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Exploration of the gut microbiome as a predictive factor for cancer treatment-induced gastrointestinal toxicity

A thesis submitted in fulfilment for the degree of

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by

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Contents

Thesis abstract

Gastrointestinal toxicity is a significant side effect of many cancer treatments. Characterised by diarrhoea, abdominal pain and bleeding, this toxicity can affect up to 80% of patients, depending on treatment regimen. Currently, there are no highly effective intervention strategies for the vast majority of people affected, thus more evidence is required to improve future management. This thesis focussed primarily on gastrointestinal toxicity stemming from two major types of cancer treatment. These are chemotherapy, the most common form of cancer treatment, and small molecule tyrosine kinase inhibitors (SM-TKIs), a class of targeted therapies, often used in combination with chemotherapy.

There is a clear gap in knowledge in understanding how these different cancer treatments cause gastrointestinal toxicity, and how the population of microorganisms in the intestine, the gut microbiome, links to these responses. This thesis therefore aimed to investigate the role of the gut microbiome in influencing the development and exacerbation of gastrointestinal toxicity stemming from cancer treatment. This was investigated in three main sections.

Firstly, I aimed to examine the interaction between the gut microbiome and the innate immune system (chiefly the innate immune receptor Toll-like receptor 4 (TLR4)), and determine how this interaction could be involved in the development of gastrointestinal toxicity following chemotherapy. In order to achieve this aim, I characterised the microbial composition of a TLR4 conditional knockout mouse model and assessed changes due to

chemotherapy treatment. There were no clear differences in the microbiome of wild type and TLR4 conditional knockout mice.

Secondly, I aimed to characterise the role of the gut microbiome in diarrhoea stemming from the SM-TKI treatment neratinib, which causes high levels of diarrhoea. I found that, in a pre-clinical model, neratinib treatment does cause changes to microbial composition, however it was unclear if these changes were a key driver of diarrhoea development or simply a side effect of this diarrhoea. Therefore I analysed diarrhoea development following an initial, antibiotic-induced perturbation, showing that addition of narrow-spectrum antibiotics caused a significant decrease in diarrhoea severity and timespan.

Finally, I clinically appraised the use of the gut microbiome in predicting risk of cancer treatment-induced gastrointestinal toxicity in two defined patient cohorts. A retrospective cohort showed that participants who went on to develop diarrhoea had significantly lower amounts of the bacterial genera *Blautia* and significantly higher amounts of the genera *Collinsella*. A longitudinal study was then developed. Pilot results did not show clear microbial clustering based on diarrhoea status.

The results of my thesis demonstrate the emerging role gut microbiome composition has on the development of diarrhoea following cancer treatment. However I was unable to definitively identify any particular bacterial type that is a key mediator of this effect. The results presented here however provide strong rationale for further research in this area using specific machine learning and metabolomic techniques to identify compositional features that may be

important in accurately predicting diarrhoea development following cancer treatment.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship and a Lion's Medical Research Foundation Scholarship.

Kate Secombe, May 2021

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Finally I would like to dedicate this thesis to my Pop, who isn't able to be here to see it, but I know would be so proud that I finally finished it.

Publications arising from this thesis

These publications are included in their original form in appendix 1:

Secombe, K. R., Coller, J. K., Gibson, R. J., Wardill, H. R. and Bowen, J. M. (2019). The bidirectional interaction of the gut microbiome and the innate immune system: Implications for chemotherapy-induced gastrointestinal toxicity. *Int J Cancer* 144(10): 2365-2376**.**

Secombe, K. R., Van Sebille, Y. Z. A., Mayo, B. J., Coller, J. K., Gibson, R. J. and Bowen, J. M. (2020). Diarrhea Induced by Small Molecule Tyrosine Kinase Inhibitors Compared With Chemotherapy: Potential Role of the Microbiome. *Integr Cancer Ther* 19: 1-12.
**invited review*

Secombe, K. R., Ball, I. A., Shirren, J., Wignall, A. D., Keefe, D. M. and Bowen, J. M. (2021). Pathophysiology of neratinib-induced diarrhea in male and female rats: microbial alterations a potential determinant. *Breast Cancer* 28(1): 99-109.

Additional studies and publications

During my PhD candidature, I published several papers that are not presented in this thesis. These publications are listed below:

Secombe, K.R., Ball, I.A., Shirren, J., Wignall, A.D., Finnie, J., Keefe, D.M.K., Avogadri-Connors, F., Olek, E., Martin, D., Moran, S. and Bowen, J.M. (2019) Targeting neratinib-induced diarrhoea with budesonide and colesevelam in a rat model. *Cancer Chemother Pharmacol* 83(3): 531-543.

Wardill H.R., **Secombe K.R.,** Bryant R., Hazenberg M.D., Costello S.P.(2019) Adjunctive faecal microbiota transplantation in supportive oncology: emerging indications and considerations in immunocompromised patients. *EBiomedicine* 44: 730-740.

Mayo B.J., **Secombe K.R.**, Wignall A.D., Bateman E., Thorpe D., Pietra C., Keefe D.M., Bowen J.M. (2020) The GLP-2 analogue elsiglutide reduces diarrhoea caused by the tyrosine kinase inhibitor lapatinib in rats. *Cancer Chemother Pharmacol* 85: 793–803

Nomenclature

This thesis contains some variations in specific terminology describing gastrointestinal toxicity following cancer treatment. This is partly due to the lack of accurate, or multiple overlapping definitions in this field. I have not changed any terminology from published chapters in order to avoid altering their content. The following list aims to provide further detail on common terms used in this thesis:

Gut and gastrointestinal tract: both referring to the small and large intestine.

CIGT (chemotherapy-induced gastrointestinal toxicity) and CID (chemotherapy-induced diarrhoea): CIGT refers to any side effect of gastrointestinal origin (i.e. diarrhoea, pain or ulceration), whereas CID specifically refers to diarrhoea.

Mucositis: used sparingly, mucositis traditionally refers to the ulceration and inflammation of any region of the alimentary tract from mouth to anus. Typically this term is used in relation to chemotherapy or radiotherapy treatment.

Cancer treatment-induced gastrointestinal toxicity: used throughout to refer to gastrointestinal toxicity developed secondary to any cancer treatment (e.g. chemotherapy, SM-TKI or radiotherapy).

HER (Human epidermal growth factor receptor), EGFR (Epidermal Growth Factor Receptor) and ErbB: interchangeable terms describing a class of proteins. In this thesis, HER is used when describing the proteins in humans,

ErbB is used to describe the class in humans or rats and EGFR is used to specifically describe the HER1/ErbB1 receptor.

List of abbreviations

For ease of reading, these abbreviations are also introduced within each chapter.

5-FU: 5-fluorouracil

AB-PAS: Alcian blue – periodic acid – Schiff's reagent

ADL: Activities of daily living

AGRF: Australian Genome Research Facility

ALP: alkaline phosphatase

ALT: alanine transaminase

ANOVA: Analysis of variance

APC: Antigen-presenting cells

ASBT: Apical sodium dependent bile acid transporter

ATP: Adenosine triphosphate

AUC: Area under the curve

AW: Albino Wistar

bp: base pairs

BRAF: gene encoding B-Raf

CaSR: Calcium-sensing receptor

CD11b: Cluster of differentiation molecule 11B

CDI: *Clostridioides difficile* infection

CFTR: Cystic fibrosis transmembrane conductance regulator

CFU: Colony forming units

CID: Chemotherapy-induced diarrhoea

CIGT: Chemotherapy-induced gastrointestinal toxicity

CMC: Carboxymethylcellulose

CRAMP: Chemoradiotherapy-associated molecular pattern

C-SAS: Chemotherapy - symptom assessment scale

D: Diarrhoea - deterioration group

DAMP: Damage-associated molecular pattern

DAPI: 4′,6-diamidino-2-phenylindole

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

DSS: Dextran sulphate sodium

EDTA: Ethylenediaminetetraacetic acid

EGFR: Epithelial growth factor receptor

EGFR-TKI: Epithelial growth factor receptor tyrosine kinase inhibitor

FACIT-D: Functional assessment of chronic illness therapy-diarrhoea

FACT-COG: Functional assessment of cancer therapy-cognitive

FDA: Food and Drug Administration

FDR: False discovery rate

FEC: Fluorouracil, epirubicin and cyclophosphamide

FEC-D: Fluorouracil, epirubicin, cyclophosphamide and docetaxel

FLT3: FMS-like tyrosine kinase 3

FMC: Flinders Medical Centre

FMT: Faecal microbiota transplant

FOLFIRI: 5-FU, folinic acid and irinotecan

FOLFOX: 5-FU, folinic acid and oxaliplatin

GF: Germ-free

GI: Gastrointestinal

GLP: Good laboratory practice

GRAFT: Guidelines for reporting animal faecal transplant

GvHD: Graft-versus-host disease

H&E: Haematoxylin and eosin

HER: Human epidermal growth factor receptor

HER2: Human epidermal growth factor receptor 2

HMGB1: High mobility group box chromosomal protein 1

HPMC: Hydroxypropyl methylcellulose buffer

HREC: Human research ethics committee

IBD: Inflammatory bowel disease

IEC: Intestinal epithelial cells

IF: Immunofluorescence

IFN: Interferon

IKK: IκB kinase

IL: Interleukin

IRAK: Interleukin-1 receptor-associated kinase

IUPAC: International Union of Pure and Applied Chemistry

KEGG: Kyoto Encyclopedia of Genes and Genomes

KIT: tyrosine-protein kinase KIT

KO: Knockout

LAL: Limulus amebocyte lysate

LC-MS/TOF: Liquid chromatography- mass spectrometry/ time of flight

LD: lactate dehydrogenase

LDA: Linear discriminant analysis

LEfSe: Linear discriminant analysis effect size

LPS: Lipopolysaccharide

MAPK: Mitogen-activated protein kinases

MASCC: Multinational Association of Supportive Care in Cancer

MD-2: Myeloid differentiation factor 2/ Protein lymphocyte antigen 96

MPO: Myeloperoxidase

mRCC: Metastatic renal cell carcinoma

mRNA: Messenger ribonucleic acid

MTX: Methotrexate

MyD88: Myeloid differentiation primary response 88

NCI CTCAE: National Cancer Institute Common Terminology Criteria for Adverse Events

ND: Diarrhoea - No deterioration group

NF-κB: Nuclear factor kappa B

NLRP6: NOD-like receptor family pyrin domain containing 6

NSTI: Nearest sequenced taxon index

OTU: Operational taxonomic units

PAMP: Pathogen-associated molecular pattern

PBS: Phosphate buffered saline

PCA: Principal component analysis

PCoA : Principal coordinate analysis

PCR: Polymerase chain reaction

PDGFR: Platelet-derived growth factor receptor

PERMANOVA: Permutational multivariate analysis of variance

PREDiCT: Personalised Risk Evaluation During Cancer Treatment

PRISMA: Preferred reporting items for systematic reviews and meta-analysis

PROs: Patient reported outcomes

PRR: Pattern recognition receptor

qRT-PCR: Quantitative real time polymerase chain reaction

RAH: Royal Adelaide Hospital

rRNA: Ribosomal ribonucleic acid

RET: Rearranged during Transfection kinase

SAGC: South Australian Genomics Centre

SCFA: Short-chain fatty acid

SEM: Standard error of the mean

SM-TKI: Small molecule tyrosine kinase inhibitor

SN-38: Active metabolite of irinotecan

SN-38G: SN-38 glucuronide

SPF: Specific-pathogen-free

STAMP: Statistical Analysis of Metagenomic Profiles

Th1: T helper 1 cells

Th17: T helper 17 cells

TIMER: Translocation, immunomodulation, metabolism, enzymatic degradation, and reduced diversity.

