with contradictory results regarding the function of GPER as to whether it is proliferative or pro-apoptotic (Hsu et al., 2019). Hence, even in TNBC, oestrogen can play a role in cancer progression, depending on levels of circulating oestrogen, in which bacteria play a role and the cancer's ability to respond via canonical or non-canonical receptors. In order

to excrete oestrogen from the body, the liver conjugates oestrogen to glucuronic acid which can be then excreted in the bile. The GM has enhanced capacity to increase systemic oestrogen levels by increasing enterohepatic circulation (Pellock and Redinbo, 2017). GM bacteria can increase levels of systemic oestrogen in two ways – first, by blocking the binding of oestrogen to glucuronic acid, reducing its inactivation (Shen et al., 2012). Secondly, oestrogens that are marked for excretion through the bile can be deconjugated by bacterial species expressing β -glucuronidases enabling their reuptake (McIntosh et al., 2012, Gloux et al., 2011). GM bacteria can also metabolise phytoestrogens from dietary polyphenols, which are thought to modulate oestrogen metabolism by inhibiting their production (blocking aromatase) of and completely binding to ERs reducing their bioavailability (Parida and Sharma, 2019).

1.2.4.2 Short chain Fatty Acids (SCFAs)

SCFAs are one of the main metabolites generated by the GM in the large intestine through anaerobic fermentation of indigestible dietary fibre and resistant starch (den Besten et al., 2013). Among these, butyrate is the most important in relation to cancer, which is produced by Firmicutes (Levy et al., 2016). Cancer driven histone deacetylase (HDAC) activity can lead to dysregulated epigenetic changes that silence tumour suppressor genes (TSG) facilitating malignant proliferation (Glozak and Seto, 2007). Butyrate shows the most potent anti-cancer properties, including anti-inflammatory effects, suppression of angiogenesis, histone deacetylase (HDAC) inhibition which can reverse silencing of tumour suppressor genes (TSGs) and apoptosis induction in tumour cells by means of mitochondrial ROS production (Liberti and Locasale, 2016, Salimi et al., 2017, Nakkarach et al., 2021, Wu et al., 2018b). A high fibre diet promotes the maintenance of butyrate producing bacteria, making it cancer protective. Conversely, the opposite effect is true; depletion of butyrate producing bacteria may promote inflammation and tumorigenesis systemically (Rea et al., 2018). Nanopore has been used in the past to detect butyrate producers their detection and abundance should be included in any biomarker score card. *Anaerostipes* of *Bacillota* phylum and *Lachnospiraceae* family are both prominent butyrate producers and have been detected using Nanopore sequencing (Low et al., 2023) using whole genome, additionally using sequence capture by hybridization (Marre et al., 2021) and a gene targeted approach (Vital et al., 2013).

1.2.4.3 Bile acids metabolites

Lithocholic acid (LCA) is a bile acid metabolite that has been found to exert cancer protective effects. It is produced exclusively in the gut by a few species of anaerobic bacteria in the genus *Clostridium* from primary bile acids (Ridlon and Bajaj, 2015). Any bile acids found in breast tissue originate from the gut (Javitt et al., 1994). LCA anticancer properties in relation to BC in general, include reductions in cancer cell proliferation and epithelial to mesenchymal cell transition inhibition acting via the G protein coupled bile acid receptor 1 (TGR5) that exerts downstream anti-inflammatory effects (Mikó et al., 2018). It also inhibits angiogenesis by inhibiting vascular endothelial growth factor (VEGF) (Sampsell et al., 2020). LCA alters cellular metabolism by inducing glycolysis and increasing mitochondrial oxidative phosphorylation in BC cells that depend on the Warburg effect (Mikó et al., 2018). The Warburg effect is a cancer specific effect, with increased glucose uptake and fermentation to lactate even in the presence of oxygen, proposed to be an evolutionary mechanism to sustain proliferative growth through the generation of essential biomolecules, byproducts of glycolysis (Liberti and Locasale, 2016b). BC patients have been found to have low levels of the LCA producing gene $7\alpha/\beta$ -hydroxysteroid dehydroxylase (*baiH*) detected in DNA extracted from stool samples in early cancer cases when compared with healthy controls (Long et al., 2017, Mikó et al., 2018). In addition, microbiota derived bile acids of GM origin accumulate in breast tumours and correlate with reduced proliferation (Wu et al., 2022b). Nanopore sequencing has been used to identify LCA producers oxidation by *Eggerthella lenta* strains C592 and DSM 2243 whole genome is required to get to strain level (Harris et al., 2018).

1.2.4.4 Polyamines

Polyamines are small polycationic molecules with a wide array of biological functions including gene regulation, stress resistance, cell proliferation and differentiation. These are mainly sourced from the GM, where they derive from bacterial amino acid metabolism (Tofalo et al., 2019). Among these, Cadaverine is a biogenic amine derived from the decarboxylation of lysine and is used by bacteria to buffer the pH of their environment. It is synthesised by lysine decarboxylase (LdcC) and cadaverine A (CadA), enzymes found in species of the bacterial genera *Enterococcus*,

Enterobacter, *Escherichia*, *Proteus*, *Streptococci*, and *Shigella* among other (Miller-Fleming et al., 2015). Cadaverine has been shown to have tumour suppressor roles in breast cancer. Its antitumour effects has been proven in a TNBC murine model (grafted 4T1 tumour cells), with cadaverine found to reduce Epithelial to mesenchymal transition (EMT), an essential process driving tumour progression and metastasis, in which epithelial cells lose their features (cell polarity and cell–cell adhesion) and gain the invasive properties characteristic of mesenchymal stem cells. Consequently, cadaverine inhibited tumour growth, reduced cellular migration and invasion, ultimately reducing metastasis. In addition, cadaverine was also found capable of reducing BC invasion by inducing mesenchymal to epithelial (MET) transition (reverting mesenchymal tumour cells to a more epithelial state cell) via the activation of trace amino acid (TAAR) cell receptors (Kovács et al., 2019). This is supported by results from clinical trials (Vattai et al., 2017). Nanopore has been used but for targeted identification of LdcC genes (Bielecka et al., 2022).

1.2.4.5 Indole derivatives

Indoles are bioactive products of the GM bacterial catabolism of tryptophan. Among these, 3-Indolepropionic acid (IPA), has been the subject of numerous studies due to its anticarcinogenic effects. IPA and other propionic acid species (PAs) derived from phenylalanine and tyrosine are synthesised through aromatic amino acid transferases and phenyllactate dehydratase found in some species of the bacterial genera *Lactobacillus*, *Akkermansia*, *Clostridium* and *Peptostreptococci* (Konopelski and Mogilnicka, 2022, Dodd et al., 2017). In general, Indoles are also key interbacterial and GM Host signalling molecules, act through steroid and xenobiotic receptors (AHRs and PXR) (Rothhammer and Quintana, 2019). Indole derivatives prevent carcinogenesis by promoting gut homeostasis (upregulating tight junction, cell turnover, mucin and AMP secretion) (Agus et al., 2018) and modulate immune tolerance by shifting immune cell polarisation towards anti-inflammatory types. IPA is a free radical scavenger and a potent antioxidant, which prevents DNA damage in non-transformed cells exposed to multiple types of oxidative damage (Karbownik et al., 2005, Konopelski and Mogilnicka, 2022). Furthermore, IPA and indoxyl sulphate (IS) exert cytostatic effects in breast cancer cells (including TNBC) *in vitro* and *in vivo*, where they reduce cancer cell stemness, their EMT and proliferation (Kovács et al., 2021). They enhance oxidative/nitrosative stress and downregulate the expression of stress response factors. In

addition, these upregulate antitumour immune responses, increase the number of tumour infiltrating lymphocytes and modulate the local microbiome composition in breast cancer models. Full 16s Nanopore sequencing has been used previously to sufficiently discern indole producers in the gut (Long et al., 2024).

1.3 THE PROXIMAL MICROBIOME AND CONTRIBUTION TO TUMOUR DEVELOPMENT

Once thought to be sterile, it is now well established that the breast has its own unique microbial signature (Urbaniak et al., 2014, Wang et al., 2017, Xuan et al., 2014, Urbaniak et al., 2016, Hogan et al., 2021). Breast and milk microbiomes are related, which makes the milk microbiome a good predictor for Breast Microbiome (BM) composition in lactating women (Eslami-S et al., 2020). Milk has been found to accommodate more than 360 species of bacteria, mainly from the *Actinobacteria*, *Bacteroidetes* and *Firmicutes* phyla (Hunt et al., 2011). Due to various exogenous factors (dietary habits, geographic locations, lactating phase and research methodologies) different studies report different microbial richness and composition at genus/species level. Despite this, *Staphylococcus* and *Streptococcus* have been present in 98.7 and 97.7% of the samples analysed, respectively, and are considered as core genera, followed by lactic acid specific bacteria (*Bifidobacterium sp.* and *Lactobacillus sp.*) (Lackey et al., 2019).

Microbes residing in breast tissue have variable origins, as these can be sourced from different body parts, through different interactions. In this regard, aerobes and facultative anaerobes from the skin and other epithelial surfaces may gain access to the breast through the nipple areolar opening. For example, bacteria from the oral mucosa may gain access during breastfeeding and/or sexual contact (OConnor et al., 2018). The growth and persistence of the infiltrated bacteria is sustained by the favourable conditions of breast tissue, featuring nutrient rich fatty tissue content, and diffuse ducts originating from the nipple and the widespread vasculature and lymphatic systems facilitating their movement (Urbaniak et al., 2014).

Conversely, the microbial translocation of strictly anaerobic bacteria, which cannot survive in the presence of oxygen (e.g., *Bacteroides*, and some *Lactobacillus* and *Bifidobacterium* species) into

breast tissue is far more complex and has been a topic of research and debate for decades. It has been suggested that some bacterial taxa may be translocated from the gut to the mammary tissue via an enteromammary pathway (Perez et al., 2007). This is supported by evidence from different studies showing that dendritic cells (DCs) can sample and engulf bacteria directly from the lumen (maintaining epithelial barrier integrity) (Rescigno et al., 2001) and transport them alive (for up to 60 h) to other lymphoid tissues (Macpherson and Uhr, 2004). This was also supported by a seminal study of the origin of human breast milk bacteria. This study revealed that during the perinatal period there is a heightened bacterial translocation to the GALT, which is followed by bacterial colonization of the breast during the immediate postpartum. Here, it was also suggested that bacterial transport was mediated by DCs, which was supported by evidence showing that: (i) the majority of mononuclear cells in the milk originated from the GALT, (ii) staining showing bacteria DCs co-localisation and (iii) culturing of viable bacteria extracted from DCs purified from maternal milk and blood. Other preclinical studies have suggested GIT translocation of certain bacteria, such as bifidobacteria, to distal tumours (Cronin et al., 2010).

Tumour colonisation by bacteria is facilitated by the increased permeability of the tumour microenvironment (TME), with leaky vasculature due to rapid angiogenesis. Tumour selective growth of specific facultative and/or anaerobic bacterial strains is supported by the highly hypoxic and nutrient rich necrotic tumour regions and its characteristic suppressed immune surveillance (Sznol et al., 2000, Forbes, 2010, Morrissey et al., 2010, Cummins and Tangney, 2013b). Several recent studies have raised convincing evidence that associates the breast microbiome with cancer; however, its role in tumorigenesis is still a subject of active investigation, as it is still unknown whether different microbial signatures are a cause or a product of tumorigenesis driven tissue remodelling (Cummins and Tangney, 2013b, Cummins and Tangney, 2013a). Nevertheless, the role of intratumoural bacteria (including in TNBC) in tumour progression is now well acknowledged and considered an enabling factor of “the hallmarks of cancer” due to their capacity to contribute to genome instability and mutation, and tumour promoting inflammation (Hanahan, 2022).

1.3.1 Unique TME signatures

In recent years various investigations have found characteristic microbial signatures associated with the breast cancer tumour microbiota (TM). In general these studies point out that the microbial

composition of breast tissue is dominated by bacteria from Proteobacteria, followed by Firmicutes, Actinobacteria and Bacteroidetes phyla, but BC tumour tissue features an altered composition in terms of abundance. In this regard, BC tumour tissue has an overrepresentation of Proteobacteria (a phylum usually associated with inflammation and disease), with higher abundances of Gammaproteobacteria, specially from the *Enterobacteriaceae* family and reduced abundance of Actinobacteria and Bacteroidetes. BC TM also has a higher abundance of pathobionts (pro-inflammatory) in the Firmicutes (e.g. *Staphylococcus* (Xie et al., 2010), *Streptococcus pyogenes* (Stevens and Bryant, 2016)) and Actinobacteria (*Micrococcus* (Lu et al., 2021), *Atopobium* (Caselli et al., 2019)) phyla. Furthermore, the BC tumour microbiota showed an increase in abundance of taxa with known carcinogenesis effects, such as those belonging to the *Fusobacterium* genus (Xuan et al., 2014, Hieken et al., 2016, Urbaniak et al., 2016, Thompson et al., 2017). In fact, taxa from the *Enterobacteriaceae*, *Streptococcaceae*, *Staphylococcaceae* and *Micrococcaceae* families have been found in pancreatic cancer tumours (Pushalkar et al., 2018) with *Staphylococcaceae* species in particular, also present in higher abundance within the tumour microbiota of prostate cancer (Cavarretta et al., 2017). Likewise, in an ovarian cancer microbiota study, the Gammaproteobacteria *Shewanella sp.* was detected in 91% of cancer tissue samples (Banerjee et al., 2017). On the contrary, bacterial taxa with known anti-carcinogenic effects, such as species from the Bacteroidetes phyla and *Lactococcus*, and *Streptococcus* genera, were less abundant in BC TM (Tzeng et al., 2021).

For TNBC in particular, the studies described in Table 1, list the microbial signatures found to be enriched in the TNBC TME for each study. The lack of congruity between studies, reflects the lack of defined protocols for the study of breast/tumour microbiomes, wherein, a myriad of variables that can influence the outcomes/results. For example, differences in sample source (fresh frozen/FFPE, ethnicity/geography), experimental protocols (collection: surgery/biopsy, DNA extraction, hybridization/sequencing), and bioinformatic workflows (Tzeng et al., 2021). Nevertheless, these studies offer insights to relevant features that are particular to the TNBC tumour microbiota. First, the TM of TNBC has the least taxonomic diversity among all BC types (Banerjee et al., 2021). Second, TNBC has higher abundance of *Aggregatibacter* and *Caulobacter* (Banerjee et al., 2015, Banerjee et al., 2018) which are biofilm forming bacterial strains that have been previously associated to localised periodontitis and endocarditis (Fine et al., 2006, Goldberg

and Katz, 2006). Furthermore, these studies found that the microbial profiles found in tumour adjacent non-cancerous (matched) tissue had a higher abundance of pathobionts and were more similar to the TM than those found in non matched healthy tissue controls. This indicates that the TME resident tumour microbiota can extend to surrounding tissues and/or that microbial profiles found in the tumour pre-existed tumour formation, which would suggest that these microbes have an active role in tumorigenesis (Banerjee et al., 2021). This can be supported by recent evidence from a study involving seven different cancer types and over 1500 FFPE tumour samples, where intratumoral bacteria were found to localise within the tumour and immune cells (Nejman et al., 2020).

All of these studies are merely taking a snapshot of the bacteria present at the time of sampling. The bacterial load is of extremely low biomass relative to the TME or the GM for that matter and issues of contamination and sampling techniques are important confounding factors. While efforts from our lab and others on focussed improvement in relevant methodology will support further studies (Walker et al., 2020b, Walker et al., 2020a, Flores Bueso et al., 2020a, Flores Bueso et al., 2020b), bacteria at the scene cannot be pinned down to having a causative effect in oncogenesis or are merely bystanders having found a niche where they can survive (Cummins and Tangney, 2013a).

Table 1. Description of studies of the TNBC microbiota. The TM of TNBC has the least taxonomic diversity among all BC types.

Authors	Banerjee et al. 2015	Banerjee et al. 2021	Tzeng et al. 2021
Year	2015	2021	2021
Patient stratification	Breast cancer receptor type	Breast cancer receptor type, Tumour grade and stage, primary site of the tumour, response to treatment, survival	Breast cancer stage, grade, subtype, receptor type, and lymph node status.

		and disease free time post treatment.	
No of TNBC samples	100	100	30
Non-cancer breast tissue	17 matched + 20 non- matched	20 matched + 68 non- matched	87 non-matched + 175 matched
Sample type	FFPE breast tumours + matched/non-matched controls	FFPE breast tumours + matched/non- matched controls	Freshly frozen breast tissue
Taxa enriched in TM vs matched controls	<i>Actinomycetaceae</i> , <i>Caulobacteriaceae</i> , <i>Sphingobacteriaceae</i> , <i>Enterobacteriaceae</i> , <i>Prevotellaceae</i> , <i>Brucellaceae</i> , <i>Bacillaceae</i> , <i>Peptostreptococcaceae</i> , <i>Flavobacteriaceae</i>	<i>Actinomyces</i> , <i>Bartonella</i> , <i>Brevundimonas</i> , <i>Coxiella</i> , <i>Mobiluncus</i> , <i>Mycobacterium</i> , <i>Rickettsia</i> , <i>Sphingomonas</i>	<i>Azomonas</i> , <i>Alkanindiges</i> , <i>Caulobacter</i> , <i>Proteus</i> , <i>Brevibacillus</i> , <i>Kocuria</i> , <i>Parasediminibacterium</i>
Reference	(Banerjee et al., 2015)	(Banerjee et al., 2021, Banerjee et al., 2018)	(Tzeng et al., 2021)

1.3.2 Protective effects

In a eubiotic state, the breast microbiota produces metabolites that may confer protection from pathogens, boost immune responses and inhibit tumourigenesis. Across different TNBC specific studies, a higher abundance of BM commensals (e.g. *Streptococcus* sp.) that synthesise cadaverine, a known BC tumour suppressor, has been found to correlate with healthy breast tissue (Kovács et al., 2019). A different study highlighted that butyrate producing strains (e.g. *Odoribacter* sp.), recognised for their anti-inflammatory effects, were absent in tumour tissues (Levy et al., 2016,

Tzeng et al., 2021). Additionally, several breast tissue resident commensals, such as *Staphylococci* sp., *Lactococcus* sp., and *Streptococcus* sp. produce lantibiotics and other bacteriocins, preventing the potential growth of pathogenic strains that could trigger chronic inflammation. Beyond this, some bacteriocins have also been found to have selective cytotoxicity toward cancer cells (Kaur and Kaur, 2015). For example, *Lactobacillus salivarius* and *L. gasseri*, have been shown to clear mastitis infections during lactation when administered as a probiotic (Jiménez et al., 2008) and their excreted products (as cell free supernatant) has been found to inhibit BC cells *in vitro* (Srikham et al., 2021). Similarly, Bovicin HC5 from *Streptococcus bovis* HC5 has been shown to induce cell death in BC cell lines *in vitro* (Yaghoubi et al., 2019).

Furthermore, breast tissue resident bacteria also have the capability to activate the immune system via MAMPs. For example, the MAMP Flagellin (and the principal component of bacterial flagella) activates Toll receptor 5 (TLR5). This is a PRR recently found to be specifically expressed in the ductal epithelium of normal breast tissues and circulating immature dendritic cells induces the secretion of pro-inflammatory cytokines and chemokines, increases tumour necrosis and neutrophil infiltration, inhibiting cell proliferation and anchorage independent growth in mouse xenografts of human BC cells (Cai et al., 2011a). In this context, *Lactococcus lactis* has been shown to recruit and activate natural killer cells (the main innate immunity cytotoxic effector cells toward cancer cells) (Urbaniak et al., 2016). This has been confirmed in a clinical trial where *Lactococcus* abundance positively correlated with the number of NK cells recruited (Li et al., 2022a). Finally, healthy breast tissue is known to harbour bacterial strains, such as species of *Lactococcus* and *Streptococcus*, genera known to produce antioxidants and ROS scavengers (e.g. indoles, IPA) that neutralise free radicals and reduce oxidative damage, preventing oncogenesis (Koller et al., 2008, Urbaniak et al., 2014). Supporting all of the protective benefits to breast health are extensive clinical studies and meta-analyses correlating the use of antibiotics with a moderately increased risk of BC (Friedman et al., 2006, Velicer et al., 2004, Simin et al., 2020).

1.3.3 Microbial contributions to oncogenesis and tumour growth

Some bacterial signatures can induce cellular and immunomodulatory changes that promote oncogenesis, tumour growth and metastasis. Here these are referred to as Pathobionts. Now, while some of these microbes may not be a pathogen in other body parts they behave as such in this

specific context, as the capacity of any microbe to act as a pathogen is context dependent, on location and state of immune activation. Some of these effects are listed in Table 2.

Table 2. Microbial contributions to oncogenesis and tumour growth

1.3.3.1	Effects	Mechanisms
	Genome damage	Some pathobionts, such as <i>E. coli</i> and <i>S. epidermidis</i> cultured from breast tissue has been shown to induce DNA double strand breaks in HeLa cells <i>in vitro</i> (Urbaniak et al., 2016).
1.3.3.2		Dysbiosis of the BM disrupts the local homeostatic levels of MAMPs and increases the levels of ROS. This triggers the expression of pro-inflammatory
1.3.3.3	Pro-inflammatory response	cytokines and the release of DAMPs, which drives a positive feedback loop towards a chronic pathogenic inflammatory response, promoting tumorigenesis or enhancing pre-existing tumour growth (Colotta et al., 2009).
1.3.3.4	Modulate immune response and survival	<p>1) <u>TLR signalling:</u> In all types of BC, TLR (microbial sensing) receptors are significantly altered. BC features upregulated TLR4 receptors, which stimulate pro-inflammatory, pro-survival pathways (e.g. NF-κB) (Wee et al., 2015, Wu et al., 2018a, Tzeng et al., 2021). In this regard, bacterial MAMPs bind to and modulate TLR4. For example LPS binds to TLR4+ monocytes and promote their differentiation to pro-tumorigenic M2 macrophages (Vitiello et al., 2019, Seifert et al., 2016).</p>
1.3.3.5		<p>2) <u>Immune cell recruitment:</u> In healthy breast tissue, the abundance of certain bacterial strains, such as <i>Streptococcus</i>, <i>Propionibacterium</i>, <i>Staphylococcus</i>, and <i>Acinetobacter</i> positively correlate with that of tumour targeting cytotoxic T-cell (e.g. CD8+), while their depletion contributes to an immunosuppressive TME typically found in BC</p>

(Tzeng et al., 2021). Conversely, the abundance of pathobionts, such as *F. nucleatum* inhibits the infiltration and effects of tumour infiltrating lymphocytes (Parhi et al., 2020)

3) Oncogene expression: Certain bacterial signatures can potentially regulate oncogene expression. For example, the presence of *Staphylococcus* has been negatively correlated with tumour necrosis factor receptor associated factor 4 (TRAF4) (Tzeng et al., 2021, Rousseau et al., 2011).

1.3.3.6	Promote Metastasis	<p>1) <u>Intracellular Pathobiont influence gene expression:</u> Certain pathobionts, such as <i>Fusobacterium nucleatum</i> invade cancer cells and induce their proliferation while effectively evading the immune system (Bullman et al., 2017). In BC, <i>F. nucleatum</i> colonisation accelerates tumour growth and metastasis (Parhi et al., 2020). Similarly, the presence of <i>Haemophilus influenzae</i>, has been correlated with the expression of pro-tumorigenic pathways (Thompson et al., 2017).</p>
1.3.3.7		<p>2) <u>MAMPs:</u> Increased levels of circulating MAMPs, such as LPS has been associated with BC metastasis by inducing a monocyte mediated endothelial adhesion of circulating cancer cells (Kostic et al., 2013) or by inducing the production of pro-metastatic neutrophil extracellular traps (NETs) (Park et al., 2016). Lymphovascular invasion is associated with a reduced abundance of <i>Oblitimonas</i> in the TM (Tzeng et al., 2021).</p>

1.4

1.5 MICROBIOME EFFECTS ON CANCER TREATMENTS

1.5.1 *Cancer Immunotherapies*

Conventionally, breast cancers were deemed to be ‘cold’ in terms of the immune response, in that they generally do not evoke a robust immune response thereby making them less likely to respond to immune checkpoint inhibitor (ICI) therapies. However, TNBC is the most immunogenic out of all the BC types (Hendrickx et al., 2017). The response to ICI can vary significantly from person to person, with only a modest subset of patients displaying increased survival rates (García-Aranda and Redondo, 2019). In a phase 1b clinical trial testing the antitumour activity of an anti-PD-L1 (programmed death receptor 1 ligand) antibody in patients with locally advanced or metastatic breast cancer, the confirmed objective response rate (ORR) for BC was 3.0% and 5.2% for TNBC. Results from this study suggest that the expression of PD-L1 by tumour infiltrating lymphocytes (TIL) may predict the response to ICI therapy in breast cancer, as patients with TILs expressing PD-L1 showed an increased ORR in the overall population (16.7% vs 1.6%), an effect that was remarkable in the TNBC subgroup, with an ORR of 22.2% vs 2.6% (Dirix et al., 2018).

Ever increasing evidence supports the GM’s role in modulating treatment and a toxic response to cancer therapy, with several recent studies precisely demonstrating the response to ICI treatments across numerous cancer types (Vétizou et al., 2015, Gopalakrishnan et al., 2018b, Matson et al., 2018, Frankel et al., 2017, Chaput et al., 2017, Routy et al., 2018a, Sivan et al., 2015b, Li et al., 2019b, Temraz et al., 2019, Fessler et al., 2019, Shui et al., 2020). Here, different studies list different species and genera associated with positive treatment outcomes, pointing to the complexity of confounding factors and interactions between the patient, the environment and their microbiome. However, in general, these studies report higher response to therapy and overall survival in patients with diverse microbiome profiles (eubiosis), containing *Bifidobacterium*, *Akkermansia* and *Enterococcus*, among others. On the other hand, patients with a reduced microbiome diversity (dysbiosis) responded poorly to this therapy (Sivan et al., 2015a, Frankel et al., 2017). This was confirmed in GF murine models. Mice receiving FMT from responders improved their response to anti-PD-LI, while those who received an FMT from non-responders developed resistance.

(Matson et al., 2018, Routy et al., 2018b). Certainly, this can be measured and provide added information to a scorecard using the Nanopore 16s platform

Interestingly, some studies have shown that the therapy can induce detrimental changes to the microbiome that may influence the development of resistance (Vétizou et al., 2015). This highlights the relevance of managing the microbiome richness during the course of treatment (Shui et al., 2020). Immunotherapies can also lead to a wide range of inflammatory side effects, including ulcerative colitis, thyroid dysfunction, pituitary inflammation, and inflammatory arthritis, among others (Naidoo et al., 2017, Martins et al., 2019). The combination of low efficacy, high cost and the risk of side effects calls for the development of effective biomarkers, potentially through GM composition. However, to date, there is not sufficient data to support this for TNBC; and thus, more studies are required.

1.5.2 Radiation therapy

Initial studies showed that cancer radiotherapy (RT) has fewer side effects on germ free mice (McLaughlin et al., 1964); further studies confirm that bacteria are responsible for the toxic effects of radiotherapy (Toucheffeu et al., 2014, Wang et al., 2015). It was also shown that during macrophage polarisation, cell metabolism is altered by shifting the balance between glucose utilisation and fatty acid oxidation, influencing the immune response in the TME. Radiotherapy response or resistance is highly dependent on the TME, and this alteration may change the radio sensitivity of cancer cells. M1 macrophages are thought to enhance the radio sensitivity of BC cells; however, M2 macrophages can activate radio resistance through IL-4/IL-13-mediated STAT6 phosphorylation and M2 polarisation (Rahal et al., 2018). One study found that the vancomycin treatment enhanced the RT efficacy and that butyrate, a metabolite generated by vancomycin depleted gut bacteria, abolished the vancomycin effect (Uribe-Herranz et al., 2020). This is another example of context dependent effects of the microbiome, wherein butyrate, which is regarded as a beneficial metabolite in the context of oncogenesis and cancer progression, can negatively impact the outcome of a cancer therapy. In addition, IPA (see 1.4.4) was found to exert an RT protective effect on mice (Xiao et al., 2020b). Patients who had a toxic reaction to RT were associated with an over abundance of *Clostridium*, *Roseosporium*, and *Phascolarctobacterium* (Reis Ferreira et al., 2019). This an example where the treatment context is all important, where a patient

is to known to have to undergo radiation thereby an over abundance of *Clostridium*, *Roseosporium*, and *Phascolarctobacterium* could be checked for in Nanopore sequencing results.

1.5.3 Chemotherapy

For patients with TNBC that has spread to the lymph nodes and is more than 1 cm in size, the American Society of Clinical Oncology (ASCO) recommends neoadjuvant chemotherapy. A lack of alternative treatments beyond initial surgeries and radiotherapy to reduce the tumour load means that chemotherapy remains the mainstay of treatment options. Common drugs include doxorubicin, cyclophosphamide, paclitaxel, methotrexate, and fluorouracil (5-FU).

The microbiome affects many of these drugs not only in their efficacy but also in their level of toxic side effects. For platins to work correctly, they have to induce double stranded DNA breaks, and the microbial production of ROS promotes these mechanisms (Iida et al., 2013). A study in germ free mice found that their cisplatin antitumor activity could be restored with the introduction of *L. acidophilus* (Armstrong et al., 2018).

A study from our lab examined the effects of bacterial species identified in breast cancer patient tumour samples on 30 standard chemotherapies *in vitro*. Results demonstrated an increase in the toxicity of six chemotherapeutic drugs, a decrease in nine, including doxorubicin and gemcitabine, and no effect in the remaining 15, with different bacterial species producing different effects (Lehouritis et al., 2015). This was also verified in an *in vivo* mouse tumour model, with inhibitory effects on gemcitabine evident in tumours colonised by *E. coli*. Such findings suggest that response to therapy in BC tumours may be improved by microbiome modulation, and/or that the microbiome profile of a patient should be considered to inform treatment choice. To this end, our lab also demonstrated a proof of concept to utilise the microbiome signature of breast biopsies to infer the malignancy status of the tissue (Hogan et al., 2021). Alternatively, in the context of bacteriotherapy (see later), another study from our lab demonstrated the ability of the natural enzymolome of introduced bacteria to mediate local tumour therapy in a murine model via activation of multiple systemically administered chemotherapeutics (Lehouritis et al., 2016).

In relation to TNBC in particular, there is only one recent study which looked at the GM of 30 patients with TNBC, identifying *Bacteroides* and *Ruminococcaceae* as taxa more abundant in

TNBC patients who achieved a complete pathological response (pCR) after treatment with neoadjuvant chemotherapy compared with those who did not achieve a pCR. Patients with a partial response had higher quantities of *Bacteroides caccae* than those without any response (Swoboda et al., 2019). Resistance to the nucleoside analogue gemcitabine was found to be induced by intracellular gammaproteobacteria in a murine model, with treatment efficacy restored through antibiotic ablation of the bacteria (Geller et al., 2017b). This level of resolution is possible using the nanopore platform and certainly would be useful information to a clinician (Taylor et al., 2020).

1.5.4 *Bacteria as theranostics*

The potentials of our microbiome can be harnessed to treat/prevent TNBC through therapeutic/life style interventions that modulate our microbiome composition. Additionally, these commensals or their by-products can be used to develop early TNBC detection systems or to build bacterial based therapies. See Figure 3.

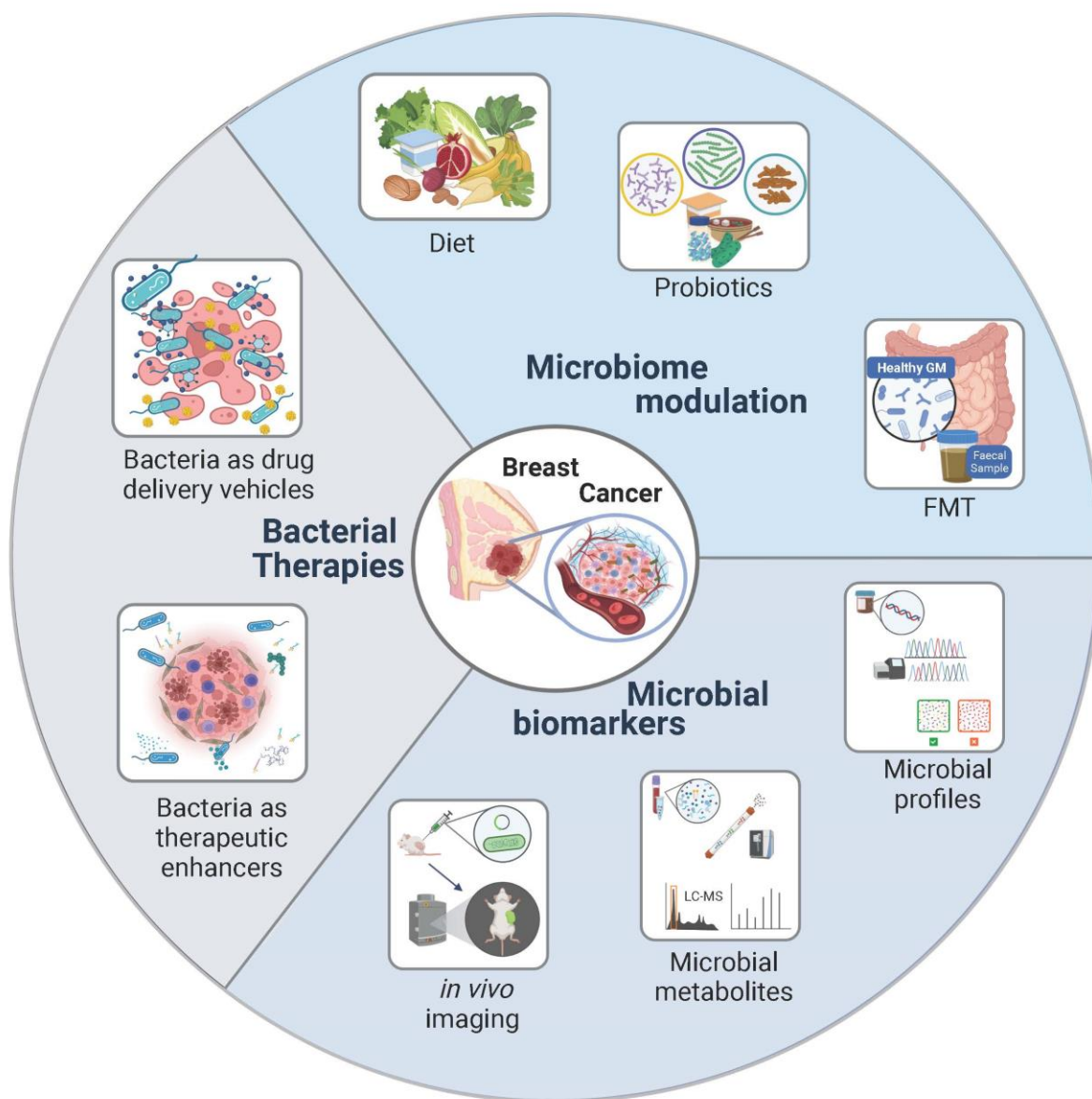


Figure 3. Bacteria as Cancer Theranostics. Harnessing the potentials of the microbiome to treat/prevent triple negative breast cancer.

1.5.5 Dietary changes to reduce the risk of cancer

Prevention is better than cure; therefore, maintaining eubiosis through diet is extremely important. A western diet rich in fat, sugar and low in fibre combined with little exercise has been associated with a higher risk of BC (Bodai and Nakata, 2020). Obesity and gut dysbiosis go hand in hand, with both being associated with chronic inflammation, increased risk of developing BC and failure

of cancer immunotherapy (Hossain et al., 2021). In a mouse model of TNBC where obesity was induced via a western diet and compared with lean mice, tumour volumes throughout the experiment were significantly higher in obese animals, as expected, with an association between obesity and enhanced TNBC growth, with significant loss of diversity in the GM, showing a decrease in *Bacteroides* species, particularly *Alistipes* (Hossain et al., 2021). Conflicting findings suggest that *Alistipes* exerts beneficial effects in colitis, has been associated with the increased efficacy of ICI and general activation of innate immunity whereas it has been found to be pathogenic in colorectal cancer (Parker et al., 2020).

Diet is crucially important in the interplay between the gut microbiome and oestrogen metabolism, thereby affecting breast cancer metastasis depending to various degrees on breast cancer type, as a western diet is associated with increased levels of β -glucuronidase feeding oestrogens back into the bloodstream. Low fibre has a compounding effect with a reduction in SCFAs such as butyrate which can help protect gut barrier maintenance and reduce inflammation, thereby adding to the pro-tumorigenic effect (Mirzaei et al., 2021). Increased levels of inflammatory proteins systemically increase insulin resistance and leptin levels, both of which promote carcinogenesis (Sánchez-Jiménez et al., 2019). A reduction in adiponectin from adipose tissue contributed to insulin resistance and increased insulin like growth factor 1 (IGF-1) levels, which can elicit increased cell proliferation (Koeth et al., 2013).

Conversely, a diet high in fibre reduces β -glucuronidase expression, lowers systemic oestrogen and increases sex hormone binding globulin (SHBG) levels with faecal excretion of oestrogen (Mirzaei et al., 2021). A high fibre diet can also drive increased intestinal alkaline phosphatase production, essential for gut barrier integrity (Singh and Lin, 2021). Butyrate is also known to inhibit histone deacetylase and tumour progression (Delage and Dashwood, 2008). Retinoic acid derived from vitamin A combined with trichostatin (a synthetic HDACi) has been found to increase the HDAC inhibitory effect in murine xenograft models of BC (Sirchia et al., 2002). A diet rich in fibre and polyphenols has been found to enhance the BC survival rate (Schultz et al., 2003, Reich et al., 2008).