TIR: Toll/interleukin-1 receptor-like

TKI: Tyrosine kinase inhibitor

TLR: Toll-like receptor

Tlr4$^{\Delta IEC}$: TLR4 epithelial conditional knockout mouse model

TNF: Tumour necrosis factor

TRAF: Tumour necrosis factor receptor-associated factor

Treg: Regulatory T cells

TRIF: TIR-domain-containing adapter-inducing interferon-β

UHPLC: Ultra-high performance-liquid chromatography

VEGF-TKI: Vascular endothelial growth factor tyrosine kinase inhibitor

VEGFR: Vascular endothelial growth factor receptor

WT: Wild-type

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Thesis introduction

1.0 Thesis background

1.1 Cancer treatment-induced gastrointestinal toxicity

Gastrointestinal toxicity is a significant side effect of many cancer treatments. Characterised by diarrhoea, abdominal pain and bleeding, this toxicity can affect up to 80% of patients, depending on treatment regimen (Lalla et al., 2014; Elad et al., 2020). In addition to a reduced quality of life; hospitalisation and need for supportive care strategies lead to an increased economic cost (Carlotto et al., 2013). Australian Medicare data suggests each episode of severe diarrhoea induced by chemotherapy costs approximately $1500 (Vouk et al., 2016). Importantly, severe levels of cancer treatment-induced gastrointestinal toxicity requires dose reductions and treatment cessation. This compromises remission and increases mortality (Di Fiore et al., 2009; Carlotto et al., 2013).

Currently, there are no highly effective intervention strategies for the vast majority of people affected by this gastrointestinal toxicity (Elad et al., 2020). Additionally, prophylactic strategies are currently not possible due to difficulties in predicting who will be most severely affected (Wardill et al., 2020). Development of toxicity depends on an interplay of multiple factors including tumour microenvironment and treatment plan, however people with similar treatments and demographics will also often develop differing levels of toxicity.

Well-coordinated research using clinically relevant models is now required to effectively predict gastrointestinal toxicity.

This thesis focusses primarily on gastrointestinal toxicity stemming from two major types of cancer treatment. These are chemotherapy, the most common form of cancer treatment, and small molecule tyrosine kinase inhibitors (SM-TKIs), a class of targeted therapies, often used in combination with chemotherapy or by themselves. Typically, these two treatment classes have been investigated separately, as they cause different types of intestinal injury – chemotherapy causes intrinsic apoptosis and atrophy of the intestinal lining, while SM-TKIs interfere with growth factor receptor signalling and thus disrupt normal cellular turnover in the intestine as well as removing survival signals (Van Sebille et al., 2015; Bowen et al., 2019). Despite the differing mechanisms of damage, the resulting symptoms of diarrhoea and intestinal ulceration manifest similarly, suggesting a unifying, common underlying mechanism. This unifying mechanism has not been thoroughly researched; in this thesis I suggest it may be linked to individual gut microbiome composition.

1.2 The gut microbiome and innate immune system in cancer treatment toxicities

The gut microbiome is defined as the collection of microbes including bacteria, viruses, fungi and phages that live in our intestinal tract. There is emerging research in both pre-clinical and clinical models describing the changes to microbiome composition and diversity in the intestine due to a range of cancer treatments. Broadly, results suggest that treatments such as chemotherapy

cause reductions in overall bacterial diversity and abundance of commensal genera including *Lactobacillus* and *Bifidobacterium* (Stringer et al., 2009a; Zwielehner et al., 2011).

The gut microbiome is also intrinsically linked to the innate immune system, with ample evidence to suggest that the host immune system is integral in selecting mucosal and luminal bacterial populations (Van den Abbeele et al., 2011). The innate immune system also utilises pattern recognition receptors (PRR) to recognise microbial molecular patterns. One type of PRR, Toll-like receptor 4 (TLR4), is present on intestinal epithelial cells as well as professional immune cells in the intestine, including dendritic cells and macrophages. The role of TLR4 in the development of chemotherapy-induced gastrointestinal toxicity has been clearly demonstrated (Wardill et al., 2016).

1.3 Predictive factors for toxicity within the microbiome

It is well demonstrated that people develop different levels of toxicity following cancer treatment, and that even within specific treatments, patients will display varying levels of toxicity (Miaskowski et al., 2014). A method of predicting toxicity would therefore be useful in prophylactically preventing gastrointestinal side effects during cancer treatment. In this thesis, I suggest that gut microbiome composition could be a common marker of toxicity following chemotherapy or SM-TKI treatment.

While it is understood that the gut microbiome changes due to cancer treatment, we have less of an understanding about the active role the microbiome or its metabolites plays in either initial development of toxicity, or in

the prolongation and exacerbation of symptoms. In addition, it is not understood how the gut microbiome is dynamically linked to the innate immune system in this way. However, previous research has shown the use of the gut microbiome in predicting cancer-related toxicities, including diarrhoea following pelvic radiotherapy (Wang et al., 2015) and gastrointestinal toxicity following the immunomodulatory treatment ipilimumab (Dubin et al., 2016). More research is required to understand whether this will also occur in chemotherapy and SM-TKI settings.

2.0 Thesis rationale

There is a clear gap in knowledge in understanding how different cancer treatments differ in ability to cause gastrointestinal toxicity, and how the gut microbiome links these responses. This thesis therefore aims to investigate whether the microbiome could be used as a targetable mechanism of cancer treatment-induced gastrointestinal toxicity in chemotherapy as well as SM-TKI treatment.

3.0 Thesis explanation, hypothesis and aims

The overarching aim of my thesis is to investigate the role of the gut microbiome in influencing the development and exacerbation of gastrointestinal toxicity stemming from cancer treatment. This is investigated in three main sections.

The first section (chapters 1-3) aims to examine the interaction between the gut microbiome and the innate immune system, and determine how this interaction

could be involved in the development of gastrointestinal toxicity following chemotherapy.

The second section (chapters 4-6) aims to characterise the role of the gut microbiome in diarrhoea stemming from SM-TKIs.

The third section of my thesis (chapter 7), aims to clinically appraise the use of the gut microbiome in predicting risk of cancer treatment-induced gastrointestinal toxicity. This is followed by a general discussion, references and appendix.

3.1 Chapter 1

Chapter 1 is my first literature review and provides an in-depth summary of the role of the microbiome in chemotherapy-induced gastrointestinal toxicity, and its links to the innate immune system. Previous research has suggested that changes to the gut microbiome occur following chemotherapy treatment. Separately, it has also been shown that innate immune system receptors may mediate the effects of gastrointestinal toxicity. However, the microbiome and the innate immune system have not been specifically linked in the context of gastrointestinal toxicity. Therefore, in this chapter, I aimed to collate current information linking the gut microbiome and the innate immune system to gastrointestinal toxicity outcomes. This chapter was published in the International Journal of Cancer *(Secombe, K. R., Coller, J. K., Gibson, R. J., Wardill, H. R. and Bowen, J. M. (2019b). The bidirectional interaction of the gut microbiome and the innate immune system: Implications for chemotherapy-induced gastrointestinal toxicity. Int J Cancer 144(10): 2365-2376).*

3.2 Chapter 2

Chapter 2 is my first original research chapter. Previous research has shown that mice with a global knockout (KO) of TLR4 develop less severe side effects of chemotherapy treatment (Wardill et al., 2016). However it is unclear whether the specific cellular localisation of TLR4 is important in mediating this effect. Therefore, in this chapter, the development of a gastrointestinal epithelium specific TLR4 KO mouse model (*Tlr4*$^{\Delta IEC}$) to assess gastrointestinal toxicity is discussed and characterised. Additionally, I aimed to add to the knowledge reviewed in Chapter 1 in understanding the links between gut toxicity and the gut microbiome. Therefore this model was then used to determine how a specific KO of TLR4 may cause changes in microbiome composition that are clinically relevant for chemotherapy-induced gastrointestinal toxicity.

Hypothesis:

TLR4 has a site-specific role in mediating microbial composition and alterations due to irinotecan treatment.

Aims:

- Generate a conditional KO model of TLR4 in the gastrointestinal epithelium for use in cancer treatment-induced gastrointestinal toxicity models.

- Assess the links between TLR4 KO status and gut microbiome composition.

3.3 Chapter 3

This chapter expands on the *Tlr4*$^{\Delta IEC}$ model developed in chapter 2. This chapter is presented as two parts that investigate faecal microbiota transplant (FMT) as a method of altering the gut microbiome in pre-clinical models. Part 1 is a systematic review that examined the methods that are currently used in FMT models of gastrointestinal disease in mice. This information was then used to establish a set of reporting guidelines for pre-clinical FMT studies. Part 2 details a pilot FMT study using the *Tlr4*$^{\Delta IEC}$ model developed in chapter 2 that was developed using these guidelines.