1.5.6 Probiotics

Probiotics can inhibit pathogenic bacteria colonisation, help maintain eubiosis, promote gut barrier maintenance, reduce gut and systemic inflammation, and enhance immune and treatment response to BC (Bhatt et al., 2017, Taherian et al., 2019, Mendoza, 2019). An *in vitro* study using the BC MCF-7 cell line found significant inhibition of cell proliferation, increased levels of apoptosis, and cell cycle arrest when using live, heat killed cells (HKC) or cytoplasmic fractions (CF) of *E. faecalis* and *S. hominis* isolated from the breast milk of healthy women (Hassan et al., 2016). Oral supplementation with a probiotic containing *Lactobacillus reuteri* alone was sufficient to inhibit BC tumourigenesis in murine models genetically predisposed to neoplasia and fed a cancer promoting western diet through microbially initiated CD4⁺/CD25⁺ lymphocytes (Lakritz et al., 2014). Moreover, researchers have found that oral administration of *L. acidophilus* can modulate immune responses via stimulation of IL-12 and promotion of Th1 production, a potent activator of NK cells, in a murine xenograft model of a breast adenocarcinoma (Yazdi et al., 2010). A further *in vivo* study showed that drinking probiotics containing *Lactobacillus helveticus* increased IL-10 and decreased IL-6 production in mice, which is vital in BC inhibition (De Leblanc et al., 2005). A Japanese population based case control study involving 306 cases of BC and 662 controls found that regular consumption of probiotics containing *Lactobacillus casei* was inversely associated with BC incidence (Toi et al., 2013). Probiotics have also been found as an alternative to antibiotic treatment in cases of mastitis during breast feeding (Arroyo et al., 2010, Fernández et al., 2016, Jiménez et al., 2008).

1.5.7 Faecal microbial transplantation

Conceivably the most radical yet efficient means to modify the gut microbiome involves faecal microbial transplantation (FMT), a black box approach, whereby the underlying mechanisms do not need to be fully understood for efficacy to be tested. The entire GM from a healthy donor, usually an exceptional responder to the treatment being applied, is transplanted into a recipient undergoing the same treatment. This technique has successfully reversed resistance to ICI treatment in two recent metastatic melanoma trials, associated with an increased abundance of Ruminococcaceae and Bifidobacteriaceae (Davar et al., 2021, Baruch et al., 2021). Besides FMT, where the entire donor GM is transplanted, other approaches focus on transplanting coalitions of

bacteria (Tanoue et al., 2019) or even components of strain specific bacteria in the case of *Enterococcus gallinarum* flagellin have been used (Lauté-Caly et al., 2019).

Microbial signatures associated with reduced treatment related toxicity can be taken advantage of using FMT or combinations of specific bacteria in all types of cancer treatments. FMT has been found to alleviate undesirable harmful effects of 5-fluorouracil based chemotherapy in murine models of colorectal cancer (Chang et al., 2020). In pre-clinical models, FMT and indole 3-propionic acid have been observed to reduce radiation associated toxicity (Xiao et al., 2020a, Cui et al., 2017). In regard to BC in general and, more specifically, TNBC, these studies still need to be carried out. Nothing specific to TNBC that can be measured by sequencing.

1.6 BIOMARKERS OF TREATMENT EFFICACY/ PROGNOSTIC BIOMARKERS OF RESPONSE TO TREATMENT

One recent study found the GM composition at BC diagnosis can serve as a prognostic marker. That α diversity was not predictive of favourable BC prognosis or side effects, and β diversity of the GM was associated with tumour grading but not BC subtype. Furthermore, the relative dominance of *C. bolteae*, *C. asparagiforme*, and *B. uniformis* in stool samples was associated with axillary lymph node invasion. (Terrisse et al., 2021). It is now evident that the GM can affect all types of cancer treatments and that bacteria associated with positive treatment outcomes can be used as biomarkers of treatment efficacy and reduced toxicity, allowing for the much touted advent of precise, tailored, personalised treatments (Panebianco et al., 2018, Gately, 2019, Wilkinson et al., 2018). Identification of these species is only possible using whole genome sequencing, so unless you were looking for these microbes in particular they would not be sufficiently resolved using full 16s Nanopore sequencing.

1.6.1 Enhance treatment efficacy

Timing is everything or could play a role in chemotherapeutic treatment; one study using metronomic chemotherapy of capecitabine which is lower doses at more frequent intervals, showed promising results with reduced side toxic effects and less drastic changes to GM diversity. They found *Blautia obeum* to be associated with significantly prolonged progression free survival (PFS) and significantly PFS with the occurrence of the *Slackia* genus (Guan et al., 2020). While

chemotherapy can change the bacterial diversity, specific microbiome composition can, in turn, modify the efficacy of chemotherapy. Therefore, it is reasonable that particular probiotic concoctions could be administered adjunctively during chemotherapeutic treatment to augment effectiveness (Bhatt et al., 2017). These can be detected by 16s Nanopore sequencing and should be paid attention to in patients undergoing chemotherapy.

Oral administration of *Bifidobacterium longum* RAPO and ICI enhanced anti-PD-1 efficacy in preclinical murine models of TNBC (Kim et al., 2021). CRISPR based phage therapy has recently been deployed to selectively wipe out nosocomial *Clostridioides difficile* infections, which are very difficult to treat without causing massive collateral damage to the patient's beneficial GM (Selle et al., 2020). This approach used in TNBC treatment, combined with restoring lost valuable members of the GM, could synergistically rejuvenate patient GM functions and pave the way for establishing the best possible microbial amalgam for positive treatment outcomes. This approach has successfully been applied in murine models of atherosclerosis, with informed modification of the GM using small peptides to remodel the GM from that of one associated with a western diet to that of a low fat diet, resulting in reduced atherosclerotic plaques and a lowered pro-inflammatory cytokine profile (Chen et al., 2020). Identification of missing valuable members of the GM could be routinely detected by sequencing and should be included on any score card biomarker.

1.6.2 Bacteriotherapy

In 1813, Vautier observed that cancer patients infected with *Clostridium perfringens* who developed gas gangrene appeared to be cured of cancer (Barbé et al., 2006). Subsequently, bacterial infections and their effects on cancer were observed over 150 years ago by two German physicians W. Busch in 1868, and F. Fehleisen in 1882 (Fehleisen, 1882, Busch, 1868), who both independently found improvements in the condition of their patient's symptoms, after a *Streptococcus pyogenes* infection. The first attempt to utilise bacteria as a cancer treatment followed shortly after, in 1893, when Dr William Coley combined the lytic compounds of *S. pyogenes* and *Serratia marcescens* and injected the mixture named Coley's toxin into the tumour tissue with partial success in some patients (Coley, 1912).

There is a tricky balance when using bacteria in any therapy to get an effective therapeutic dose without toxic side effects on normal tissues. The advantage of using bacteria to deliver a genetic

therapeutic cargo is their natural tumour tropism when systemically administered with high levels of local replication and the ability to persist within the TME. Furthermore, invasive species can invade tumour cells and, upon bacterial lysis, deliver a therapeutic DNA payload in the form of a bacterial plasmid which can be expressed directly in the tumour cell (Baban et al., 2010). Various payloads can allow for the direct killing of tumour cells or immunomodulation of the host immune system, increasing tumour antigenicity and promoting clearance by the immune system (Amer, 2014).. Non-pathogenic *Salmonella* engineered with a quorum sensing (QS) switch naturally hone to tumours and only express their therapeutic payload specifically within the TME once they have reached a particular critical density, thereby destroying cancerous tissue only (Swofford Charles et al., 2015). In preclinical models, activation of TLR5 by *S. typhimurium* flagellin in BC cells activates innate pro-inflammatory response for effective anti-tumour clearing (Cai et al., 2011b). Bacteria can also be engineered to produce intrinsic bacterial biomolecules with known tumoricidal effects, some of which have been proven effective in human breast cancer cell lines see Supplementary Table 1 <https://acrobat.adobe.com/id/urn:aaid:sc:EU:37f8ef25-fab5-4b46-80f5-7cd2fa2d2691>. These biomolecules include bacterial peptides, bacteriocin compounds, enzymes or toxins.

Despite the large potential of bacteria based mediated cancer treatments, the risks of adverse unmanageable side effects have tempered their use. Currently, the BCG vaccine is the standard for treating patients suffering from the NMIBC (Sieow et al., 2021). 50 to 70% of patients have a positive outcome, with approximately 5% suffering adverse effects, including sepsis (Alexandroff et al., 1999). Clinical trials with positive results include intra-tumoral administration with spores of the attenuated strain of *Clostridium novyi* (*C. novyi* NT) to treat one patient suffering from advanced leiomyosarcoma, with promising results (Roberts et al., 2014). Attenuated *L. monocytogenes* have also been found to be safe and effective in treating patients with advanced mesothelioma, lung, pancreatic, and ovarian cancers (Maciag et al., 2009, Le et al., 2012). Pre-clinical studies using *Bifidobacterium* expressing cytosine deaminase (CD), which converted prodrug 5-fluorocytosine (5-FC) into chemotherapeutic agent (5-FU), was found to be effective in treating breast cancer when administered systemically in animal models (Fujimori, 2008, Fujimori, 2006, Sasaki et al., 2006).

1.7 CONCLUSION

The associations between cancer and the microbiota are intricate, not yet fully understood, but undeniable. It is still not apparent whether changes in the GM and BM microbial composition are drivers of carcinogenesis or a response to tumour development. The effects are bidirectional, making it much more challenging to tease out these complex relationships. Furthermore, another issue is that these studies are just a snapshot in time. Bacteria have a short lifespan and quick generation time, changing over the course of the day with the body's own circadian rhythms. Guilty microbes may have been and gone, whilst their effects rage on, especially in the case of DNA damage or epigenetic changes to the hosts' genome, enabling cancer progression. Context is also critical concerning space and time; a bacterium in the gut may be beneficial but, when found in the TME, may exert the opposite effect. This also applies to different types of cancer. Bacterial metabolites can act as hormones, are highly pleiotropic and context dependent, and can be produced distal to the tumour site while having systemic immune modulatory effects.

Despite associations having been found in various microbiome wide association studies, very little is known regarding the actual biochemical mechanisms. In the context of TNBC, even less is known, but lessons learned from studies involving other BC types are undoubtedly valuable. FMT also shows that even without the underlying mechanisms being fully understood, bacteria can still be used safely to improve treatment efficacy and reduce the toxic side effects of all types of cancer treatments currently used in TNBC.

More extensive clinical studies are explicitly required to further elucidate the microbiome's role and see past all of the confounding factors. Indeed, dietary changes should be taken on board to prevent cancer first, with probiotics (symbiont microbes) playing a role in returning the body to a state of normobiosis. Known associations can now be used as therapeutic and prognostic biomarkers, with enormous potential. The promise of completely personalised theranostics, however, is still a way off. Bacteriotherapy beyond FMT is further away, with plenty of evidence in *in vitro* and murine studies but lacking any clinical trials because of safety concerns. Genetic manipulation to reduce potential risks continues to make progress which, when matched to bacteria's ability to selectively replicate within the TME, may in time overcome treatment hesitancy.

Indeed, monitoring of cancer patients' microbiomes may become standard practice where feasible, with modulation to a more beneficial state where possible. Altering the microbiome has potential to be the most unintrusive and safe means to change the body's metabolism, improve treatment efficacy and reduce toxic side effects. Large scale profiling of the GM and the associated metabolome of cancer patients relative to that of healthy individuals will allow the development of theranostic biomarkers and treatments to improve the clinical prognosis and quality of life for cancer patients. Every cancer and treatment regime requires further investigation using observational studies adequately powered to gain further insights. Even though sequencing technologies have advanced to such a degree, with the amount of data they produce, there is lag in time to processing the data and getting useful information to the clinic. Greater sequencing resolution is key.

1.8 CHAPTER CONCLUSION

The value of biomarkers in precision oncology is unquestionable, and increasing the range of biomarker strategies will benefit cancer diagnosis, treatment and research. This chapter validates the potential of bacteria in cancer biomarker settings, while this thesis studies both bacteria and proteins produced from bacteria for exploitation as cancer biomarkers.

2 CHAPTER 2:

Synthetic protein protease sensor platform

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2.1 ABSTRACT

Protease activity can serve as a highly specific biomarker for application in health, biotech and beyond. The aim of this study was to develop a protease cleavable synthetic protein platform to detect protease activity in a rapid cell free setting. The protease sensor is modular, with orthogonal peptide tags at the N and C terminal ends, which can be uncoupled via a protease responsive module located in between. The sensor design allows for several different readouts of cleavage signal.

A protein ‘backbone’ (Green fluorescent protein (GFP)) was designed *in silico* to have both a C-terminal Flag-tag and N-Terminal 6x histidine tag (HIS) for antibody detection. A protease cleavage site, which can be adapted for any known protease cleavage sequence, enables the uncoupling of the peptide tags. Three different proteases - Tobacco Etch Virus (TEV), the main protease from coronavirus SARS-COV-2 (Mpro) and Matrix Metalloproteinase 9 (MMP9) - a cancer selective human protease were examined. A sandwich Enzyme Linked Immunosorbent Assay (ELISA) was developed based on antibodies against the HIS and Flag tags. As an alternative readout, a C-terminal quencher peptide separable by protease cleavage from the GFP was also included. Purified proteins were deployed in cell free cleavage assays with their respective protease. Western blots, fluorescence assays and immunoassay were performed on samples.

Following the design, build and validation of protein constructs, specific protease cleavage was initially demonstrated by Western blot. The novel ELISA proved to afford highly sensitive detection of protease activity in all cases. By way of alternative readout, activation of fluorescence signal upon protease cleavage was also demonstrated but did not match the sensitivity provided by the ELISA method.

This platform, comprising a protease responsive synthetic protein device and accompanying readout, is suitable for future deployment in a rapid, low cost, lateral flow setting. The modular protein device can readily accommodate any desired protease response module (target protease cleavage site). This study validates the concept with three disparate proteases and applications – human infectious disease, cancer and agricultural crop infection.

2.2 INTRODUCTION

Synthetic protein design and technology is a rapidly evolving field (Walker et al., 2021). As the technology progresses, opportunities increase for exploitation of designer proteins in detecting targets of interest (Flores Bueso and Tangney, 2017). More traditional proteins (antibodies) are already commonplace as part of rapid ‘in the field’ tests, such as lateral flow tests used for the detection of biomarkers including microbial antigens (e.g. Covid-19 antigen tests) (Candel et al., 2020) or hormones (pregnancy tests) (Leuvering et al., 1980). Novel strategies, however, need not be restricted to the immune binding of targets, with the potential to design proteins as ‘sensors’ of biological or chemical activity.

Proteases can act as highly specific indicators of the activity of multiple biological processes and can therefore serve as useful as biomarkers for a wide range of targets of value in health, food and other key sectors of global social and economic importance. Every living organism has associated proteases involved in functions such as digestion, waste degradation, cell signalling and post-translational modification. These processes are usually highly regulated, with dysregulation being an indicator of disease. The human genome has over 600 proteases, and these are involved in virtually every stage of a cell’s life cycle (Bond, 2019). Proteases associated with human disease range from dysregulated endogenous proteases involved in cancer progression or inflammatory disease to prokaryotic and viral pathogens. The majority of human disease causing viruses encode at least one protease (Sharma and Gupta, 2017). The ability to rapidly detect and amplify this signal of protein cleavage could prove invaluable in a diagnostic setting for a wide array of human diseases.

Proteases hydrolyse peptide bonds and can be divided into six classes based on the mechanism of action. Aspartic, glutamic and metalloproteases use an activated water molecule as the nucleophile to attack the peptide bond. In contrast, with cysteine, serine, and threonine proteases, the nucleophile is the amino acid residue, after which they are named (López-Otín and Bond, 2008). The substrate specificity is based on protein/protein interactions between the substrate and protease in the binding pocket of the enzyme, with substrate side chains accommodated in side pockets (Schauperl et al., 2015). Typically, proteases recognise six to eight amino acid residues with varying levels of specificity ranging from the most stringent with single peptide bond recognition

in the case of angiotensin converting enzyme to proteinase K, which is broad spectrum (Zhou et al., 2020). Knowing the cleavage site amino acid sequence of any given protease means it can be engineered as part of a synthetic protein. Cleavage site sequence information can be found on the MEROPS database (Rawlings and Bateman, 2021). The proteases chosen for this study have substrates that do not overlap (although cleavage by proteases with overlapping substrate specificity cannot be ruled out completely (Schauperl et al., 2015)).

Currently, protease activity can be detected using laboratory instruments using a number of methods, reviewed elsewhere (Chung and Lin, 2020, Ong and Yang, 2017). Briefly, these include mass spectrometry based assays (Ritorto et al., 2014, Yan et al., 2011); colourimetry based chemistry (Lou et al., 2010, Guarise et al., 2006); fluorescent protein based techniques (Nicholls et al., 2011, Callahan et al., 2010, Heim et al., 1994, Zhang, 2004, Stawarski et al., 2014); bioluminescence based enzymes (Wigdal et al., 2008, Yao et al., 2007); quantum dots (QDs) (Medintz et al., 2006); nanoparticles (Laromaine et al., 2007, Loynachan et al., 2019, Jin et al., 2012, Song et al., 2013, Berdichevsky et al., 2003). More recent developments involve using liquid crystal (LC) based protease assays which can allow for naked eye detection of protease activity (Jannat and Yang, 2018). Transcription based assays have also been described and offer extremely high sensitivity (Fink and Jerala, 2022, Liu et al., 2021b) In terms of existing diagnostic kits, these can be divided into two broad categories: nucleic acid based molecular diagnosis and antigen/antibody based immunoassays. Nucleic acid quantification methods include quantitative reverse transcription polymerase chain reaction (qRT-PCR), loop mediated isothermal amplification (LAMP) based assays, clustered regularly interspaced short palindromic repeats (CRISPR) associated protein (Cas) system, and RNA sequencing, with qRT-PCR currently deemed the gold standard for COVID-19 detection. For a test which could be used at home, a lateral flow assay (LFA) which works on the same principle as the sandwich ELISA presented in this study, could be adapted quite easily using the same antibodies (Yalow and Berson, 1960). Pregnancy tests, and more recently, the Covid-19 pandemic, have brought LFAs to the fore of at home tests worldwide.

This study describes the design, build and test of a novel protein based platform for potential use as a rapid test for any protease, comprising a synthetic protein with a sensor, readout modules and accompanying readout. A quenched GFP was chosen as the backbone of the sensor, allowing for

confirmation of cleavage through fluorescent signal detection. The incorporation of opposing distinct peptide tags allowed for protease activity to be detected by sandwich ELISA using well characterised commercially available antibodies, significantly increasing its sensitivity. Three unrelated protease cleavage sequences were chosen to validate this approach's versatility - TEV, MMP9, and Mpro. Tobacco Etch Virus (TEV) is one of the most routinely used proteases for in research and industry (Kapust et al., 2001). Mpro is the viral protease from the SarsCov-2 virus (Jin et al., 2020), and MMP9 is a ubiquitous cancer associated protease involved in cancer mediated tissue remodelling and metastasis (Page-McCaw et al., 2007), well studied as a cancer selective biomarker. For each protease, a protease specific protease sensor featuring its cognate protease specific cleavage peptide was designed, with only the amino acid sequence of the cleavage site varying between constructs (Figure 1a).

While there are tests for proteases that exploit ELISA, a key aim of this study is to progress the technology that is synthetic proteins as 'smart' tools for biotech, in particular the potential to design proteins as 'sensors' of biological activity. Here, we provide proof of concept for a design strategy of a synthetic protein as a sensor of biological activity, where the readout parts are 'uncoupled' by the target activity (protease in this case). This design has potential value in multiple settings *in vivo* or *in vitro*, for research, diagnostic or therapeutic functions. This study generates proof of concept data through validation of its use *in vitro* when coupled with an ELISA detection strategy.

2.3 MATERIALS & METHODS

2.3.1 *In silico* protein design

All synthetic proteins were designed and modelled *in silico* to predict suitability for wet lab use and redesigned where necessary. The process for *in silico* modelling used was as previously described by our lab (Yallapragada et al., 2020). Briefly, *in silico* features, including Molecular Weight, Theoretical pI, Hydrophobicity (GRAVY) and Instability Index were calculated using the ProtParam facility, hosted by the Swiss Bioinformatics Resource Portal (expasy.org). The protein tertiary structures were predicted using I-TASSER suite (v5.1). This suite of tools also provides solvent accessibility estimates per residue and prediction of active sites. This additional information was combined using R to find the solvent accessibility of the active site. All generated data points were then integrated and used to rank the *in silico* designed constructs in terms of predicted performance.

A green fluorescent protein (GFP) was chosen as a protein ‘backbone’. The GFP protein chosen was GFP S65T (PDB-ID 1Q4A), is 27 KDa in size, has an S TO T mutation at the residue number 65 from wild type *Aequorea victoria* GFP (PDB-ID 1EMA) and has excitation /emission spectra of 490 and 520 nm, respectively. A previously described version of this protein, fused at the carboxyl terminus to a 27 AA peptide originating from the M2 influenza proton channel was used for design (Pinto and Lamb, 2006). This peptide promotes the tetramerisation of GFP, disrupting its maturation, consequently leading to a loss of signal and is hereafter referred to as the quencher (Nicholls et al., 2011).

For the study described here, a protease cleavage site for a given protease was incorporated module between the GFP and its quencher, to permit the restoration of fluorescence following protease cleavage. A Flag-tag was added to the amino terminus and a 6 x HIS-tag was added to the carboxyl terminus of the protein backbone to facilitate the detection of protein cleavage.

2.3.2 DNA Design

Following *in silico* validation of test sequences, the finalised constructs' AA sequences were reverse translated into their corresponding DNA sequences using the backtranseq feature on the EBI website (https://www.ebi.ac.uk/Tools/st/emboss_backtranseq/). The DNA sequences were codon optimised for expression in *E.coli* using the codon optimisation tool available on the IDT website (<https://eu.idtdna.com/codonopt>). Gene Blocks were purchased from Integrated DNA Technologies (IDT, Belgium).

New England Biolabs (NEB, Ipswich, MA, USA) and SnapGene's Gibson assembly simulators were used to design the homologous arms to facilitate Gibson assembly. Amplification and sequencing primers were designed using Primer3Plus. All primers were sourced from IDT (Belgium).

2.3.3 DNA Assembly

Conventional PCR:

25 µl reactions were performed using Taq 2X Master Mix (NEB, Ipswich, MA, USA) and 0.25 µM of each primer. Cycling conditions included: an initial denaturation for 30 sec at 95 °C. 25 – 35 cycles of denaturation at 95°C for 10 sec, annealing for 15 sec at the primers' optimal temperature (specified by NEB's calculator for Taq DNA polymerase), 20-40 sec of extension at 68 °C (20 sec for 200bp amplicons and 40 sec for 400-500 bp amplicons), and a 5 min final extension at 68 °C. 10 µl of amplified products were loaded to a 1.5% agarose gel, run at 180 V for 40 min, and imaged with Gel Doc EZ System (Bio-Rad, California, USA).

Plasmids:

The pRSFDuet-1 plasmid (NOVAGEN Darmstadt, Germany) was used for cloning and expression of the protease sensors. The plasmid encodes two multiple cloning sites (MCS), each preceded by a T7 promoter, lac operator, and ribosome binding site (RBS). The plasmid also carries the pRSF1030 replicon, *LacI* gene, and kanamycin resistance marker.

Bacterial transformation: *E. coli* BL21 cells were made competent using Cohen *et al.* 1972 protocol (Cohen *et al.*, 1972). 100 ng plasmid was mixed with 30 µl of competent *E. coli* BL21 cells and were placed on ice for 20 min. The suspension was subjected to heat shock at 42 °C for 20 min. The cells were again placed on ice for 2 min and 1 ml LB broth was added. 100 µl transformed cells were plated on LB agar containing kanamycin at 50 µg/ml. Colonies were subcultured and stored in 25 % glycerol at - 80 °C for further use. For plasmid extraction, overnight subcultures of the transformed bacteria were processed through a Monarch Plasmid miniprep kit (New England Biolabs, Ipswich, MA, USA) using the manufacturer's protocol.

Restriction digest:

Restriction enzymes used with pRSFDuet-1 were NcoI, AvrII, NdeI and BglIII (NEB), as per manufacturer instructions. Following restriction digest, DNA was purified using a PCR purification kit (Qiagen, Hilden, Germany) protocol, and the digest was verified by Agarose gel electrophoresis. DNA concentration was determined using a NanoDrop spectrophotometer (ThermoFisher, Waltham, MA, USA).

Gibson Assembly:

Gibson Assembly was carried out using the Gibson Assembly master mix described by DG Gibson *et al.* (Gibson *et al.*, 2009). Plasmid and DNA gene blocks were mixed in a 1:3 ratio in a Gibson Assembly master mix and incubated at 50 °C. *E. coli* BL21 cells were transformed with the assembled plasmids and plated on LB agar medium with kanamycin at 50 µg/ml. Selected colonies were added to the NEB PCR master mix with 2.5 µl of corresponding primers. PCR was carried out as per NEB Q5 polymerase PCR protocol. Sanger sequencing (GATC light run) was also performed on the selected colonies to confirm the assembly (Eurofins Genomics, UK Ltd).

2.3.4 Protein Expression and Purification

Bacterial protein production:

All expression constructs described were transformed into *E. coli* strain BL21 and grown at 37 °C to an optical density (O.D) A600 of 0.6. Cultures were then induced using 1 mM isopropyl β-D-1-

thiogalactopyranoside (IPTG) and the temperature reduced to 25 °C for three h of expression. Cells were harvested by centrifugation at 5,000 x g for 15 min and lysed under native conditions using BugBuster (NOVAGEN Darmstadt, Germany) or under denaturing conditions using a lysis buffer containing 8 M urea, 100 mM NaH₂PO₄, 10 mM Tris HCl and 10 mM imidazole at pH 8.0. Clarified lysates were prepared by centrifugation at 15,000 x g for 40 min. For use as a cleaved protein size comparison, a GFP-only protein was employed (lacking the quencher module), for which the gene block was obtained from IDT (Belgium). This was purified using HIS-tag/Ni-NTA system affinity liquid chromatography. Under native conditions, the column was washed with a buffer composed of 50 mM imidazole, 300 mM NaCl, and 50 mM NaH₂PO₄ pH 8.0. Proteins were eluted using a buffer of 300 mM imidazole, 300 mM NaCl, and 50 mM NaH₂PO₄ pH 8.0, aliquoted and stored at – 80 °C.

All protease sensors containing the M2 fluorescence quencher required purification under denaturing conditions using the following buffers; Denaturing wash buffer (wash buffer 1) - 8 M urea, 100 mM NaH₂PO₄, 150 mM NaCl and 20 mM imidazole at pH 8.0. Native wash buffer (wash buffer 2) - 50 mM NaH₂PO₄, 500 mM NaCl and 20 mM imidazole at pH 8.0. Elution buffer 50 mM NaH₂PO₄, 500 mM NaCl and 250 mM imidazole at pH 8.0.

Protein concentration, diafiltration, desalting and buffer exchange:

Purified proteins were dialysed using a Slide-A-Lyser ® from Thermo Scientific in a storage buffer containing 50 mM Tris and 150 mM NaCl at PH 8.0 and concentrated using an Amicon ® Ultra Centrifugal Filter Devices 10K filter device, all as per manufacturer's instructions.

2.3.5 Protein Analyses

2.3.5.1 SDS PAGE and Western Blot:

An estimate of protein concentration was determined by measuring A_{280 nm} using a Thermo Scientific NanoDrop 2000 spectrometer prior to SDS page and Western blot. Concentrated proteins were solubilised in loading buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 41.7 mM dithiothreitol,

10% glycerol, 0.01% bromphenol blue). Then boiled for 10 min at 70°C and resolved on a 4-12% gradient SDS-PAGE gel. After running, the gel was fixed in a solution of 50% methanol and 10% acetic acid and stained with EZBlue™ gel staining reagent. The gel was imaged after staining, and subsequent washes using a Bio-Rad EZ Gel imager and analysed using the Image Lab (BioRad California, USA).

SDS-Page gels were electro-transferred to PVDF membranes for immunoblotting using Trans-Blot® Turbo™ (BioRad California, USA) reagents and equipment. The membranes were blocked with 5% skim milk and incubated with indicated primary antibodies overnight at 4°C. Primary α -Flag M2 antibody was used for the detection of Flag fusion proteins (1:1,000). This mouse raised monoclonal antibody was purchased from Merck (Darmstadt, Germany). Primary mouse monoclonal [HIS.H8] recognises HIS-tagged recombinant proteins (1:1,000). The secondary Antibody used was IRDye® 800CW Goat anti-Mouse IgG from Li-Cor (1:15,000). Odyssey® DLx from Li-Cor was used to image and analyse the blots. A 10–250 kDa Protein ladder Precision Plus Protein Dual Colour Standards from BioRad was used to estimate protein size. The protein standard used for estimating protein concentration was an Amino-terminal FLAG-BAP™ Fusion Protein.

2.3.6 *Cleavage Assays*

SARS-CoV-2 Mpro: The assay was performed with a buffer consisting of 50 mM Tris-HCl and 1 mM EDTA (pH 7.3) at 30°C for various lengths of time. 1 μ g of Mpro (Merck Darmstadt, Germany) of 33.8 kDa in size with 50 μ g of protease sensor 33.1 kDa.

MMP9: The assay was performed with a buffer consisting of 50 mM Tris/HCl, 150 mM NaCl, 10 mM CaCl₂, 20 μ M ZnCl₂, 0.05% Brij35 at pH=7.5. 1 μ g of MMP9 catalytic domain (Abcam, Cambridge, UK) of 40 kDa combined with 50 μ g of protease sensor 33.1 kDa for various lengths of time at 37°C.

TEV: Carried out as per manufacturer's instructions (NEB, Ipswich, MA, USA). 1 µl of TEV Protease per 15 µg of substrate in the provided TEV reaction buffer and incubated at 30°C for various lengths of time.

Fluorescence assays

Protease sensor samples containing 50 µg protein with or without associated protease were prepared at time zero to assess the ability of the protease to cleave directly. At the initiation of the reaction, 200 µl aliquots of the digest and controls were added to a Costar 96-well black plate. The fluorescence was measured at various time points (Ex. 475 nm/Em. 512 nm) using a BMG FLUOstar® Omega multi-mode microplate reader.

2.3.7 Enzyme linked immunosorbent assay (ELISA)

Antibodies, reagents, and material

Antibodies used in this study were sourced from ABCAM (Netherlands) and were previously validated for use in ELISA. These are listed in Supplementary Table 1. 3,3',5,5'-tetramethylbenzidine (TMB) substrate was acquired from Surmodics (US, Cat. No. TMBS-1000-01). All other reagents were prepared as per Supplementary Table 2 <https://acrobat.adobe.com/id/urn:aaid:sc:EU:37f8ef25-fab5-4b46-80f5-7cd2fa2d2691>. ELISAs were performed on NUNC MaxiSorp™ 96 well plates (Sigma-Aldrich, Darmstadt, DE, Cat. No. M9410).

Standards

Standards used for these assays were purified as per protocols in section 2.4. Proteins were verified by immunoblotting and their purity and quantity assessed via PAGE gels and spectroscopy. The full protease sensor was used as a standard to quantify uncleaved proteins and the cleaved sensor as a standard for measuring cleaved fractions. Seven points (1/5 dilutions) standard curves, ranging from 1 to 5,000 ng of protein, were used for optimisation of the assay (antibody titration and

defining sample load; sFigure 1). Six point standard curves covering a range of 10 – 1,000 ng of protein (2/5 dilutions) were used for quantifying cleavage.

2.3.8 Samples & plate layouts

The products of cleavage reactions with or without Mpro, TEV, or MMP9 (samples) were eluted in EB to a concentration of 1.25 ng/μl, and 125 ng (100 μl) was loaded into each well. For each protease tested, cleavage was quantified by loading a plate that included: (a) Six replicates of each standard (full and cleaved sensor); (b) Twelve replicates of each, matched cleaved and uncleaved reaction products, three replicates to be analysed with each primary detection antibody (α-HIS vs α-GFP) and standard.

Assay

General practices: All incubation steps, unless specified, were performed at 20 °C, shaking at 350 rpm. All the solutions added to the wells were pipetted using a multichannel pipette, avoiding over drying of the wells and were removed by swiftly inverting the plate on top of a sink and blotting against low lint absorbent paper. All washes were performed with 300 μl of the corresponding wash solution as specified below.

Procedure: a) *Coating:* Wells were coated with 100 ng of Goat α-Flag in 100 μl PBS overnight at 4 °C. Unabsorbed antibody was removed from the plate and the wells were washed three times with PBS. b) *Blocking:* Wells were blocked with 250 μl of blocking solution for 1.5 h and the excess was removed by washing the plate three times with PBS. c) *Antigen binding:* Samples and standards were loaded to the respective wells and incubated for 2 h. Unbound antigens were removed from the wells with four PBS-T washes. d) *Antibody & conjugate labelling:* 8 ng of α-GFP or 20 ng of α-HIS antibodies in 100 μl of EB, were added to the wells and incubated for 2h. The unbound antibody was removed with four PBS-T washes. This was followed by a 1.5 h incubation with 8 ng of α-Rabbit HRP-conjugated antibody. After which, the plates were thoroughly washed with five PBS-T washes. e) *Detection:* 100 μl of TMB substrate was added to the wells and the plate was incubated for 15 min in the dark and the reaction was stopped with 100 μl of Sulfuric acid (1M). e) *Reading:* The plate was read within 5 min of adding H₂SO₄, with 450

nm and 570 nm absorbance filters, following the default settings of a BMC Omega plate reader. See the illustration of components in Figure 4a.

Data interpretation and analysis

The preparation of raw ELISA data was performed with the BMG Omega – data analysis software. Here, the background was removed from the raw data by subtracting the values obtained for 570 nm absorbance. Four standard curves were plotted per plate (see sFigure 1), one per sensor (cleaved/full) detected with each antibody (α -GFP/ α -HIS). These were plotted using the average of three replicates and fitted using the five parameter logistic (5PL) regression model to account for differences in curve symmetry. Standard curves with goodness of fit ($R^2 \geq 0.98$) were deemed suitable for analysis. The resulting raw quantity of protein detected for each reaction was exported to Excel, where it was tabulated in a format compatible with R. Further analysis was performed in R using Tidyverse packages. Raw and tabulated data and R-scripts used for the analysis can be found in the Zenodo repository DOI 10.5281/zenodo.7022826. To account for differences in antibody detection (α -GFP/ α -HIS) signals, the quantity of protein detected for each replicate was normalised using the uncleaved controls as normalisers. The resulting values were then plotted using ggplot2 and statistical significance was tested with Welch Two Sample t-tests.

2.4 RESULTS

2.4.1 *In silico design and validation of protease sensor platform*

The entire 33.1 KDa construct, consisting of all the described parts (Flag + GFP + Protease cleavage signal peptide + Quencher + HIS-tag), is hereafter referred to as the protease sensor (Figure 1a). For each protease, a protease specific protease sensor featuring its cognate protease specific cleavage peptide was designed, with only the amino acid sequence of the cleavage site varying between constructs shown in magnified circle of (Figure 1a). Figure 1b is a Western blot showing the sensors of the correct size.

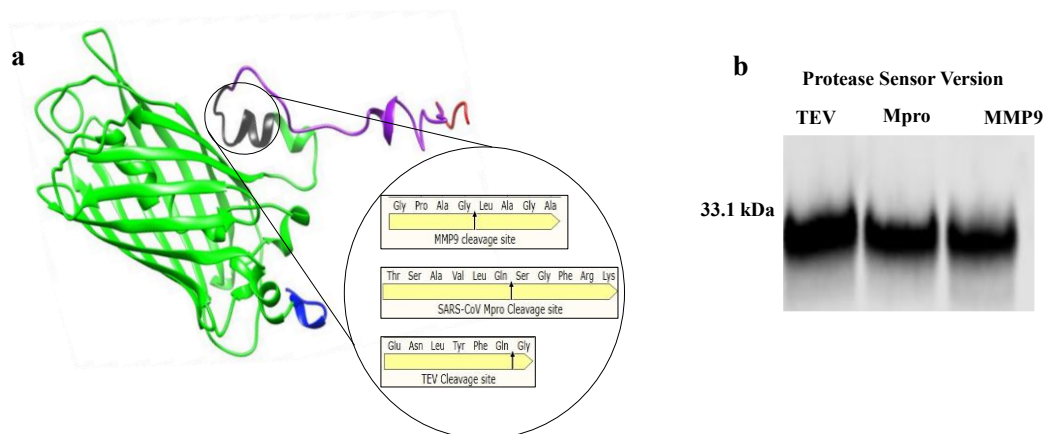


Figure 1. Protease sensor Design. *a)* 3D model of engineered protease cleavable synthetic protease sensor (33.1 kDa), showing Flag-tagged N-terminus (blue), 27 kDa GFP beta-barrel (green) with cleavage site in (black), M2 quencher (purple) and HIS tagged C terminus (red) Magnified cleavage site with amino acid cleavage sequence for each of the three chosen proteases (TEV, MMP9 and Mpro) *b)* Western blot α -Flag showing each of the 33.1 kDa protease sensor versions TEV, Mpro and MMP9.

2.4.2 Experimental Validation of Protein Sensors

Specific cleavage of protease sensors as evidenced by Western blot:

The modular protein device can readily accommodate any desired protease response module by incorporating the target protease cleavage site. Cleavage assays were performed on protease sensors using Western blot as readout (Figure 2). α -Flag antibody was employed to detect the cleavage assay products of the three selected proteases, along with a control for a size comparison of the cleaved product of the sensor. The cleavage of each sensor protein version was shown to be specific to its cognate protease only. Included in each cleavage assay were negative controls, including no protease/buffer only and two additional negative controls with two non-specific

proteases. For example, in the case of the TEV cleavage assay, the two non-specific proteases would have been Mpro and MMP9. For each assay, cleavage was evident by densitometry at levels of 22% for TEV, 29% for Mpro and 24% for MMP9 sensors.

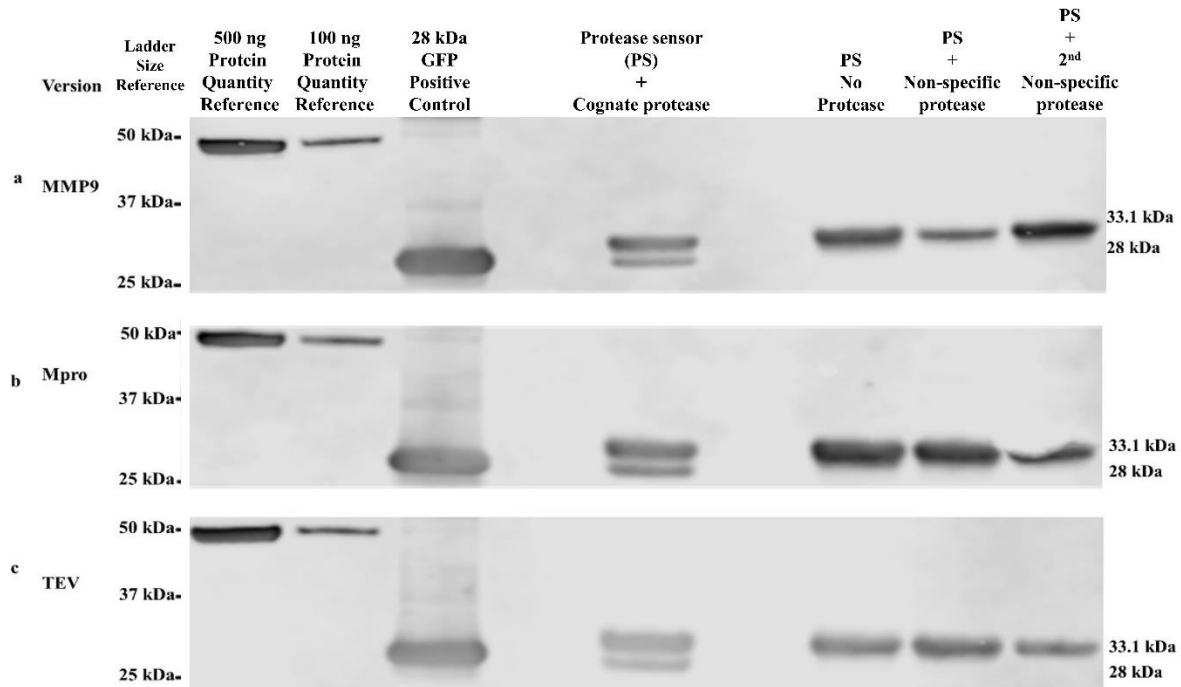


Figure 2. Western blot validation of protease specific cleavage of protease sensors. α -Flag antibodies were used in the detection and size determination of protease sensors +/- cleavage. **a)** MMP9 version, **b)** Mpro version, **c)** TEV version. Intact protease sensor corresponds to 33.1 kDa, while cleaved protease sensor (HIS-bearing terminus removed by protease cleavage) corresponds to 28 kDa.

2.4.3 Fluorescence as a readout of protease sensor response to protease

While not the aim of this study, fluorescence was used as an alternative rapid method of cleavage validation. Once specific cleavage was shown for each construct, fluorescence as a readout was examined. Having previously shown specificity of cleavage and lack of cross reactivity of the sensor versions, one exemplar construct was chosen to proceed. The Mpro sensor was chosen as it

showed the most efficient cleavage ratio by densitometry. The Mpro protease sensor was incubated with Mpro protease with samples of the cleavage assay and fluorescence readings taken over a 24 h period. Within less than 15 min, ~ 20% of the sensor was cleaved as measured by densitometry (Figure 3a). Figure 3b shows the percentage of the protease sensor cleaved over time as evidenced by Western blot densitometry. There was a delay in fluorescent signal which we hypothesise to be associated with maturation of the cleaved sensor between 15 min and 45 min, consistent with the known maturation for GFP S65T of 27 min *in vitro* (Figure 3c) (Iizuka et al., 2011). Cleavage of the M2 quencher is followed by fluorophore maturation, possibly contributing to the apparent delay between cleavage signal and fluorescence with cleavage dynamics for each protease also a contributory factor. Complete cleavage was not apparent by 24 h and was found to be the case with all three sensor versions and hypothesised to be a consequence of M2 tetramerisation whereby access to the cleavage site is obstructed in a sterically hindered manner. A significant ($P < 0.001$) gain of fluorescent signal was detected on cleavage, corresponding to 5.5 times the background fluorescence of the sensor in *in vitro* assays after 1.5 h incubation with Mpro (Figure 3d).

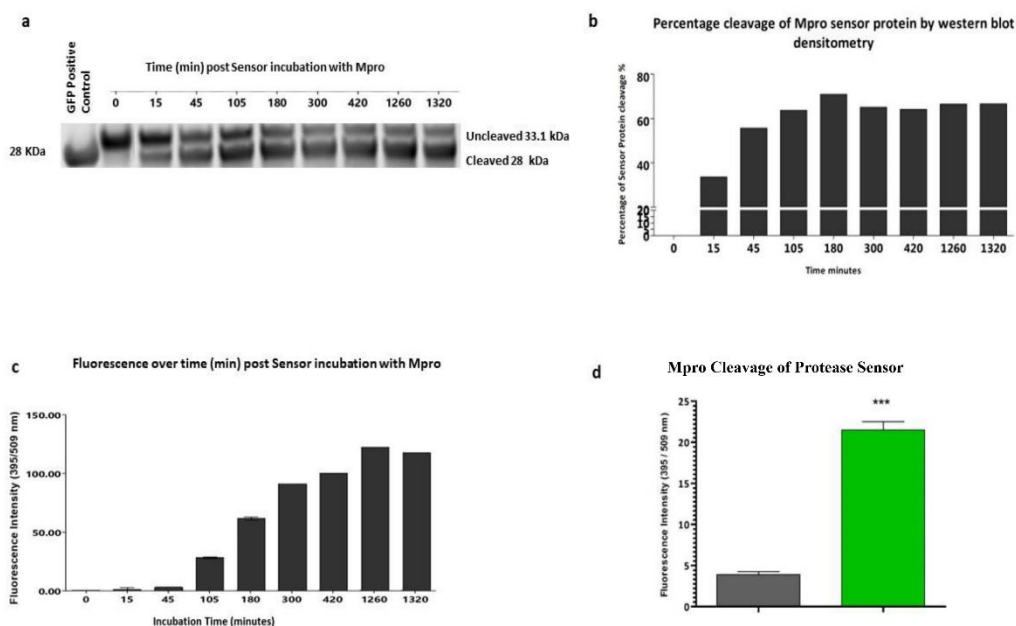


Figure 3. Fluorescence readout of protease sensor activity. All assays have the same components +/- test protease. **a)** Western blot analysis of cleavage of protease sensor by Mpro protease from 0 min to 22 h post addition of protease. **b)** Percentage cleavage by densitometry analysis of protease sensor over time. **c)** Gain of fluorescent signal over time, with background uncleaved signal removed. Cleavage precedes the appearance of fluorescence with a ~ 45 min lag ($n = 3$). **d)** Comparison of the fluorescent signal after 1.5 h incubation of sensor with (grey) or without (green) Mpro protease.

2.4.4 Immunoassay detection of tag uncoupling

The sandwich ELISA based readout detects the synthetic tags on either side of the protease cleavage module, providing a quantitative readout of uncleaved (dual tag-positive) vs cleaved (single tag-positive) device. Cleavage detection involves two separate Sandwich ELISAs using already well characterised antibodies, both of which use α -Flag antibody as the capture antibody. Labelling primary antibody differs between the two ELISAs, whereby α -HIS is used for the uncleaved sensor and α -GFP for both cleaved and uncleaved sensors.. The secondary labelling

antibody is α -rabbit conjugated to horseradish peroxidase (HRP) in order to produce a chemiluminescent signal as a readout. The cleavage site for each sensor is located post the GFP module (Figure 4a), where, upon cleavage, the HIS tag will be separated from the Flag-tagged GFP. Protease sensors cleaved in the presence of their specific protease will show a reduced HIS ELISA signal when compared with uncleaved. Figure 4b illustrates the sandwich ELISA components, with Figure 4c depicting scenarios providing detectable signals. Figure 4d depicts diagrammatically cleavage of the protease sensor resulting in the removal of the HIS tag which leads to a corresponding reduction in the α -HIS ELISA signal.

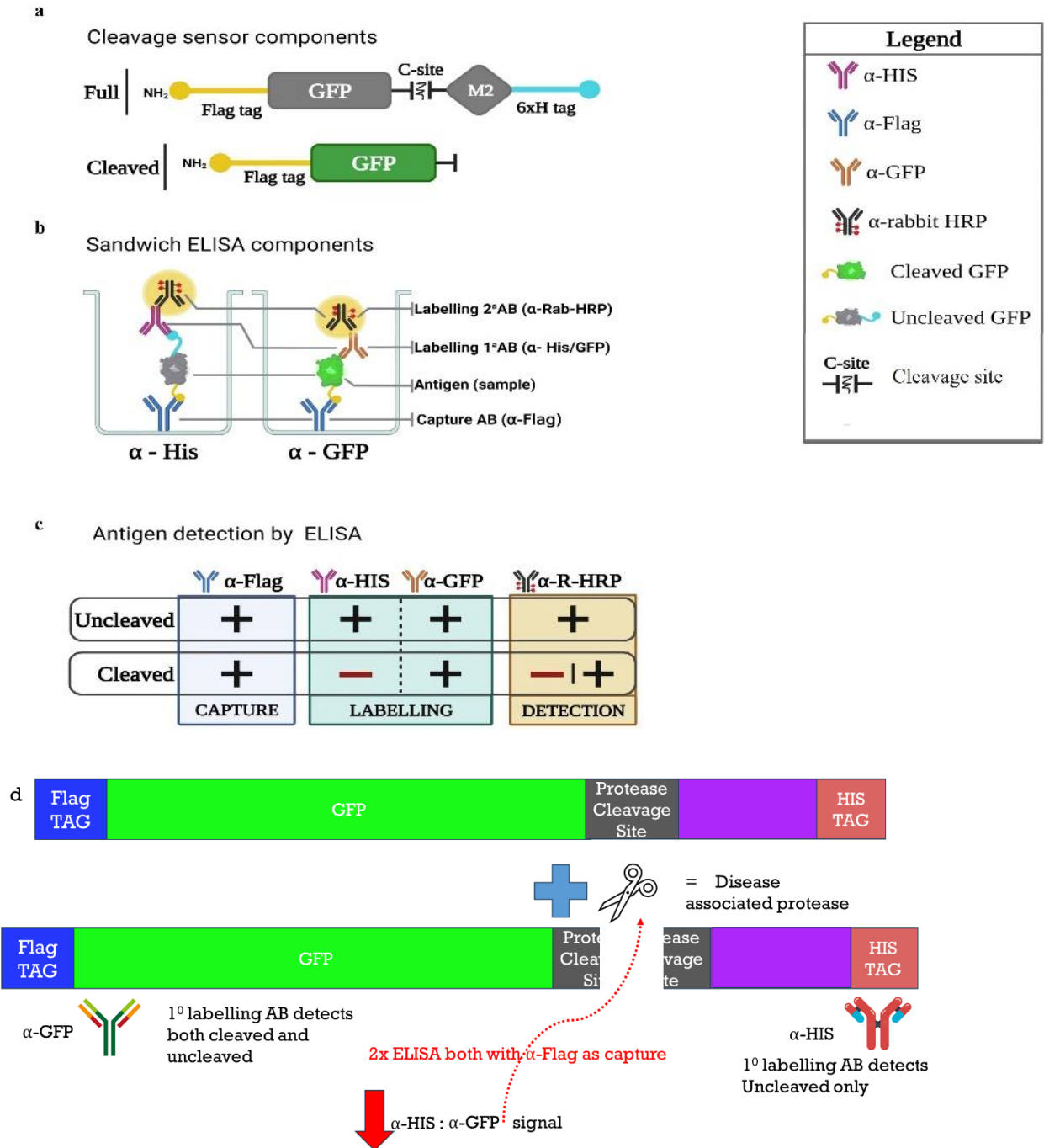


Figure 4. Immunoassay components. *a)* Protease sensor diagram comparing full sensor to sensor post cleavage with HIS tagged removed *b)* Sandwich ELISA components. Antibodies used for the reactions: α-HIS antibody detects the full sensor and α-GFP detects both cleaved and uncleaved sensors. *c)* Antigen detection by ELISA. Illustration of sensor detection (full or cleaved with α-

HIS or GFP). Cleaved sensors would only be detected with α -GFP and not α -HIS. d) Cleavage of protease sensor results in removal of HIS tag with corresponding reduction in α -HIS signal.

A reduction in the α -HIS signal of cleaved protease sensors was detected and quantified for each of the three sensor versions. Numbers reported are percentage reduction in protein quantities after normalisation to uncleaved controls, i.e. uncleaved control as 0% reduction. Mpro and TEV versions displayed statistically significant signal reduction of 74% ($p = 0.01039$) and 80% ($p = 0.0205$) respectively. The MMP9 version showed a signal reduction of 14%, although not statistically significant ($p = 0.2719$) (Figure 5). The ELISA proved to be capable of detecting protein quantities as low as 10 ng, as shown in each of the standard curves (sFigure 1).

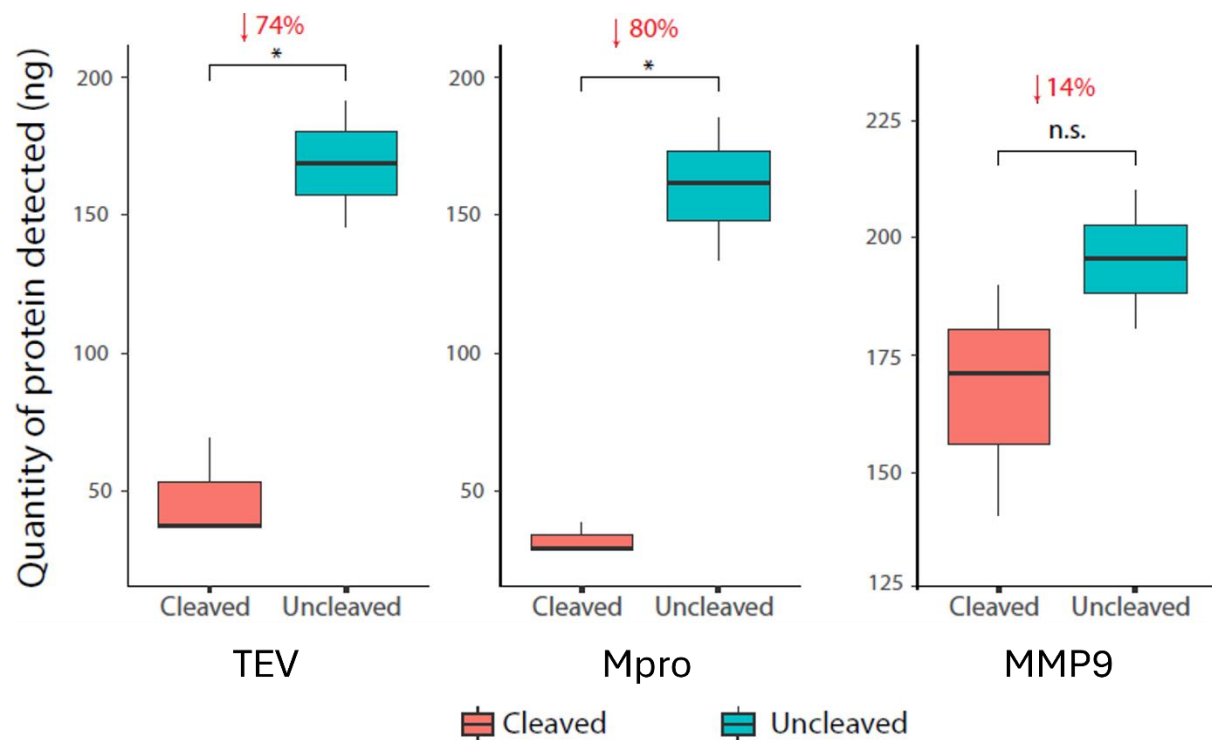


Figure 5 Immunoassay readout of various protease sensors. Cleaved (HIS-Flag+) and uncleaved (HIS+Flag+) protein was quantified by the immunoassay +/- test protease for each sensor

construct. The assay successfully detected cleavage (reduction in α -HIS signal) in all cases, with cleavage patterns matching Western blot analysis (Figure 4).

2.5 DISCUSSION

As all life on the planet is protein based, proteases are universally distributed throughout all living organisms. Their activity is specific and, as a result, can consequently be detected precisely. This work combines the sequence specificity of any given protease with the simplicity of protein production and the sensitivity of a sandwich ELISA in a novel and straightforward fashion, using already well characterised antibodies. Protease activity is detected by discriminating between signal reductions in decoupled cleaved dual tagged positive devices relative to uncleaved.

A synthetic biology approach was used to design the DNA constructs necessary to recombine a protease responsive sensor element separating two peptide tags. This study has shown its adaptability by using 3 disparate proteases, 2 viral and 1 human, associated with 3 different types of disease –a plant pathogen, human cancer, and Covid 19. The results were validated with 3 different readouts: Western blot, fluorescence, and a purpose designed sandwich ELISA.

While Western blot densitometry demonstrated approximate meaningful differences between cleavage ratios, the ELISA, which was performed in triplicate and measured against individual standard curves for each sensor, revealed cleavage ratios in a more sensitive and accurate manner. Densitometry is useful for approximating and comparing relative amounts of protein, whereas an ELISA is far more sensitive and quantitatively accurate (Butler et al., 2019, Oh et al., 2017). Limits of detection (LOD) were determined to be 20 ng for the α -HIS and 8 ng for the α -GFP (sFigure 1).

Warren *et al.* described a sandwich ELISA similar to that described in this study, albeit using different capture antibodies, also to detect protease activity (Warren et al., 2014). A crucial difference is the fact that the peptide protease substrate was conjugated to a synthetic iron oxide “nanoworm” with additional synthetic elements including a non-degradable spacer composed of D-isomer amino acids, meaning that this type of reporter cannot be genetically encoded. The fact

that the protease sensor in our study can be produced in *E. coli* makes production simple and cost effective.

The assays and components employed in this study to validate the strategy provide proof of concept for the applicability of such an approach. This study employed ‘industry standard’ well described tags and matched antibodies. Other tag antibody combinations could readily be employed. 6xHIS is immunogenic and while suitable for *ex vivo* assays as described here, could be replaced by alternative tags and matched antibodies for *in vivo* application.

Future iterations might better avoid the fluorescent element with a quencher examined here to facilitate easier protein purification and increased cleavage ratio. Indeed, we encountered some difficulties due to the M2 quencher, combined with a high copy number plasmid resulting in accumulation in inclusion bodies necessitating time consuming denaturing purification steps. The M2 quencher also reduced cleavage ratios because of the tetramerisation of the protease sensor. In the future, these elements would be replaced with neutral non-functional peptide spacers of optimum length for cleavage discernment and antibody detection.

Recent Ebola, Zika and COVID-19 epidemics have illustrated the need to develop cost effective rapid diagnostics (Kelly et al., 2022). Diseases which are idiopathic in nature, e.g. Lyme disease, are generally difficult to diagnose and lead to avoidable complications if left undiagnosed (Rogalska et al., 2021). Orphan diseases, which affect no more than 0.05% of the population (Schaaf et al., 2020) and where there is less commercial incentive to develop diagnostics, are another area where synthetic biology approaches could offer the potential for innovative solutions.

The pervasiveness of proteases in living organisms means that a device based on this principle could be universally applied to detect protease activity across all domains of life. Once the cleavage sequence is known, this platform is adaptable for any given protease. The specificity of proteases, by their very nature of amino acid sequence recognition, endows this platform with the same level of precision for any given particular protease. Any protease associated with a disease state, to be deployed in this type of protease responsive synthetic protein technology, would need to be approached individually, taking into account its level of specificity and any overlap with other proteases in its testing environment.

As time goes by more efficient and economical methods of mapping protease specificity are being developed (Zhou et al., 2020, Ratnikov et al., 2021, Adachi et al., 2006a). Armed with this increasing knowledge index of a given proteases proclivity for its substrate profile, these factors can be taken into account and when used in conjunction with an array of tests for a specific disease can lend credence to a more accurate diagnosis with higher confidence.

Finally, the platform's simplicity using pre-existing, well characterised antibodies make this sensor device highly applicable. This novel method could be could potentially be applied to any biological liquid sample, such as blood or saliva for detection of protease activity to reveal pathogens, disease biomarkers etc. It could also be applied in environmental settings such as waste water from industry, hospitals or homes to detect environmental biological contaminants. Development as an at home test means it could be used as a stand alone or as a prelude to other types of testing. Further development and optimisation are required to produce a rapid, cost effective and easy to operate test. Still, as outlined in this work, it is within the bounds of current technologies. Large scale screening, with high population coverage and early diagnosis, is critical to the commencement of appropriate treatment or intervention with the ability to do this outside of the laboratory setting, at home or in developing countries where resources for healthcare are limited, there is the potential to save many lives.



Devoy, C. 2024. Bacteria and bacterial-produced proteins as biomarker technology for precision oncology. PhD Thesis, University College Cork.

Please note that Chapter 3 (pp. 88-119) is unavailable due to a request by the author.

CORA Cork Open Research Archive <http://cora.ucc.ie>

4 CHAPTER 4:

A study within a study: Logistics of patient sample acquisition in academic clinical microbiome studies: Identification of determinant factors of success.

4.1 ABSTRACT

Introduction: Clinical microbiome wide association studies (MWAS) play a crucial role in understanding the relationship between gut microbial communities and cancer development and treatment outcomes. However, challenges in the study setup phase (SU), patient management (PM), and sample acquisition remain significant factors that influence the success of these studies. This chapter investigates the determinants of success or failure in patient and sample acquisition across three hospitals, focusing on comparative analysis.

Aims: This study aims to analyse factors influencing patient participation, SU efficiency, PM systems, and their impact on patient and sample acquisition in clinical microbiome studies. Through comparative analysis, this chapter seeks to provide insights for optimising logistics and enhancing reliability in future academic clinical microbiome research.

Methods: Three MWAS Clinical Observational Studies (COS) were established in the course of this thesis work. Data from these studies, relating to three separate hospital sites, one private and two public, were compiled and analysed.

Results:

Hierarchy and number of team members: Variation in team composition and hierarchical structures substantially impacted SU time and patient/sample accrual rates. In the context of an observational clinical trial, "hierarchical structures" refer to the organisational framework within which the trial is conducted, especially concerning the roles, responsibilities, and relationships between different members of the clinical team. This structure determines how decisions are made, how information flows, and how tasks are allocated and executed within the trial.

Patient flow through the study: Patient recruitment and retention strategies, including the role of clinical personnel, influenced study continuity and sample acquisition.

Overall patient numbers: Differences in patient enrolment rates were observed, with Site C demonstrating continuous growth compared with Site B's staggered pattern and Site A's premature termination.

Overall sample numbers: The sample collection intervals varied between sites, influenced by treatment regimens and study dynamics. Site C exhibited continuous sample accumulation, surpassing other sites despite a later start, with the treatment regime and the timing of sample collection playing a significant role.

Overall clinical site rates: Site C emerged as the most successful, with higher sample and patient acquisition rates, emphasising the importance of proactive recruitment strategies.

Delays and Impact on Study SU Timelines: Delays in SU were observed across all sites, attributed to various factors including regulatory processes and team dynamics.

Overall key findings include that the involvement of dedicated research nurses in the PM phase increases success (sample rates), and the SU team size is inversely proportional to the length of time taken for clinical study SU.

Conclusion: Understanding and addressing factors influencing patient and sample acquisition are crucial for the success of clinical microbiome studies. Through comparative analysis, this report provides insights into optimising logistics and enhancing reliability in future research endeavours. While anecdotal evidence similar to the outputs of this chapter exists, to the authors' knowledge, this is the first study to analyse such data from studies systematically, certainly in an Irish context.

4.2 INTRODUCTION

Clinical microbiome-wide association studies (MWAS) have become crucial for understanding the complex relationships between gut microbial communities and cancer development, including treatment outcomes (Aggarwal et al., 2023). However, challenges from the initial SU to patient enrolment and sample acquisition significantly impact the success and reliability of these studies. Clinical research of value is characterised by its relevance to pertinent medical problems, significant results, pragmatic approach, patient-centred focus, cost-effectiveness, feasibility, and transparency. Unfortunately, many clinical studies or trials do not meet all these criteria (Ioannidis, 2016).

Observational clinical studies, much like randomised trials, face numerous challenges in setup and recruitment. The concept of a Study Within A Trial (SWAT) is relevant here (Treweek et al., 2018). A SWAT is an embedded research study within a host trial that aims to evaluate or explore alternative methods of delivering or organising a specific trial process, addressing methodological questions outside the main trial's objectives. This report goes beyond a conventional SWAT by comparing three similar studies to identify key factors of success and improve the studies as they progress, highlighting the importance of conducting trials effectively (Treweek et al., 2018). Unfortunately, a lack of evidence on optimising trial methodologies leads to uncertainties for researchers, funders, and participants regarding trial efficiency and effectiveness.

Most of the literature emphasises patient willingness to participate in clinical trials, which is essential for ensuring study success and applicability of findings. Difficulty in recruiting patients can lead to study abandonment and selection bias, compromising the reliability of results (Cullati et al., 2016, Galea and Tracy, 2007). Previous studies reveal that sicker patients and those with altruistic motivations are more likely to participate (Trauth et al., 2000, Locock and Smith, 2011). Principal investigators (PIs) believe that staff support, and informatics driven approaches enhance participant recruitment (Idnay et al., 2023). The enthusiasm and motivation of the consultant, research nurses, and junior doctors significantly influence patient consent rates (Kurt et al., 2017). Patients value the public release of trial results and believe that the informed consent process should address potential non-publication (Jones et al., 2016). Prior ethics committee approval and public release of results increase participation, while a lack of study feedback negatively affects

patient perception (Madsen et al., 1999). Clear study information and addressing logistical barriers, such as additional costs, can improve patient willingness to participate (Mattson et al., 1985, Moorcraft et al., 2016). Conversely, complex or inadequate study information and personal inconvenience such as extra appointments and the burden of intervention, deter participation (Gayet-Ageron et al., 2020, Locock and Smith, 2011). Financial incentives may motivate adherence to study protocols (Green et al., 2019, Thompson et al., 2022). Certain tests, especially those requiring stool samples, can deter participation, particularly among very ill patients (Thompson et al., 2022, Bolte et al., 2021). Understanding patient motivations and addressing participation barriers are essential to achieve target sample sizes and ensure clinical study success.

However, patient willingness may not be the primary barrier to clinical study success. Before laboratory work, two critical, yet often underappreciated, and under recorded stages require attention: i) the SU, involving administrative document preparation and regulatory requirements, and ii) patient and sample management (PM). This chapter first compares the initial SU process, considering the hierarchical structure, time taken, unforeseen delays, and lessons learnt. Second, it analyses the PM systems used in different hospitals, specifically contrasting the use of nurses versus specialist registrars (SpRs) for PM from consent to the completion of the trial. While one private and one public hospital utilised nurses for patient consent, another public hospital relied solely on SpRs. This chapter also examines the effects of investing in additional nursing resources and using an electronic data capture platform.

Through this comparative analysis, our goal is to elucidate the impact of various factors, including the hospital setting (private vs. public), PM (with and without nurses), and additional upfront investment on the efficiency and success of patient and sample acquisition in clinical studies. Moreover, the majority of studies on trial participation come from high-income countries, particularly the United States, limiting the generalisability of findings to countries with different health systems and resources. No relevant studies were identified in Ireland as of 2016, and this situation has remained largely unchanged to date. In the hierarchy of research designs, randomised controlled trials are considered the gold standard, while observational studies are often seen as less valid due to the potential overestimation of treatment effects (Concato et al., 2000). The term 'conditional altruism' describes the willingness to help others that initially motivates people to participate in trials, but practical participation hinges on the perceived personal benefits.

Recognising this concept is crucial for planning recruitment communications to promote informed and voluntary participation (McCann et al., 2010). The patients taking part in this study will not see any benefits from the study themselves, and having been consented to take part, are doing so in the hope that their contribution will help cancer patients in the future.

Identifying these determinant factors will provide valuable insights for optimising logistics and enhancing the reliability of future academic clinical microbiome research. This report does not aim to cast blame on any individual or organisation. Clinical investigations require teamwork and dedication, involving significant time and financial investment, and patients' hope to contribute to future cancer treatments. We owe it to the patients and all stakeholders to ensure the success of these studies. The rare opportunity to establish and dissect three studies during this PhD project is invaluable for extracting critical success factors.

4.3 METHODS

This chapter follows the STROBE checklist guidelines for reporting observational studies, as it is a report covering three studies at separate sites (Vandenbroucke et al., 2007). The methodology included measuring the time from the initial SU of COS to obtaining the consent of the first patient while analysing all the stages and obstacles encountered throughout the process. Furthermore, we examined all the individuals, systems, and institutions involved in this process hierarchically. The PM stage of the study involved measuring the rates and total number of patients and sample uptake. Since this chapter compares three studies, a basic understanding of the COS design and methodology is also necessary.

4.3.1 *COS design*

Key elements of the COS design are common across all three studies, and important differences will be addressed in the discussion. The primary endpoint is to sequence the gut microbiome (GM) at three time points in the treatment regime of a cancer patient and correlate these findings with

the efficacy of the treatment to find biomarkers of positive treatment results or negative side effects. Secondary endpoints include the effect of treatment on GM composition.

At the time of consent, patients received three OMNIgene®•GUT (OMR-200) home testing kits to collect a stool sample, each labelled with their unique study identifier number. The stool collection kits streamline DNA collection for gut microbiome analysis, allowing easy self-sampling at home. They rapidly stabilise DNA at collection, ensuring an accurate representation of *in vivo* microbiota profiles. With barcoded traceability, these kits maintain DNA integrity for up to 60 days at ambient temperature. Their benefits include improved donor compliance, minimising bias from microbial growth, and DNA degradation. Standardised volumetric samples eliminate weighing and aliquot bias, maintain stability in ambient temperature fluctuations, and provide reliable microbiota profiles for analysis. Consenting patients received three stamped addressed envelopes in the appropriate packaging to send stool samples directly to the Cancer Research @UCC (CR@UCC) laboratory for analysis. At each time point, approximately 500 mg of stool sample were collected using the kit. The lab for analysis is common to all three studies. On receipt of the sample, it is immediately frozen in a -80 °C freezer until the DNA extraction step.

4.3.2 Sites

All study sites involve cancer care centres in the Republic of Ireland. Site A is a public hospital where a clinical study was already in progress. Therefore, all that was required for the initial SU was an amendment to the original ethics. The initial SU began in March 2020, and the study ended in October 2022, having completely stopped recruiting. Site B, the second public hospital began the initial proceedings in November 2021 and is still recruiting. Site C, the only private hospital, also began in November 2021 and is still recruiting. Participants eligibility criteria are shown in Table 1.

Table 1. Inclusion and exclusion criteria by site

Site A

Inclusion Criteria		Exclusion Criteria	
a)	Histologically or cytologically confirmed malignant advanced solid tumours due to commence ICI therapy	g)	Prior surgery, radiation, chemotherapy, or other anti-cancer therapy within 4 weeks prior to enrolment in the study. Endocrine/hormonal treatments may be continued.
b)	Documented tumour progression as defined by clinical, biochemical, or radiological (RECIST) criteria.	h)	All patients who have received prior immunotherapy / ICI must be discussed with the sponsor regarding eligibility to enrol on this translational study
c)	Measurable disease (clinical or radiological)	i)	Diagnosis of immunodeficiency or receiving systemic steroid therapy or any other form of immunosuppressive therapy within 7 days before enrolling in the research study. The use of physiologic doses of corticosteroids may be approved after consultation with the chief investigator.
d)	Life expectancy greater than 3 months	j)	No known history of human immunodeficiency virus (HIV) (known HIV1 / 2 antibodies positive), no known active hepatitis B (defined as known positive HBsAg) or hepatitis C (defined as known positive Hep C Ab and known quantitative HCV RNA results > lower limits of detection of assay)
e)	Age ≥18 years	k)	Active autoimmune disease that has required systemic immunosuppressive treatment in the past 2 years (i.e. with the use of disease modifying agents, corticosteroids or other immunosuppressive drugs). Replacement therapy (e.g. thyroxine, insulin, or physiologic corticosteroid replacement therapy for adrenal or pituitary insufficiency, etc.) is not considered a form of systemic treatment.
f)	Signed informed consent	l)	Active infection requiring systemic therapy
		m)	No previous allogeneic tissue / solid organ transplant

Site B

Inclusion Criteria

Exclusion Criteria

a)	Patients due to commence neoadjuvant chemotherapy or chemoradiation for documented histologically confirmed early-stage locally advanced breast cancer or early-stage locally advanced GI cancers diagnoses. Including:	h)	Previous diagnosis of cancer that was treated with chemotherapy, radiation therapy, chemoradiation combination therapy or immunotherapy before or any other anticancer treatment.
b)	Breast Cancer – all HER-2 positive, ER and PR positive subtypes and TNBC treated with neoadjuvant treatment, including monoclonal antibodies	i)	Active infection requiring systemic antibiotic therapy
c)	Rectal Cancer treated with neoadjuvant therapy	j)	Previous allogenic tissue/solid organ transplant
d)	Oesophageal Cancer treated with a neoadjuvant approach including gastro-oesophageal junctional carcinoma		
e)	ECOG Performance Status 0-2		
f)	Age $\geq$ 18 years old		
g)	Willingness and consent to comply with study protocol with signed informed consent obtained		

Site C

Inclusion Criteria		Exclusion Criteria	
a)	Able to give written informed consent	f)	Less than 18 years of age
b)	Be over 18 years of age.	g)	Patients who, in the opinion of the clinician, would not be able to comply with the study requirements or who would be distressed by being invited to participate
c)	Life expectancy greater than 3 months	h)	Previous ICI treatment
d)	Scheduled to receive any line of palliative ICI alone or in combination with Tyrosine Kinase Inhibitors (TKI) and/or cytotoxic chemotherapy	i)	Patients on clinical ICI trials (academic or commercial) are excluded unless the trial sponsor gives written permission
e)	Able to provide stool samples at home and post to the CR@UCC laboratory		

Bias The protocol dictates three sampling time points per patient to address, to an extent, sampling bias. Additionally, the OMR-200 kits contain preservatives to minimise bias from microbial growth and DNA degradation. Standardised volumetric samples eliminate weighing and aliquot bias.

Study size Sites A had a study target of 200 patients, Site B 120 patients, while Site C had a goal of 50 patients.

4.4 RESULTS

4.4.1 Hierarchy and number of team members

To compare the three studies, it is important to first compare the systems in place during SU and PM. Figure 1 illustrates the hierarchical structures in place for every level at each of the study sites.

Each site had a team. The number of people on a site team varied (variable). The number of ‘tasks’ required was deemed to be the same for all sites (constant). Task owners varied in number and nature between sites and are represented as Full-Time Equivalent staff (FTE) (variable).

Constants: Commonalities among task owners that exist across all studies are i) the consultant medical oncologist, ii) the university PI; and iii) the university Ph.D. student.

Variables: Some sites and tasks had more than one team member available to execute a task.

Site A:

SU phase: This involved an amendment to an existing study, where stool was to be added as an additional sample.

SU team size: 3. Consultant medical oncologist, university PI, university Ph.D. student.

Dedicated FTE: 0. Tasks were shared among individuals with significant additional responsibilities, including the consultant medical oncologist, the scientific PI, and a science PhD student.

PM phase: *PM team size:* 1. A clinical study nurse was available for a portion of the study timeline.

Site B:

SU phase: New study.

SU team size: 5. Consultant medical oncologist, biostatistician, research contracts officer, programme manager - cancer clinical trials & research unit, SpR, and a clinical data manager / translational research coordinator.

Dedicated FTE: 4 (devoted to multiple trials)

PM phase: *PM team size:* 1

Site C:

SU phase: New study.

SU team size: 4. Consultant medical oncologist, biostatistician, data manager, and UCC CTG academic studies coordinator.

Site C did not have a dedicated SU team to begin with, and it was assembled over time, with the specialised academic unit joining halfway through the SU process.

It is important to emphasise the pivotal role of the ‘Academic Studies Coordinator’, who had a strong understanding of the interconnections between academic and clinical settings. This role facilitated the biostatistician interaction from an early SU stage to build a clinical patient database using the Castor software.

Unique to this study was a second, university-based biostatistician for developing the clinical trial platform Castor database.

Dedicated FTE: 4 (devoted to multiple trials)

PM phase: *PM team size:* 4. Private hospital consultants involved in patient consent.

This site had the largest PM team, including a rota of four consultant oncologists and four research nurses (not all working at the same time).