Hypothesis:

Altering microbial composition of mice prior to irinotecan treatment will alter the development of gastrointestinal toxicity.

Aims:

- Determine, via systematic review, what methods are currently used in FMT models in mice.

- Develop an FMT model in *Tlr4*$^{\Delta IEC}$ mice.

3.4 Chapter 4

This chapter, my second literature review, was an invited review that generated one of the key hypotheses of my thesis, suggesting that the microbiome plays a similar role in the development of gastrointestinal toxicity from both chemotherapy and SM-TKI treatment. There is currently a lack of published

research showing common factors in the toxicities of these two agents, and this review aimed to suggest why the microbiome is a key common factor. This chapter was published in Integrative Cancer Therapies *(Secombe, K. R., Van Sebille, Y. Z. A., Mayo, B. J., Coller, J. K., Gibson, R. J. and Bowen, J. M. (2020). Diarrhea Induced by Small Molecule Tyrosine Kinase Inhibitors Compared With Chemotherapy: Potential Role of the Microbiome. Integr Cancer Ther 19: 1-12).*

3.5 Chapter 5

This chapter introduces the SM-TKI medication neratinib, which is Food and Drug Administration (FDA) approved for the extended adjuvant treatment of early-stage human epidermal growth factor receptor 2 (HER2) positive breast cancer, and in combination with capecitabine for advanced and metastatic HER2 positive breast cancer. While it has been established in multiple clinical trials that neratinib causes high levels of diarrhoea (Chan et al., 2016), it is unclear by what mechanism this occurs. This study demonstrated the development of a model of neratinib-induced diarrhoea in male and female rats, and used archived caecal content samples from rats treated with neratinib or vehicle control to examine changes in the gut microbiome. The animal study presented in this chapter was completed before my PhD. However, during my candidature I analysed and synthesised all data, completed histological staining and scoring of intestinal sections and completed all work related to 16S microbial sequencing. This chapter was published in Breast Cancer (*Secombe, K. R., Ball, I. A., Shirren, J., Wignall, A. D., Keefe, D. M. and Bowen, J. M.*

(2021). Pathophysiology of neratinib-induced diarrhea in male and female rats: microbial alterations a potential determinant. Breast Cancer 28(1): 99-109).

Hypothesis:

Neratinib will cause high levels of diarrhoea in rats, and will cause significant changes in the gut microbiome.

Aims:

- To develop a reproducible model of neratinib-induced diarrhoea in male and female rats.

- To characterise intestinal damage caused by neratinib.

- To use 16S pyrosequencing techniques to characterise the microbiome of rats treated with neratinib compared to control rats.

3.6 Chapter 6

This chapter expands on the work done in chapter 5, to further determine the role of the gut microbiome in neratinib-induced diarrhoea. In the previous chapter, caecal microbiome samples were taken for analysis at the completion of the study. Here, to further understand the links between SM-TKI toxicity and the microbiome, the microbiome was altered using antibiotics before neratinib treatment began. This experiment was designed in order to investigate pre-treatment microbiome composition as an eventual predictor of diarrhoea development. Rats were treated with neratinib following microbial depletion with different classes of antibiotics (vancomycin, neomycin and a broad-spectrum

antibiotic cocktail), to observe differences in the development of gastrointestinal toxicity.

Hypothesis:

Antibiotic treatment will alter diarrhoea from neratinib treatment.

Aims:

- To characterise clinical markers of diarrhoea and gastrointestinal damage following treatment with neratinib and either vancomycin, neomycin or an antibiotic cocktail.

- To examine microbial changes following neratinib and antibiotic treatment.

3.7 Chapter 7

This chapter reports on two pilot clinical studies. Here, I aimed to understand, in a clinical cohort, whether there was a relationship between microbiome composition before cancer treatment and eventual diarrhoea development. Firstly, archived faecal samples taken before a chemotherapy infusion, mid-way through treatment, were analysed, as well as diarrhoea status as assessed by clinical staff. In the second study, people who were cancer treatment naïve and scheduled to receive chemotherapy-based treatment donated stool samples before and during treatment. Patient reported outcomes (PROs) pertaining to gastrointestinal toxicity were analysed along with 16S sequencing of pre-treatment stool samples.

Hypothesis:

Gut microbiome composition prior to cancer treatment will be different between people who develop diarrhoea and people who do not develop diarrhoea.

Aims:

- To recruit participants before beginning cancer treatment to donate stool samples.

- To characterise the microbial composition of these samples.

- To collect PROs related to diarrhoea severity and overall side effects of treatment.

- To explore relationship between symptoms of gastrointestinal toxicity with pre-treatment microbial composition.

Statement of Authorship

Title of Paper	The bidirectional interaction of the gut microbiome and the innate immune system: implications for chemotherapy-induced gastrointestinal toxicity
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Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	27/04/2021

Chapter 1: The bidirectional interaction of the gut microbiome and the innate immune system: implications for chemotherapy-induced gastrointestinal toxicity

Chapter one is my first literature review and aims to provide an in-depth summary of the role of the gut microbiome in chemotherapy-induced gastrointestinal toxicity. This chapter was published in the International Journal of Cancer (*Secombe, K. R., Coller, J. K., Gibson, R. J., Wardill, H. R. and Bowen, J. M. (2019b). The bidirectional interaction of the gut microbiome and the innate immune system: Implications for chemotherapy-induced gastrointestinal toxicity. Int J Cancer 144(10): 2365-2376*). This chapter is presented in its original publication format. The referencing style, spelling and figure/table numbers have been modified to maintain consistency throughout this thesis.

1.1 Abstract

Chemotherapy-induced gastrointestinal toxicity (CIGT) occurs in up to 80% of all patients undergoing cancer treatment, and leads to symptoms such as diarrhoea, abdominal bleeding and pain. There is currently limited understanding of how to predict an individual patient's risk of CIGT. It is believed the gut microbiome and its interactions with the host's innate immune system plays a key role in the development of this toxicity and potentially other toxicities, however comprehensive bioinformatics modelling has not been rigorously performed.

The innate immune system is strongly influenced by the microbial environment and vice-versa. Ways this may occur include the immune system controlling composition and compartmentalisation of the microbiome, the microbiome affecting development of antigen-presenting cells, and finally, the NLRP6 inflammasome orchestrating the colonic host-microbiome interface. This evidence calls into question the role of pre-treatment risk factors in the development of gastrointestinal toxicity following chemotherapy.

This review aims to examine evidence of a bidirectional interaction between the gut microbiome and innate immunity, and how these interactions occur in CIGT. In the future, knowledge of these interactions may lead to improved personalised cancer medicine, predictive risk stratification methods and the development of targeted interventions to reduce, or even prevent, CIGT severity.

1.2 Introduction

Gastrointestinal toxicity is a significant and often dose-limiting adverse event of many chemotherapeutic agents used in cancer treatment, and is currently without a widely effective preventative or treatment strategy. Chemotherapy-induced gastrointestinal toxicity (CIGT) covers a constellation of cancer treatment-related adverse events often referred to as mucositis, as inflammation of the mucosa is a key aspect of tissue injury (Lalla et al., 2014). Characterised by painful ulcerative lesions along the entire gastrointestinal tract from mouth to anus, CIGT affects up to 80% of patients, depending on treatment regimen (Lalla et al., 2014). This leads to a heightened risk of

adverse events such as infection and diarrhoea (Carlotto et al., 2013). Symptoms such as abdominal bleeding and abdominal pain are common, and result in increased hospital stays and the need for parenteral nutrition (Lalla et al., 2014). These interventions, as well as use of pain management medication, results in a significantly increased economic cost, with Medicare data from Australia suggesting a cost of $1500 per episode of severe diarrhoea (Vouk et al., 2016). Available economic evidence substantially underestimates the larger societal burden and loss of quality of life of CIGT, caused by loss of productivity, need for informal care arrangements and increases in anxiety and depression levels (Tarricone et al., 2016). Severe complications of CIGT such as bacteraemia and sepsis cause chemotherapy dose reductions and in profound cases, treatment cessation, compromising remission and increasing mortality (Di Fiore et al., 2009). Subsequently, CIGT presents as a major clinical and economic burden (Carlotto et al., 2013).

Activation of transcription factors and upregulation and release of pro-inflammatory cytokines, in response to initiating events, are integral in the pathobiology of CIGT. More recent reports have focussed on an altered gut microbiome and damaged epithelial cells that produce cellular damage signals, causing activation of the innate immune system (Vasconcelos et al., 2016; Wardill et al., 2016). These types of damage signals are recognised by receptors in the innate immune system, present on gastrointestinal tract cells (Jacobs et al., 2014).

It is now known that cancer treatments cause a raft of changes to the microbiome and host innate immune system (Alexander et al., 2017). There is

significant heterogeneity in the microbiome and in immune function, and as such, this emergent data represents a chance to personalise cancer treatment, and allow the identification of patients at risk of severe symptoms (Dubin et al., 2016; Zhu et al., 2017). However there is a clear gap in knowledge in translating these results from studies in radiotherapy- and immunotherapy-based patients into those with chemotherapy. The symptoms of CIGT and other cancer treatment-induced toxicities (including radiotherapy) are similar, and while occur from different initiating events, have a similar timeline of pathogenesis (Sonis, 2004b). These radiotherapy studies are beyond the scope of this review, however chemotherapy and radiotherapy are often concurrently used and these studies have been included in this review, in order to best assess available evidence. In addition to translating results into chemotherapy studies, it is not yet known if there is a specific gut microbiome profile that establishes risk of acute CIGT, and the role of the host immune system in maintaining or changing that profile. This review will provide evidence for an updated mechanistic hypothesis of CIGT wherein the bidirectional interaction of the host innate immune system and native microbiome may predict the severity of gastrointestinal toxicity a patient will suffer following chemotherapy. A semi-structured search of PubMed for full-text articles in English found more than 1000 studies investigating the microbiome and/or innate immune system in chemotherapy-induced gastrointestinal toxicity. Search terms included: ‘microbiome’, ‘chemotherapy-induced gastrointestinal toxicity’, ‘chemotherapy-induced mucositis’, ‘cancer treatment diarrhoea’ and ‘chemotherapy microbiome’. Findings of key studies are summarised in tables 1-3.

1.3 Pathobiology of CIGT

Although CIGT is a significant concern in the treatment of cancer, the underlying mechanisms remain unclear. Sonis introduced a model in 2004, consisting of five continuous and overlapping phases. This model elegantly showed the integral role of transcription factor activation and subsequent upregulation and release of pro-inflammatory cytokines, in response to initiating events (Sonis, 2004b). These initiating events may be the innate immune system's recognition of a Pathogen-Associated Molecular Pattern (PAMP) (e.g. lipopolysaccharide (LPS)), Damage-Associated Molecular Pattern (DAMP) (e.g. high mobility group box chromosomal protein 1, heat shock proteins) or ChemoRadiotherapy-Associated Molecular Pattern (CRAMP) (Sonis, 2010). There are several cellular mediators critical in these developmental events. These include nuclear factor kappa B (NF-κB), which causes the upregulation of up to 200 genes possibly involved in CIGT development (Sonis, 2004b). Pro-inflammatory cytokines (primarily tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, IL-18 and IL-33 (Logan et al., 2008b; Guabiraba et al., 2014; Lima-Junior et al., 2014)) are significantly elevated, leading to an amplification of apoptosis and epithelial damage (Sonis, 2004b).

Since this model was introduced, more recent research suggests that the large population of bacteria resident in the small and large intestines plays a fundamental role in CIGT development (figure 1.1). With recent advances in 'omics' technology, our ability to understand the unique idiosyncrasies of the microbiome is vastly improving. This onslaught of information highlights the

need for improved translational integration of the role of gut bacteria in the pathobiological model.

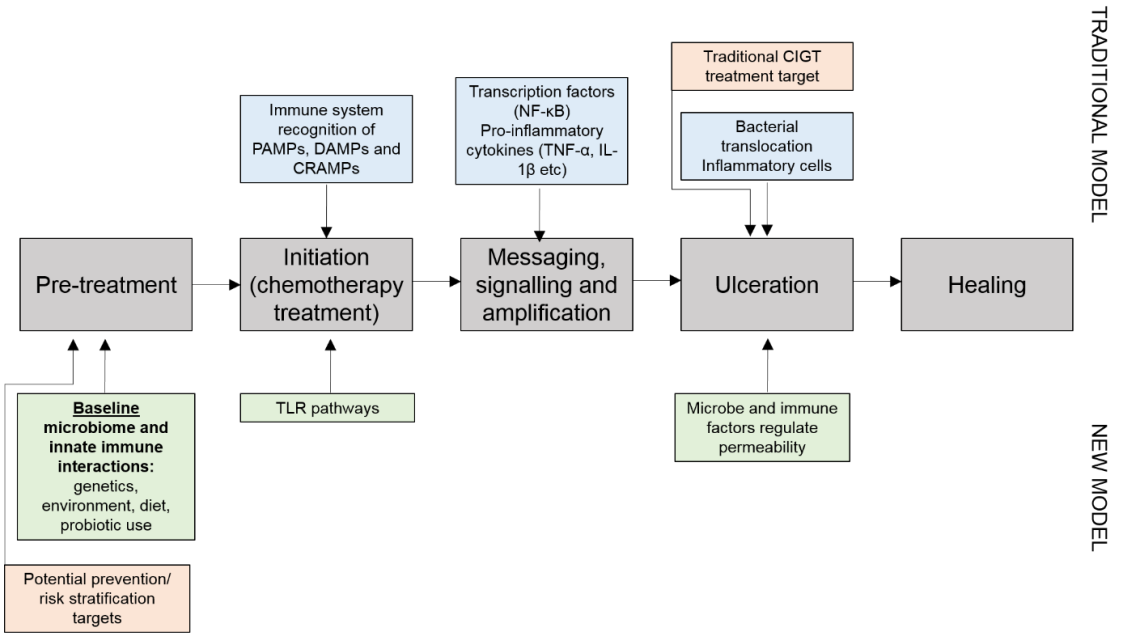

Figure 1.1: Pathobiology of CIGT. Chemotherapy treatment leads to direct DNA damage and release of Reactive Oxygen Species, causing transcription factor activation and release of pro-inflammatory cytokines. This leads to an amplification of apoptosis and epithelial damage, eventually causing intestinal ulceration and severe pain, in some cases caused by bacterial translocation through these ulcers. Removal of chemotherapy treatment will allow healing. Since the five-phase model was introduced in 2004 (Sonis, 2004b), significant research has been done to improve this model. We now suggest that bacterial and immune cells not only play a role in the ulceration phase, but also have an important baseline role in setting up risk of severe toxicity.

1.4 The gut microbiome

The gut microbiome, defined here as the collection of bacteria and other microorganisms present in and around tissues from the mouth through to the anus, is made up of almost one hundred trillion microorganisms (Ley et al., 2006). Functions include protective and immunological actions, supporting energy metabolism and triggering mucous barrier formation (Purchiaroni et al., 2013; Jacobs et al., 2014). The importance of the microbiome in normal development and functioning can be observed in germ-free mice, which are completely devoid of a microbiome raised under special conditions so they have no bacterial population. As a consequence of this, they have a variety of developmental differences including immature immune systems and altered digestive enzyme development, and are thus extremely susceptible to infection (Roy et al., 2017).

Although the composition of the human gut microbiome is dependent on many factors such as diet, sex and ethnicity, much of its composition is determined in birth and infancy (Roy et al., 2017). New findings also suggest that microbiome composition is not significantly associated with genetics, and is often more associated with environment (Rothschild et al., 2018). While each individual's gut microbiome is unique; the overall framework is often similar with the *Firmicutes* and *Bacteroidetes* phyla comprising over 90% of the gut microbiome (Van den Abbeele et al., 2011). Most bacteria belong to the *Clostridium* and *Bacteroides* genera, with major commensal (species which cohabitate with mutual benefit) species being *Lactobacillus* spp. and *Bifidobacterium* spp. (Purchiaroni et al., 2013). Additionally, there are marked differences between

the oral, gastric, small and large intestinal bacterial populations (Donaldson et al., 2016), reflecting physiological differences such as oxygen gradient, pH and presence of antimicrobial peptides. The oral microbiome is dominated by *Streptococcus* spp., the small intestine by *Lactobacillaceae* and *Enterobacteriaceae*, and the colon by species such as *Bacteriodaceae* and *Prevotellaceae* (Donaldson et al., 2016; Lloyd-Price et al., 2016).

The gastrointestinal epithelium is constantly in contact with adherent bacteria. A sensitive balance exists, with continual cross talk between the microbiome, immune cells and the mucosal barrier to maintain homeostasis (Wells et al., 2011). A disruption in this balance, known as dysbiosis, has been shown to have a role in multiple autoimmune diseases such as multiple sclerosis (Miyake et al., 2015). Additionally, the inter-individual differences in the gut microbiome are now considered to be one of the key contributors to immune response in humans. This is thought to be via influence of an individual's cytokine response (with strongest effects on interferon gamma (IFN-ɣ) and TNF-α production), and therefore disease susceptibility and overall immune function (Schirmer et al., 2016).

1.5 The innate immune system

The innate immune system is the first line of response to bacterial invasion or an aseptic tissue injury. It responds to danger signals by recruiting immune cells to the injury site, inducing inflammation and activating the adaptive immune system. The innate immune system is vital in the gastrointestinal tract, with the luminal gastrointestinal surface of being one of the largest common

surface areas between host and environment. With a diverse range of microbes living so close to the host, the innate immune system is critical in maintaining immune tolerance to commensal microbes, whilst ensuring the rapid initiation of an immune response following invading pathogens.

The innate immune system utilises many systems in the gastrointestinal tract in order to maintain homeostasis, including separation mechanisms including epithelial and mucosal layers, and compounds such as antimicrobial peptides and antibodies. Additionally, the innate immune system utilises a system of pattern recognition receptors that recognise microbial molecular patterns.

One type of pattern recognition receptor, Toll-like receptors (TLRs), are particularly important in sensing molecular patterns from gut microbes. TLRs are highly conserved transmembrane receptors and are members of the Toll/Interleukin 1 Receptor signalling pathway (Akira et al., 2004). TLRs are present in the gastrointestinal mucosa on basolateral and apical surfaces of epithelial cells, professional immune cells, enteric neurons and glia (Barajon et al., 2009; Abreu, 2010). There are eleven TLRs found in humans, recognising diverse ligands including RNA, DNA and LPS (Akira et al., 2004). TLR4 is a particular focus of recent research due to its known expression changes following chemotherapy, and its ability to recognise patterns released by chemotherapy-damaged cells and therefore will be the focus here (Wardill et al., 2016). TLR4 can be activated by exogenous and endogenous danger signals such as LPS, high mobility group box chromosomal protein 1 and heat shock proteins (Akira et al., 2004), causing a downstream signalling pathway of

transcription factor (e.g. NF-κB) upregulation and pro-inflammatory cytokine release.

The role of TLR4 in CIGT development is now well known, with TLR4 knockout mice having less diarrhoea, weight loss and histological damage in response to irinotecan treatment (Wardill et al., 2016). However, there is some disparity surrounding the role of TLR4. One study where TLR4 was blocked using naloxone was unable to reduce irinotecan-induced gastrointestinal damage (Coller et al., 2017), whereas other research has shown that TLR4 agonist LPS can protect intestinal crypts from other insults such as radiation (Riehl et al., 2000). Studies have shown protection and exacerbation, possibly due to differences between acute and chronic injury (Zheng et al., 2017) or chemotherapeutic agents, and therefore the role of TLR4 appears to be complicated and context-specific. Immunologic cell death, a form of cell death where dendritic cell activation leads to a specific T cell response, is caused by some anti-cancer agents such as oxaliplatin and is characterised by release of DAMPs. As TLR4 is of vital importance in binding DAMPs, it is possible that immunogenic cell death may contribute to modulating the role of TLR4 in CIGT.

1.6 A bidirectional interaction: innate immunity and the gut microbiome

The innate immune system is strongly influenced by the microbial environment (Schirmer et al., 2016). However, there is growing evidence that the reverse is also true, and that the microbial environment is similarly influenced by the innate immune system (Jacobs et al., 2014; Thaiss et al., 2016). This

bidirectional interaction between microbiome and immune system has been described by Hooper et al. (2012) as ‘inside-out’ and ‘outside-in’ interactions, and is observed in a number of ways.