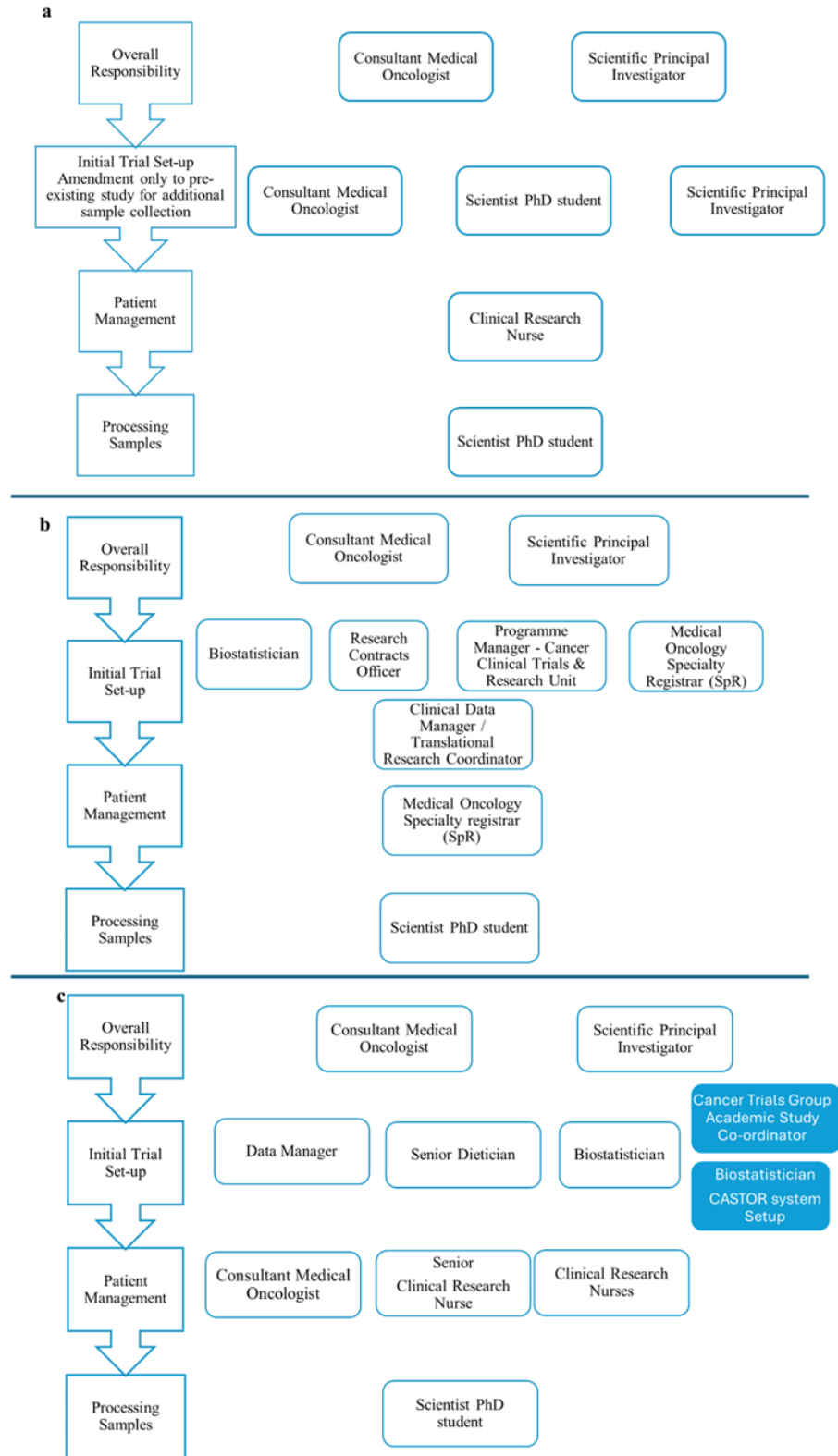


Figure 1. Hierarchical structure of each COS by study site

4.4.2 Timelines of the SU phase

The length of time taken to complete the SU of the study is a crucial factor that must be measured with careful consideration of delays to identify potential system improvements. Figure 2 shows a timeline for each of the studies, from the decision to collaborate to the first or last sample collected, depending on whether the study is still in operation. Currently, sites B and C are still enrolling patients and collecting samples. Table 2 tabulates the time for key milestones and delays. The required paperwork for a study is as follows:

1. Clinical Research Ethics Committee (CREC) application
2. Observational clinical study protocol
3. Patient information letter (PIL)
4. Letter to Patient's GP
5. Data Protection Impact Assessment (DPIA) plan
6. Material Transfer Agreement (MTA)
7. Patient consent form

The ethics application committee meets monthly; hence a missed submission deadline results in a one month delay. Data here indicate an inverse relationship between SU team size and the number of rounds of CREC approval meetings.

Site A took the longest to complete SU, even though it was an amendment to an existing study that entailed extra details being added to the study protocol and patient consent form, plus an amendment form that had to be approved by CREC. Mitigating factors to explain some of the delays were COVID-19 and the HSE ransomware attack. The ethics amendment consequently took more than a year to be approved, at 390 days, and an additional 263 days to obtain the first patient sample. The study then collapsed 82 days after starting, once the study nurse left.

Site B had the ethics application submitted in the shortest time, less than one month, only to have a delay of 329 days on the academic side for the MTA, which stretched the ethics approval to 338

days. The first subsequent patient sample was 51 days, the fastest of all studies. As of March 31, 2024, it has been running for 317 days.

Site C, the only study conducted in a private setting, took the longest to set up when compared to Site B, the only other site requiring a full application. The extended time needed to assemble the SU team members led to a slow start, with an overall duration of the SU of 459 days and a time to the first sample of 135 days. The UCC Cancer Trials Group (UCC CTG¹), which included dedicated team members, especially the academic coordinator, joined the SU phase to expedite the process, completing it in 185 days. Site C also invested extra time developing a food questionnaire and a clinical platform with the necessary database, which occurred concurrently with the CREC application. This parallel development contributed to some delays in obtaining ethical approval. As of March 31, 2024, Site C has been operational for 214 days.

¹ The UCC Clinical Trials Group (CTG), established in 2022 and funded by the Health Research Board (HRB), provides critical infrastructure to support the high-quality execution of patient-focused research at University College Cork (UCC) and its affiliated hospitals within the South and South-West Hospital Group (SSWHG). Nestled within the academic environment of CR@UCC, the group is strategically positioned to drive advancements in cancer research.



Figure 2. Timeline for each of the COS, from the decision to begin the study, to the first patient consent or sample, highlighting key events. Events are in chronological order but not to scale a) Study Site A. b) Site B. c) Site C.

Table 2. Length of time for key milestones and delays.

Site A		Site B		Site C	
Step	Days	Step	Days	Step	Days
Ethics Amendment	390	Ethics Application	338	Ethics Application	459
Time to 1st sample post approval	263	Ethics approval to 1st sample	51	Ethics application to 1st sample	135
Trial Duration	82	Trial duration to 31/03/24	317	Trial duration to 31/3/24	214
Covid and ransomware delays	~200	MTA delay on academic side	329	CTG time to CREC approval	185

4.4.3 Patient flow through the study

Once overall approval had been reached from the necessary bodies, cancer patients undergoing treatment at the various sites can be assessed and approached to join the study and they may agree to provide stool samples with informed consent. A patient flow chart illustrates the patient's journey from eligibility considerations to the data reporting and publication stage, detailing the sample collection points relative to the treatment schedule (Figure 3.). This basic structure is common to all studies, with three samples to be collected per patient. Treatment time between samples will vary depending on the type of treatment regimen. The selection of eligible patients is carried out by the consultant medical oncologist, who determines if the patient is well enough to participate and meets all eligibility criteria. Dropout points for this type of study can occur at any point up to the last sample collection. Common barriers to consenting despite being eligible or dropping out would be the impact on a person receiving a cancer diagnosis, plus that many people would find collecting a stool sample to be understandably off-putting, in agreement with other studies (Thompson et al., 2022). Many cancer patients are quite elderly, and depending on the state of the disease, could be quite unwell. This is the main reason for dropouts post consent.

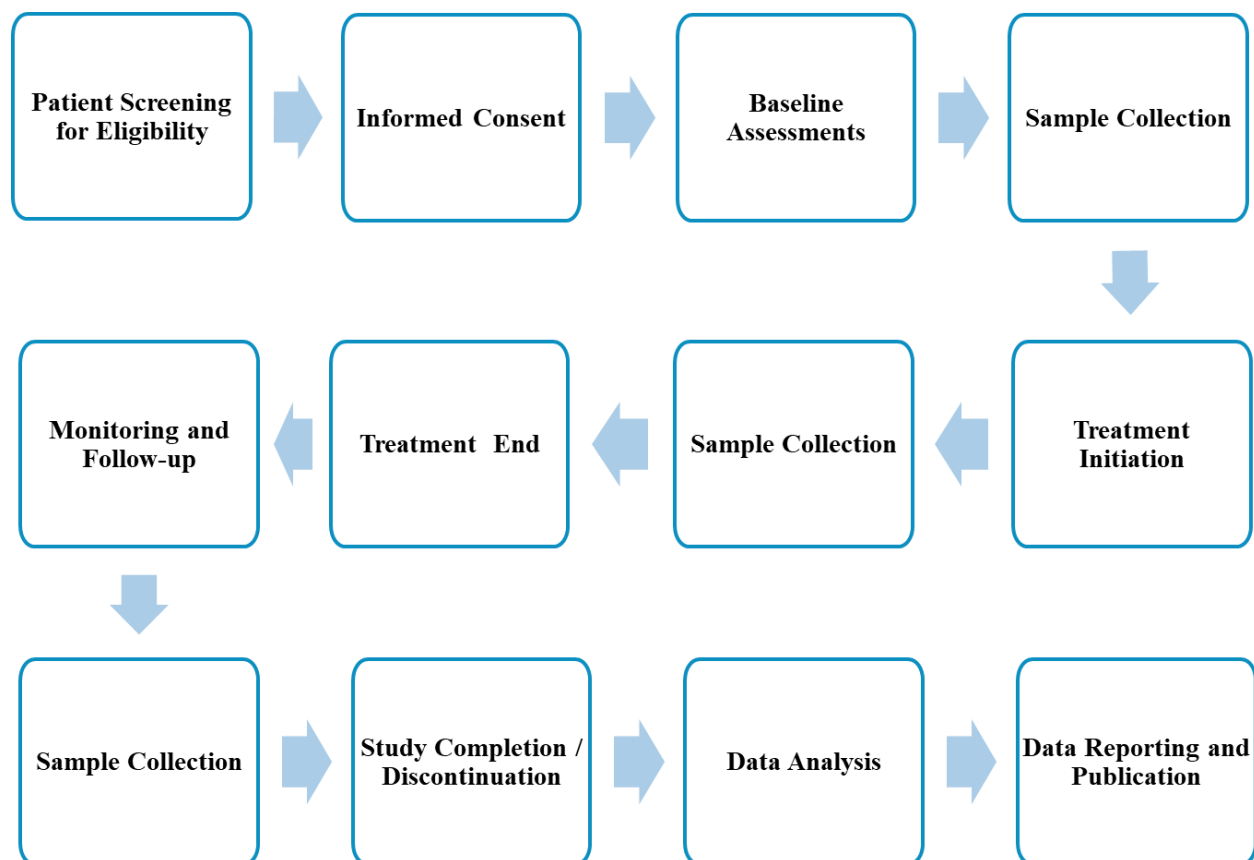


Figure 3. Patient Flow Diagram. Detailing the patient’s journey through the various stages from patient screening after diagnosis to final report and publication. The diagram details the sample collection points relative to the course of treatment. For this type of study drop-out points can occur at any time, up to the last sample collection point. The time between sample collection points varied between studies, depending on the treatment regime.

4.4.4 Overall patient numbers

Once patients are enrolled in the study, they receive OMR-200 stool collection kits from DNAgenotek, which allows stool sampling in the patient’s own home with subsequent posting of the sample directly to the CR@UCC lab for processing. The kits are common in all study sites. The patients are anonymised and the kits they receive are bar-coded in order to match kits to patients at any stage. Consented patient numbers are tabulated monthly, see Figure 4.

Figure 4a details the timeline of consented patients who provided at least one sample from the first consented patient in January 2022 to the present. Patients that were consented and subsequently dropped out prior to providing any sample, are not included in these total numbers. Site A consented four patients in total before it finished, just over two months later. Up to the point where the only study nurse left, the number of patients accrued was very steady, and the study looked promising. Site B was the next to commence, consenting two patients immediately, followed by two months of zero growth. Growth then continued until October 2023, where there was another zero-growth period followed by one more consented patient in December 2023 and another zero-growth period until the end of March 2024, resulting in a staggered pattern. Site C had continuous levels of growth at varying rates, but with no breaks the in continuity of the study.

Comparing the studies from their starting month (M1), Site C was the best performing (Figure 4b). At M3, where all studies were active, Site C had six patients, Site A had four, and Site B had two. Continuing to the next time point possible for comparison, at M7, Site C had 13 patients compared with 10 for Site B for the same amount of time.

Patient numbers by category are shown in Figure 4c. Categories include total screened for eligibility, missed screening, eligible patients, patients who were deemed eligible but who declined, consented patients, and finally patients who were consented and provided a sample. The only site at present that has provided all of these numbers is Site C, highlighting that there were 30 potential patients that could go on study; six missed screening because of limited PM resources, five did not meet the inclusion criteria, and three declined to take part. 15 consented, with two subsequently dropping out before providing a sample. 13 are currently on study, with one more patient being screened as of the 31st of March 2024.

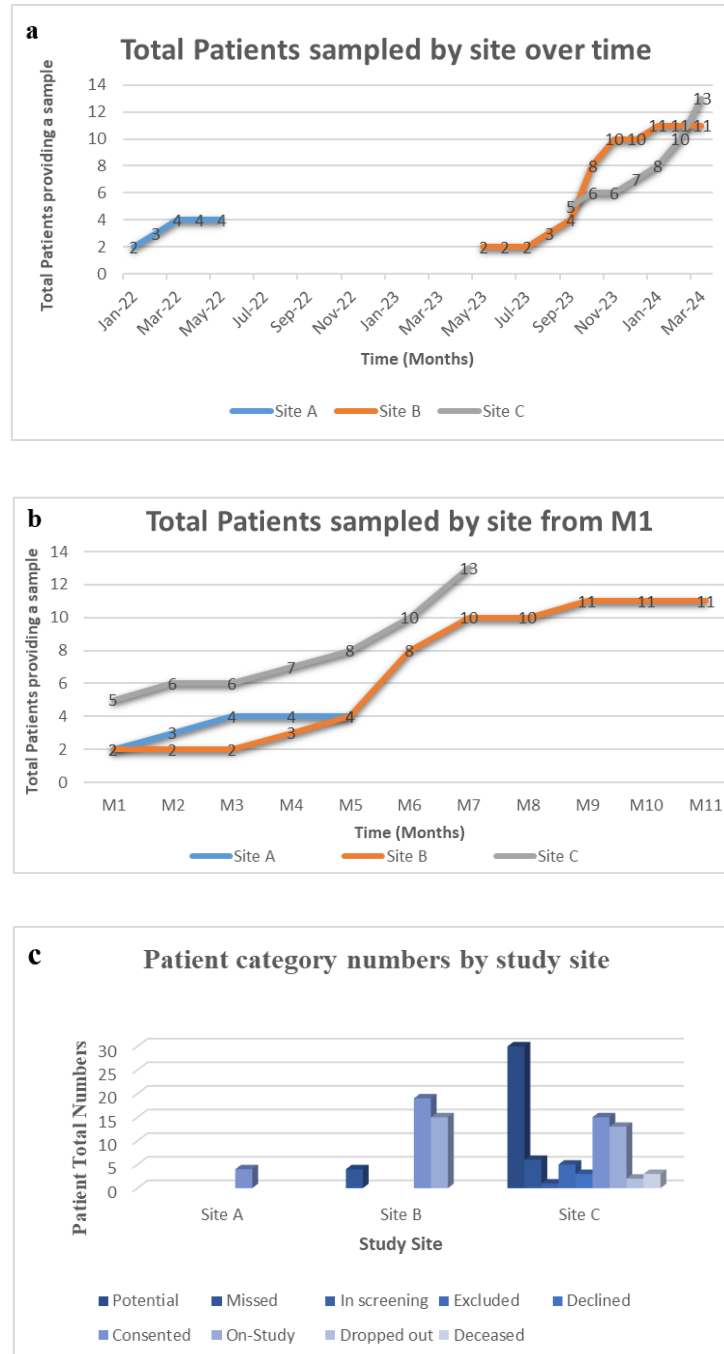


Figure 4. Total number of patients. a) Over time, from the beginning of the first study to the end of March 2024. b) Relative to each study from M1 to the starting month. c) Total patient numbers to the end of March 2024 broken down by category from screened to having provided a sample.

4.4.5 Overall sample numbers

Consented patients, on request by the PM team, provide samples prior to treatment, during treatment, and once treatment has been completed. Site A had four patients in total, three of whom provided the three samples, with the average time for the three patients between sample one and sample two being 33 days, and the time between sample two and sample three being 47 days. Site B had five second samples, and only one patient with all three samples, partly explained by the longer treatment regime (the actual treatment schedule is unavailable at the time of writing). Site B had an average time between time points one and two of 88.6 days, with a wide range of 40 to 162 days. Site C had an average of 23.7 days between the first and second samples, with an average interval of 59.6 days between the second and third samples.

The graphs for each site show the same patterns for sample accrual as for patient recruitment previously. Site A showed steady accrual until failure. Site B started well and followed a staggered pattern with three distinct breaks in continuity where growth flatlined. Again, site C performed the best, with continuous growth and no interruptions in continuity (Figures 5 a, b, and c). Figure 5d indicates that even though Site C commenced three months later than Site B, it rapidly surpassed Site C in sample numbers. Part of this can be explained by the shorter treatment regime, since Site B has two more patients than Site C, but five fewer samples. When comparing the sites with each other from M1 (Figure 5d), it is evident that, according to the patient graphs, site C is the best performing, with site A showing initial promise, while site B has a discontinuous staggered pattern. Looking at relative sample accumulation from the M1 starting month, by M5, Site A had 10 samples, Site B had five samples, and Site C had 17 samples. By M7, Site C had a total of 22 samples, almost double Site B's total of 12 samples.

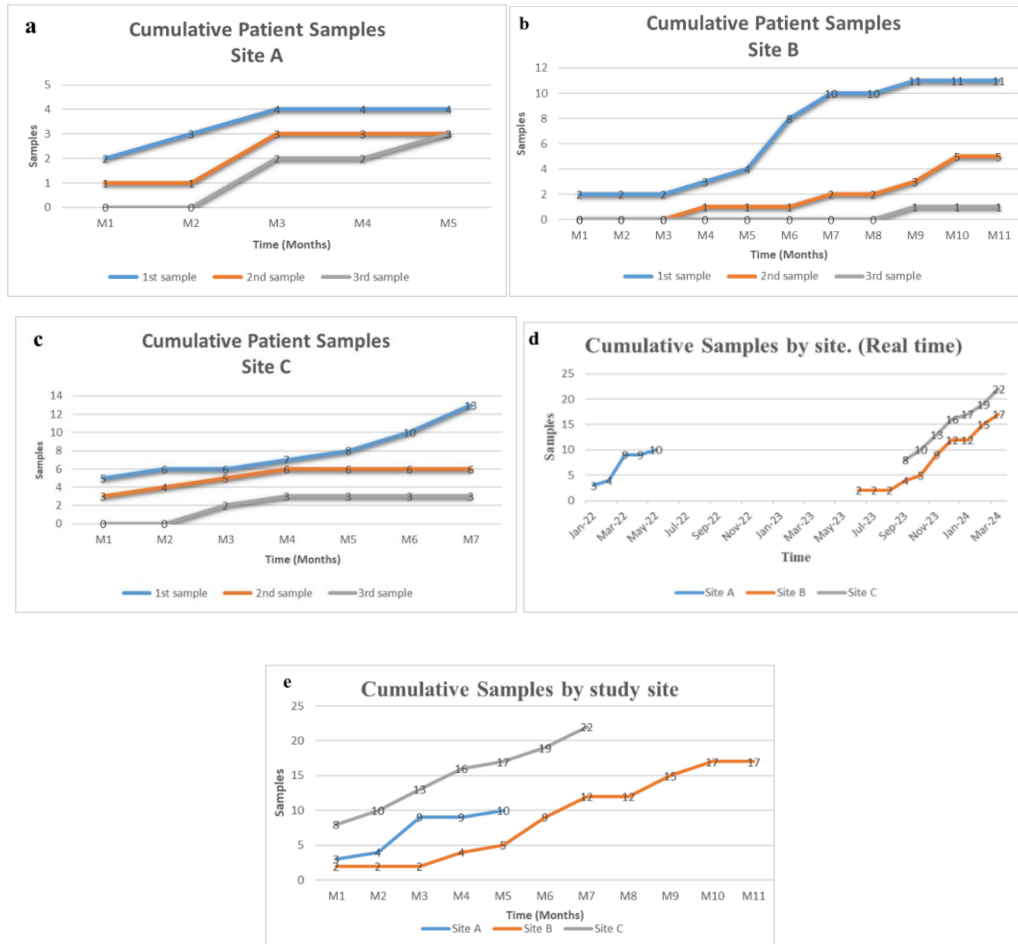


Figure 5. Total sample numbers. a. b. and c.) Cumulative sample numbers over time broken down by sampling number (1st, 2nd, 3rd) for each site. d) Illustrates cumulative sample collection for all sampling time points from the beginning of the first study to the end of March 2024. e) Relative to each study from M1, the starting month.

4.4.6 Overall site rates

Knowing the numbers of patients and samples accumulated over time, it is possible to calculate the rates of patient and sample growth per month for each site (Figure 6a). Here, it is evident that study site A, for the brief period of time that it was operating, had comparable patient accrual rates with site C and had the highest rate of sample collection of all studies, and no other numbers are provided for attrition. Site B has the lowest rate of sample accrual at an average rate of 1.55 samples per month and a low average monthly patient recruitment rate of 1.18. There have been rates as high as four per month, but during the study, there have been four months of zero growth, leading to a low average. Of the two studies that are currently running, Site C is the most successful of the two when it comes to rates, with an average of 3.14 samples per month and 2.14 patients with an average attrition rate of 0.29 patients per month, with two patients of the 15 who consented to have dropped out.

Based on these current rates, it is possible to calculate the estimated completion time of the study (Figure 6b) for the 120 patient target set for Study Site B, which at current rates would not finish until November 2031. Site C, at current rates and a target of 50 patients, is due to finish in August 2025, if Site B were to reduce its target to 50 patients, it would complete in December 2026. Increasing recruitment rates by one patient per month would reduce the completion time by 3.5 years for the 120 target at Site B and six months for the 50 patient target at Site C (Figure 6c).

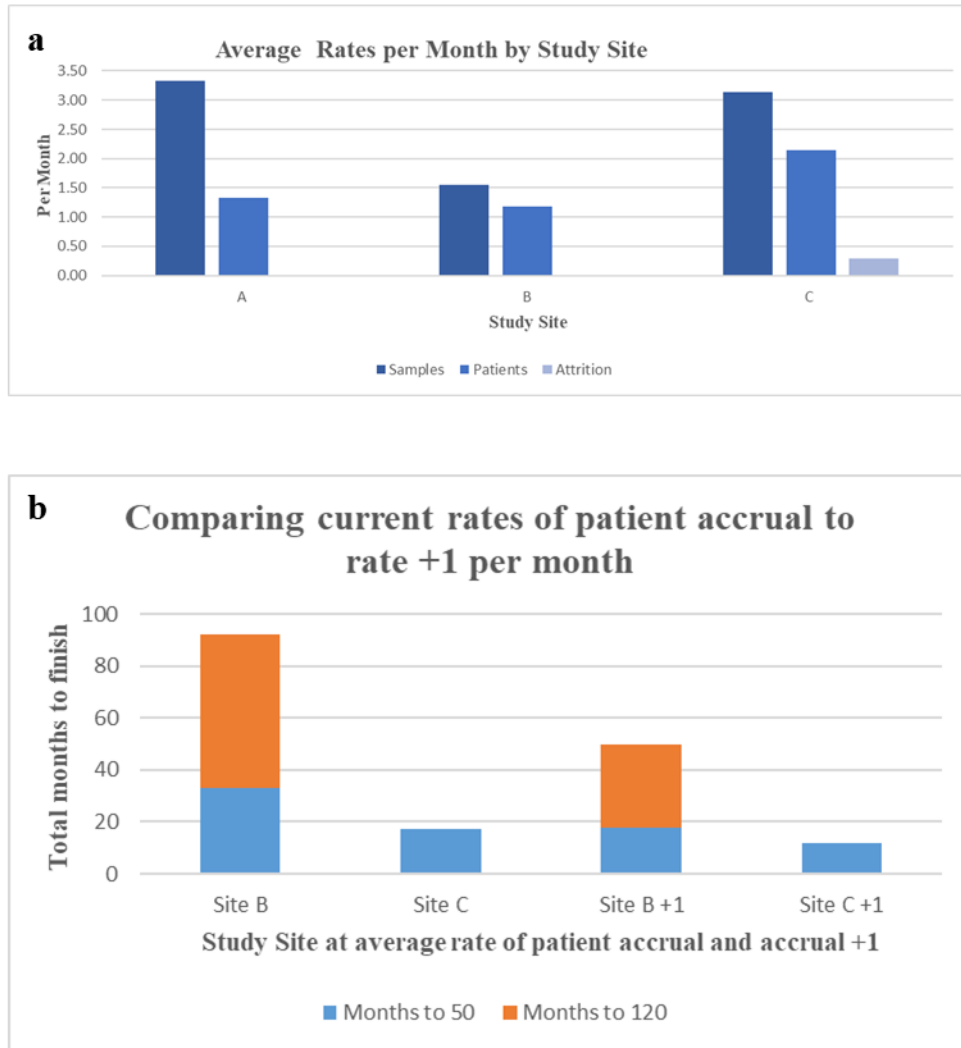


Figure 6. Examination of rates. a) Rates of patients, samples, and attrition per month by study site. b) Total months to finish the study if current accrual rates are maintained or with a +1 patient per month rate for a total goal of 50 patients (blue) according to the goals of Site Cs and for 120 patients according to the goals of Site Bs, but also showing a goal of 50.

4.4.7 Interpretation of Combined Results

Overall, key findings include that the involvement of dedicated research nurses in the PM phase increases success (sample rates), and the SU team size is inversely proportional to the length of time taken for clinical study SU.

Hierarchical structure and number of team members: The number of team members is the most important factor to reduce the time of SU and increase the patient and sample accrual rates in the PM phase. The number of dedicated team members, varied during the SU and PM phases, with a direct relationship to the speed of SU and rates of accrual. Site C exhibited an acceleration of the whole SU process, with the CTG academic coordinator joining the team.

Patient Flow Through the Study: Patient recruitment and retention strategies are critical, with common barriers including patient reluctance and health status. Sites A and C employed specialised clinical nurses on top of consultant medical oncologists and SpRs to enrol and manage patients, leading to greater levels of continuity for the duration of the study and increased patient and sample accrual. Continuity is the next most important factor.

Overall Patient Numbers: Consented patient numbers vary among sites, with Site A experiencing steady accrual until termination due to staffing issues. while Site B demonstrates a staggered growth pattern. Site C maintains continuous growth with effective screening and consent processes, resulting in higher patient enrolment. The impact of numbers in PM personnel is evident, underscoring the importance of adequate staffing for study continuity and success.

Overall Sample Numbers: Sample collection intervals vary between sites, influenced by treatment regimens and study dynamics. Site B exhibits discontinuous growth, while Site C demonstrates continuous accumulation, as did Site A until it ended prematurely. Shorter treatment regimens contribute to rapid sample accumulation at Site C, surpassing other sites despite a later start.

Overall Clinical Site Rates: Site A exhibited comparable patient accrual rates with Site B and led in sample collection. Site B shows the lowest sample accrual rate and patient recruitment, while Site C emerges as the most successful, with higher sample and patient acquisition rates. Adjusting recruitment rates can significantly impact study timelines, highlighting the importance of proactive recruitment strategies.

Delays and Impact on Study SU Timelines: The delays at site A can be attributed somewhat to COVID-19 and, to a lesser extent, the HSE ransomware attack. However, the majority of delays were due to poor communication and the lack of a cohesive team. Site B experienced significant delays due to legal processing in the academic setting, delaying the SU by 329 days despite having

all the paperwork on the study site. in order within a month. Site C faced delays in SU due to slow team assembly and initially low number of team members, which improved with the participation of the academic coordinator. SU at site C was less hurried and was able to include additional elements, such as Castor and the food questionnaire.

4.5 DISCUSSION

Reading clinical protocols might suggest that such studies are straightforward: obtaining full approval, consenting patients to send samples from home at their convenience, processing the samples, and publishing the findings. However, the reality is far more complex. The significant involvement of multiple personnel in the SU phase, the extensive paperwork, and the collaboration across institutions with outdated systems unsuitable for efficiently setting up observational studies reveal the challenges involved. While anecdotal evidence similar to the outputs of this chapter exists, to the authors' knowledge, this is the first study to analyse such data from these studies systematically.

The findings highlight the importance of hierarchical structure and the number of dedicated team members in managing clinical studies, particularly during the SU and PM phases. The number of team members and redundancy levels are crucial in expediting SU and enhancing patient and sample accrual rates during the PM phase. The variation in team member numbers across different phases directly correlates with the speed of SU and accrual rates. For instance, Site C experienced an accelerated SU process with the inclusion of a UCC CTG academic coordinator, demonstrating the positive impact of specialised personnel on study efficiency.

Effective patient recruitment and retention strategies are critical to study success. Site A and C's employment of specialised clinical nurses, alongside consultant medical oncologists and SpRs, improved continuity and increased patient and sample accrual. A study on clinical research nurses found the role particularly challenging, with conflicts in roles as both researcher and nurse, obstacles in obtaining cooperation from clinical nursing staff to adhere to trial protocols, and

struggles in maintaining personal motivation identified as the main challenges (Spilsbury et al., 2008). Continuity emerges as a critical factor, underscoring the importance of robust PM teams for study progression. Site C reported missing six patients due to a lack of resources, despite being the most efficient in terms of patient accrual.

Estimated numbers for the other sites are currently unavailable. It is assumed that Site B had only one person at the PM stage, which may explain some system errors and lower recruitment continuity. Despite restrictions within public hospital systems and workloads, the hard work of SpRs resulted in noteworthy recruitment rates. The SpR-only system appears to depend on exceptionally dedicated individuals. If more personnel were involved in consenting, zero growth months in terms of patient and sample accrual would not be expected. This indication is supported by a recent Irish report that found that most hospital-based clinicians surveyed viewed research as 'essential,' with career advancement as the primary motivator, alongside the desire to improve patient outcomes. However, the absence of dedicated research time was the most significant obstacle. Most did not see the hospital environment as conducive to research participation. Gender disparities were not evident, and 63% held an advanced academic degree. Engaging in research as a student did not correlate with increased publication success, whereas holding an MD/PhD degree did (Kyung Ha et al., 2022).

Securing adequate funding throughout the duration of a study poses significant challenges for investigator-initiated trials. Common issues include budgetary oversights by PIs, insufficient funding for feasibility assessments, overly optimistic trial initiation with inadequate funding, and the inability of PIs to acquire additional funds during the trial. Participant recruitment frequently exceeds projected timelines, yet budgets for investigator-initiated trials rarely account for potential delays. Consequently, when PIs cannot secure supplemental funding, trials often have to be prematurely terminated (Briel et al., 2021).

The timing of consent is another critical factor. Site C reported that the window for obtaining consent from patients is typically limited to a week from identification at the clinic, planning meetings, or multidisciplinary team (MDT) discussions. Patients are contacted within this time frame to provide them with relevant information and arrange a visit for consent. Most patients are approached during pre-screening for their treatment, typically 2-4 days before their scheduled visit

to the day ward. In our studies, upon obtaining consent, patients are provided with the necessary sampling kit to collect the required samples at home. Although some patients may provide a sample on the same day as their visit, time constraints and concurrent studies often make collection of samples the same day infeasible. Patients often prefer not to attend study visits alone and may decline participation if they are unable to arrange a convenient meeting time. Efficient communication and scheduling are essential to optimise patient recruitment and participation. A study found that conflicts arising from communication breakdowns between researchers and clinical teams were key aspects of trial failure (Donovan et al., 2014).

Delays in the study SU timelines, due to prevalent systemic challenges and the need for streamlined legal processes, were observed across sites. Major delays, such as those encountered by Site B due to legal processing in the academic setting, significantly impacted study initiation and progression. The studies aimed to collect stool samples from patients who had provided their informed consent. This should be the only document required for this type of non-invasive observational study. The SU phase should ensure the availability of the required resources for the study's duration to achieve its stated goals. This, combined with informed consent of the patient, should be sufficient for a study. Experiences in other European jurisdictions have found bureaucratic burdens to be a common factor in study failure (Mills et al., 2006).

Efforts to improve future study efficiency and reduce costs should prioritise increasing personnel numbers and optimising PM systems. Motivation, feedback mechanisms, and stakeholder engagement are crucial for study success. Addressing barriers to patient recruitment and optimising recruitment strategies are essential to achieving study goals. Future trials should incorporate specialists, streamline legal processes, and ensure adequate personnel cover to mitigate the risk of study failure. Collaboration between stakeholders, regular reporting, and effective communication are also essential to address challenges and improve study outcomes. Feedback from the Site C PM team highlights the importance of positive interactions and enthusiasm, creating a conducive environment for motivation and morale among team members.

Implementing more regular reporting and improving feedback mechanisms at meetings is essential to address issues comprehensively across all levels of any future study. Improved communication fosters transparency and collaboration, mitigating the risk of communication breakdowns, as

exemplified by Site A, which can lead to negative feedback loops and frustration. Such breakdowns may have cascading effects, diminishing motivation and morale, and ultimately impacting study outcomes. Recognising the impact of both positive and negative feedback loops on motivation levels is essential.

An important success factor common to all sites is the OMNIgene-GUT kit from DNA Genotek, which has cost and time implications due to its perishable nature. This kit, selected four years ago to ease collection and processing for stakeholders and improve results while reducing sampling bias, is now the industry standard for GM DNA and RNA analysis. From the sample collection and processing aspects, these kits have been highly effective. Feedback from PM teams indicated that the kits reduce reluctance to donate stool samples, a finding supported by a previous study where patients were reluctant to sample at home due to temporary storage in their home freezer (Bolte et al., 2021). The kit can keep bacterial DNA stable for up to 60 days at ambient temperatures. There have been no system failures regarding home sampling and sending back to the CR@UCC laboratory, where samples are frozen at -80 ° C until processing. The only issue with the kits is their expiration date. Due to delays and the eventual failure of Site A, 80% of the kits were wasted. Each kit costs €26.00 per unit delivered, including customs charges. To give them to patients, their barcodes had to be recorded and each kit was placed into its own envelope with a postage fee of €3.80. Once a batch of recorded and packaged kits is ready, they must be distributed to the various study sites, potentially involving further postage costs. This was done for Site A, and after the study failed, the kits were returned in the hope they could be used before expiration. Eighty kits subsequently expired, resulting in a monetary waste of more than € 2,400. Learning from this costly mistake, kits are now purchased in smaller batches of 50 with a two-week lead time on delivery.

Additional investments and insights into differences between private and public settings reveal several key points. Although Site C, the private study site, was the slowest to set up, it experienced less pressure compared to public settings. However, the dedication and substantial work at Site B were evident. One of the SpRs at Site B remained involved even after moving hospitals twice and continues to be involved nearly two years after the start of SU.

Since Site C was a private hospital, the study budget had to cover all additional foreseen costs to remain cost-neutral, with pre-agreed PM fees of €304.50 per patient. The impact of this up-front agreement on the study's success or its potential implementation in the public setting remains to be seen. Speculatively, Site A might have finished if these resources had been applied within the public system, and Site B's patient and sample accrual rates could have increased significantly. The data generated here suggest this.

The less stressed private setting and less hurried SU at Site C allowed for additional elements like a food questionnaire and the adoption of the Castor clinical platform database, costing approximately €2,000 per annum. This fee included SU and maintenance by UCC's Statistics, Data, & Analysis Unit (SDAU), streamlining clinical data entry and subsequent data interrogation, reducing errors, and enhancing efficiency in the final data reporting and publication stages. Despite numerous attempts over the past two years, retrieving clinical data from Site A has been challenging, emphasising the importance of efficient data management systems.

In conclusion, the success of clinical trials depends on robust hierarchical structures, adequate team members and redundancy levels, continuous accrual, and effective PM strategies. Addressing systemic challenges and optimising study processes are imperative for advancing clinical research and improving patient outcomes. Successful trials are characterised by their flexibility and robustness in addressing unexpected challenges (Campbell et al., 2007). A focus on improving patient clinical outcomes is a key driver of clinical research in Ireland. Perceived barriers include a lack of integration of research into healthcare provision, a lack of protected time for clinicians, staffing shortages, especially of research nurses and clinical research professional supports, such as regulatory support, and funding deficits (Ward and Kennelly, 2019). Several factors impact the initiation and progression of trials in Ireland, with delays in obtaining contracts and ethics approval being major barriers. Specialist support in ethics and regulatory affairs and the availability of specialised staff in areas like statistics and data management are key actions to enhance clinical trial activity in Ireland (Leddy et al., 2020). While large, single improvements are rare, marginal gains in processes can add up across a whole system.

5 CHAPTER 5:

Preliminary sequencing of the gut microbiome of patients undergoing cancer treatments

5.1 ABSTRACT

Introduction: Humans are symbiotic communities of organisms, combining the stable diploid somatic genome of ~ 20,000 protein coding genes with the everchanging gut microbiome (GM), estimated to possess over 100 times more, whose composition is impacted by multiple influences. The GM includes all bacteria, viruses, protozoa, fungi, and archaea present in the gut. To generalise, it is essential for the maintenance of gut health, digestion, immune function, and the detoxification of many harmful metabolic byproducts. Dysbiosis, or the disruption of GM homeostasis, can lead to diseases including cancer, cancer progression effects, altered treatment outcomes and negative treatment side effects. The GM has been shown to significantly influence the efficacy of various cancer treatments examined, including chemotherapy, radiotherapy, and, in particular, modern immunotherapies, with immune checkpoint inhibitor (ICI) treatment the most studied to date. Previous studies have found that the GM plays a major role in determining ICI treatment response, and that certain species can be used as biomarkers of treatment response and potential negative side effects.

Aims: The primary objective is to analyse faecal samples from cancer patients undergoing various treatments and to identify biomarkers for better patient selection and treatment strategies. The secondary objective is to investigate the impact of the type of therapy on GM composition.

Methods: For this chapter, samples from Sites B and C were analysed. Patient Recruitment: At Site B, 11 participants with early-stage breast and gastrointestinal (G.I.) cancers were recruited to provide stool samples before, after, and six months post-treatment. At Site C, 13 cancer patients receiving immune checkpoint inhibitors (ICIs), with or without additional treatments, provided stool samples at three time points. Collected stool samples were frozen, processed in batches, and microbial DNA extracted using a standardised kit. The 16S rRNA gene was amplified and sequenced. Bioinformatic analysis evaluated alpha diversity, Shannon Diversity Index, and differential abundance across samples.

Results: Preliminary sequencing of 39 patient samples revealed the relative abundance of the top 20 bacterial genera. *Blautia* was the most prevalent genus across all samples. Notable changes in genus representation were observed in different cancer types and treatment stages. Alpha diversity, measured by the Shannon Diversity Index, varied with treatment. Differential abundance analysis

indicated significant shifts in bacterial genera that highlight the dynamic nature of the GM and have been found previously in other GM association studies.

Conclusion: While the low patient number at this early stage of the study was not expected to yield statistically significant comparisons, the analyses performed in this pilot validate the process and capability to achieve the long term aim of elucidating potential correlations between patient GIT microbiome characteristics and disease or treatment outcomes.

5.2 INTRODUCTION

Humans possess two sets of genomes: the inherited human diploid genome and the metagenomic collection of haploid genomes of our GM. Unlike the host genome, which remains inherently stable throughout life, the microbiome is hyperdynamic and can be influenced by factors such as diet, host genetics, age, hormone cycles, physical health, and medications, to name but a few. The GM consists of all genomic elements of the microbes residing in the human gut, including bacteria, viruses, protozoa, fungi, and archaea. The human genome is composed of up to 20,000 genes (Amaral et al., 2023). With over 1,000 bacterial species and over 3 million genes, the microbiome plays a critical role in human physiology, immune system development, digestion, detoxification, and regulation of the immune system (Rinninella et al., 2019). The GM behaves like a virtual organ and is vital for various bodily functions (O'Hara and Shanahan, 2006). It aids in the microbial fermentation of complex non-digestible dietary carbohydrates and host derived glycans, pathogen suppression, and immune system maintenance (Valdes et al., 2018). Dysbiosis, a disruption in the GM composition, can lead to disease through a loss of beneficial bacteria and an increase in pathogenic species, leading to inappropriate immune responses. This in turn promotes a proinflammatory environment, which can in turn initiate oncogenesis, affect treatment efficacy, and increase negative side effects through immune system interactions (Jain et al., 2021).

GM & chemotherapy or radiotherapy: The GM's role in the efficacy of chemotherapy treatment is well established, especially for cancers of the gut itself (von Frieling et al., 2018, Heshiki et al.,

2020, Si et al., 2021, Zheng et al., 2019). Studies on colorectal carcinoma patients and those undergoing chemotherapy for non-Hodgkin's lymphoma have shown significant changes in GM composition, associated with treatment side effects and efficacy (Warren et al., 2013). Chemotherapy has been shown to disrupt GM homeostasis by direct killing of beneficial OTUs, allowing potentially harmful bacteria to thrive, with chemotherapeutic induced diarrhoea (CID) being a very common side effect that can result in death or, at the very least, discontinue treatment. This has been shown to significantly reduce alpha diversity, promote dysbiosis, shifting the GM towards an unhealthy state, and promote disease with positive inflammatory feedback loops (Montassier et al., 2015, Lin et al., 2012, Maroun et al., 2007). Outside of cancers of the G.I. tract, there is a lack of comprehensive animal and clinical studies examining the correlation between GM changes during chemotherapy and chemoradiation and the resulting pathological responses. Even where studies of sufficient size and power have been undertaken, mechanistically, very little is understood due to the level of intrinsic complexity.

GM & immunotherapy: One of the hallmarks of cancer is immune system evasion, essentially the ability of cancer cells to hide their neoplastic intentions and appear benign (Hanahan, 2022). Some of the mechanisms employed by cancer cells are mediated through the interaction of checkpoint molecules. Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD1) are immune checkpoint proteins expressed on T cells. To avoid unwanted autoimmunity, non-malignant cells express various checkpoint ligand molecules on their cell surface, such as the well described PD-L1. These molecules interact with their cognate T-cell checkpoint receptors and regulate the immune response in an inhibitory manner by dampening down the body's reaction, acting as the brakes of immune activation (Jacob et al., 2021). Cancer cells that have managed to express these checkpoint molecules have a selective growth advantage through this type of immunosuppression.

Recent research has highlighted the GM's influence on cancer treatment outcomes, especially with ICI (Gopalakrishnan et al., 2018b, Routy et al., 2018a, Matson et al., 2018). However, the efficacy of these treatments can vary greatly between patients and is on average only 50% effective, with many studies implicating the role played by the GM in determining the level of treatment response (Westdorp et al., 2021). Numerous clinical trials have shown that different operational taxonomic groups (I) of bacteria are associated with responders and non-responders, alluding to the

complexity of interactions between the host, GM, and environment (Zhang et al., 2023). The cost effectiveness of ICI is a major factor driving MWAS in general, as pharmaceutical companies are looking to increase the number of patients receiving ICI whilst reducing the level of negative side effects and justifying their rapacious profits. The rise of immunotherapy to combat cancer has contributed massively to more than doubling of per capita spending in Ireland from €29/person to €64/person in the decade from 2008 to 2018 (Hofmarcher et al., 2020).

This study: This study aims to explore the relationship between GM alterations and the efficacy of cancer treatment, not just for immunotherapy but also for traditional chemotherapy, radiotherapy or combined therapies, through metagenomic analysis of stool samples.

Study Site B focuses on early-stage breast cancer (BC) patients undergoing neo-adjuvant chemotherapy and early-stage G.I. cancer patients (rectal and oesophageal cancer) receiving neo-adjuvant chemoradiation. Samples are collected before and after treatment, and the GM's association with the cancer's responses to treatment will be evaluated based on histologically confirmed outcomes post-operatively.

This study also seeks to further scrutinise the interaction of the GM and its effect on ICI. Frequently, patients are treated with a combination of ICI and cytotoxic chemotherapy, and these patients are also eligible. Treatment outcomes will be determined by computed tomography (CT) scanning and then associated with GM OTUs, sequenced from patient's stool samples collected prior to, during, and post ICI treatment.

Study Site C involves cancer patients scheduled to receive any line of palliative ICI alone or in combination with Tyrosine Kinase Inhibitors (TKI) and/or cytotoxic chemotherapy for disease types including cancers of the lung, including adenocarcinoma, small cell and non-small cell, cholangiocarcinoma (cancer of the bile ducts), metastatic melanoma, renal carcinoma, and metastatic squamous carcinoma.

Advances in 16S ribosomal RNA gene (rRNA) and sequencing have enabled the identification of previously unculturable bacterial strains, facilitating GM biomarker identification of positive treatment outcomes and minimal side effects. These biomarkers can be used to improve patient treatment selection, reduce toxic side effects, and enable interventions like the introduction of

beneficial bacterial species prior to treatment through faecal microbiome transplants (FMT). Initial clinical trials involving FMT have shown promise in overcoming resistance to ICIs, improving treatment outcomes, and reducing negative side effects (Angrish and Pezo, 2022).

5.3 MATERIALS & METHODS

5.3.1 *Patient recruitment*

At Site B, this study aims to recruit 120 participants: 60 participants with early-stage BC receiving neoadjuvant chemotherapy +/- monoclonal antibodies, and 60 participants with early-stage GI receiving neoadjuvant chemotherapy alone or chemotherapy combined with radiation. (This will include all GI cancers.). Participants are asked to undergo a two-stage assessment to examine the GM before and after neo-adjuvant chemotherapy/chemoradiation. They are also invited to have the GM examined six months post treatment to assess the long-term changes in the GM from baseline. This involves patients being provided with three home stool sample kits with instructions on how to retrieve the samples and how to place them in the pre-stamped postage envelopes to be sent to this researcher for assessment of the GM.

At Site C, cancer patients scheduled to receive any line of palliative ICI alone or in combination with tyrosine kinase inhibitors (TKI) and/or cytotoxic chemotherapy are asked to partake in the study, with a total aim of recruiting 50 patients over two years. Upon consent, the patient is supplied three stool collection kits (Ilett et al., 2019) and envelopes with postage paid to allow for samples to be returned to this researcher for processing. Patients are requested to supply stool samples at three time points with their informed consent and agreement (see Table 1 and Figure 1 below). At each time point, c. 500 mg of stool is collected by the patient at home using a home collection kit.

1. Baseline (prior to receiving immunotherapy/immune checkpoint inhibitor therapy (ICI))
2. After 2-4 weeks of immunotherapy ICI treatment

3. At tumour response to immunotherapy / ICI therapy

OR

4. At unequivocal tumour progression on immunotherapy / ICI therapy

Essentially, the number of samples at site B and C is the same, but the timing is dictated by the individual patients' treatment schedule.

Time point	Definition of time point	Classification of sample	Tumour biopsy
Before ICI	Registration Within 14 days of commencing ICI	Stool Sample A	Surplus standard of care archival tumour sample will be analysed as part of this research study.
During ICI	Prior to 2 nd course of ICI, after 2-4 weeks of initial ICI therapy	Stool Sample B	Any other tumour biopsy or sampling as part of standard patient clinical care will be requested for surplus tissue to be offered for this research project. No additional biopsy samples will be taken as part of this research study.
At disease response to treatment	Radiological or clinical confirmation of tumour response to ICI therapy as defined by response as assessed by treating clinician as CR, PR, SD, pseudo progression, or PD. At roughly 3 months post initial ICI treatment	Stool Sample C	
At disease progression	Radiological or clinical confirmation of tumour response to ICI therapy as defined by response as assessed by the treating clinician as CR, PR, SD, pseudo progression, or PD. At cessation of ICI treatment, within less than 3 months (for clinical progression or for G3 or higher	Stool Sample C	

	immune adverse (irAEs))	related events		
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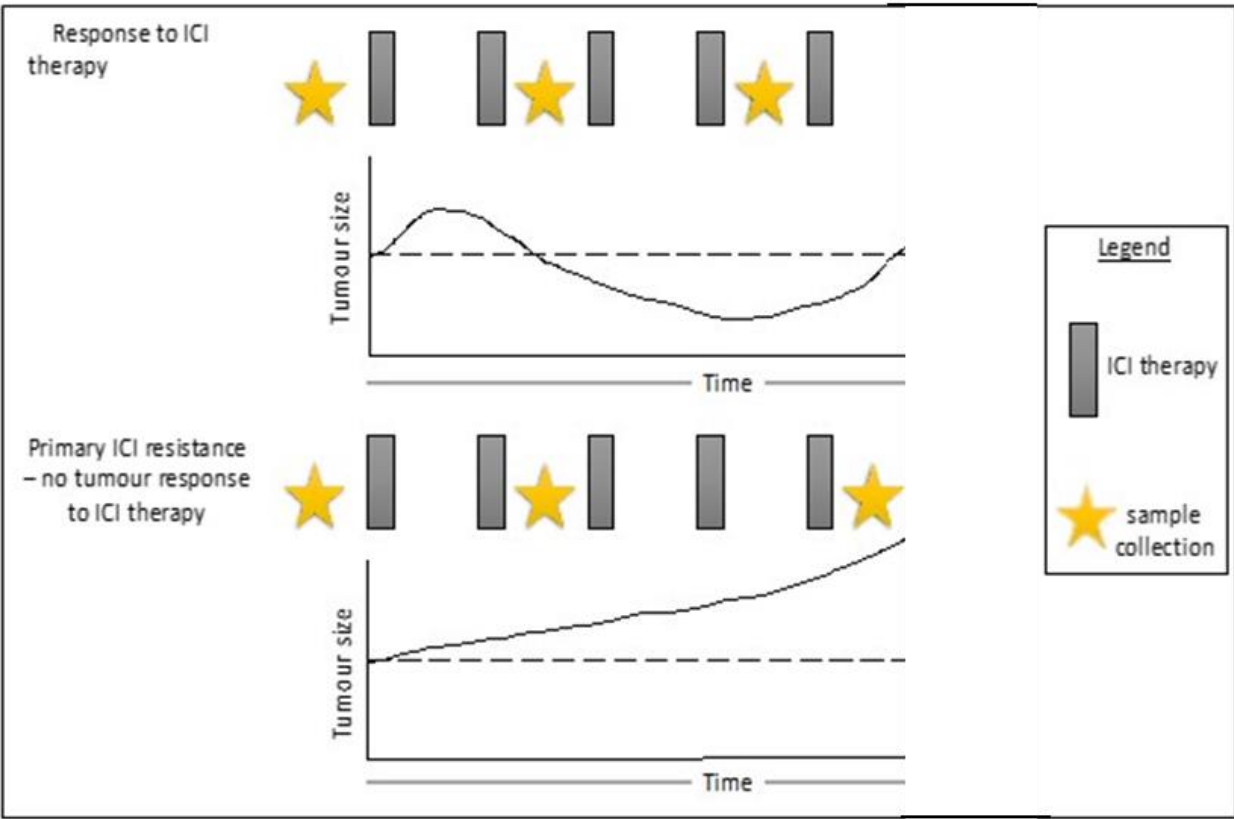


Figure 1. Timing of stool sample collection in relation to treatment, time, and tumour size

5.3.2 Sample Processing

Samples were frozen, immediately upon receipt at the Cancer Research @UCC laboratory in a - 80 °C freezer and processed in a batch manner.

Microbial DNA extraction

Faecal samples were collected and stabilised using the OMR200 kit for subsequent extraction of microbial DNA using the QIAGEN QIAamp PowerFaecal Pro DNA Kit. Prior to purification, the

samples were vigorously vortexed for 10 s. The PowerBead® Pro tube was spun briefly to ensure that the beads had settled at the bottom. 250 µL sample from the OMR200 tube was transferred to the PowerBead Pro Tube. 800 µL Solution CD1 was added to the PowerBead Pro Tube and vortexed gently to mix. From this point on, the QIAamp PowerFaecal Pro DNA Kit procedure was followed, starting at step 2 (bead vortexing). All work was carried out in laminar flow hood class II. For every DNA extraction run, two negative controls were run through the entire extraction process, beginning with an empty OMR200 tube, to control for potential contamination.

The full 16S ribosomal gene was amplified using primers provided in the 16S Barcoding Kit 24 V14 (SQK-16S114.24) by Oxford Nanopore Technologies. DNA quantity and purity were checked using a Qubit (Invitrogen) and DNA agarose gel to confirm the correct amplified size of ~ 1,550 bp.

5.3.3 Library preparation; 16s Barcode Preparation and Sample Processing

One 96-well plate containing 16S barcodes was used. A set of barcodes (1-24) was broken away from the plate, and the rest were returned to storage. The 96-well plates were designed to break in one direction only, allowing strips or multiple strips of eight wells/barcodes to be removed simultaneously. The desired barcodes were thawed at room temperature, briefly centrifuged in a microfuge to ensure the liquid was at the bottom of the tubes and placed on ice.

The LongAmp Hot Start Taq 2X Master Mix was thawed, spun down briefly, mixed well by pipetting, and placed on ice. DNA was prepared in nuclease-free water. Each genomic DNA sample (10 ng) was transferred into a 0.2 ml thin-walled PCR tube, and the volume was adjusted to 15 µl with nuclease-free water. The samples were mixed thoroughly by flicking to avoid unwanted shearing and briefly spun down in a microfuge.

In each 0.2 ml thin-walled PCR tube containing a sample, the following mixture was prepared:

Reagent	Volume
10 ng input DNA (from previous step)	15 μ l
LongAmp Hot Start Taq 2X Master Mix	25 μ l
Total	40 μ l

The components were thoroughly mixed by pipetting and briefly spun down. Clean pipette tips were used to carefully pierce the foil surface of the required barcodes, with a new tip used for each barcode to avoid cross-contamination. The barcode numbers run for each sample were noted.

Using a multichannel pipette, the 16S barcodes were mixed by pipetting up and down 10 times, and 10 μ l of each 16S Barcode was transferred into respective sample-containing tubes. The components were thoroughly mixed by pipetting the contents of the tubes 10 times and briefly spun down.

PCR amplification was performed using the following cycling conditions:

Cycle Step	Temperature	Time	No. of Cycles
Initial denaturation	95 °C	1 min	1
Denaturation	95 °C	20 secs	25
Annealing	55 °C	30 secs	25

Cycle Step	Temperature	Time	No. of Cycles
Extension	65 °C	2 mins	25
Final extension	65 °C	5 mins	1
Hold	4 °C	∞	

5.3.4 Reagent Preparation

Reagents were thawed at room temperature, briefly spun down using a microfuge, and mixed by pipetting or vortexing as indicated:

Once thawed, all reagents were kept on ice. EDTA (4 µl) was added to each barcoded sample, mixed thoroughly by pipetting, and briefly spun down. The samples were incubated for 5 minutes at room temperature. Each barcoded sample (1 µl) was quantified using a Qubit fluorometer for QC check.

All barcoded samples were pooled in equimolar ratios in a 1.5 ml Eppendorf DNA LoBind tube. The AMPure XP Beads (AXP) were resuspended by vortexing. To the pool of barcoded samples, a 0.6X volume ratio of resuspended AMPure XP Beads (AXP) was added and mixed by pipetting:

Volume of barcoded sample pool	Volume of AMPure XP Beads (AXP)
37.5 µl	22.5 µl
75 µl	45 µl

Volume of barcoded sample pool	Volume of AMPure XP Beads (AXP)
150 µl	90 µl
300 µl	180 µl
600 µl	360 µl

The mixture was incubated on a Hula mixer (rotator mixer) for 5 minutes at room temperature. Fresh 80% ethanol in nuclease-free water (2 ml) was prepared. The sample was briefly spun down and placed on a magnetic rack until the supernatant was clear and colourless. While keeping the tube on the magnetic rack, the supernatant was pipetted off.

The beads were washed with 1 ml of freshly prepared 80% ethanol without disturbing the pellet, and the ethanol was removed using a pipette and discarded. This step was repeated. The sample was spun down, placed back on the magnet, and any residual ethanol was pipetted off. The beads were allowed to dry for ~30 seconds without drying to the point of cracking.

The tube was removed from the magnetic rack, and the pellet was resuspended by pipetting in 15 µl Elution Buffer (EB). The sample was spun down and incubated for 5 minutes at room temperature. The beads were pelleted on a magnet until the eluate was clear and colourless, for at least 1 minute. The eluate (15 µl) was transferred into a clean 1.5 ml Eppendorf DNA LoBind tube, retaining the eluate containing the DNA library. The pelleted beads were discarded.

The eluted sample (1 µl) was quantified using a Qubit fluorometer. Fifty fmol of the eluted sample was transferred into a clean 1.5 ml Eppendorf DNA LoBind tube, and the volume was adjusted to 11 µl with Elution Buffer (EB). In a fresh 1.5 ml Eppendorf DNA LoBind tube, the Rapid Adapter (RA) was diluted as follows and mixed by pipetting:

Rapid Adapter (RA) 1.5 µl

Adapter Buffer (ADB) 3.5 µl

Total 5 µl

Diluted Rapid Adapter (RA) (1 µl) was added to the barcoded DNA, mixed gently by flicking the tube, and spun down. The reaction was incubated for 5 minutes at room temperature.

5.3.5 Sequencing and analysis

Sequencing was performed by the researcher using the nanopore long-read sequencer MinION™. Oxford Nanopore Technologies system. The sequencing run was started using the MinKNOW software, which collected raw data from the device and converted these data into base-called reads. Taxonomic classification of single reads was carried out using the Epi2me platform provided by Oxford Nanopore Technologies. Subsequent bioinformatic analysis was conducted in Rstudio and Python to assess Alpha diversity, Shannon Diversity Index (SDI), and differential abundance analysis across patient samples. With 11 patients at Site B and 13 patients at Site C, this was as far as the analysis could be taken.

5.4 RESULTS

5.4.1 Patient Accrual

From the three clinical studies that were initiated, only patient samples from sites B and C were sequenced, as the trial at Site A was discontinued and no clinical data were available. All patient samples received up to March 31 2024 had their GM full ~1,550 bp 16S rRNA gene sequenced and analysed by Nanopore sequencing.

A total of 11 patients at Site B and 13 patients at Site C consented and provided at least one sample. Site B collected a total of 17 samples, with only one patient providing samples at all three time points. Site C collected 22 samples in total, with three patients providing samples at all three time points. Additionally, the patients were spread across 10 distinct cancer types: breast, oesophageal, rectal, adenocarcinoma lung, small cell lung, non-small cell lung, renal carcinoma, metastatic squamous carcinoma, metastatic melanoma, and cholangiocarcinoma. The highest number of patients with more than one sample for any single cancer type was for rectal cancer, with three patients providing two samples each.

5.4.2 Sequencing Results

5.4.3 Relative Abundance of Bacteria at the Genus Level

Patient samples, 39 in total, had their full 16s gene sequenced, with the top 20 relatively abundant genera across both sites identified, and, for each sample, the relative abundance of these top 20 genera was tabulated and graphed in a stacked bar plot (*Figures 2a and 2b*). Any genus outside of the top 20 was included in the “Other” category. The most prevalent genus across all patient samples was *Blautia*, except for one rectal patient, BH12, with two samples, BH12.1 and BH12.2. For the most part, the top 20 genera were well represented across all samples. Only two samples had a greater than 70% “Other” category. BON12.1 is composed of 50% *Enterobacter* at the genus level, and BON9.2 is composed of 35% *Christensenella*.

5.4.3.1 *BC*

Common to all BCs, which included five patients with six samples, were the genera *Blautia*, *Faecalibacterium*, *Anaerostipes*, *Mediterranibacter*, *Dorea*, and *Clostridium*, except BH14, which was missing *Clostridium*. *Walteria* was common to all except patient BH10.

5.4.3.2 *OC*

OC had only one patient, with a reduction in the overall number of genera with each sampling time point. There was a loss of *Walteria*, *Vescimonaes*, *Eubacterium*, *Copracoccus*, *Ruminococcus*, and a gain of *Escherichia* by sampling time point three.

5.4.3.3 *Rectal*

Rectal with five patients and eight samples, were well represented by the top 20 genera, except for patient BH12. All of the genera represented by their two sampling time points included only four genera, namely *Streptococcus*, *Shigella*, *Escherichia*, and *Clostridium*. BH5.1 had other genera outside of the Top 20 as comprising around 50% of bacteria at the genus level. This percentage was not predominated by any one genus but appears to be the most diverse at the genus level out of all samples, with the most abundant genera outside of the top 20 being *Peptostreptococcus*, *Ruthenibacterium*, *Dialister*, and at the family level, *Fennollaria*, which were not further classified to the genus level.

5.4.3.4 *Cholangiocarcinoma*

Cholangiocarcinoma had two patients, one with the full three sampling time points and one patient with only one sample. The top 20 genera were well represented, all above 75%. For patient BON11, there was a loss of *Escherichia* and *Eubacterium* and a large relative expansion of *Gemmiger*, *Fusicantebacter*, and *Faecalibacterium* post ICI treatment.

5.4.3.5 *Metastatic melanoma*

Metastatic melanoma had just one patient with two samples, almost identical except for the inclusion of an unclassified-Lachnospiraceae during ICI treatment. For metastatic squamous

carcinoma, there was just one sample, with 25% of the genera contained in the other category; Intestinibacter accounted for 5% and Terrisporobacter 4%.

5.4.3.6 *Adenocarcinoma*

Adenocarcinoma lung had two patients with three samples and two patients with one sample. Except for patient BON12 sample number BON12.1, the remaining samples were well represented by the Top 20 genera, with less than 20% having genera belonging to any other category. All but one patient sample had Waltheria in common.

5.4.3.7 *Non-small lung cancer (NSLC)*

NSLC had one patient who provided two samples and had the lowest representation in the "Other" category. Sample one contained approximately 5% of genera in the "Other" category, while sample two had around 10%. Between the first and second sampling time points, there was a loss of Ruminococcus, Vescimonas, and Roseburia, along with an increased relative abundance of Escherichia during ICI treatment.

5.4.3.8 *Small cell lung cancer*

Small cell lung cancer had two patients with two samples each. All samples had Streptococcus, Ruminococcus, and Clostridium in common. Sample BON9.2 showed the biggest relative abundance change between time points.

5.4.3.9 *Renal*

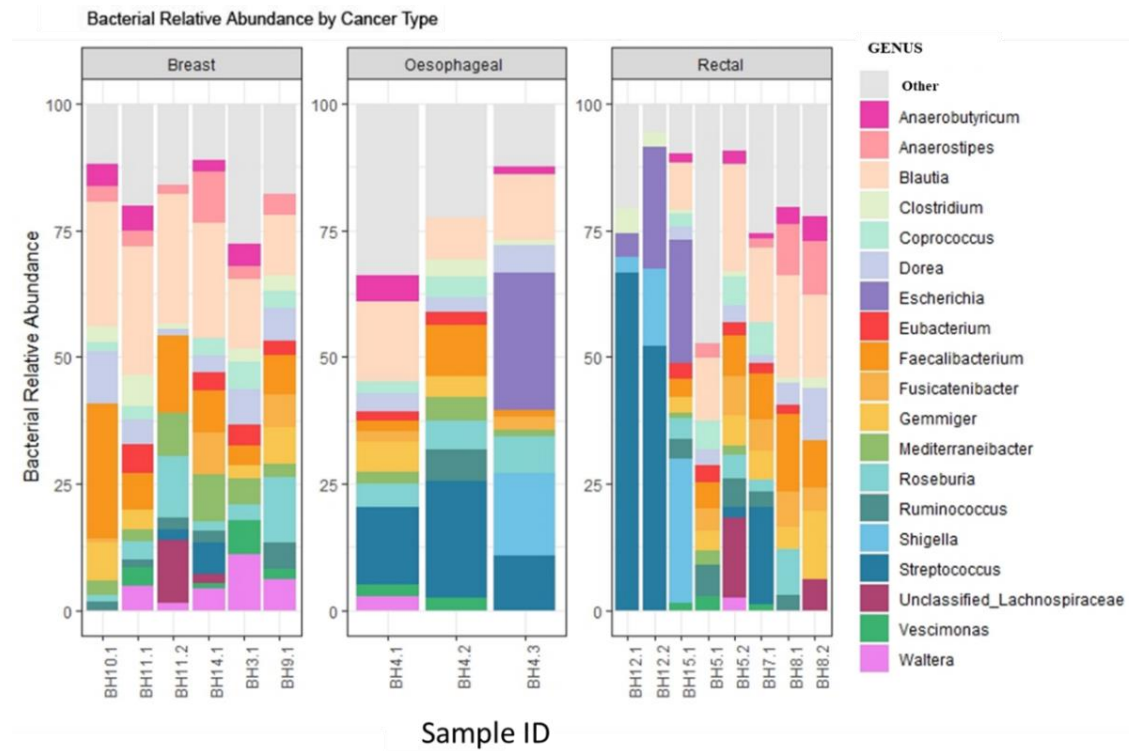
Renal carcinoma had one patient with two samples, and the samples showed almost identical relative abundances within the top 20 genera, with only the loss of Ruminococcus and the gain of Clostridium during treatment.

5.4.3.10 *Metastatic Squamous Carcinoma*

Metastatic Squamous Carcinoma had only one patient with one sample, which contained 75% of its relative abundance within the overall top 20 genera. The most dominant genera were

Feacalibacterium at 20%, and just outside of the top 20 were Terrisporobacter and Intestinibacter at 5% each.

a



b

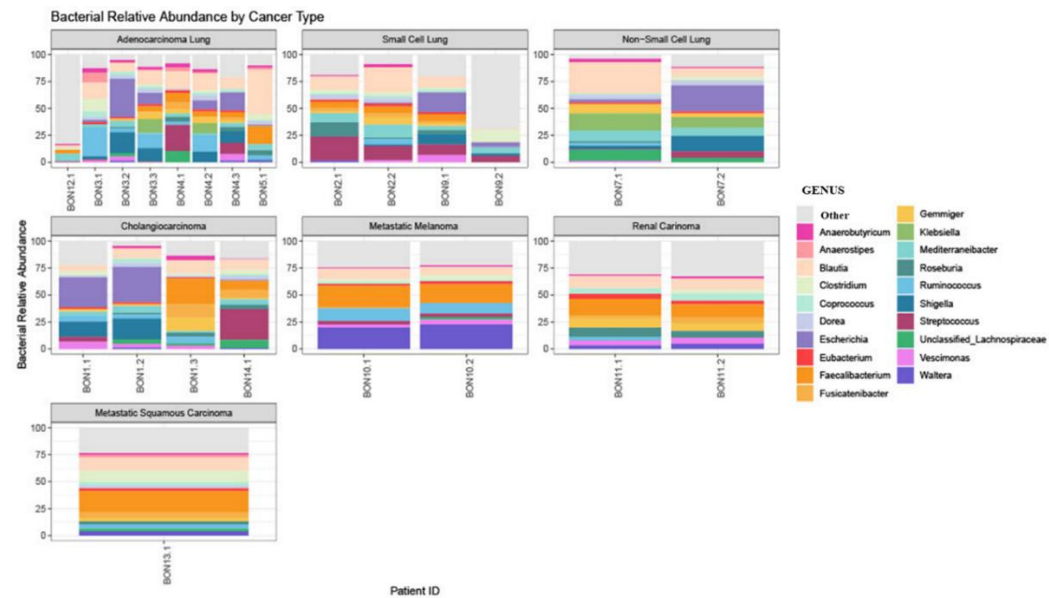


Figure 2. Relative abundance of the top 20 bacterial genera present in the stool samples of patients with different cancer types. Patient number is followed by a full stop; sample number is either 1, 2, or 3 which correspond to sequential sampling timepoints. a) Patients from Site B. b) Patients from Site C

5.4.4 Alpha diversity of patient samples over time

Alpha diversity, as measured by the Shannon Diversity Index (SDI), was calculated for each patient sample. Figure 3 displays patients with more than one sample and the SDI score is broken down by patient and cancer type. Both patients suffering from adenocarcinoma showed an upward trend in alpha diversity post ICI treatment. The single BC patient with two samples had decreased alpha diversity post neoadjuvant chemotherapy. The SDI of the single patient with cholangiocarcinoma also went down during ICI treatment and then trended back up slightly post treatment. Alpha diversity between timepoints one and two went up for patients with metastatic melanoma, non-small cell lung cancer, and renal cell carcinoma. The three rectal patients, all with two samples each, showed the greatest range of alpha diversity, with patient BH12 starting with a score below two and trending even lower post treatment. Patient BH5 had the highest alpha diversity score of four, which was reduced post treatment. Unlike the other two patients, BH8 had increased alpha diversity post treatment. For oesophageal cancer, the diversity score went up initially and then trended lower post treatment below the initial reading for timepoint one. Finally, the two patients with small cell carcinoma of the lung provided two samples each, with patient BON2 showing no change between timepoints and BON9 having a relatively large decrease by timepoint number two.

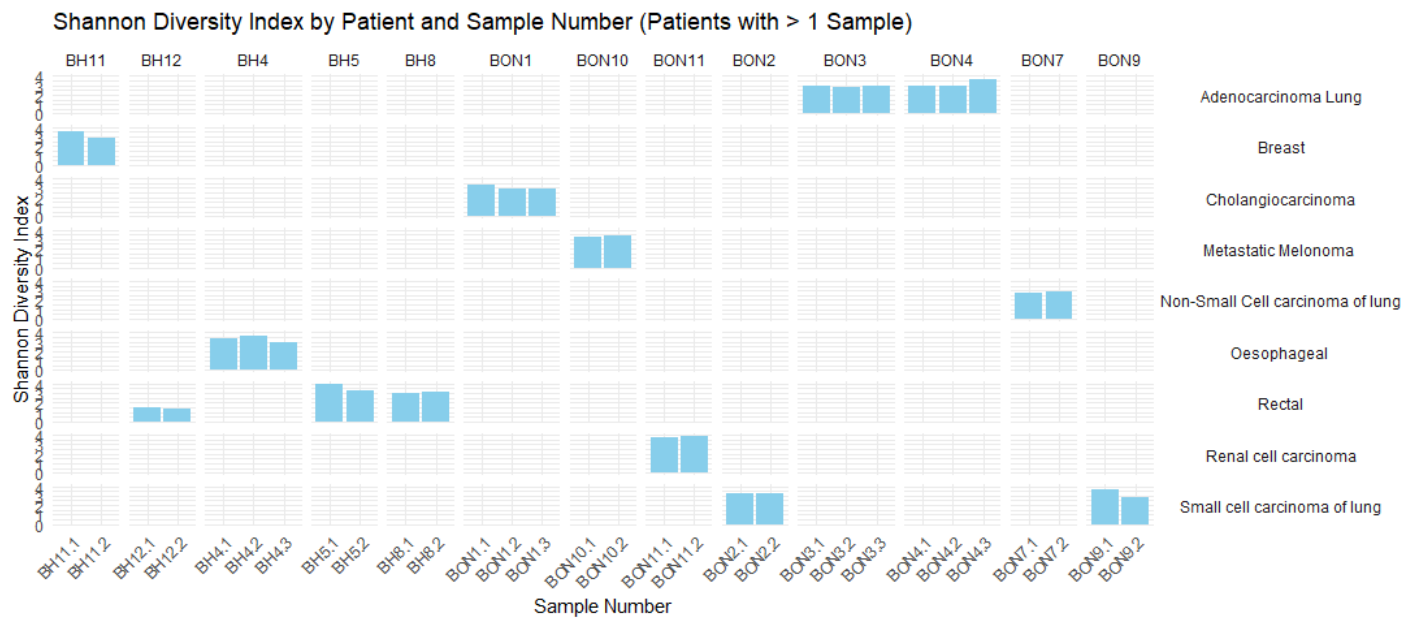


Figure 3. Alpha diversity as measured on the SDI for patient samples from patients with more than one sample, broken down by patient and cancer type.

5.4.5 Differential Abundance

Knowing the relative abundance at each timepoint permit changes from one sampling timepoint to another for the same patient to be tabulated (Appendix Table–1 <https://acrobat.adobe.com/id/urn:aaid:sc:EU:1b1b4d10-f288-43b0-85b1-7a932e6a9e15>) and visualised using volcano plots (Figures 4a-4q).

5.4.5.1 BC

Figure 4a shows the changes from sampling timepoints one to two for BC patient BH11, currently the only BC patient on trial with more than one sample for comparisons to be made. The operational taxonomic unit (OTU) at the genus level showing the most significant levels of change on the positive side were Wujia, Unclassified_Lachnospiraceae, and Drancourtella, with Gemmiger, Phascolarctobacterium, and Anaerobutyricum representing the most significant negative log-fold decreases.

5.4.5.2 *OC*

Figures 4b and c represent the only patient with OC with timepoints one to two and two to three, respectively. For the first transition from timepoints one to two, the top ten most significant fold changes were in the positive direction, with the top three being *Romboutsia*, *Terrisporobacter*, and *Dialister* the only negative fold changes were *Phascolarctobacterium*, and *Unclassified_Lachnospiraceae*. For the second transition, the opposite case was found, where most of the significant log-fold changes were negative; however, increases in *Escherichia*, *Shigella* had the highest log-fold changes, positively speaking. *Unclassified_Lachnospiraceae* and *Haemophilus* showed the most significant negative log-fold change.

5.4.5.3 *Rectal*

Rectal patients represent the largest group of patients on trial as of March 31, 2024, with a total of three patients with two samples each (Figures 4d, e, and f). With some commonality between patients, *Peptostreptococcus* was found to be significantly reduced post treatment for all three patients. Two of the patients, BH5 and BH8, were found to have *Unclassified_Lachnospiraceae* with a significant log-fold increase. *Gemella* and *Aneareococcus* were found to be commonly reduced between patients BH5 and BH12.

5.4.5.4 *Cholangiocarcinoma*

Patient number BON1 was the only enrolled patient with multiple samples, suffering from cholangiocarcinoma, and has provided all three samples. Figures 4g and 4h represent the differential abundances going from timepoints one to two and two to three, respectively. Four out of the top-10 significantly differentially abundant genera were positive for the first transition, the top four being *Perlabentimonas*, *Waltera*, *Unclassified_Lachnospiraceae*, and *Butyricimonas*. Significant decreases included *Ruthenibacterium*, *Pseudomonas*, *Gorbachella*, *Megasphaera*, and *Akkermansia*. For the remaining transition, of the top-10 differential abundances, eight showed a decrease, with only *Anaerocolumna* and *Tractidigestivibacter* showing an increase. The most significantly reduced were *Ihubacter* and *Anaerophaga*, with notable reductions also observed in *Escherichia* and *Shigella*.