The immune system controls composition and compartmentalisation of the microbiome (Hooper et al., 2012). There is ample evidence to suggest that the host immune system is integral in selecting mucosal and luminal bacterial populations, ensuring there is minimal direct contact between the gastrointestinal surface and bacteria, keeping penetrant bacteria constrained to the lumen (Van den Abbeele et al., 2011; Jacobs et al., 2014). Van den Abbeele et al. (2011) hypothesised that a distinct mucosal-associated microbial community has many immune-regulating effects with large potential biological outcome. Conversely, bacteria likely to be targeted by host defences are restricted to the lumen. This paper also suggested an outer colonic mucus layer, situated between the inner mucus layer and the lumen, may contain a ‘backup’ of microorganisms, which could act as an inoculum to restore the initial microbial balance after a perturbation, ensuring continual stability. In contrast, the colonic inner mucus layer is effectively devoid of bacteria (Hansson et al., 2010). The small intestine does not have these two distinct layers, and instead relies on antimicrobial peptides / receptors such as RegIIIɣ and TLRs to minimise bacterial penetration of the mucus layer (Donaldson et al., 2016).

An alternative mechanism explaining the interaction between the microbiome and immune system is through the development of antigen-presenting cells (APCs) such as dendritic cells and macrophages. Gastrointestinal dysbiosis has been shown to reduce infiltrating mature APCs (Xu et al., 2017).

Additionally, commensal bacteria can regulate dendritic cell activity. When investigating the role of the microbiome in APC development, it was found there were less gastrointestinal, but not systemic, dendritic cells in germ-free animals, and subsequent mono-colonisation of the gastrointestinal tract with *E. coli* caused gastrointestinal dendritic cell recruitment (Williams et al., 2006; Haverson et al., 2007). Additionally, microbe-derived adenosine triphosphate has been shown to stimulate CD70 and CX3CR1 expressing dendritic cells, which can then go on to induce differentiation of Th17 cells (Atarashi et al., 2008).

The NLRP6 inflammasome is key in orchestrating the colonic host-microbiome interface (Wlodarska et al., 2014), and is important in production of pro-inflammatory IL-18. Mice deficient in NLRP6 have been shown to be more susceptible to enteric infection (Wlodarska et al., 2014), have an altered microbial biogeography and to have a dysbiotic bacterial profile more likely to cause colitis-like symptoms (Elinav et al., 2011). A recent paper has shown microbiome-modulated metabolites are able to regulate the NLRP6 inflammasome and subsequent IL-18 production (Levy et al., 2015). These microbial metabolic products are also known to regulate the role of colonic regulatory T cell homeostasis among a raft of other effects (Shapiro et al., 2014).

1.7 The gut microbiome in CIGT

The gut microbiome plays a variety of roles in the development of CIGT. One study showed that compared to germ-free mice, conventional mice treated with

the same dose of irinotecan had more lesions within the jejunal intestinal epithelium and higher gastrointestinal permeability (Pedroso et al., 2015). This was also reflected in another study, which showed that diarrhoea was more common in conventional mice compared to germ-free mice, which also had a lowered intestinal damage score (Brandi et al., 2006). Rigby et al. (2016) also showed the role of gastrointestinal bacteria in mediating doxorubicin-induced gastrointestinal damage by showing that germ-free mice did not display the changes in crypt depth and proliferative cell numbers that conventional mice treated with doxorubicin showed. Closely linked are the inflammatory pathways that are markedly upregulated in all cases of CIGT. It is now known that microbiome-host interactions modulate inflammatory cytokine production capacity (Schirmer et al., 2016). Dysbioses of gut microbes are often linked to aberrant immune responses, often complemented by abnormal production of inflammatory cytokines. Additionally, commensal bacteria have protective effects on the integrity of the gastrointestinal mucosal barrier, including interactions with tight junctions and regulation of mucous layer (Reinoso Webb et al., 2016).

Research in pre-clinical models conducted over the past decade has shown a variety of changes occur to microbiome composition and diversity in the gastrointestinal tract due to chemotherapy treatment. There are some limitations to using pre-clinical microbiome models with 85% of bacterial sequences seen in a mouse representing genera not detected in humans (Ley et al., 2005). However, there is also significant similarity in the distal gut microbiome between human and mice at a divisional level, and both have the

same two most abundant bacterial divisions (*Firmicutes* and *Bacteroidetes*). Therefore pre-clinical models are often routinely used in this field.

Pre-clinical studies show a decrease in commensal species following chemotherapy, which causes reduced protective effects and decreased resistance to pathogenic colonisation (table 1.1). This increase in pathogenic species also corresponds to an increase in gram negative species, which release LPS that is known to initiate the inflammatory pathways involved in CIGT development (Akira et al., 2004). These pre-clinical studies generally show decreases in *Lactobacillus* and *Bifidobacterium* and increases in *Escherichia coli* (*E. coli*) and *Staphylococcus* (Stringer et al., 2009b). However, many of these studies used polymerase chain reaction (PCR) or microbial culture techniques to delineate species and changes. While these methods were standard at the time, with more sophisticated pyrosequencing techniques now the norm, these results may have limited reproducibility.

A small number of clinical studies have also been conducted with patients undergoing chemotherapy, often replicating what has been shown in pre-clinical studies (table 1.1). However, due to a focus on clinical outcomes (e.g. diarrhoea severity), these studies have failed to conclusively link pre-treatment to post-treatment microbiome composition. Results overall have included a decrease in total bacteria numbers and diversity (Touchefeu et al., 2014; Montassier et al., 2015). At a species specific level, findings have shown increases in *Bacteroidetes*, *Clostridium* cluster IV and *E. coli* (Zwielehner et al., 2011; Montassier et al., 2015) and decreases in *Lactobacillus*, *Bifidobacterium* and *Clostridium* cluster XIV (Zwielehner et al., 2011; Stringer et al., 2013).

Attempts to ameliorate chemotherapy-induced changes to the microbiome profile have been variably successful in lowering damage severity. Administration of probiotics in pre-clinical models have attenuated gastrointestinal damage from chemotherapy by preventing apoptosis, reducing barrier disruption and promoting crypt survival (table 1.2) (Bowen et al., 2007; Southcott et al., 2008). However there have been inconsistencies, with level of diarrhoea reduction varying due to probiotic strains, dosing and treatment plan. For example in pre-clinical studies using *Streptococcus thermophiles*, one study showed promising results, with non-tumour bearing rats treated with methotrexate having attenuation of gastrointestinal damage (Tooley et al., 2006), while another study utilising the same probiotic and chemotherapeutic in tumour bearing rats showed no benefit (Tooley et al., 2011). These studies additionally demonstrate a potential role of the tumour itself in regulating CIGT and gut microbiome changes, however further research is required to more fully understand this.

Multiple clinical trials have used probiotics in patients undertaking chemotherapy, with *Lactobacillus* species have been a particular focus (table 1.2). For example, Osterlund et al. (2007) showed that *Lactobacillus* supplementation led to less severe diarrhoea, less abdominal discomfort and fewer dose reductions after 5-fluorouracil (5-FU) treatment for colorectal cancer. Subsequently, the Multinational Association for Supportive Care in Cancer (MASCC) released new clinical guidelines in 2014 suggesting the use of “probiotic agents containing *Lactobacillus* species for the prevention of chemotherapy and radiation-induced diarrhoea in patients with a pelvic

malignancy” (Lalla et al., 2014). Despite this, a recent meta-analysis found insufficient current evidence to support widespread implementation of probiotics after chemotherapy (Wardill et al., 2018).

Clinical trials have also investigated the impact of the antibiotic neomycin in combination with irinotecan, and results have shown less diarrhoea (Kehrer et al., 2001; Alimonti et al., 2003). However, a larger study conducted by de Jong et al. (2006) did not find a substantial role for neomycin in reducing diarrhoea severity. Following inadequate / conflicting evidence, a 2013 systematic review was conducted and concluded that no clinical guideline for the use of neomycin was possible (Gibson et al., 2013). Another study administering antibiotics showed compromised anti-tumour efficacy of chemotherapy and an increase in potentially pathogenic bacteria (van Vliet et al., 2009). These results may suggest that the removal of the entire microbiome with broad spectrum antibiotics does more harm than good, and that restoring microbial diversity is more important in maintaining damage-defence mechanisms.

Table 1.1: Summary of studies investigating gut microbiome changes due to cancer treatment. Increase and decrease columns refer to bacterial species that changed after cancer treatment.

Study Type/ Sample Size	**Cancer treatment**	**Study population age (years)**	**Detection Method**	**Increases**	**Decreases**	**Outcome/ commentary**	**Reference**
Pre-clinical (tumour bearing rats) n=30	Irinotecan		RT-PCR, PCR-denaturing gel electrophoresis	*Clostridium* cluster XI (0.5 log) and *Enterobacteriaceae* (1.5 log) ($P < 0.05$)	Total bacteria number (by day 3, 1 log), *Clostridium* cluster XIVa (1-3 log), *Lactobacillus* and *Bifidobacterium* ($P < 0.05$)	Pathogenic bacteria increased	Lin et al. (2012)
Pre-clinical (mice) n=100	Irinotecan		Differences in gastrointestinal toxicity response between germ-free and conventionalised mice. Conventionalised mice = increased inflammation, lesions of gastrointestinal epithelium and increased permeability. Conventionalisation of germ-free reversed phenotype to conventionalised.				Pedroso et al. (2015)
Pre-clinical (rats) n=81	Irinotecan		Microbiological culture	*Escherichia* spp., *Clostridium*, *Enterococcus*, *Serratiam Staphylococcus* (qualitative results)	*Peptostreptococcus, Bifidobacterium* (qualitative results)	Microbiome changes corresponded with diarrhoea incidence	Stringer et al. (2007)
Pre-clinical (rats) n=81	Irinotecan		RT-PCR	*E. coli*, *Staphylococcus* spp. ($P < 0.05$)	*Lactobacillus* spp. ($P < 0.05$)	Species that decreased were beneficial, major components of gut microbiome	Stringer et al. (2008)

Pre-clinical (rats) n=48	Irinotecan, 5-FU or oxaliplatin		16S pyrosequencing	Irinotecan: *Fusobacteria* (relative abundance 13-fold) and *Proteobacteria* (relative abundance 17-fold)		Also noted changes in serum and urine metabolome.	Forsgard et al. (2017)
Pre-clinical (rats) n=27	Irinotecan		Microbiological culture and RT-PCR	*E. coli* (qualitative results)	*Bifidobacterium* (qualitative results)	Changes also observed in mucous secretion and release	Stringer et al. (2009b)
Pre-clinical (rats) n=75	5-FU		Microbiological culture	Large intestine: Gram-negative facultatives (1-fold change in proportion)	Large intestine: Gram-positive facultatives (9-fold change in proportion)	Small intestine: shift from domination by Gram-positive cocci to Gram-negative rods	Von Bultzingslowen et al. (2003)
Clinical n=16	Various chemotherapies	Cohort 1: mean= 71 (range 36-82), Cohort 2: mean= 63 (range 40-77)	Microbiological culture, qRT-PCR	*E. coli* and *Staphylococcu*s spp. (no statistics due to low patient number)	*Lactobacillus* spp., *Bifidobacterium* spp., *Bacteroides* spp., and *Enterococcus* spp.		Stringer et al. (2013)
Clinical n=9	Various chemotherapies + antibiotics	Paediatric cohort, age not reported	PCR-denaturing gel electrophoresis fingerprinting and *in situ* hybridisation	Enterococci (100-fold)	Anaerobic bacteria (10,000-fold). Commensal species (*Bacteroides* spp., *Clostridium* cluster XIVa, *Faecalibacterium*	Prophylactic and therapeutic antibiotic use did not explain changes in	van Vliet et al. (2009)

					prausnitzii and *Bifidobacterium* spp., 3000-6000-fold)	microbiome composition	
Clinical n=17	Various chemotherapies +/- antibiotics	Treatment group: mean= 59 ± 13, healthy group: mean= 65 ± 18	TaqMan qPCR and gel electrophoresis fingerprinting	Bacteroides (2%), *Clostridium* cluster IV (2%)	*Bifidobacteria* (0.9%) and *Clostridium* cluster XIVa (22% to 19%)	Decrease in total number and abundance of bacteria	Zwielehner et al. (2011)
Clinical n=8	Carmustine, etoposide, aracytine and melphalan	Mean= 50.5 ±10.8	16S rRNA 454 high throughput-pyrosequencing	Bacteroidetes (32%), Proteobacteria (14%) (P = 0.008)	Firmicutes (56%) and Actinobacteria (5%) (P = 0.008)	Reduced diversity and microbiome metabolic capacity	Montassier et al. (2014)
Clinical n=9	Pelvic radiotherapy (concurrent chemotherapy in subset of patients)	Range= 35-63	16S rRNA 454 high throughput-pyrosequencing	Fusobacteriaceae (6-fold) and Streptococcaceae (P < 0.05)	Firmicutes (10%)	Number of species-level taxa significantly reduced after therapy	Nam et al. (2013)

qRT-PCR = qualitative real time polymerase chain reaction, 5-FU = 5-fluorouracil

Table 1.2: Summary of studies investigating probiotics and antibiotics in significantly modulating cancer treatment-induced gastrointestinal toxicity

Study Type/ Sample Size	**Cancer treatment**	**Study population age (years)**	**Probiotic/ antibiotic**	**Administration**	**Endpoint**	**Outcome**	**Reference**
Pre-clinical (rats) n=48	Irinotecan		VSL#3	Oral gavage, different schedules: 7, 21, 28 days pre-, pre- and post- or post-treatment	Clinical and histological injury markers	Probiotic reduced weight loss compared to irinotecan alone (5.3% vs 12.6%, $P < 0.05$), increased crypt proliferation ($P < 0.05$), inhibited apoptosis ($P < 0.05$)	Bowen et al. (2007)
Pre-clinical (rats) n=8	MTX		Cow or sheep milk yoghurt with probiotics	Oral gavages twice daily 7 days prior to MTX and for remainder of trial	Intestinal barrier function, lactulose/mannitol ratio	Sheep yoghurt probiotic improved histological damage and small intestinal permeability ($P < 0.05$)	Southcott et al. (2008)
Pre-clinical (rats) n=27	MTX		*Streptococcus thermophilus*	7 oral gavages from 48h prior to MTX to 72 hours after	MPO levels, 13(C) sucrose breath test, histological injury markers	High-dose probiotic partially attenuated mucositis (↓ MPO, ↓ histological damage, $P < 0.05$)	Tooley et al. (2006)
Pre-clinical (tumour bearing rats) n=36	MTX		*Streptococcus thermophilus*	7 oral gavages from 48h prior to MTX to 72 hours after	MPO levels, 13(C) sucrose breath test, histological injury markers	No difference between groups	Tooley et al. (2011)

Clinical n=15	Irinotecan + 5-FU/ leucovorin	Not reported	Neomycin + bacitracin	Oral, 3 x daily, from second chemotherapy cycle, days 2-5 and 16-19 of each cycle	Diarrhoea reduction or resolution	Antibiotic = complete resolution of diarrhoea from 2^{nd}-4^{th} chemotherapy cycle in all patients	Alimonti et al. (2003)
Clinical n=62	Irinotecan	Range= 36-80	Neomycin	Oral, 3 x daily for 3 days, starting 2 days before Irinotecan	Grade 3 diarrhoea levels	No difference between groups	de Jong et al. (2006)
Clinical n=7	Irinotecan	Median=57, Range= 49 – 71	Neomycin	Oral, 3 x daily, if developed diarrhoea in first chemo course, oral, days -2 to 5 of other cycles	Diarrhoea reduction	Diarrhoea ameliorated in 6 of 7 patients (P = 0.033)	Kehrer et al. (2001)
Clinical n=150	Adjuvant 5-FU (colorectal cancer patients)	Median= 60, Range= 31–75	*Lactobacillus rhamnosus*	Oral, 2 x daily, 24 weeks of treatment	Reduction of severe diarrhoea	Less severe diarrhoea (22 vs 37%, P = 0.027), less abdominal discomfort (2 vs 12%, P = 0.025), less dose reductions (22% vs 47%, P = 0.0008)	Osterlund et al. (2007)
Clinical n=42	Various chemotherapies-paediatric population	Range= 14 months –13 years 4 months	*Bifidobacterium breve* strain Yakult	Oral, 3 x daily, 2 weeks before treatment, 6 weeks after (8 weeks total)	Effect on infectious complications	Reduced need for antibiotics (3.2 vs 6.9 days, P = 0.04) and frequency of fever (44 vs 68%)	Wada et al. (2010)
Clinical n=63	Pelvic radiotherapy with cisplatin	Placebo group: median= 52, treatment group: median= 47	*Lactobacillus acidophilus* + *Bifidobacterium bifidum*	Oral, 2 x daily, 7 days before treatment then every day of treatment	Diarrhoea reduction	Less grade 2 and 3 diarrhoea (9 vs 45% control, P = 0.002)	Chitapanarux et al. (2010)

Clinical n=85	Adjuvant radiotherapy: cervical or endometrial cancer + concomitant cisplatin	Placebo group: median= 59.34 ±12.77, treatment group: median= 60.91 ± 11.8	Lactobacillus casei DN-114001	Oral	Diarrhoea reduction	No difference	Giralt et al. (2008)

5-FU = 5-fluorouracil, MTX = methotrexate

1.8 Gastrointestinal toxicity, the microbiome and the innate immune system

Modifications to both immune function and bacterial profile may influence severity of gastrointestinal injury following chemotherapy (Wardill et al., 2017). This could possibly occur via an altered capacity to mount an immune response. For example, commensal bacteria are able to induce CD4+ T cell differentiation. Specifically, *Bacteroides fragilis* can induce the development of a systemic Th1 response through polysaccharide A molecules (Mazmanian et al., 2005). It has been well established that *Bacteroides* genus levels are decreased by chemotherapy in both pre-clinical and clinical models (Stringer et al., 2009b; van Vliet et al., 2009; Lin et al., 2012). This therefore leads to a potential decreased ability to mount a Th1 response after chemotherapy, which may affect the severity of gastrointestinal injury.

The TIMER (translocation, immunomodulation, metabolism, enzymatic degradation, reduced diversity) model was recently proposed by Alexander et al. (2017) to show the various ways the gut microbiome can influence chemotherapy efficacy and toxicity. It is possible that each part of this model is reliant on the microbiome's interaction with the innate immune system, and thus the gut microbiome and innate immune system henceforth should be investigated together as much as practicable.

There are a variety of ways this interaction could be studied in future. Currently, much CIGT research is undertaken in animals or relatively rudimentary cell cultures. Additionally, there are limitations to using animal pre-clinical models

for microbiome models, with many strains present in the mouse microbiome not present in humans as discussed previously (Ley et al., 2005). Reproducible and scientifically robust *in vitro* and *ex vivo* models are therefore needed to effectively study the microbiome in intestinal models. This may include using human tissue samples or stem cells to grow organoids, or the development of gut-on-a-chip technology to incorporate microbiome changes (Arnold et al., 2016). Additionally, recent trials of ingestible electronic capsules have showed the ability to sense gases produced by the microbiome in the gastrointestinal tract (Kalantar-Zadeh et al., 2018). This may represent a real-time method of monitoring gut microbiome changes that could be paired with other analyses.

1.9 Future opportunities for risk prediction and modification

A personalised approach could soon be taken to manage CIGT, where a patient's individual risk of toxicity could be managed early in their treatment plan. This approach is supported by observations relating genetic testing on DNA extracted from saliva to severe CIGT, in which key immunogenetic factors relating to the *TNF-a* and *TLR2* have been correlated with more severe toxicity (Coller et al., 2015). Whether the patient's gut microbiome profile pre-cancer treatment could also predict toxicity severity is largely unknown, particularly in the setting of chemotherapy-induced damage. However, this idea was first postulated by Touchefeu et al. (2014), and more recently by Wardill and Tissing (2017). Use of the microbiome as a predictive marker is gaining support in a variety of fields, including recent prediction of chemotherapy-related bloodstream infection (Montassier et al., 2016). Studies may employ methods such as machine learning or metabolomics techniques to create an algorithm or

predictive model in which a patient's microbiome profile can be input to test risk of disease (Montassier et al., 2016).