5.4.5.5 *Metastatic melanoma*

For metastatic melanoma (Figure 4i), there was only one patient with two samples, the most differentially abundant genera being *Ruminococcoides* and *Unclassified_Lachnospiraceae* and *Novisyntrophococcus* and *Enterococcus* being the most significantly reduced.

5.4.5.6 *Adenocarcinoma*

Adenocarcinoma is the next largest group, with two patients, both of whom have provided the full complement of three samples each (Figures 4j to 4m). For patient BON3, there was a significant increase in *Unclassified_Lachnospiraceae*, *Perlabentimonas*, and *Butyricimonas*, only to have those gains reversed, moving to timepoint three. Similarly, genera significantly reduced from timepoint one to two, namely *Intestinibacter*, *Congobacterium*, and *Zhenhengia*, were found to be significantly differentially increased moving onto timepoint three, along with *Klebisella*. BON4 shared increases from timepoints one to two, with only *Escherichia* being in common within the top-10, otherwise some genera moved in the opposite direction, including *Unclassified_Lachnospiraceae* and *Congobacterium*. For timepoints two to three, *Butyricimonas*, *Yokenella*, and *Perlabentimonas* all went in opposite directions to the BON3 differential abundances for the same time points, with *Klebisella* showing the biggest reduction, a reversal of the timepoint one to two findings.

5.4.5.7 *Small cell lung*

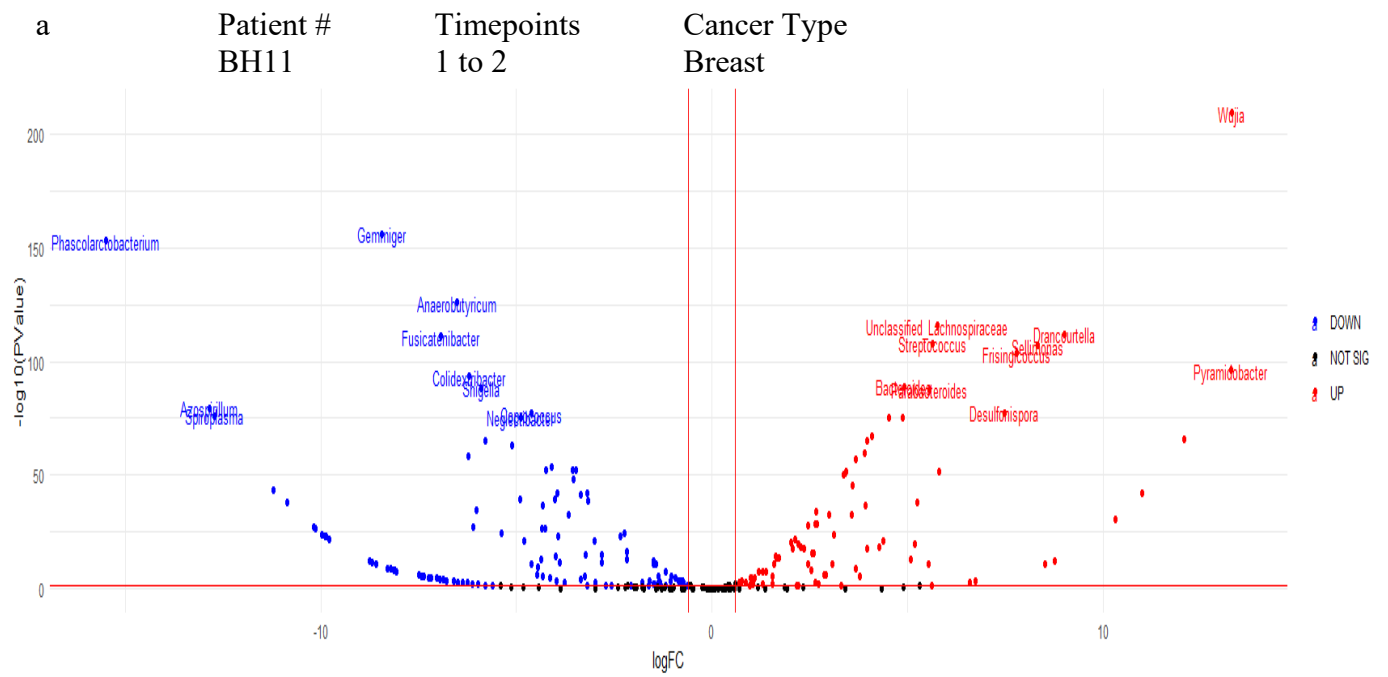
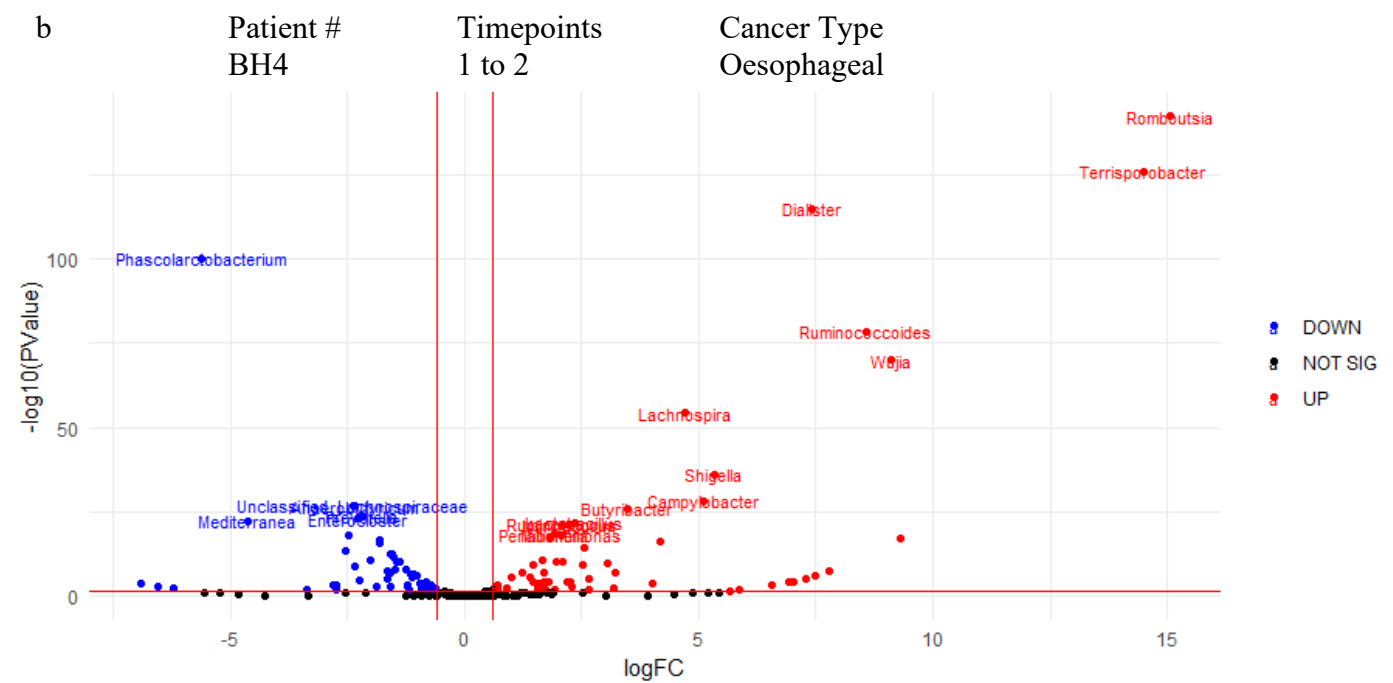
Representing small cell carcinoma of the lung, there were two patients, BON2 and BON9, with two samples each (Figures 4n and 4o). Of the top-10 significantly differentially abundant genera, none were in common between patients sampling timepoints. *Megamonas* and *Ruminococcus* were on the positive side of the graph for BON2, with *Waltera* and *Colidextribacter* found to be the top two with significantly reduced relative abundance. For patient BON9, *Faecalibacterium* and *Gemmiger* were the most significantly reduced with, *Christensenella* and *Fusibacter* being the most significantly increased from timepoints one to two.

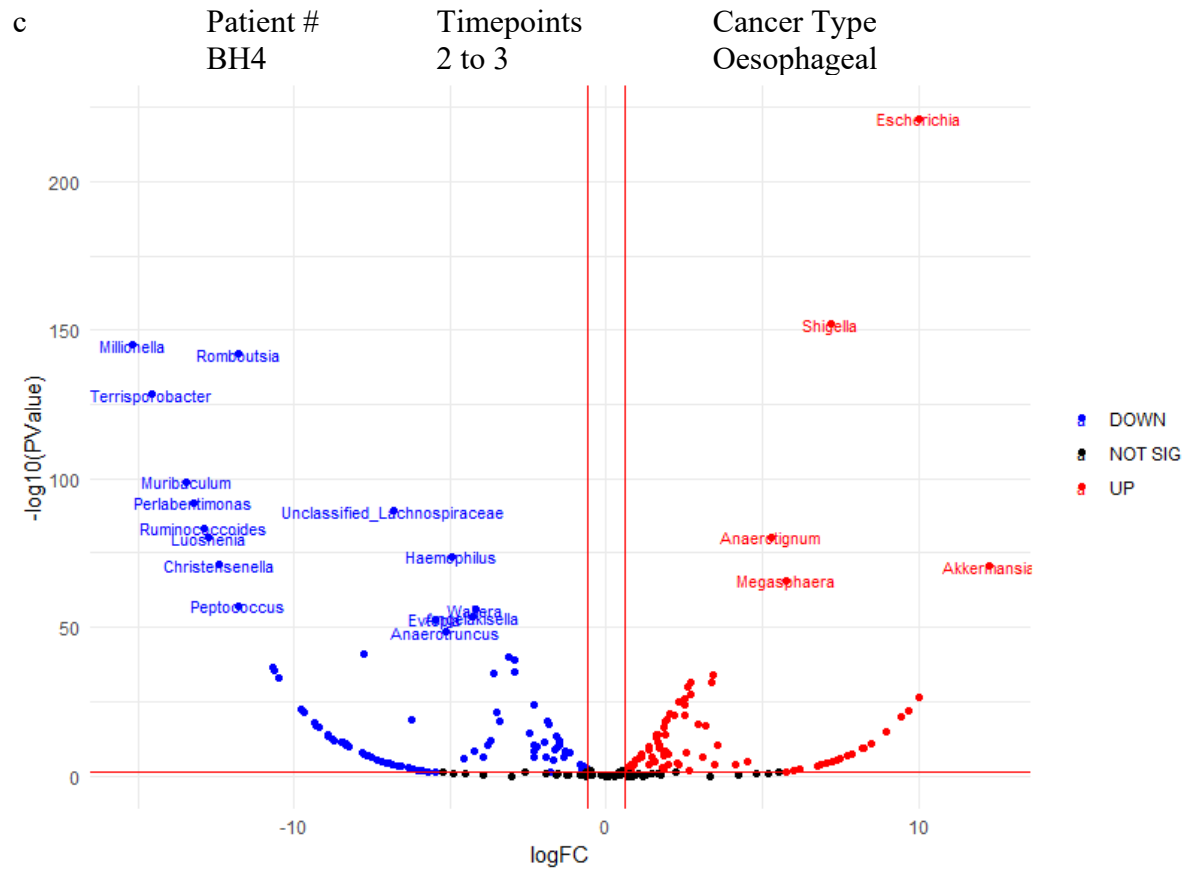
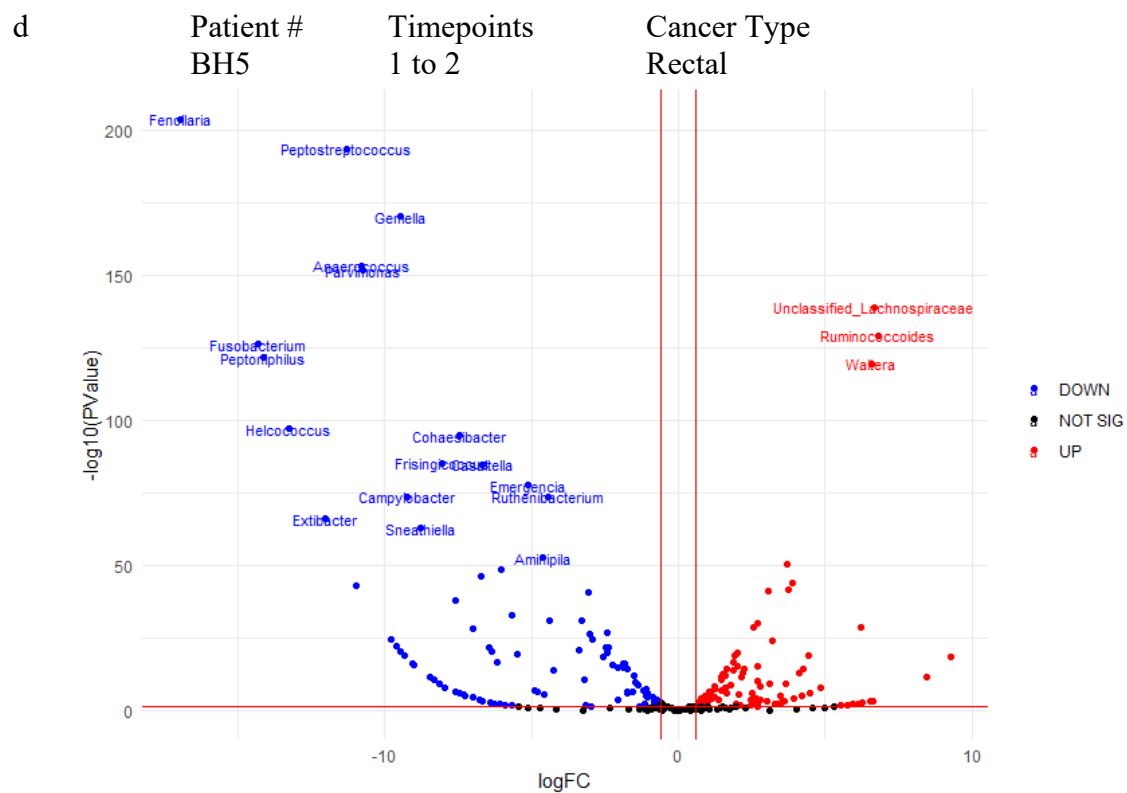
5.4.5.8 *NSLC*

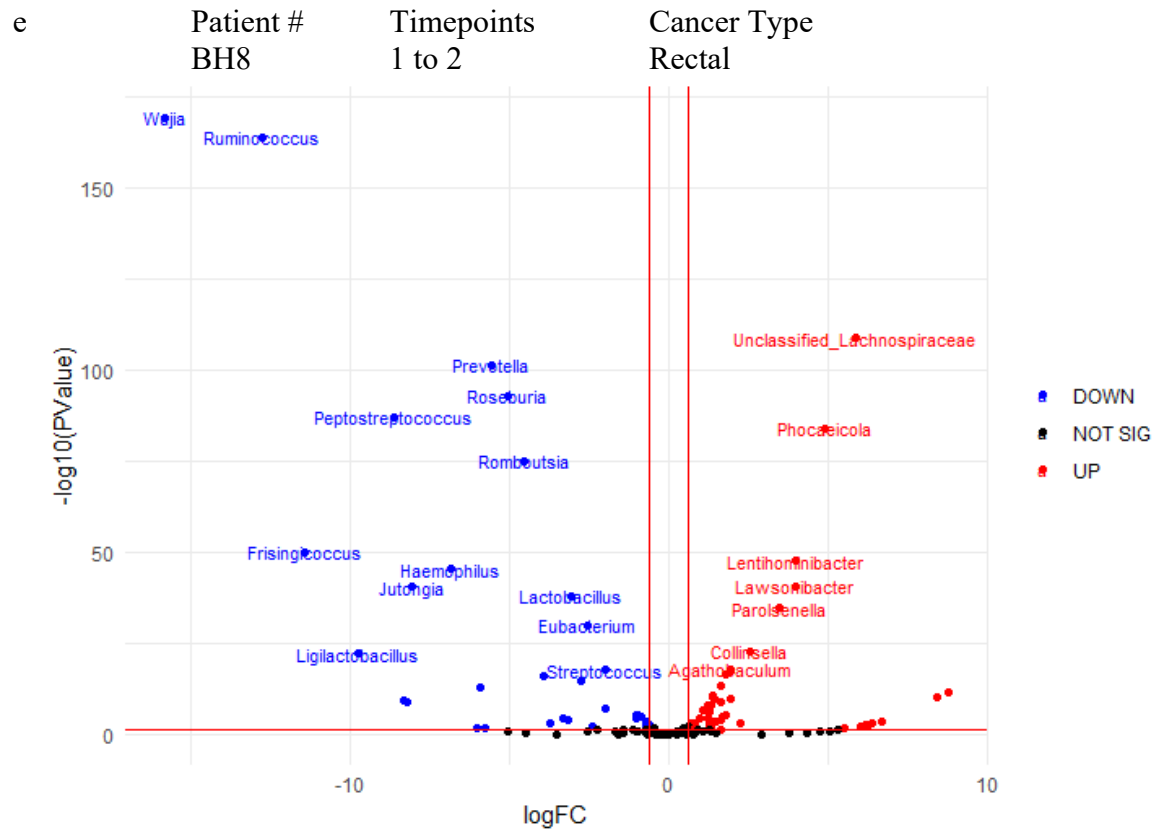
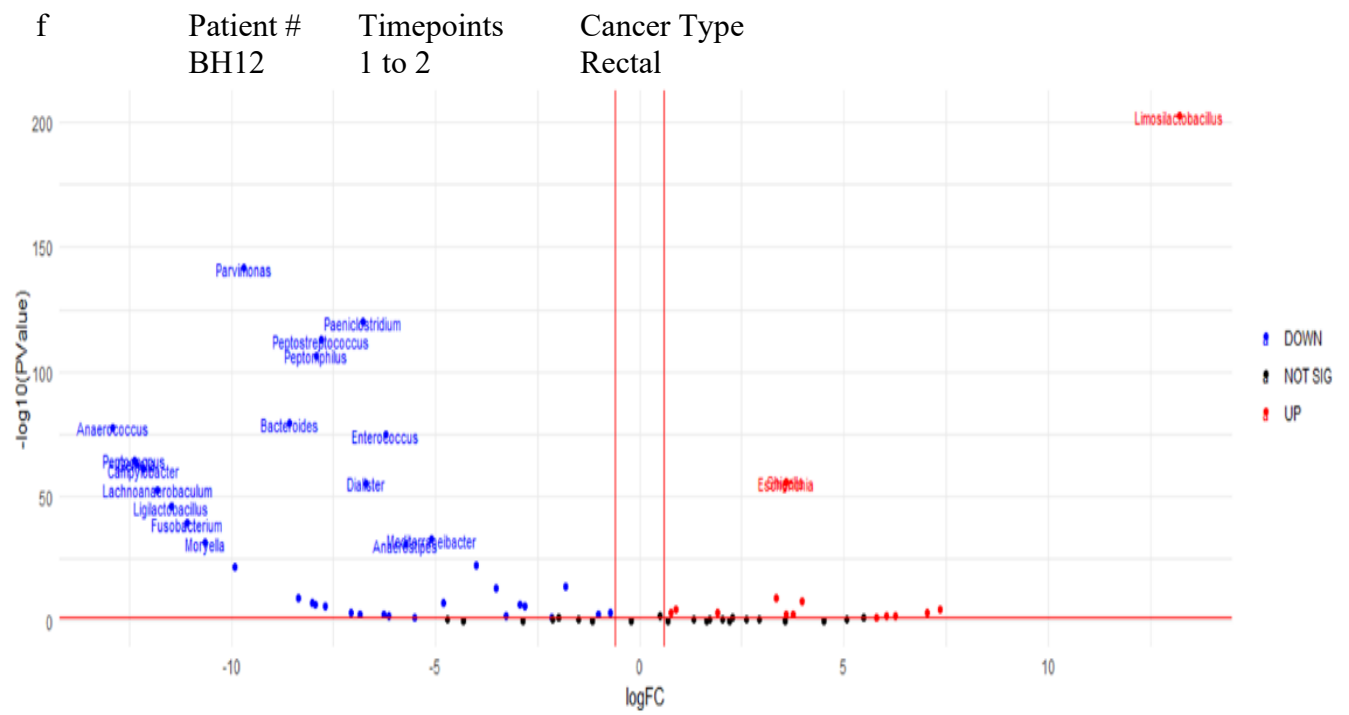
The remaining two cancer types had one patient with two samples each. Figure 4p represents the changes from timepoint one to two for patient BON7 with non-small lung cancer, *Limosilactobacillus* and *Veillonella* showed the most significant increases, whereas *Ruminococcus* and *Luoshenia* showed the greatest decreases between sampling time points.

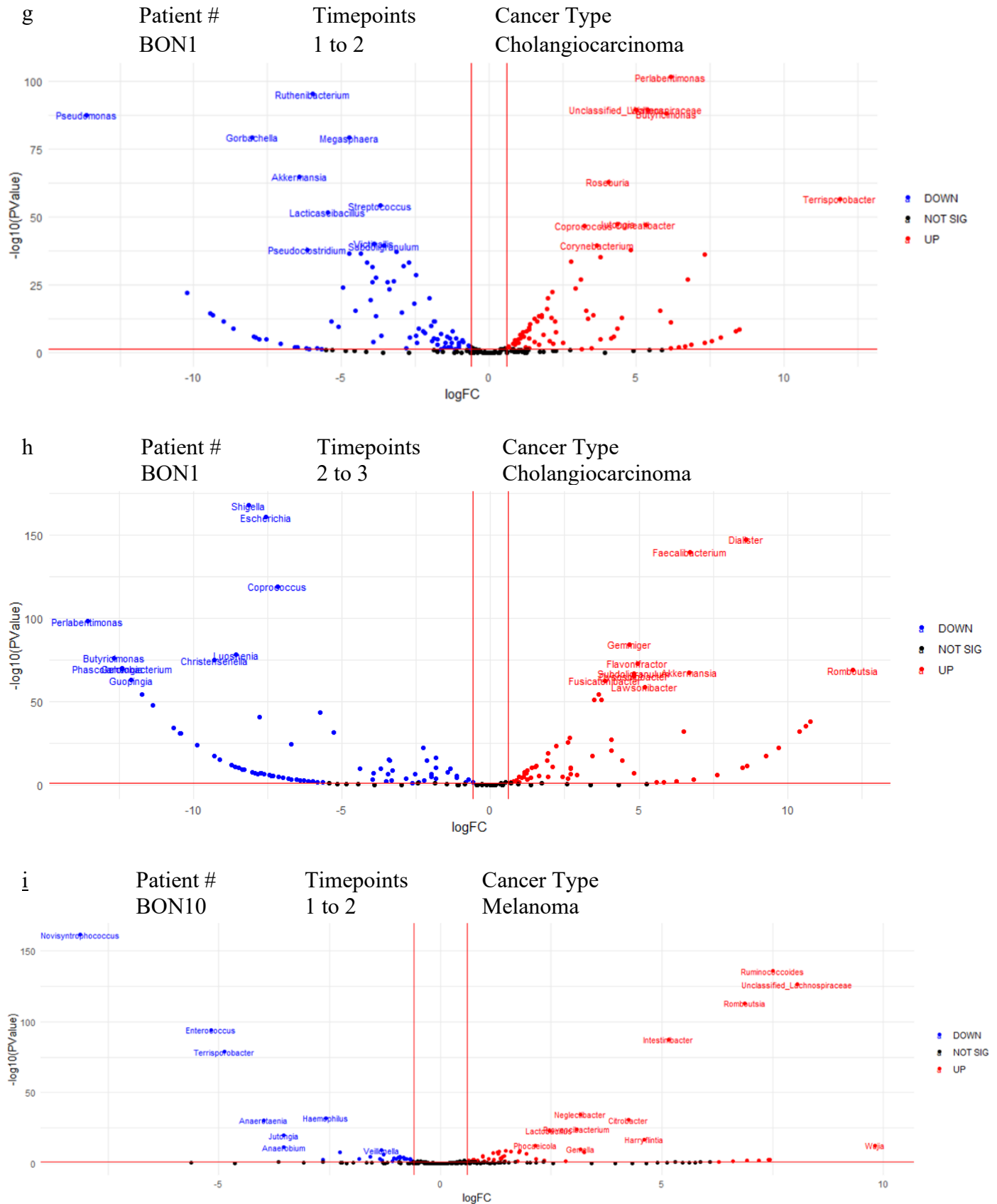
5.4.5.9 *Renal*

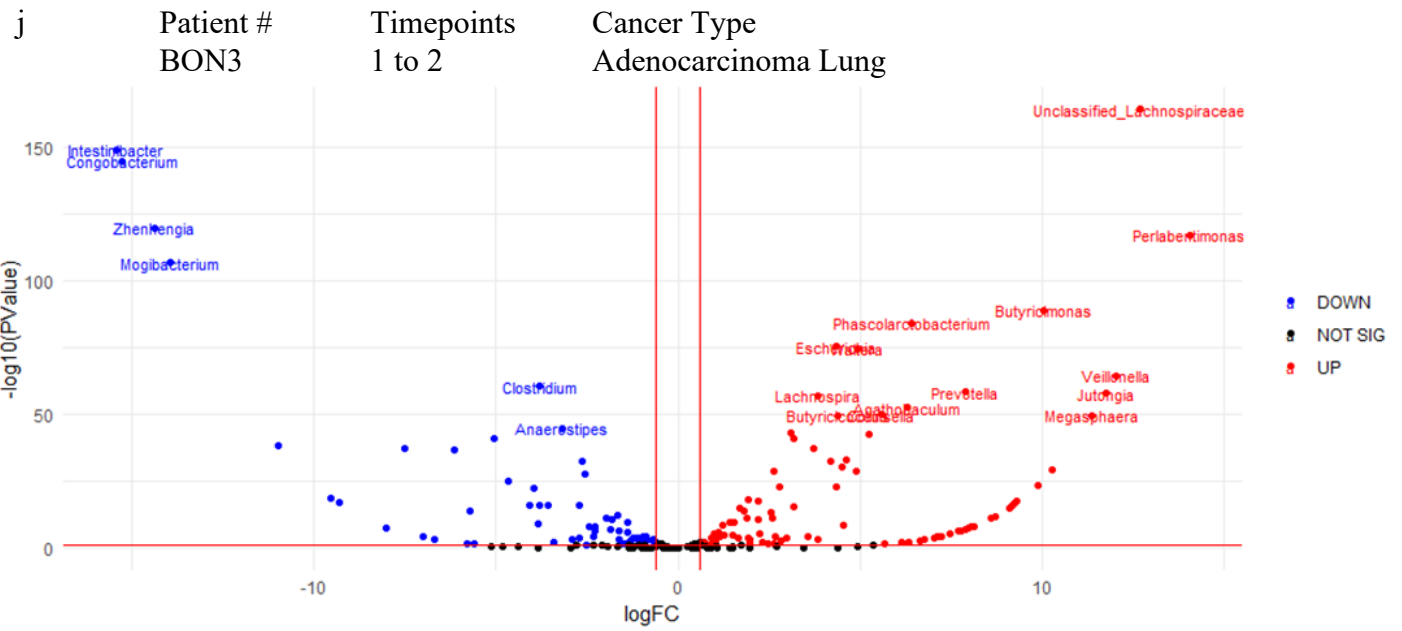
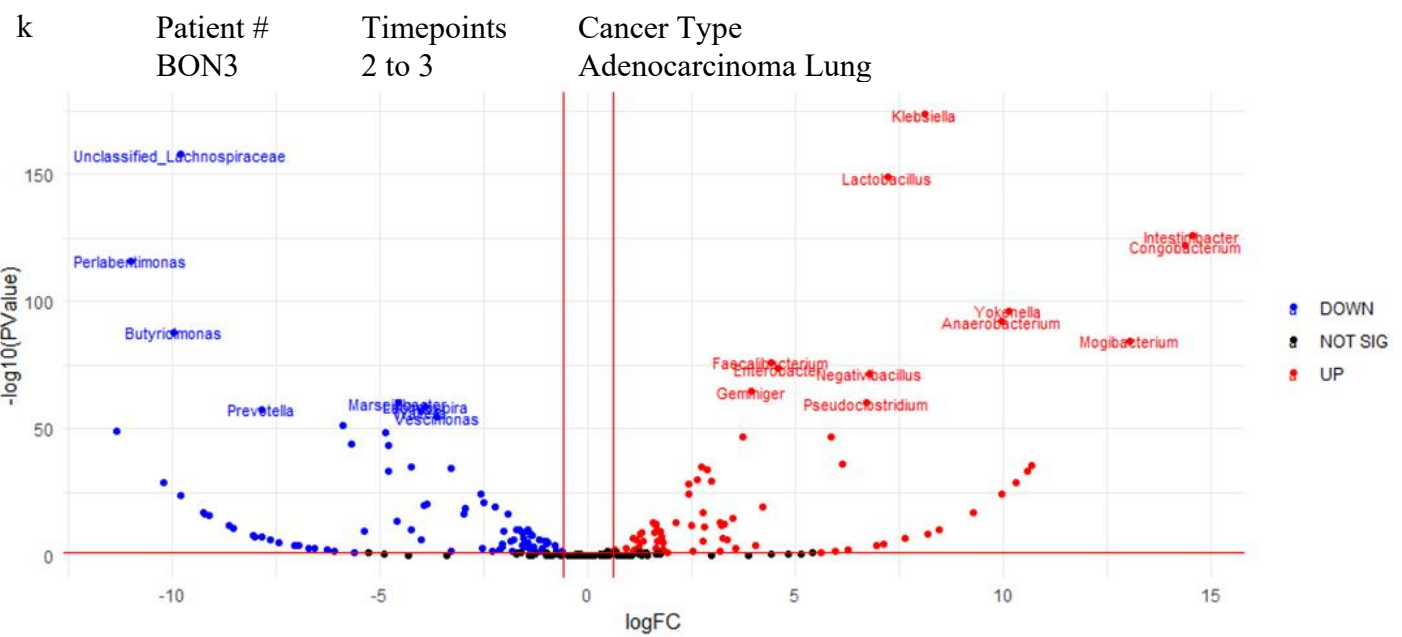
Finally, patient BON11 with renal cell carcinoma provided two samples, with *Bacillus* and *Weissella* showing the most significant increases. *Limosilactobacillus* and *Unclassified_Oscillospiraceae* had the most significant reductions in relative abundance.



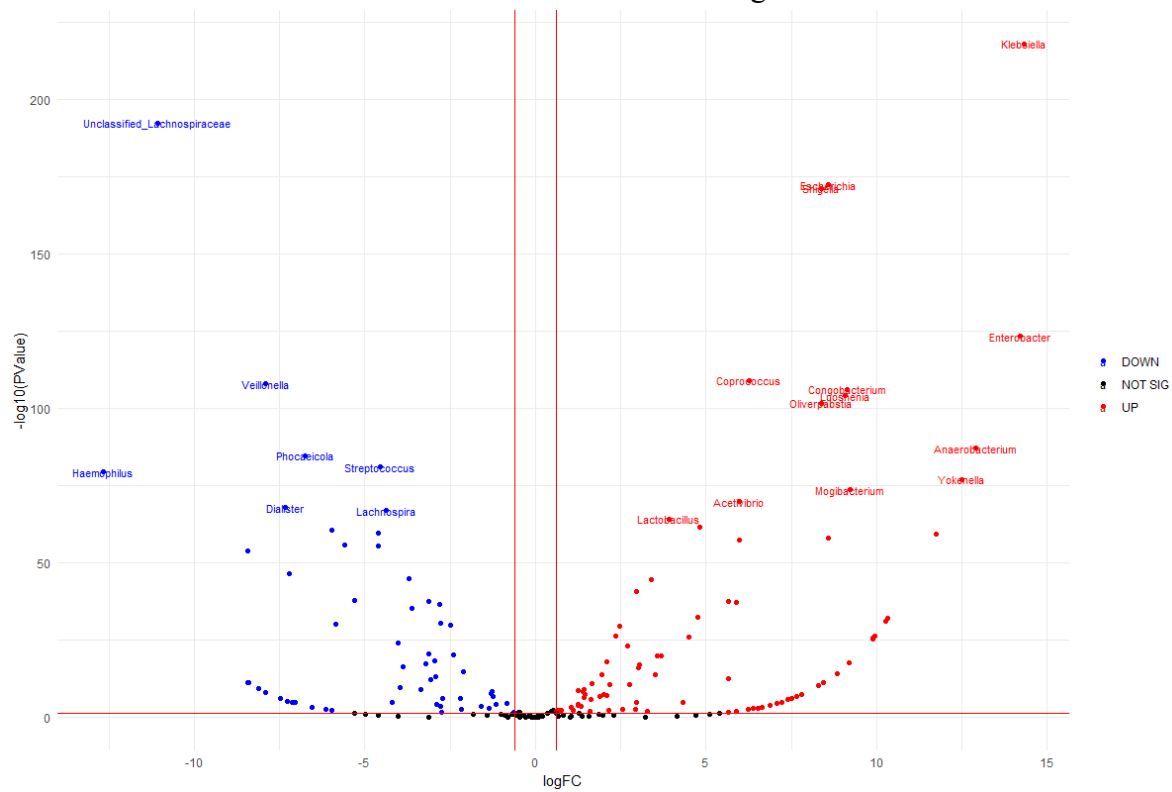




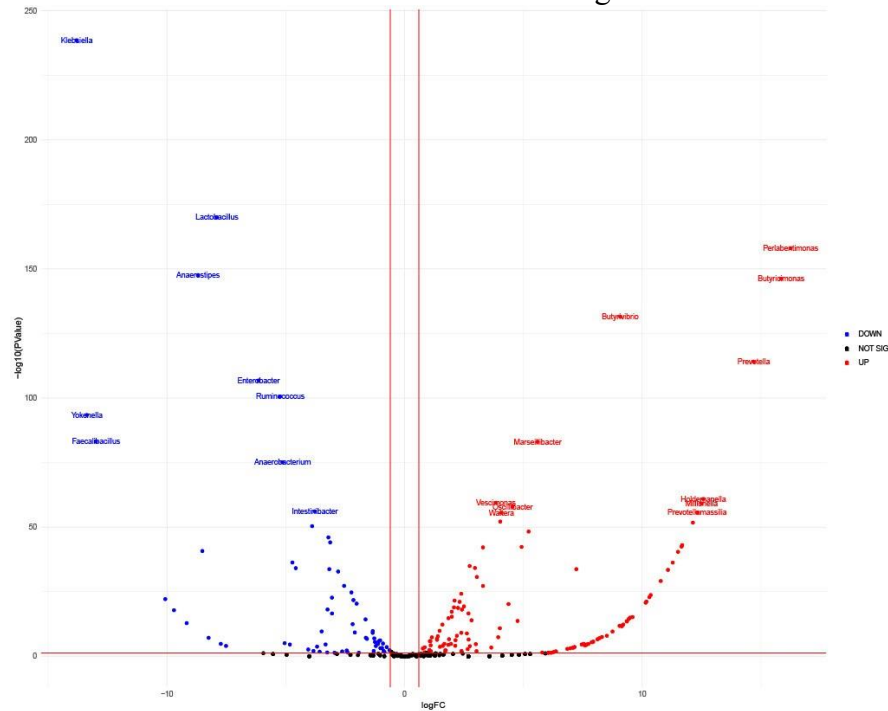


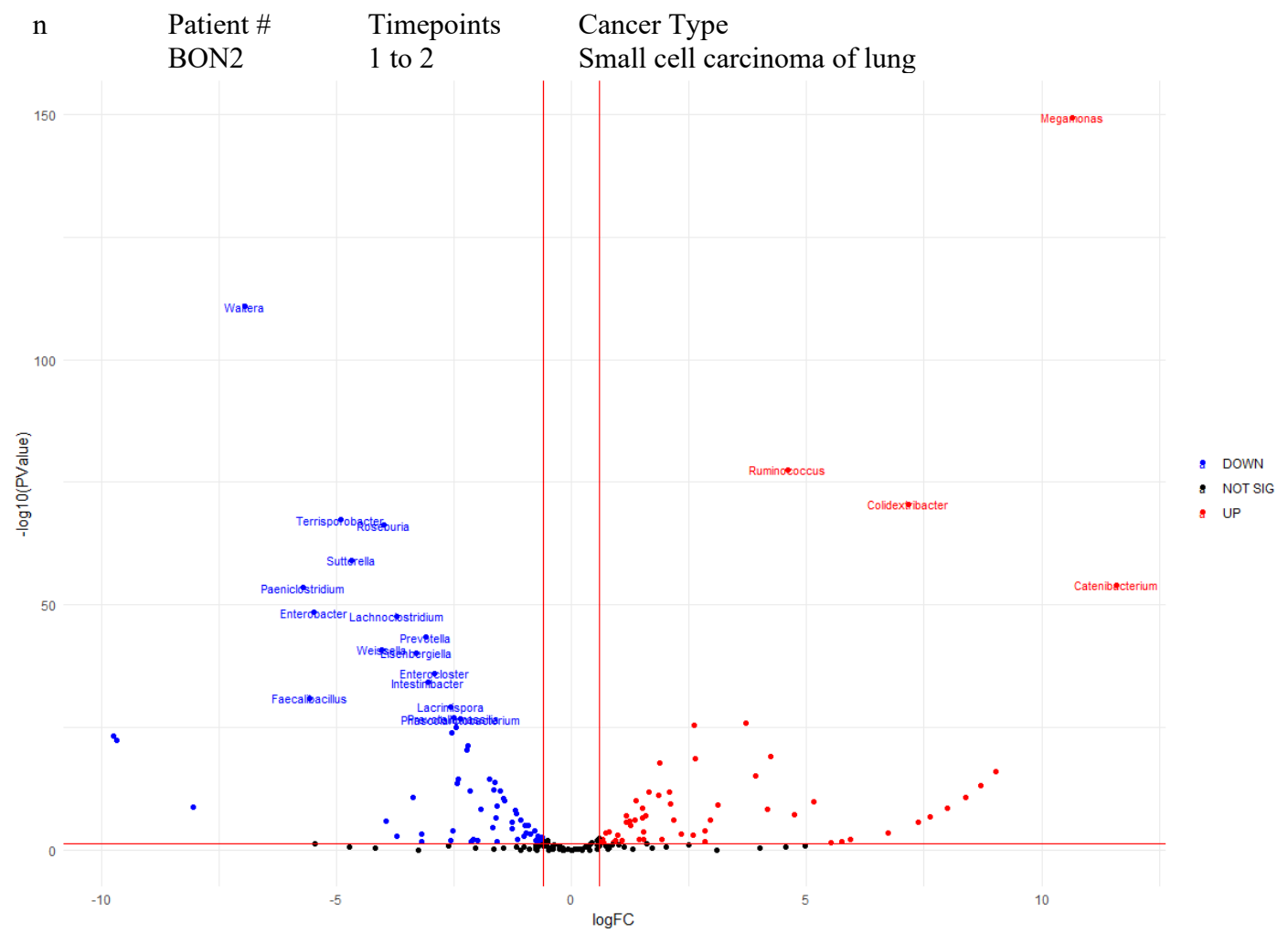


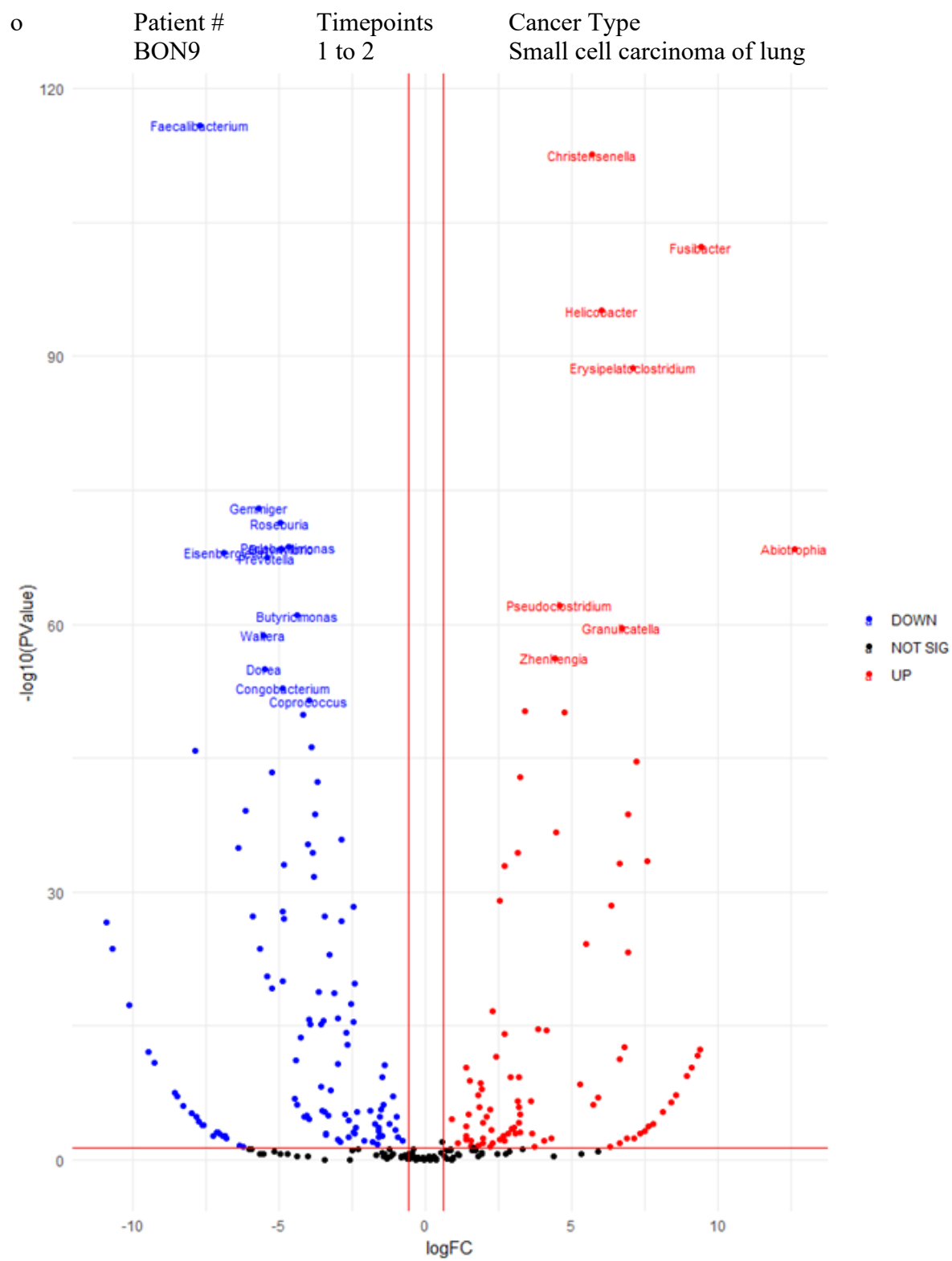
1 Patient # Timepoints Cancer Type
BON4 1 to 2 Adenocarcinoma Lung

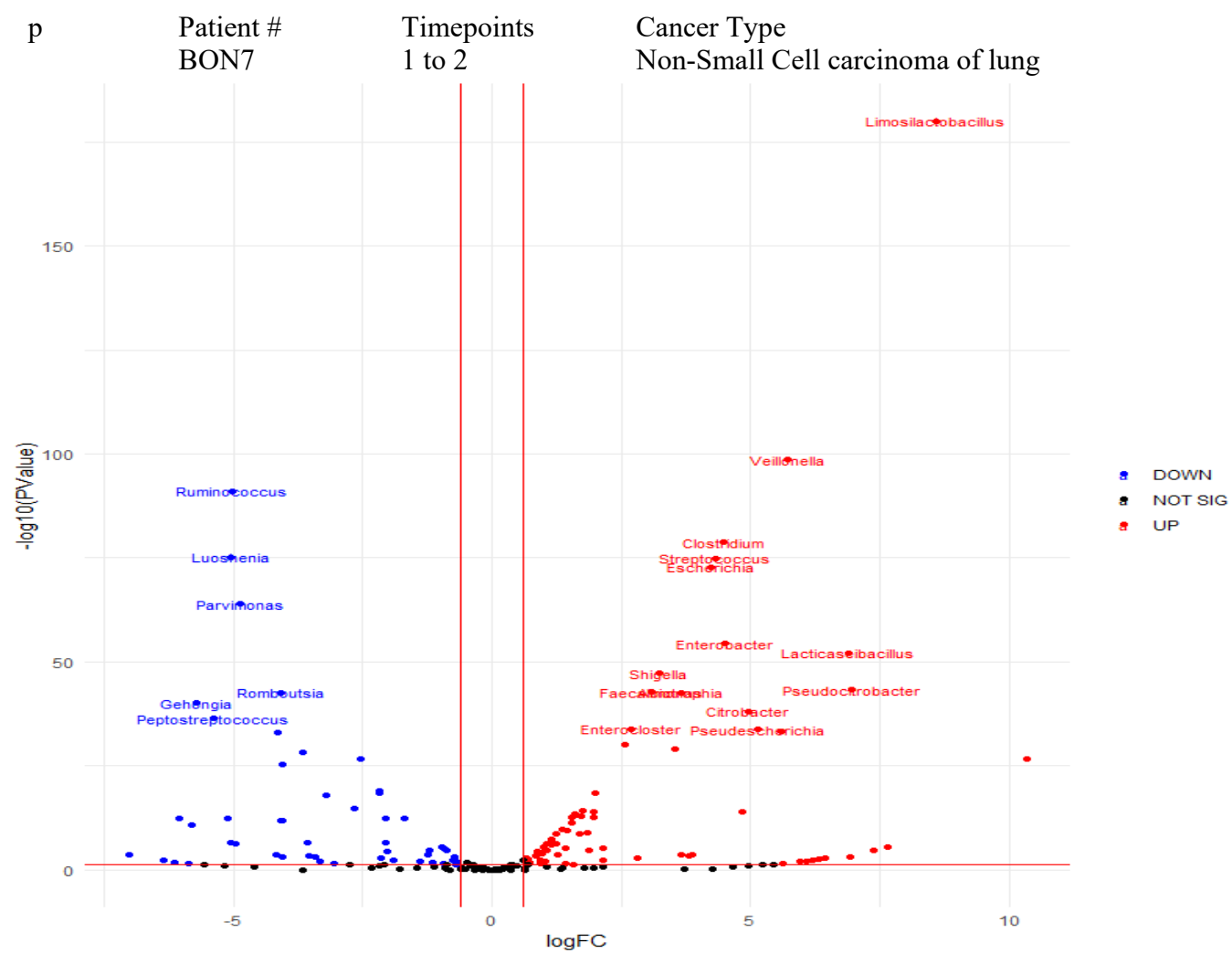


m Patient # Timepoints Cancer Type
BON4 2 to 3 Adenocarcinoma Lung









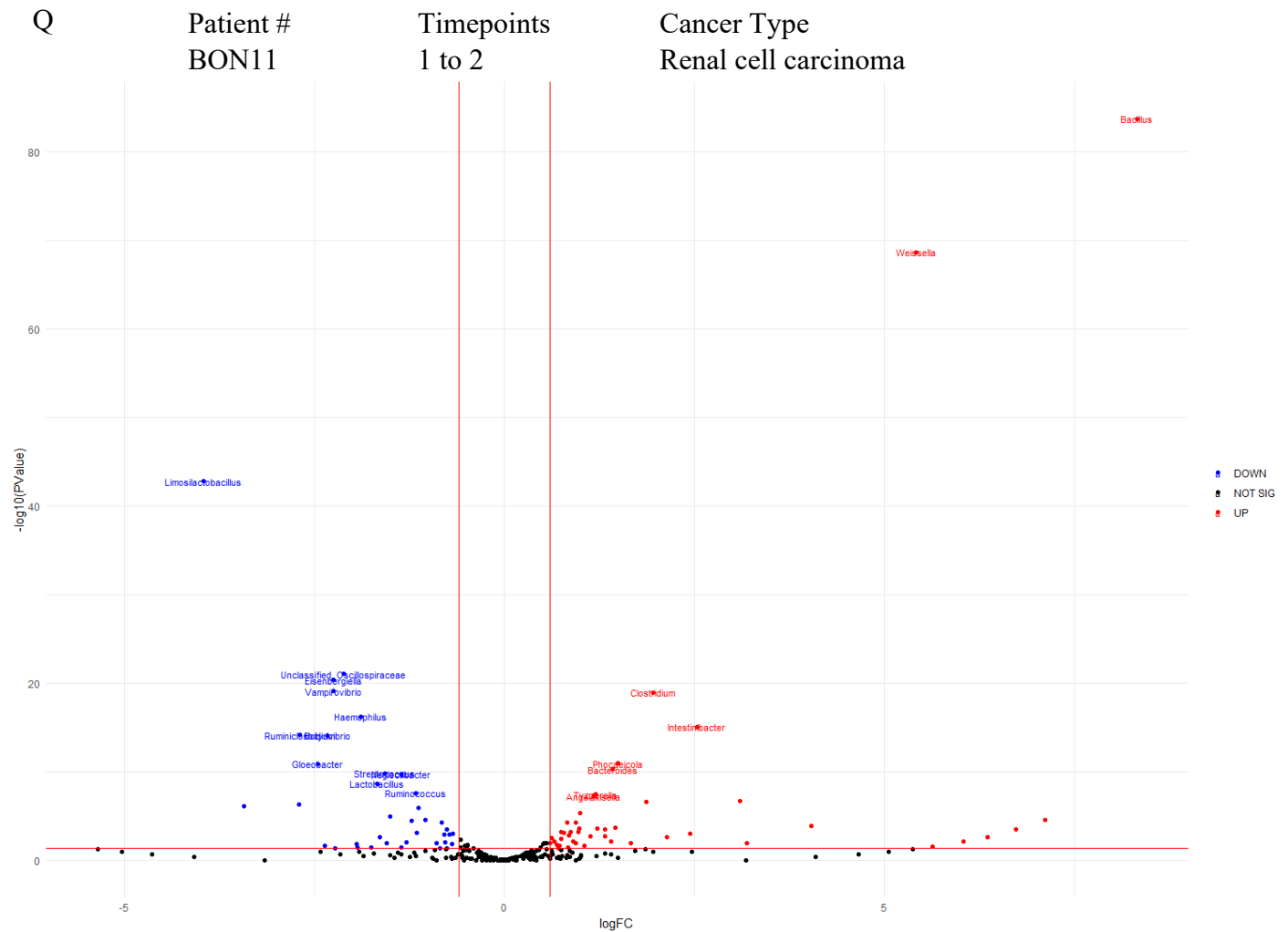


Figure 4. Differential abundance of genera between sampling timepoints. Volcano plot showing OTU fold changes on the X-axis and the negative logarithm (base 10) of the Bonferroni adjusted p-value on the Y-axis, illustrating the differentially abundant bacterial genera between timepoints one and two or timepoints two and three as indicated. Genera with increased relative abundance are shown in red, while those with decreased abundance are shown in blue. The top 20 differentially abundant genera between samples are labelled.

5.5 DISCUSSION

The gut microbiota has been implicated in cancer and shown to modulate the efficacy of anticancer treatment, accentuate or diminish negative side effects, and directly affect GM composition. Historically, clinical studies into the GM and cancer have found inconsistent association patterns. This low concordance between studies is due to the sheer number of confounding factors, including, but not limited to, host genetics, dietary habits, drug treatments, intestinal motility, stool moisture content, and the thousands of potentially occurring bacterial OTUs and their metabolites (Vujkovic-Cvijin et al., 2020). More importantly, the compositional nature of microbiome investigations (Gloor et al., 2017), with significant bacterial load variation having been found between samples in healthy individuals, means that identifying causal relationships between the GM and pathology is nuanced to say the least (Vandeputte et al., 2017). A recent investigation found that conclusions drawn from sample collection times as little as four hours apart can differ greatly, and poor reproducibility in microbiome research may be partially explained by inconsistent sample collection times (Allaband et al., 2024). This is almost impossible to control for, especially in a large MWAS.

In this chapter, at this preliminary stage, there are not enough patient numbers to carry out any sort of meaningful statistical analysis on the clinical data regarding treatment outcome to carry out principal component analysis (PCA) and identify particular genera of bacteria as biomarkers of positive clinical treatment responses or indeed biomarkers of negative side effects. However, preliminary sequencing results have been generated, and they can be discussed within the context of published study findings.

5.5.1 *Relative abundance*

5.5.1.1 *Blautia*

The most prevalent bacterial genus found within the studies patient samples was *Blautia*, a genus of anaerobic bacteria first described in 2008 and commonly found in the gut of mammals (Maturana and Cárdenas, 2021). It is reported to have probiotic properties that include inhibiting pathogenic bacteria, gut barrier maintenance, immune system modulation, and inflammation reduction (Liu et al., 2021c). Reclassification of species originally thought to belong to the genera

Clostridium and *Ruminococcus* resulted in 20 new species of *Blautia* being described in 2020 (Liu et al., 2021c). Despite its potential probiotic benefits, our understanding of *Blautia* diversity remains incomplete, particularly concerning its role in cancer.

Recent research has highlighted a possible association between *Blautia* and increased susceptibility to cutaneous melanoma (CM). Six distinct GM taxa, including *Blautia*, have also been linked to CM (Bao et al., 2024). Additionally, higher relative abundance levels of *Blautia*, specifically the species *Blautia producta*, has been associated with reduced progression free survival (PFS) in melanoma patients (Peters et al., 2019). *Blautia*'s prevalence has also been associated with hepatocellular carcinoma. In a study comparing the GM of 44 primary liver cancer patients with 76 healthy individuals a higher prevalence of *Blautia* was found in cancer patients (Pomyen et al., 2023). However, the exact mechanisms underlying these associations are unclear.

In BC patients undergoing metronomic chemotherapy with capecitabine, the abundance of certain bacterial species, including *Blautia obeum*, was correlated with increased PFS (Guan et al., 2020). *Blautia obeum* might serve as a microbial marker for predicting the overall prognosis of capecitabine treatment. Furthermore, a study on colorectal cancer (CRC) revealed gender specific differences in the GM, with *Blautia*, *Barnesiella*, and *Anaerostipes* being the most significant discrepant genera between males and females with CRC (Yang et al., 2023a).

Mouse models treated with 5-fluorouracil (5-FU) showed an increase in the abundance of *Blautia*, among other species, suggesting potential interactions between these bacteria and chemotherapy (Yuan et al., 2018). Certain species of *Blautia*, such as *Blautia hansenii* and *Blautia producta*, were inversely associated with visceral fat accumulation, indicating their role in metabolic health (Ozato et al., 2022).

In the context of CRC, *Blautia*, along with *Bifidobacterium* and *Faecalibacterium*, were found to be reduced in the mucosa adherent microbiota of patients, while *Fusobacterium* were enriched (Chen et al., 2012). The prevalence of *Blautia* underscores the necessity of going beyond the genus taxonomic level to the species, or even strain level.

5.5.1.2 BC

Blautia has shown significant variation according to the clinical staging and tissue prognosis grading of BC. Notably, among patients with different histoprognostic grades, the abundance of *Blautia* species increases markedly in parallel with the grade of BC (Luu et al., 2017). Studies have also found that the absolute numbers of *Bifidobacterium* and *Blautia* varied significantly according to the clinical stages of BC patients, with other bacterial counts varying with BMI (Bard et al., 2015).

This chapter study so far does not have the resolution to identify *Blautia* to species level; however, it will be interesting to compare the overall relative abundance of *Blautia* with clinical data when it is received. From the results so far, patients BH10, BH11, BH12, and BH14 all had abundance levels of roughly 25%, whereas patients BH3 and BH9 had abundance levels between 10 and 20%.

The genus *Walteria*, family *Lachnospiraceae*, was first proposed by Wylensek *et al.* in 2020 (Wylensek et al., 2020), and even though it is associated with the BC group more closely than any other cancer type, there have been no published associations to date, to the author's knowledge.

BC development is influenced by both oestrogen dependent and non-oestrogen dependent functions of the GM involving the production of bioactive metabolites. Dysbiosis of the intestinal microbiota has been linked to BC, as certain gut bacteria can alter the production of beneficial anticancer metabolites and disrupt oestrogen metabolism in the gut (Mikó et al., 2019).

Microbial β -Glucuronidase (GUS) can play a role in oestrogen reactivation and BC (Sui et al., 2021). GUS producing bacteria, such as *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Citrobacter*, *Escherichia*, *Faecalibacterium*, and *Propionibacterium*, can increase GUS deconjugation activity, which is dependent on gut microbial composition and diet (Dabek et al., 2008, Bard et al., 2015, Alizadehmohajer et al., 2020). Postmenopausal women seem to be particularly sensitive to oestrogen levels, and their BC risk is directly and significantly associated with a high level of exposure to oestrogens (Key et al., 2002). *Clostridium* and *Faecalibacterium* were both found within the top 20 genera in all BC samples except BH14, which was lacking *Clostridium*.

Short chain fatty acids (SCFAs), such as butyrate, propionate, acetate, and lactate, are key bacterial metabolites impacting BC progression. SCFAs are primarily produced by species colonising the

colon, such as *Eubacterium rectale*, *Clostridium leptum*, and *Faecalibacterium prausnitzii*, as well as by lactate utilising species like *Eubacterium hallii* and *Anaerostipes* (Jaye et al., 2022). Evidence has shown that sodium butyrate has promising anti-tumour activity on BC cells, both alone and in combination with other anti-cancer agents like the anti-HER2 antibody trastuzumab (Chen et al., 2011). *Eubacterium*, *Clostridium*, *Faecalibacterium*, and *Anaerostipes* were found in the majority of BC samples, but they were not resolved to species level.

The reclassification of many *Ruminococcus* species to the genus *Mediterraneibacter* in 2018 may account for the lack of literature regarding associations between *Mediterraneibacter* and any type of cancer in GM association studies (Togo et al., 2018). *Ruminococcus gnavus* has previously been found to be associated with BC (Long et al., 2023).

5.5.1.3 Rectal cancer

A research study that examined host gene expression data has revealed stronger microbe host interactions in early-onset colorectal cancers compared to late-onset cancers. This suggests that these interactions are age dependent, with more pronounced microbial host interactions at the tumour site in early-onset CRC. The results imply a direct role of the GM in tumourigenesis via cancer related pathways in this age group (Adnan et al., 2024).

Several GM pathogens have emerged with specific signatures linked to colorectal cancer (CRC) associated with excessive body weight (EBW). These species include *Anaerobutyricum hallii*, *Clostridioides difficile*, and *Fusobacterium nucleatum* (Zhu et al., 2024). The genus *Fusobacterium* was found at the 1% relative abundance level in patient BH5's first sample but not at the next timepoint. Patient BH12 also had *Fusobacterium*, but at a lower level of 0.1%. Further investigation would be necessary to get to species level. The issue with pathogenic bacteria associated with cancer initiation and stool sampling is that the causal bacteria may have come and gone, but their negative effects in terms of cancer may be life threatening long after they are no longer detectable. *Fusobacterium nucleatum*'s primary niche is the oral cavity, where it is associated with periodontal disease (Chen et al., 2022a).

Sulfidogenic bacteria, such as *Fusobacterium*, *Desulfovibrio*, and *Bilophila wadsworthia*, are implicated in CRC development through the production of hydrogen sulphide, which may

contribute to tumourigenesis (Dahmus et al., 2018). Dating back to 1966, *Streptococcus bovis* was one of the first bacteria linked to CRC (Trichopoulos and Polychronopoulou, 1986).

Compared with healthy controls, studies have consistently shown that patients with CRC have a significantly higher relative abundance of *Fusobacterium nucleatum* and decreased overall alpha diversity (Ahn et al., 2013). The bacterial genera *Bacteroides fragilis*, *Escherichia coli*, *Fusobacterium nucleatum*, *Enterococcus faecalis*, and *Streptococcus gallolyticus* have all been linked to CRC (Alhinai et al., 2019).

The occurrence of pathogenicity islands (PAIs) significantly influences the virulence of various bacteria and their association with colorectal cancer (CRC). These genomic regions, acquired through horizontal gene transfer, enhance the pathogenic capabilities of bacterial strains, thereby contributing to disease progression, including carcinogenesis (Hacker and Kaper, 2000).

The pathogenicity island polyketide synthase (pks) encodes a set of enzymes that synthesise the genotoxin colibactin (Chagneau et al., 2022), and *E. coli* strains carrying the pks pathogenicity island are notably prevalent in the colonic mucosa of colorectal cancer patients. The prevalence of pks+ *E. coli* was found to be about 67% in CRC patients compared to 21% in healthy individuals (Arthur et al., 2012; Buc et al., 2013). This stark contrast highlights the potential involvement of pks+ *E. coli* in the development of CRC.

One investigation has discovered a unique mutational signature in human intestinally derived organoids exposed to genotoxic pks+ *E. coli* (Pleguezuelos-Manzano et al., 2020). The same pattern has been detected in CRC patients, suggesting a prior exposure to *E. coli* carrying the pks pathogenicity island. The presence of this mutational signature in both experimental models and human cancer patients highlights the carcinogenic potential of pks+ *E. coli*.

The relative abundance for sample BH12.1 was primarily composed of only four genera, with *Streptococcus* comprising over 60% of the relative abundance followed, by *Clostridium*, *Escherichia*, *Shigella*, and 20% in the “Other” category. The second sample, BH12.2, had the same four genera: an expansion of *Escherichia*, *Shigella*, a reduction of *Streptococcus*, and the “Other” category. This patient had poor gut health, which was getting worse as time progressed.

Gender differences in the GM of CRC patients have also been observed. The top three bacterial genera for men with CRC were found to be, *Bacteroides*, *Eubacterium*, and *Faecalibacterium*, whereas in women, the top three were *Bacteroides*, *Subdoligranulum*, and *Eubacterium*. The abundance of *Escherichia*, *Eubacteriales*, and *Clostridia* was also higher in males compared to females. This highlights the importance of taking gender into account when using gut bacteria to treat and predict CRC (Yang et al., 2023b). Without clinical data, no inferences can be made at this stage regarding gender.

The oral microbiome member *Peptostreptococcus anaerobius*, has been found to increase colon dysplasia in mouse models of colorectal cancer (CRC). This bacterium interacts with the Toll-like receptors TLR2 and TLR4 on colon cells, leading to elevated levels of reactive oxygen species. This, in turn, promotes cholesterol synthesis and cell proliferation, which are factors in tumour development and exacerbate CRC development (Tsoi et al., 2017). Sample one from patient BH5 had 6% *Peptostreptococcus* relative abundance.

This bacterium induces intratumoural myeloid derived suppressor cells (MDSCs) and enhances their immunosuppressive activities whilst impairing effective T-cell responses. (Liu et al., 2024). Other evidence implicates the gut microbiota in CRC, with *P. anaerobius* selectively enriched in the faecal and mucosal microbiota of CRC patients. *P. anaerobius* drives CRC via a cell signalling axis, involving the putative cell wall binding repeat 2 (PCWBR2) integrin axis as a potential therapeutic target for CRC (Long et al., 2019).

Microbial dysbiosis in the non-invasive diagnosis of laterally spreading tumours (LST) is characterised by a high relative abundance of the genera *Lactobacillus* and *Streptococcus* and the species *enterotoxigenic Bacteroides fragilis* (ETBF), *Peptostreptococcus stomatis* (*P. stomatis*), and *Parvimonas micra* (*P. micra*). The faecal microbiome signature (ETBF–*P. stomatis*–*P. micra*) can diagnose LSTs with high accuracy. These bacteria are related to malignant LST, and *P. stomatis* exhibits a high predictive value for adenoma recurrence after LST resections. Thus, the faecal microbiome signature of LST could provide a non-invasive alternative for early detection of LST and predicting adenoma recurrence risk post resection (Shen et al., 2021b). Lacking resolution and clinical data, no inferences can be made at present.

The GM composition is closely associated with the pathological response after preoperative chemoradiation in patients with rectal cancer. Specifically, the complete response (CR) rate was found to have a positive linear relationship with *Dialister pneumosintes* and a negative linear relationship with *Anaerostipes hadrus*, *Bacteroides dorei*, and *Alistipes senegalensis* (Jang et al., 2020).

Contrasting this, but at a higher taxonomic level, *Dialister* was found among 17 bacterial OTUs identified as potential biomarkers for colorectal cancer (CRC) recurrence and poor patient prognosis (Huo et al., 2022). *Dialister invisus* and *Fusobacterium nucleatum* were found to indicate a high risk of CRC progression (Huh et al., 2022). Patient BH5's first sample, BH5.1, had a *Dialister* relative abundance of 4%, which was reduced to 1% by sample BH5.2, and sample BH7.1 from patient BH7 was at the 1% level. Again, species level resolution is vitally important.

The GM exhibits distinct compositions in patients with colorectal cancer (CRC) compared with healthy controls, with specific microbial taxa being exclusively detected in CRC patients. *Fenollaria massiliensis*, along with *Porphyromonas*, *Peptoniphilus*, *Finegoldia*, and *Ezakiella*, were identified solely in individuals with CRC, particularly in stages II and III. These microbial taxa could potentially serve as biomarkers for CRC at these stages (Messaritakis et al., 2024). Patient BH5 sample BH5.1 had 6% *Fenollaria* relative abundance and was one of only two patient samples found to have any trace of *Fenollaria*. Interestingly, the only other sample was another rectal patient, sample BH12.1, with a 0.001% relative abundance. BH5.2 did not contain any *Fenollaria* reads.

Additionally, *Ruthenibacterium lactatiformans*, a known lactate producer, has been reported at heightened levels in CRC patients. This bacterium, along with other CRC associated bacterial species, contributes to the unique microbial profile observed in CRC, potentially influencing disease progression and offering insight into microbial interactions in the tumour microenvironment (Adnan et al., 2024). Sample BH5.1 had the genus *Ruthenibacterium* at 3%, reducing to 0.008% by the second sample. The remaining rectal patients all had trace amounts except for patient BH12, who has already been described here as having the least diverse GM of all samples in this study.

5.5.1.4 Cholangiocarcinoma

Cholangiocarcinoma (CCA) at the global level is frequently associated with *Opisthorchis viverrini* infection. Since this is a food borne trematode parasite, a southeast Asian liver fluke, it is unlikely to be significantly relevant in cancer patients in an Irish context. However, clinical reports have frequently observed an increase in *Helicobacter* species in stool samples from these patients (Elvevi et al., 2023). Overexpression of the *Helicobacter* genes *CagA* and *CagE* has been documented in CCA. The proteins derived from these genes can migrate across the plasma membrane, causing phosphorylation of sarcoma family kinases. This activity may serve as a transduction signal to promote fibrosis and inflammation of the bile ducts (Deenonpoe et al., 2017).

Increases in *Veillonellaceae*, *Alistipes*, *Enterobacteriales*, and *Firmicutes* have been suggestively associated with higher intrahepatic cholangiocarcinoma (ICC) risk, while increases in *Anaerostipes*, *Paraprevotella*, *Parasutterella*, and *Verrucomicrobia* appeared protective. Bioinformatics analysis revealed that differentially expressed genes near GM associated loci might influence ICC through regulating pathways and the tumour immune microenvironment (Chen et al., 2023). Another study found associations at the genus level with *Alloscardovia*, *Peptostreptococcaceae*, *Actinomyces*, and *Lactobacillus* all being found to be enriched compared to healthy controls (Jia et al., 2020).

In the study samples, the genus *Megasphaera*, a member of the *Veillonellaceae* family, was the seventh highest relatively abundant genus in the second sampling timepoint of patient BON1. Sample BON14.1 did contain the genus *Veillonella*, another member of the *Veillonellaceae* family, ranking as the ninth highest relatively abundant genus. There were no reads for any *Helicobacter* spp.. Causally-associated bacteria such as *Helicobacter* may have been a transient infection in the past. None of the genera *Alloscardovia*, *Peptostreptococcaceae*, *Actinomyces* or *Lactobacillus* were found to be enriched in any of the ICC patient samples.

5.5.1.5 Metastatic melanoma

Outside of the associations with *Blautia* mentioned at the start of the discussion, the majority of melanoma GM association studies are carried out in relation to ICI treatment, and the GM members associated with better treatment outcomes. As regards microbes with causal effects, Vitali *et al.*

found that gut fungal profiles are significantly different in melanoma patients compared to controls. Specifically, the GM of melanoma patients was found to have a higher relative abundance of yeasts belonging to the order *Saccharomycetales* and the bacterial species *Prevotella copri* (Vitali et al., 2022).

The study provided evidence potentially implicating the GM in the pathogenesis of melanoma. The authors observed that the composition of the GM in early-stage melanoma changes along a gradient from *in situ* to invasive and then to metastatic melanoma. Changes in the GM and mycobiome are correlated with the histological features of early-stage melanoma, the clinical course, and the response to immune therapies in advanced stage melanoma through direct or indirect immunomodulation (Vitali et al., 2022). Whilst at this preliminary stage the mycobiome has not been sequenced, this work will be carried out in the future before the study ends with amplification of the internal transcribed spacer (ITS) rRNA (Schoch et al., 2012).

5.5.1.6 Metastatic squamous carcinoma

Similar to melanoma, there are few causal associations between metastatic squamous carcinoma, which is commonly referred to as neck cancer, and GM OTUs other than a slight association with the human papilloma virus (HPV) (Desai et al., 2009). The majority of studies focus on ICI treatment and the effect of GM composition on treatment outcomes and negative side effects. Viruses are not expected to be investigated during this study.

Adenocarcinoma Lung Cancer (LADC)

Previous studies have identified specific gut microbial signatures for the potential prediction of early-stage lung cancer, including *Bifidobacterium*, *Aecalibacterium*, *Bacillus*, *Streptococcus infantis*, and *Veillonella* (Zheng et al., 2020). Patients with LADC exhibit reduced α -diversity and altered β -diversity in their GM compared with healthy controls. Notably, the GM of LADC patients showed higher abundances of *Flavonifractor*, *Eggerthella*, and *Clostridium*. An increased abundance of *Flavonifractor plautii* was linked to advanced-stage LADC and a higher metastasis rate. Additionally, *Haemophilus parainfluenzae* was the most frequently shared taxon in both the lung and gut microbiota of LADC patients. Functional pathways involving leucine, propanoate, and fatty acids were associated with LADC progression (Guo et al., 2023).

Except for one sample (BON12.1), the remaining samples from patients with LADC were well represented in the top 20 genera, with less than 20% belonging to other categories. Sample BON12.1 was composed of 50% *Enterobacter*, which is known for its involvement in opportunistic infections and endotoxin production (Fei et al., 2020). *Enterobacter spp.* and *Escherichia coli* are common in lung cancer tissues (Laroumagne et al., 2011, Georgiou et al., 2021). Other researchers have found that lung cancer patients exhibited lower levels of *Enterobacter* in stool samples compared with healthy controls (Zhang et al., 2018).

5.5.1.7 Non-Small Cell Lung Cancer (NSCLC)

In other studies of early-stage NSCLC patients, significant alterations have been found in 26 genera and 123 metabolites. More abundant genera include *Agathobacter*, *Blautia*, *Clostridium*, and *Muribaculacea*. For different grades of invasive pulmonary adenocarcinoma (IPA), *Blautia* and *Marinobacter* were characteristic of grade type 1, *Dorea* of grade type 2, and *Agathobacter* of grade type 3 (Ni et al., 2023). In this chapter, with only one patient and two samples, *Blautia* abundance did decrease from 25% to just under 10%.

Distinct host microbe interactions have been observed during different phases of NSCLC. Specific forms of the GM and SCFAs serve as valuable biomarkers and therapeutic targets for NSCLC diagnosis and treatment. A biomarker panel including *Faecalibacterium*, *Bifidobacterium*, *Butyricicoccus*, *Klebsiella*, *Streptococcus*, and *Blautia* successfully distinguished patients in the early lung cancer (ELC) with brain metastases (BM) stages from healthy controls (Jiang et al., 2024a). Additionally, a significant positive correlation was found between the genera *Romboutsia* and *Alistipes* in both lung tumour biopsies and stool samples from NSCLC patients, with higher bacterial richness in stool samples correlating with prolonged overall survival (Ankudavicius et al., 2024). Patient BON7 had all of the biomarkers for patients with brain metastases except *Bifidobacterium* and was highly positive for both *Romboutsia* and *Alistipes*.

5.5.1.8 Small Cell Lung Cancer (SCLC)

Several associations with SCLC include protective effects linked to the *Eubacterium ruminantium* group, *Barnesiella*, the *Lachnospiraceae* family, and *Butyricimonas*. Conversely, *Intestinibacter*, *Eubacterium*, *Bilophila*, and *Bacillales* potentially promote the onset of SCLC (Yang et al., 2024).

Sample two from patient BON9 (BON9.2) showed the most significant relative abundance change, with an increase to 35% *Christensenella*. This bacterium is associated with controlling insulin resistance and positively impacting metabolic disorders like diabetes and obesity, as well as inflammatory bowel disease (Pizzo et al., 2022, Ignatyeva et al., 2023). However, *Christensenella* has also been found in lung tissue from idiopathic pulmonary fibrosis (IPF) and lung cancer, highlighting the importance of context in bacterial associations with cancer. This example also offers insight into the additional role of bacteria outside of the GM, either in the proximal or intratumoural microbiomes, where bacterial roles and phenotypes can vary greatly depending on the environmental setting.

5.5.1.9 Renal cancer

The majority of studies looking at associations between renal cancer and the GM are in relation to ICI treatment outcomes and side effects. Five genera - *Blautia*, *Streptococcus*, *Ruminococcus*, *Romboutsia*, and *Eubacterium* were all found to be significantly overrepresented in stool samples from the clear cell renal carcinoma (ccRCC) group, whereas *Prevotella*, *Lachnospira*, *Lachnospirillum*, and *Roseburia* were the most abundant microbiota in healthy controls (Chen et al., 2022b). One of the only other studies looking in particular at associations with the GM found enrichment and proliferation of sulphide degrading bacteria, *Desulfovibrionaceae*, to be associated with clear cell renal carcinoma (ccRCC) when compared with a healthy control group (Yang et al., 2023a).

5.5.1.10 Oesophageal cancer (OC)

Associations between bacteria and OC initiation have been known for some time. One notable example is *Fusobacterium nucleatum*, which primarily resides in the oral cavity and is known to cause periodontal disease. This bacterium can contribute to aggressive tumour characteristics in oesophageal cancer by activating chemokines such as CCL20, which, together with its ligand partner CCR6, is responsible for the chemoattraction of immature dendritic cells (DC) in oesophageal cancer tissue (Baba et al., 2017, Schutyser et al., 2003). DC cells have been found to facilitate tolerance towards transformed cells (Dudek et al., 2013). Similarly, *Campylobacter* species, which have a potential pathogenetic role, are significantly present in both Barrett's

oesophagus and adenocarcinoma, indicating their possible involvement in tumour progression (Man, 2011).

Gastroesophageal reflux disease (GERD) is another important risk factor for oesophageal adenocarcinoma. GERD can cause erosive oesophagitis, which, following an abnormal healing process, can lead to metaplastic changes in the oesophagus (Wheeler and Reed, 2012).