There is some initial evidence that such an idea could also prove true in relation to other cancer treatment-induced toxicities (table 1.3), although as yet no study has investigated this following chemotherapy. One study of patients undergoing pelvic radiotherapy (Wang et al., 2015) found that patients who went on to develop diarrhoea had lower bacterial diversity and a higher *Firmicutes* / *Bacteroidetes* ratio. A study of patients suffering from oral mucositis after radiotherapy for head and neck cancers was also able to build a predictive model for the aggravation of oral mucositis (similar to CIGT in the oral cavity) (Zhu et al., 2017). Only one study has identified a protective phenotype of the gut microbiome for gastrointestinal toxicity (Dubin et al., 2016). This study measured development of checkpoint blockade-induced colitis in melanoma patients receiving the immunomodulatory therapy ipilimumab. Using 16S rRNA pyrosequencing techniques, it was shown that an increased representation of bacteria in the *Bacteroidetes* phylum before treatment was associated with resistance to development of colitis.

Finally, one study (Covington et al., 2012) did not use a bacterial sequencing approach, but rather used the relatively novel method of an electronic nose and the Field Asymmetric Ion Mobility Spectrometry method, to analyse stool samples. Gases and other metabolic by-products of microbiome fermentation emitted from the samples were analysed. The patient pre-radiotherapy samples were successfully separated, using principal component analysis and linear discriminant analysis, into those who suffered from gastrointestinal toxicity and

those who did not. This method represents a translatable clinical test, where pre-treatment samples could be analysed, and future cancer treatment and supportive care measures adapted to suit.

This emergent data on the role of the microbiome and the immune system in determining severity of cancer treatment-induced toxicities represents an additional opportunity to personalise cancer treatment, and allow the identification of patients at risk of severe symptoms. Alexander et al. (2017) summarised evidence of being able to adapt the microbiome profile of the gastrointestinal tract using an enzyme inhibitor such as a β-glucuronidase inhibitor (Wallace et al., 2010), changes in diet (Faber et al., 2011) or probiotics (Bowen et al., 2007). Of these, diet modification has been shown to be one of the most consistent and predictable ways of remodelling the microbiome, able to induce rapid shifts in composition and subsequent function (David et al., 2014). An enzyme inhibitor may be particularly useful for patients undergoing irinotecan treatment, as the enzyme β-glucuronidase is crucial in irinotecan toxicity. SN-38, the active form of irinotecan, is conjugated in the liver to a less toxic metabolite, SN-38G. When excreted to the gastrointestinal tract via bile, it is hydrolysed back to the toxic SN-38 form by microbe-derived β-glucuronidase (Kehrer et al., 2001; Stringer et al., 2008). As discussed above, probiotics have previously been used in CIGT studies, with mixed results (table 1.2). There is currently a renewed call for carefully planned studies with new types of probiotics to better understand the potential benefits they could play in modulating CIGT risk (Ciorba et al., 2015). Also required is better characterisation of the microbial profiles associated with toxicities caused by

different agents to properly identify and develop the ideal microbial protectant. Finally, the developing area of faecal microbiota transplants and even faecal capsules represent methods of directly modifying the gut microbiome yet to be investigated in this context.

Table 1.3: Evidence of pre-treatment microbiome as a predictive factor in cancer treatment-induced gastrointestinal toxicity. Increase and decrease columns refer to differences in bacterial species from healthy controls or low toxicity groups that led to gastrointestinal toxicity symptoms

Study Type/ Sample Size	**Cancer treatment**	**Study population age (years)**	**Detection method**	**Predictive factor**	**Pre-therapy significant differences compared to controls**		**Outcome**	**Reference**
					Increases	**Decreases**		
Clinical n=20	Pelvic radiotherapy	Median= 51 years, range= 41–64	16S rRNA pyrosequencing	Development of diarrhoea	*Bacteroidetes, Dialister, Veillonella* (P < 0.05)	*Clostridium* cluster XI and XVIII, *Faecalibacterium, Oscillibacter, Parabacteroides, Prevotella* (P < 0.05)	Patients who developed diarrhoea: lower bacterial diversity (Shannon's index= 2.74 vs 3.78, P < 0.01), higher *Firmicutes* / *Bacteroidetes* ratio (P < 0.05)	Wang et al. (2015)
Clinical n=90	Radiotherapy - head and neck cancers	Mean= 47.2, range= 22–75	16S rRNA pyrosequencing	Severe oral mucositis	*Actinobacillus* during erythema	-	Healthy controls: greater diversity of oropharyngeal bacteria. Built predictive model (AUC=0.89) for	Zhu et al. (2017)

							mucositis aggravation i.e. mild progressing to severe. Could not construct model to predict aggravation prior to irradiation.	
Clinical n=34	Ipilimumab	Range= 28-85	16S rRNA pyrosequencing	Checkpoint blockade-induced colitis development	-	*Bacteroidetes*	Only study finding a protective phenotype. Model had sensitivity of 70% and specificity of 83%.	Dubin et al. (2016)
Clinical n=23	Pelvic radiotherapy - gynaecological malignancies	Mean= 71.5	Used electronic nose and Field Asymmetric Ion Mobility Spectrometry	Gastrointestinal toxicity (Irritable Bowel Syndrome scale)	-	-	Clear separation between high and low toxicity groups after 4 weeks of treatment (90% accuracy of reclassification).	Covington et al. (2012)

AUC= area under the curve

1.10 Conclusion

Effective treatments or predictive strategies for gastrointestinal toxicity caused by chemotherapy are urgently required. This under-reported but common adverse event has a substantial effect on quality of life and economic burden. Understanding the effect the gut microbiome and the innate immune system has on CIGT is key to developing treatment and prevention strategies. This review has summarised the key interactions between the gut microbiome and the innate immune system, and how these interactions may adversely affect gastrointestinal toxicity following chemotherapy. We have also drawn emphasis to new data suggesting that the bidirectional interaction between microbiome and immune system is distinct to each patient and how we may be able to predict high-risk patients, thereby adjusting supportive care measures to each person. It is hoped that through pragmatic and rigorous scientific investigation, effective CIGT treatment may be within reach.

Chapter 2: Assessment of the gut microbiome in Toll-like receptor 4 knockout models

Chapter two is my first original research chapter and details the development of a gastrointestinal epithelium-specific TLR4 knockout (KO) mouse model and characterisation of the microbiome of the model. This chapter is written as a traditional thesis chapter.

2.1 Abstract

Introduction: Toll-like receptor 4 (TLR4) has previously been implicated in the development of gastrointestinal toxicity following irinotecan treatment. However, TLR4 is broadly expressed in the gastrointestinal tract, and it is unknown whether TLR4 expressed on specific cell types has the key role in this toxicity. Here, a conditional TLR4 knockout (KO) was generated, where TLR4 is knocked out in intestinal epithelial cells (IECs) only.

Methods: A Vil-cre/ floxed knockout technique was used to developed the conditional knockout model (*Tlr4*$^{\Delta IEC}$). Initially, the microbiome of global TLR4 knockout mice was assessed. Then, the microbiome of wild-type (WT) and *Tlr4*$^{\Delta IEC}$ mice was assessed in two studies. Firstly, mice were genotype-specifically housed, before being co-housed for 4 weeks to assess any changes in microbiome composition. Secondly, the microbiome was assessed in WT and *Tlr4*$^{\Delta IEC}$ mice at baseline, following injection with MC-38 colorectal tumour cells, and following irinotecan treatment.

Results: Global TLR4 knockout mice showed significant differences in microbiome composition compared to WT mice. However, these differences were not replicated when comparing *Tlr4*$^{\Delta IEC}$ and WT mice. Additionally, there were no significant differences in composition following MC-38 inoculation or irinotecan treatment.

Conclusion: This study suggests that changes in epithelial TLR4 expression does not cause changes in the gut microbiome. This may suggest that epithelial TLR4 is a passive regulator of the microbiome.

2.2 Introduction

2.2.1 Toll-like receptor 4

Toll-like receptors (TLRs) are a group of evolutionarily conserved transmembrane pattern recognition receptors forming part of the innate immune system (Abreu, 2010). They recognise molecular patterns of both exogenous and endogenous danger signals. TLRs have an extracellular leucine-rich repeat domain responsible for ligand recognition, as well as the intracellular toll/interleukin-1 receptor-like (TIR) domain, important in signal transduction (Akira et al., 2004; Abreu, 2010).

Toll-like receptor 4 (TLR4) has a key role in maintaining gastrointestinal homeostasis (Burgueno et al., 2020). Along with the protein lymphocyte antigen 96 (MD-2), intestinal TLR4 recognises danger signals including bacterial lipopolysaccharide (LPS), high mobility group box chromosomal protein 1 (HMGB1) and heat shock proteins (Akira et al., 2004). Following the recognition

and binding of these signals, TLR4 homodimerises or heterodimerises, and initiates signal transduction via two pathways, Myeloid differentiation primary response 88 (MyD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF) (Kawasaki et al., 2014). MyD88 binding is the predominant TLR4-signalling pathway and leads to activation of interleukin-1 receptor-associated kinase (IRAK), which cause multiple downstream signalling cascades (figure 2.1). This chiefly activates nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPKs) and subsequent pro-inflammatory cytokine release (Akira et al., 2004; Deguine et al., 2014). Less common is TRIF signalling, which as well as activating NF-κB and MAPKs, also upregulates genes including Chemokine (C-C motif) ligand 5 and type I and II interferons (Yamamoto et al., 2003).