The relationship between the gut microbiome and OC, however, remains poorly explored. Recent studies have begun to shed light on this relationship. A small study involving 15 patients found a substantial increase in *Bacteroides vulgatus* and *Escherichia coli* in stool samples from patients with squamous cell carcinoma and adenocarcinoma of the oesophagus compared to healthy controls (Ishaq et al., 2021). A similarly sized study involving 23 patients, all with OC but not subtyped, found clear differences in the microbial communities between oesophageal cancer patients and controls. These differences were primarily due to the enrichment of several genera in cancer patients, including *Streptococcus*, *Bifidobacterium*, *Subdoligranulum*, *Blautia*, *Romboutsia*, *Collinsella*, *Paeniclostridium*, *Dorea*, and *Atopobium* (Deng et al., 2021).

The single patient BH4 did exhibit an *Escherichia* increase in relative abundance of 30% by sampling timepoint three. *Bacteroides* were at low levels, below 1% for all samples. All samples were enriched for *Streptococcus*, with an average of 20% relative abundance across all three sampling timepoints. The average for *Blautia* was 15%, *Dorea* 10%, and *Romboutsia* was 2%, but only for samples BH4.2 and BH4.3. *Prevotella* was at 10% abundance for sample one, BH4.1, which has been found to be enriched in OC tumours (Greathouse et al., 2024).

5.5.2 Alpha diversity as measured by SDI

Alpha diversity of the GM, as measured using SDI, reflects the richness and abundance of the organisms present within a sample or community. This summative measure may correlate better with cancer treatment outcomes than the presence or absence of specific microorganisms (Pierrard and Seront, 2019). Studies have shown that higher alpha diversity is associated with better response rates to ICI (Gopalakrishnan et al., 2018b, Salgia et al., 2020, Hakoziaki et al., 2020). Conversely, patients treated with antibiotics before ICI therapy exhibited lower alpha diversity

compared with controls (Hakozaki et al., 2020). Overall, the use of antibiotics had a significant negative impact on patients' responses to ICI treatment, suggesting that the use of antibiotics in this patient population should be carefully considered (Angrish and Pezo, 2022).

The relationship between alpha diversity and cancer treatment outcomes can vary. For instance, no significant differences in alpha diversity were observed between responders (R) and non-responders (NR) in the GM of gastric cancer patients (Zhang et al., 2024). In the context of colorectal cancer (CRC), there is growing evidence that patients exhibit decreased gut microbiota diversity (Yachida et al., 2019). Similarly, a decrease in species diversity is one of the manifestations of gut dysbiosis in lung cancer (LC) patients (Han et al., 2023).

In BC patients, most studies show a significant reduction in alpha diversity compared to cancer free controls, representing a loss of compositional complexity within the GM (Gaber et al., 2024). This reduction in microbiome richness and evenness is consistent with unhealthy gut environments, ageing, and disease states (Hou et al., 2021). Restoration of a healthy gut microbiota through faecal microbiota transplantation (FMT) is a potentially beneficial prospective approach that could enhance the therapeutic efficacy of ICIs and ameliorate immune related adverse events (irAEs) (Angrish and Pezo, 2022).

As a result of the low patient numbers per cancer type, it is impossible to distinguish any patterns of alpha diversity change on average because of cancer or treatment type using the SDI. Stepping back and taking all 13 patients with more than one sample, six trended upwards and seven down with successive sampling points. Combined with the lack of clinical data or outcomes, there is little more that can be said.

5.5.3 Differential abundance in relation to treatment type

5.5.3.1 Chemotherapy

Capecitabine, an orally delivered prodrug of 5-fluorouracil (5-FU), is commonly used in combination with platinum based chemotherapy to treat advanced metastatic breast and colorectal cancers. It is converted to its cytotoxic form, 5-FU, by thymidine phosphorylase, primarily in

tumour cells and the liver, where it exerts its anti-tumour effects (Yu and Di, 2017). The relative abundance of GM changes significantly during chemotherapy. For example, lung cancer patients undergoing chemotherapy showed a higher relative abundance of *Eubacterium*, *Ruminococcus*, and *Faecalibacterium* compared to healthy subjects, while the abundance of *Prevotella*, *Streptococcus*, *Enterococcus*, and *Roseburia* decreased. These changes were also evident at the phylum level, with *Firmicutes* and *Euryarchaeota* dramatically increasing post-chemotherapy. Specific GM compositions at baseline could predict G.I. reactions in lung cancer patients during chemotherapy (Zheng et al., 2020).

SCFAs, such as butyrate and propionate, may enhance tumour sensitivity to chemotherapeutic agents and augment anti-tumour immune responses. Decreased abundance of SCFA producing taxa like *Coprococcus*, *Dorea*, and uncultured *Ruminococcus* is linked to lower efficacy of neoadjuvant chemotherapy in BC patients (Li et al., 2022b). Additionally, microbial enzymes can induce drug transformations that inactivate cytotoxic compounds. For example, intratumoural γ -*proteobacteria* can modulate the toxicity of gemcitabine by transforming it into its inactive metabolite through the enzyme cytidine deaminase, a function also found in *Escherichia coli* (Geller et al., 2017a). Similarly, *E. coli* can inhibit or enhance the antineoplastic activity of various chemotherapeutic drugs (Lehouritis et al., 2015).

An increased abundance of mucosal pathobionts, such as *Fusobacterium nucleatum*, is associated with resistance to oxaliplatin and 5-FU in colon cancer cell lines. This resistance is mediated by the activation of the autophagy pathway through targeting TLR4 and MYD88, which orchestrates a downstream molecular network of microRNAs (Yu et al., 2017). Reactive oxygen species induced by commensal bacteria dependent inflammatory responses also play a crucial role in maintaining the cytotoxicity of oxaliplatin (Li et al., 2023b). Conversely, some bacteria promote chemotherapy resistance; *F. nucleatum*, for instance, is linked to chemotherapy resistance in colorectal cancer patients (Yu et al., 2017).

Chemotherapy also influences gut microbiota composition. For instance, after capecitabine plus oxaliplatin treatment, there is an increase in pathogenic bacteria and a decrease in probiotics such as *Dorea*, *Streptococcus*, and *Roseburia* (Kong et al., 2019). Notably, butyrate, a widely studied

SCFA, is found at higher levels in colorectal cancer chemotherapy responders, suggesting it has potential as a predictor of chemotherapy response (Sánchez-Alcoholado et al., 2021).

5.5.3.2 BC

The single BC patient with more than one sample was BH11, and this patient underwent docetaxel, carboplatin, Herceptin (trastuzumab), and Perjeta (pertuzumab) (TCHP) treatment. Docetaxel inhibits spindle formation and so arrests mitosis at G2 phase, leading to apoptotic cell death (Pienta, 2001). Carboplatin inhibits DNA synthesis by cross linking DNA molecules (Schoch et al., 2020). Both Herceptin and Perjeta are monoclonal antibodies that block different regions of the HER2 receptor, blocking oestrogen driven growth signals (Maadi et al., 2021, Nami et al., 2018). A study investigating this exact treatment regime found significant increases in *Verrucomicrobia* (genus *Akkermansia*) but decreases in *Bacteroidetes* (genus *Alistipes*) (Wu et al., 2022a). Again, unfortunately, the resolution to species level is not there, but even at the genus level for this single patient with two samples, it was not apparent (Figure 4a). The genus with the most significant positive log-fold change was *Wujia*, with a p-value of 6.13E^{-210} . This genus, named after a Chinese scientist, has no known association with cancer to my knowledge (Liu et al., 2021a). At the other end of the scale, the genus with the most significant loss of relative abundance with a p-value of 1.01E^{-153} was *Phascolarctobacterium*, which is a known SCFA producer and a beneficial microbe (Wu et al., 2017). The next biggest negative log-fold change was in the genus *Gemmiger*, another loss of a beneficial butyrate producer (Kircher et al., 2022). Although some members of the *Gemmiger* genus, such as *Gemmiger formicilis*, have been associated as pathogens with Crohn's disease (Mondot et al., 2016). Just to note, the p-values are so large because the genus has gone from being completely absent to being present, or *vice versa*.

5.5.3.3 OC

The single OC patient with more than one sample underwent 5-fluorouracil, leucovorin, oxaliplatin, and docetaxel (FLOT) treatment. 5-fluorouracil is an analogue of the nucleotide uracil and inhibits translation by inhibiting rRNA and tRNA production. Leucovorin increases the activity of 5-fluorouracil and allows a lower dose to be used (Mishima et al., 2006). Oxaliplatin has the same mode of action as carboplatin (Szikriszt et al., 2020). To my knowledge, there have been no MWAS studies involving this specific treatment type.

For the single patient BH4, who had three samples, there was an increase in alpha diversity from timepoint one to two, accompanied by a positive overall differential abundance log-fold change (Figures 4b and 4c). The two most significant increases in relative abundance were observed in the genera *Romboutsia* and *Terrisporobacter*. Notably, *Romboutsia* has been identified as a genus that can distinguish healthy controls from OC (Deng et al., 2021). Another example of low concordance between studies or insufficient taxonomic resolution, a more recent study in China found that a decreased risk of oesophageal cancer was associated with an increased abundance of *Romboutsia* (Gao et al., 2023b). Genus *Terrisporobacter* has been associated with an increased risk of oesophageal ulcers (Wu et al., 2024).

From sample BH4.2 to BH4.3, the main movers were within the top 20 genera and have previously been discussed in the relative abundance section, except for the genus *Haemophilus*, which was the second most highly significant drop in relative abundance after Unclassified_*Lachnospiraceae*. The unclassified_*Lachnospiraceae* exhibited the largest decrease in relative abundance, however, this is not particularly informative. As research indicates, while *Lachnospiraceae* are significant producers of SCFAs, different taxa within this family are pathogenic (Vacca et al., 2020). *Haemophilus* is one of the most abundant genera in the human lung (Dickson et al., 2016). Previous studies have implicated *Haemophilus* with colorectal cancer recurrence (Huo et al., 2022). The relative abundance of *Haemophilus* in the GM could distinguish healthy controls from LC, which does not mean that *Haemophilus* presence is causative (Shen et al., 2021a).

5.5.4 Radiotherapy

Radiotherapy causes bidirectional interactions with the gut microbiota, influencing tumour radiosensitivity and treatment efficacy while also causing radiation toxicity, which leads to side effects like mucositis, diarrhoea, and fatigue (Li et al., 2023c, Wang et al., 2015, Gerassy-Vainberg et al., 2018). Certain gut microbiota can resist radiation side effects (Guo et al., 2020). For example, *Coprococcus* is significantly enriched in patients with radiation enteritis prior to radiotherapy, and high counts of *Clostridium* IV, *Roseburia*, and *Phascolarctoxin* are associated with radiation enteritis (Reis Ferreira et al., 2019). The pathophysiological mechanism of G.I. mucositis involves several stages, including initiation, primary damage response, signal amplification, ulceration, and healing (Sonis et al., 2004). The GM participates in the pathogenesis of radiation induced G.I.

mucositis by regulating inflammatory processes, oxidative stress, intestinal permeability, mucus layer composition, and immune responses (Van Vliet et al., 2010). There were no patients being treated with radiotherapy alone.

5.5.5 Chemoradiotherapy (CRT)

CRT is also associated with changes in GM composition. For instance, patients with diarrhoea due to CRT show significantly lower alpha diversity, suggesting a link between gut microbiota imbalance and diarrhoea (Manichanh et al., 2008). Patients experiencing fatigue after CRT had increased bacterial abundances of *Eubacterium*, *Streptococcus*, *Adlercreutzia*, and *Actinomyces*, along with increased microbial sucrose degradation pathways (González-Mercado et al., 2021). The abundance ratios of *Bacteroidetes* and *Blautia* differed between symptomatic and asymptomatic subjects (Gonzalez-Mercado et al., 2021). At present, pathways have not been looked at in this study, but the bacterial DNA extracted from the stool samples could be used to look for the presence or abundance of specific enzymatic pathway genes in the future.

Significant microbiome alterations have been observed during neoadjuvant chemoradiotherapy (nCRT) for locally advanced rectal cancer (LARC), with a decrease in LARC related pathogens and an increase in *Lactobacillus* and *Streptococcus*. Microbiota related to butyrate production, such as *Roseburia*, *Dorea*, and *Anaerostipes*, were overrepresented in responders, whereas *Coriobacteriaceae* and *Fusobacterium* were more prevalent in nonresponders. These differences could help predict treatment responses (Yi et al., 2021).

5.5.5.1 Rectal

Rectal patients all received capecitabine CRT, followed by CAPOX treatment. Capecitabine is a precursor of the cytotoxic moiety 5-fluorouracil (5-FU), and CAPOX is a combination of capecitabine and oxaliplatin (Carrato et al., 2008). All three provided two samples, and this represented the largest group of patients on trial as of March 31, 2024. This allows comparisons to be made between patients as to possible treatment effects on the GM and the identification of any previously described GM associations, *Peptostreptococcus* was found to be significantly reduced post treatment for all three patients. Two of the patients, BH5 and BH8, were found to have *Unclassified_Lachnospiraceae* with a significant log-fold increase (Figures 4d and 4e). *Gemella*

and *Anearooccus* were found to be commonly reduced between patients BH5 and BH12. Having previously gone into detail in the relative abundance section regarding BH5's genera occurring in the "Other" category, *Peptostreptococcus* has already been discussed. Common fold increases in Unclassified_*Lachnospiraceae* is not very informative because of the mix of pathogenic and beneficial bacterial genera within the group. *Anearooccus* to my knowledge have no known associations with cancer and are generally considered commensals and opportunistic pathogens (Murphy and Frick, 2013).

Gemella has been identified as a prominent CRC associated bacteria (Ternes et al., 2020). Studies have found that *Gemella* species are significantly enriched at tumour sites (Sakamoto et al., 1999), highlighting their potential involvement in colorectal cancer (CRC). Within this genus, *Gemella morbillorum* has been found to regulate IL-12 production, which plays a role in immunoregulation within the context of CRC (Ribeiro Sobrinho et al., 2002). This regulation of IL-12 indicates that *G. morbillorum* might influence the immune environment of the tumour, potentially affecting cancer progression and patient outcomes.

Furthermore, *Gemella haemolysans* has been noted for its ability to cleave IgA1, thereby diminishing the protective function of the adaptive immune response at the mucosal surface. While no direct role of *G. haemolysans* in CRC development has been reported, its impact on the immune system suggests it could play a contributory role in the disease's progression (Lomholt and Kilian, 2000). So, the reduction in its presence post treatment could be a good sign for patients BH5 and BH8.

As regards treatment associations previously found in other studies involving chemoradiation, after treatment, as in *Roseburia*, *Dorea*, and *Anaerostipes*, were overrepresented in responders. Patient BH5 had *Roseburia* increase in relative abundance from 1.5% to 4%, *Dorea* went from 2% to 3%, and *Anaerostipes* decreased from 2% to 0.8%, possibly a good indicator of treatment response. Patient BH8 showed increases across the board for these three genera: *Roseburia* 8% to 0.2%, *Dorea* 4% to 9%, and *Anaerostipes* 9% to 10% relative abundance, which augurs well (Figure 4f). Without clinical outcome data at this stage, conclusions cannot be drawn.

5.5.6 Immune Checkpoint Inhibitor Treatment

The remaining cancer patient groups have all received various types of ICI treatment. Among these groups, the maximum number of patients providing more than one sample is two per cancer group. Gut microbiota composition is also crucial in predicting responses to ICI treatments. Microbial signatures enriched for *Lachnospiraceae* spp. and *Streptococcaceae* spp. are associated with favourable and unfavourable clinical responses, respectively, in melanoma patients treated with anti-PD-1 (McCulloch et al., 2022). Enrichment of *Bifidobacterium longum*, *Alistipes putredinis*, and *Prevotella copri* is linked to improved immunotherapy efficacy (Jin et al., 2019). Notably, taxa such as *Phascolarctobacterium*, *Bifidobacterium*, *Akkermansia muciniphila*, and *Rothia* are associated with good responses to ICIs across different cancer types (Derosa et al., 2022, Gopalakrishnan et al., 2018a, Lili and Ye, 2020, Wind et al., 2020). Conversely, *Actinomyces* genus members are consistently enriched in non-responders for hepatobiliary cancers and metastatic melanoma, while *Faecalibacterium prausnitzii* is increased in responders (Frankel et al., 2017, Mao et al., 2021).

5.5.7 Antibiotic use and ICI

The use of antibiotics has a significant negative impact on patients' responses to ICI treatment, necessitating careful consideration of their potential risks and benefits (Angrish and Pezo, 2022). The GM of non-small cell lung cancer (NSCLC) patients who responded well to ICIs were enriched with *Akkermansia muciniphila*, *Eubacterium hallii*, and *Bifidobacterium adolescentis* and had a more inflamed tumour microenvironment. Antibiotic use coincided with resistance to ICIs in patients with a relative dominance of *Akkermansia* and *Clostridium* (Derosa et al., 2022). The synergistic activity of several bacteria genera, including *Akkermansia muciniphila*, *Alistipes indistinctus*, *Bacteroides*, *Bifidobacterium*, and others, were found to be associated with enhanced immunotherapy efficacy, whereas *Blautia obeum*, *Roseburia intestinalis*, and certain antibiotics compromised it (Routy et al., 2018a). To date, the clinical data for patients enrolled in the study has not been released.

5.5.7.1 Adenocarcinoma

Adenocarcinoma patients BON3 and BON4 each provided three samples whilst undergoing treatment with pembrolizumab, carboplatin, and pemetrexed. Pembrolizumab blocks the PD-1 pathway, helping to prevent cancer cells from evading the immune system (Kwok et al., 2016). Notably, these patients exhibited significant log-fold changes in similar genera, yet these changes moved in opposite directions, suggesting contrasting treatment responses.

From timepoints one to two, BON3 showed significant increases in *Unclassified Lachnospiraceae*, *Perlabentimonas*, and *Butyricimonas*, while *Intestinibacter*, *Congobacterium*, and *Zhenhengia* decreased (Figure 4j). This contrasted with BON4 who showed a reduction in *Unclassified_Lachnospiraceae* and an increase in *Congobacterium* (Figure 4l). From timepoints two to three, BON4 exhibited significant increases in *Intestinibacter*, *Congobacterium*, *Zhenhengia*, and *Klebsiella* (Figure 4m). During this period, *Butyricimonas*, *Yokenella*, *Perlabentimonas*, and *Enterobacter* significantly decreased in BON4, opposite to the trends seen in BON3. Notably, *Klebsiella* showed the largest reduction, reversing the findings from timepoints one to two (Figure 4k). These polar opposite shifts in the GM composition highlight the disparate responses to ICI treatment in these two patients. It will be interesting to find out if the level of treatment response corresponds accordingly.

The responder group has frequently been associated with an increased relative abundance of *Butyricimonas* in cancer patients treated with anti-PD-1 combined with chemotherapy (Wu et al., 2022b). In non-small cell lung cancer patients treated with anti-PD-1 immunotherapy, *Butyricimonas faecalis* was significantly more abundant in those with long PFS (Dora et al., 2023). In hepatocellular carcinoma patients treated with sorafenib, a higher abundance of *Butyricimonas* was associated with the absence of diarrhoea (Yamamoto et al., 2020). Moreover, *Butyricimonas* was enriched in patients with major pathological response (MPR) undergoing neoadjuvant camrelizumab and chemotherapy for thoracic oesophageal squamous cell carcinoma (Xu et al., 2022).

It has been found that *Intestinibacter bartlettii* has been strongly associated with irAEs, such as ICI induced colitis. These findings have been replicated across different cohorts of metastatic melanoma patients treated with anti-CTLA-4, anti-PD-1, or combination ICI therapies (Andrews

et al., 2021). However, it also exhibited higher abundances in patients with long PFS in advanced melanoma undergoing ICI therapy, indicating a dual role in contributing to both irAEs and improved clinical outcomes (Björk et al., 2024).

5.5.7.2 *Small Cell Lung Cancer*

The small cell lung cancer group had two patients, BON2 and BON9, with two samples each. Both patients were undergoing atezolizumab, etoposide, and carboplatin treatments. Atezolizumab works by targeting the immune checkpoint protein PD-L1 (Horn et al., 2018), and etoposide is a topoisomerase II inhibitor that causes DNA double strand breaks and inhibits repair mechanisms (Montecucco et al., 2015). *Enterococcus faecalis* has been identified as an etoposide metaboliser through O-demethylase activity, thereby playing a role in its bioavailability and effectiveness (Tripathi, 2022). There were no *Enterococcus* detected in any BON9 samples and just a few hundred reads in patient BON2, but other GM members may have similar enzymatic abilities. Notably, BON9 and BON2 had no significantly differentially abundant genera in common between sampling timepoints (Figures 4n and 4o).

For patient BON2, *Megamonas* was among the top significantly differentially abundant genera, showing a positive differential abundance. *Megamonas* has been linked to adverse G.I. reactions in lung cancer patients (Zhang et al., 2020). The genus *Ruminococcus* was similarly significantly increased in BON2. This genus has been observed to increase after chemotherapy, reflecting its adaptive response to cancer treatment (Zhang et al., 2020).

Colidextribacter showed a significant reduction in relative abundance in patient BON2. Previously, this genus has been noted to decrease the efficacy of CTLA-4 blockade in a mono-colonised mouse model (Mager et al., 2020). Additionally, responder associated amplicon sequence variants (ASVs) belonging to *Colidextribacter* were identified in non-small cell lung cancer patients responding to PD-1 blockade (Newsome et al., 2022).

The genus *Faecalibacterium* was one of the most significantly reduced genera for patient BON9. The species *Faecalibacterium prausnitzii* is a major butyrate producer and is clinically significant in treating inflammatory bowel diseases and colorectal cancer due to its anti-inflammatory properties (Dikeocha et al., 2022).

5.5.7.3 NSCLC

Two samples were provided by patient BON7 while receiving Pembrolizumab, carboplatin, and Paclitaxel treatment (Paz-Ares et al., 2018). Figure 4p illustrates the microbiome changes between the two time points. Notably, there was a significant increase in *Limosilactobacillus* and *Veillonella*, while *Ruminococcus* and *Luoshenia* exhibited the most substantial decreases.

At the species level, *Limosilactobacillus fermentum* has been recognised for its potential therapeutic role in preventing leaky gut syndrome (De Gregorio et al., 2023). In pre-clinical trials using a cyclophosphamide (CPP) mouse model, researchers demonstrated the immunomodulatory efficacy of *Limosilactobacillus fermentum*, which included enhanced natural killer (NK) cell activation, increased nitric oxide (NO) production, phagocytic capacity, and cytokine secretion in mice with CPP induced immunosuppression (Park et al., 2024).

One study found significantly higher levels of *Limosilactobacillus gorillae* and *Streptococcus salivarius* in the faecal microbiota of subjects with NSCLC compared to healthy subjects. However, this finding does not necessarily imply that these microbes contribute to tumour occurrence and development (Feng et al., 2024). Enrichment of *Veillonella parvula* in the lung microbiome is strongly associated with NSCLC and has been shown to accelerate the progression of lung adenocarcinoma *in vivo*, though its precise role and mechanism remain unclear (Zeng et al., 2023). Other studies have corroborated the strong association between *Veillonella* and NSCLC, suggesting that *Veillonella* significantly promotes lung cancer progression (Zeng et al., 2022). Previously, patients who experienced diarrhoea had a higher relative abundance of *Veillonella* (Liu et al., 2019). A prospective study involving 63 patients with advanced NSCLC undergoing anti-PD-1 therapy found that *Veillonella* was more prevalent in patients with PFS < 6 months (Song et al., 2020). In another study with 42 metastatic melanoma patients, *Veillonella parvula* was among the abundant bacteria found in patients who responded to anti-PD-1 treatment (Matson et al., 2018). This may not just be a case of contradictory findings, but of a different context in terms of cancer type.

The results confirmed that *Ruminococcus* was significantly more abundant in the faeces of responders compared to non-responders in 71 stool samples from patients with advanced NSCLC receiving immunotherapy treatment (Ren et al., 2024). *Ruminococcus* spp. were also found to be

enriched in patients with better clinical responses and longer PFS in two independent studies using 16S rRNA gene sequencing and metagenomics shotgun sequencing on faecal samples of NSCLC patients (Routy et al., 2018a, Hakozaiki et al., 2020). The polyphenol castalagin has been shown to improve the CD8⁺/FOXP3⁺CD4⁺ ratio in the tumour microenvironment, and this in turn is associated with enhanced resistance to anti-PD-1 therapy. This polyphenol has been shown to induce metabolic changes, including increased levels of taurine conjugated bile acids. NSCLC responders enriched with *Ruminococcus* were able to metabolise castalagin, suggesting that this bacterium plays a role in enhancing the anti-tumour response (Messaoudene et al., 2022).

The recently described *Luoshenia tenuis* has been shown to have probiotic properties. It produces SCFAs and other volatile metabolites with known anti-inflammatory and anti-tumour characteristics (Jiang et al., 2024b). However, it has not been associated with cancer in any MWAS, positively or negatively, to the best of my knowledge.

The cholangiocarcinoma group has enrolled just one patient, number BON1, with a total of three samples collected. They received a treatment regimen of Durvalumab, Cisplatin, and Gemcitabine. Durvalumab is a PD-1 monoclonal antibody (MAB), and Gemcitabine is an analogue of deoxycytidine (Fung and Syed, 2023). Figures 4g and 4h represent the differential abundances between sampling timepoints one to two and two to three, respectively. Four out of the top ten significantly differentially abundant genera increased during the first transition, including *Perlabentimonas*, *Waltera*, *Unclassified_Lachnospiraceae*, and *Butyricimonas*. Significant decreases included *Ruthenibacterium*, *Pseudomonas*, *Gorbachella*, *Megasphaera*, and *Akkermansia*. During the second transition, eight out of the top ten differentially abundant genera decreased, with *Anaerocolumna* and *Tractidigestivibacter* being the only genera to increase. Notably, *Faecalibacterium* and *Dialister* exhibited very significant increases, just outside the top ten, while *Ihubacter* and *Anaerophaga* showed the most significant reductions.

Previous studies have found *Perlabentimonas gracilis* to be enriched in colorectal tumour tissue, although the genus *Perlabentimonas* has not been otherwise associated with cancer (Fukuoka et al., 2023). This suggests a potential, albeit unclear, role in cancer pathology, with discrimination to species level necessary.

With regard to *Butyricimonas*, one study found that patients with stage III hepatocellular carcinoma (HCC) showed a reduction in *Butyricimonas* relative abundance (Liu et al., 2023). At the species level *Butyricimonas synergistica* in particular has been associated with responders to immune checkpoint inhibitor (ICI) treatment in melanoma (Lee et al., 2022).

The presence of *Ruthenibacterium lactatiformans*, along with other species such as *Eubacterium limosum* and *Faecalibacterium ulcerans*, has been shown to contribute to improving ICI efficacy (Tanoue et al., 2019). On a contrary note, a study on advanced melanoma patients found that those with a PFS of less than 12 months were enriched with *Ruthenibacterium lactatiformans* across all study visits (Björk et al., 2024).

A novel genus, *Gorbachella*, based on the discovery of *Gorbachella massiliensis*, has no current associations with cancer (Durand et al., 2017). *Tractidigestivibacter* is a relatively new genus, discovered in 2015, has no current associations with cancer (Zgheib et al., 2021). Another recently discovered genus, *Ihubacter*, has with no known associations to cancer (Wu et al., 2020). The species *Novisyntrophococcus fermenticellae* has been identified as a key species in the EBW-CRC but has no published associations with cancer to date (Li et al., 2023a).

As a biomarker, *Megasphaera* has been identified as a predictive marker for gastric cancer (Zhang et al., 2021). Other studies have found that the combination of PD-L1 with *Megasphaera* secondary metabolites has been shown to enhance anti-tumour effects *in vivo* (Huang et al., 2022b). *Faecalibacterium prausnitzii* has been linked to a favourable clinical response to ICI therapy in patients with advanced cancer (Chang et al., 2024). *Intestinal Akkermansia muciniphila* predicts clinical response to PD-1 blockade in advanced non-small cell lung cancer patients (Derosa et al., 2022).

Elevations in bile acid and bilirubin levels, along with poor liver function, were associated with *Dialister* species, which were also significantly linked to mild diarrhoea (Mao et al., 2021). In chemotherapy, *Anaerocolumna* was found to have increased relative abundance in patients undergoing gemcitabine and nab-paclitaxel treatments compared to untreated controls (Panebianco et al., 2023)

The opportunistic pathogen *Pseudomonas* is one of the top three biomarkers for perihilar cholangiocarcinoma (pCCA) at the genus level (Ye et al., 2023). Their potential pathogenicity was highlighted by a rare case of *Pseudomonas aeruginosa* enteritis induced by pembrolizumab in a patient with hypopharyngeal carcinoma and lung metastasis (Miyamoto et al., 2023).

5.5.7.4 Metastatic Melanoma

In the metastatic melanoma group (Figure 4i), there was only one patient with two samples treated with a combination of Ipilimumab and Nivolumab, which are CTLA-4 and PD-1 inhibitors, respectively (Larkin et al., 2019). The highest differentially abundant genera were *Ruminococcoides* and *Unclassified_Lachnospiraceae*, while *Novisyntrophococcus* and *Enterococcus* were the most significantly reduced.

The recently discovered SCFA producer *Ruminococcoides bili* resulted in the introduction of a new genus, *Ruminococcoides*, to the Ruminococcaceae family. This bacterium was isolated from human liver bile, and although it has no known associations with cancer, its ability to produce SCFAs suggests beneficial properties (Kim et al., 2024). All *Enterococcus* strains and species that express active peptidoglycan hydrolase protein, specifically SagA orthologs, have been found to promote ICI cancer immunotherapy in mouse models (Griffin et al., 2021). In a large metagenomic study involving 981 metagenomic samples, *Novisyntrophococcus fermenticellae* was associated with the EBW-CRC community (Zhu et al., 2024), but there are no associations with melanoma.

5.5.7.5 Renal Cancer

There was just one patient, BON11, with two samples provided. Their treatment for renal cancer involved Nivolumab, a PD-1 checkpoint inhibitor (Motzer et al., 2015). The analysis showed that *Bacillus* and *Weissella* exhibited the most significant increases, while *Limosilactobacillus* and *Unclassified_Oscillospiraceae* had the most significant reductions in relative abundance (Figure 4q). Many species within the *Bacillus* genus are enteric pathogens capable of producing cytolethal oncogenic toxins. These toxins have been identified in enteric pathogens such as *Escherichia* and *Bacillus* spp. that could promote cancer initiation (Zhao et al., 2023). Although most species of *Weissella* are considered candidate probiotics, some species, like *Weissella confusa*, can act as opportunistic pathogens (Kim et al., 2023, Ahmed et al., 2022). Another recently added genera,

Limosilactobacillus, previously classified as a member of the *Lactobacillus* genus, has many probiotic properties, including antimicrobial and anti-inflammatory abilities, but has not been identified in any MWAS studies to date (Verhoef et al., 2023).

5.5.8 Other Drugs

The impact of other drugs on the GM includes analgesics like paracetamol and opiate medication, which have been found to lead to an increase in the relative abundance of *Streptococcaceae* (Jackson et al., 2018, Rueda-Ruzafa et al., 2020). The use of proton pump inhibitors (PPIs) have been found to increase the relative abundances of enterococci, streptococci, staphylococci, and *E. coli* species, predispose patients to *Clostridioides difficile* infections, and generally increase the risk of enteric infections (Jagdish and Kilgore, 2021, Imhann et al., 2016). Clinical patient data will help to explain some of the relative abundance patterns observed thus far.

5.6 CONCLUSION

The interactions between cancer treatments and the GM are complex and bidirectional, significantly impacting treatment efficacy and patient outcomes. The composition of the GM influences the development and progression of all cancers to varying degrees, with specific bacteria playing crucial roles in modulating immune responses, skewing signalling pathways, and the production of beneficial microbial metabolites. Conversely, certain bacteria or even certain relative abundances of bacteria can promote malignant growth through the production of genotoxins or other mechanisms and can be associated with negative treatment outcomes and side effects. The mechanisms remain poorly understood, so it is pretty much a black box for the most part, with individual OTUs being reduced to good or bad bacteria categories.

Alpha diversity, such as the SDI, is only one of many such measures that can show general trends in GM species richness changes across different cancer types and treatments. In this chapter, the limited number of patients per cancer type restricts the ability to draw definitive associations. All things going according to plan, the studies will hit their recruitment targets and will be sufficiently

statistically powered to gain more comprehensive insights. Future work on these studies will consider other measures of alpha diversity to gain a more nuanced understanding.

Many of the bacteria found to be significantly differentially abundant have not been found in previous association studies. These previously unassociated bacteria may play roles in cancer initiation, progression, and treatment response but might not have been identified in earlier studies due to confounding factors or sampling bias. On top of this, many of the genera identified in these preliminary findings have only recently been discovered. The dynamic and ephemeral nature of bacterial colonisation in the gut, along with the presence of proximal microbiomes at other body sites plus intra-tumoural microbes, adds another layer of complexity to these interactions.

Future work will focus on completing patient recruitment and clinical data collection. Principal component analysis (PCA) will identify key factors related to treatment response, and machine learning tools may help analyse the diverse cancer types studied. Additionally, investigating fungal members of the mycobiome could provide further insights into the GMs role in cancer therapy. The most important next step is to improve patient recruitment.

6 CHAPTER 6:

Conclusion

It is worth explaining any perceived disconnect between chapters 4 and 5 (clinical microbiome focus) and the other chapters (synthetic protein lab work) of this thesis. This thesis project commenced before the pandemic lockdown. The early intention was to focus primarily on microbiome work, facilitated by clinical sample collection from a single hospital followed by wet -and dry-lab analyses. The pandemic and subsequent cyber-attack delayed sample acquisition to such an extent that relying on clinical samples entirely put the thesis at risk. To mitigate this risk, the described lab work (Chapter 2 and 3) was designed and pursued while awaiting clinical samples.

Despite these challenges, the thesis project proceeded to study both bacteria and proteins produced from bacteria for exploitation in cancer biomarker technology. Chapter 2 describes the development of a detection system of biomarker activity, produced from bacteria; Chapter 3 describes development of a platform for deployment of biomarker detectors, produced from bacteria; while Chapter 4 describes clinical processes for examining bacteria themselves as biomarkers.

Both natural/endogenous biomarkers (bacterial microbiome) and exogenous strategies (synthetic proteins) were examined here. In general, sources of biomarkers/sampling strategies include blood, urine, exhaled breath, tissue biopsies and stool. This thesis investigates sample types which are non-invasive and have potential for ‘at-home’ use – urine and stool.

Chapter 2 describes a bacterially produced synthetic protein platform for disease detection, including cancer. Protease activity can serve as a highly specific biomarker for application in health, biotech and beyond. This work combines the sequence specificity of any given protease with the simplicity of protein production and the sensitivity of a sandwich ELISA in a novel and straightforward fashion, using already well-characterised antibodies. This novel method could be applied to any biological liquid sample, such as urine for detection of protease activity to reveal cancer biomarkers. Development as an at-home test means it could be used as a stand-alone or as a prelude to other types of testing. Further development and optimisation are required to produce a rapid, cost-effective and easy-to-operate test. Large-scale screening, with high population coverage and early diagnosis, is critical to the commencement of appropriate cancer treatment or intervention with the ability to do this outside of the laboratory setting, at home

or in developing countries where resources for healthcare are limited, there is the potential to save many lives.

Urine serves as an ideal biological sample due to its non-invasive nature and ease of collection, allowing for unlimited repeated sampling, including in the at-home setting. Chapter 3 describes the engineering and production of a stable, detectable protein that safely transports itself to urine when made available systemically in the body. This strategy serves as a proof of concept for a synthetic protein platform for urine-based diagnostics, serving as a template for designer proteins which may be edited for biomarker detection *in vivo*. A key advantage of MUPt is that the protein family on which it is based has evolved to be excreted through the urinary system. Future iterations in humans could be bacterially produced within tumours, based on human lipocalins.

A cancer patient's collection of bacteria (microbiome) has potential to act a biomarker in precision oncology. Clinical microbiome wide association studies (MWAS) play a crucial role in such development. There is a perception that observational studies of this nature are straightforward - get study ethical approval, consent the patients so that they can send the samples from home when it suits them best, process the samples and publish the findings. As can be seen in Chapter 4 from the amount of people involved in the set-up and the sheer volume of paperwork plus collaboration across different types of institutions with archaic systems unsuitable for efficiently setting up observational studies, it becomes more apparent that the reality is far from straight forward. Through comparative data analysis, this chapter provides insights into optimising logistics and enhancing reliability in future research endeavours. While anecdotal evidence exists related to the outputs of this chapter, this is the first such study involving analyses of such data from studies, to the authors' knowledge. Some key findings include the return on investment in dedicated research nurses, which should be given higher priority in project costings (arguably above any laboratory personnel), and core dedicated staff for clinical study set-up. Put simply, no samples, no project.

With Chapter 5, delays prevented sufficient patient sample acquisition to generate meaningful sequencing bioinformatics data within the timeframe of this thesis project. Nonetheless, all samples accrued were processed in the lab and sequenced in-house by NanoPore technology, which has potential in development of POC testing. Microbiome data presented are not statistically

powered but display the strategy and usefulness of metataxonomic characterisation of cancer patient stool to inform and potentially predict patient responses to treatments, and outcomes.

The value of biomarkers in precision oncology is unquestionable, and increasing the range of biomarker strategies, such as studied here, will benefit cancer diagnosis, treatment and research.

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