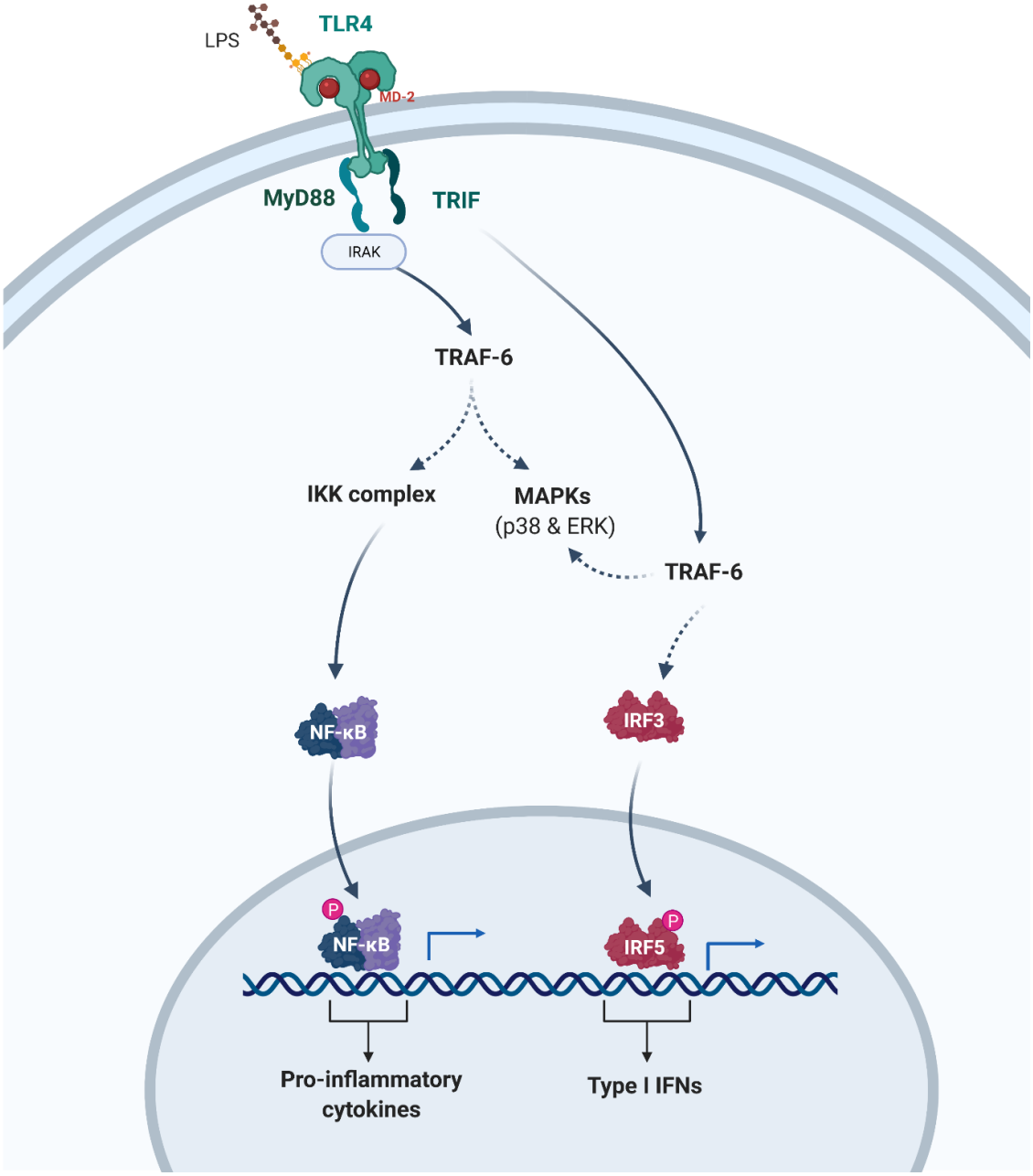

Figure 2.1: TLR4 signalling pathway. Activation by danger signals (e.g. bacterial LPS) causes a downstream signalling pathway mediated by MyD88 and TRIF. MyD88 binding recruits interleukin-1 receptor-associated kinase (IRAK), which when phosphorylated, enables binding of tumour necrosis factor receptor-associated factor 6 (TRAF6). A signalling cascade then occurs leading to the phosphorylated of the IκB kinase (IKK) complex, which leads to the activation of NF-κB and subsequent production of pro-inflammatory cytokines. TIR-domain-containing adapter-inducing interferon-β (TRIF) and mitogen-activated protein kinase (MAPK) signalling will also lead to release of type I interferons (IFNs). Adapted from “TLR4/5/7/8 Signalling Cascade”, by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

TLR4 is normally expressed at low levels on human intestinal epithelial cells (IECs) including enterocytes, Paneth cells, goblet cells and enteroendocrine cells (Abreu, 2010; Price et al., 2018). In human enterocytes, TLR4 has been shown in foetal tissue to predominantly localise to the basolateral surface (Fusunyan et al., 2001). However, in mouse tissue, TLR4 has been shown to be apically localised in ileal epithelium, basolaterally in the colon, and also in the Golgi compartments of small intestinal epithelial cells, suggesting TLR4 can be expressed in a variety of cellular locations (Hornef et al., 2002; Ortega-Cava et al., 2003; Chabot et al., 2006). In addition, TLR4 is also expressed by various immune cells including macrophages and dendritic cells, as well as B cells and T cells (Ganley-Leal et al., 2010; Gonzalez-Navajas et al., 2010; Vaure et al., 2014).

Expression of TLR4 changes in both amount and location during intestinal inflammation (Cario et al., 2000; Hausmann et al., 2002). Cario et al. (2000) showed colonic IECs isolated from patients with inflammatory bowel disease (IBD) had higher overall expression levels of TLR4 compared to samples from healthy tissue. The same study also showed TLR4 expression was highest at the apical pole of IECs in people with Crohn's disease, while tissue from people with ulcerative colitis showed higher expression at the basolateral surface (Cario et al., 2000). Another clinical study has shown inflammation causes higher TLR4 (and TLR2) levels in the intestinal macrophages and dendritic cells, as well as all inflammatory cells of the lamina propria (Hausmann et al., 2002). However it should be noted that the control tissue used in this study was

from patients with colonic carcinomas, so was unclear whether there were any changes in the structure of this control tissue.

2.2.2 TLR4 in chemotherapy-induced gastrointestinal toxicity

The role of TLR4 in the development of gastrointestinal toxicity following chemotherapy has been gaining attention over the past decade. In pre-clinical studies, increased TLR4 messenger ribonucleic acid (mRNA) levels were shown in the intestine following chemotherapy treatment with irinotecan, 5-fluorouracil (5-FU) and methotrexate (MTX) (Ferreira et al., 2012; Hamada et al., 2013; Gibson et al., 2016). A further study of BALB/c mice with a global knockout (KO) of TLR4 treated with irinotecan showed less diarrhoea, weight loss and histological damage compared to wild-type (WT) mice (Wardill et al., 2016). Additionally these TLR4 KO mice had less severe intestinal barrier dysfunction and lowered levels of inflammatory markers.

Despite these promising findings, broad-spectrum targeting of TLR4 may not translate clinically to reduce chemotherapy-induced gastrointestinal toxicity (CIGT) due to potential alterations in chemotherapy efficacy. One study of irinotecan in rats used naloxone to inhibit TLR4 (Coller et al., 2017) based on previous research showing its TLR4 antagonist properties (Wang et al., 2016b). In this study, naloxone was unable to reduce weight loss or gastrointestinal damage, and in fact led to increased tumour growth and significantly reduced efficacy of irinotecan (Coller et al., 2017). However, whether this was due to a direct TLR4-mediated effect was not studied, as TLR4 signalling levels were not measured. TLR4 KO mice have also shown increased basal tumour growth and

overall decreases in chemotherapy efficacy compared to WT mice (Apetoh et al., 2007; Fukata et al., 2007), although this finding has been disputed in another model (Makkar et al., 2019). Clinically, patients with breast cancer who carried a TLR4 loss-of-function allele relapsed more quickly after chemotherapy than patients with a normal TLR4 allele (Apetoh et al., 2007). These results demonstrate broad targeting of TLR4 may not be a useful method of reducing CIGT.

While limited studies have shown TLR4 signalling is important for chemotherapy treatment outcomes, additional work has been conducted in models of colonic inflammation such as colitis. The features of experimental colitis mimic many of the features of chemotherapy-induced injury and thus provide insight into possible relationships between TLR4 and CIGT. Previous work in dextran sodium sulphate (DSS) rodent models have demonstrated the double-edged sword of TLR4 function in the gastrointestinal tract, where both low and excessive TLR4 signalling can promote intestinal inflammation in colitis models (Dheer et al., 2016). In a mouse model with constitutively active TLR4, mice had increased susceptibility to colitis (Fukata et al., 2011). Inversely, another study showed mice with global KO of MyD88 or TLR4 had increased mortality and weight loss stemming from DSS administration (Rakoff-Nahoum et al., 2004). These results further demonstrate broad targeting of TLR4 may not be a useful method of reducing CIGT, and hence altering TLR4's interactions with gut bacteria may be an alternative strategy.

2.2.3 TLR4 and the microbiome

As summarised in chapter 1, TLR4 and intestinal microbes have a two-way relationship that enables regulation of each through a variety of feedback mechanisms. The interaction between microbial-derived ligands (e.g. LPS) and TLR4 induces CD8+ T cell activation and polarises CD4+ T cells towards the T helper 17 (Th17), T helper 1 (Th1) and regulatory T cells (Treg) subsets (Valentini et al., 2014). TLR4 is also critical in the microbial regulation process, as humans with a MyD88 deficiency develop recurrent pyogenic bacterial infections (von Bernuth et al., 2008). However, it is not currently understood how these interactions link to each other in the context of CIGT. It is also currently not known what role the host immune system plays in the development of particular microbiome profiles, and capacity for repairing the microbial balance post-chemotherapy.

2.2.4 New models for CIGT

While previous global TLR4 KO models have provided evidence to support TLR4 signalling as a potential therapeutic target (Wardill et al., 2016), it is yet to be determined whether the crucial signal is derived from epithelial cells expressing TLR4, or the immune signal from TLR4 expressed on mucosal immune cells (figure 2.2). In pre-clinical studies, irinotecan causes epithelial cell death within approximately 6 hours of treatment (Keefe et al., 2000; Wardill et al., 2016), which corresponds with an increase in interleukin-6 (IL-6) (Wardill et al., 2016; Khan et al., 2018). A loss of barrier integrity, and associated pathological symptoms occurs around 24 hours post-treatment, corresponding

with increases in cytokines such as IL-1β and tumour necrosis factor alpha (TNF-α) (Wardill et al., 2016). Epithelial TLR4, which is usually present at low levels, could be activated by either, or both, of these events (early cell death or later loss of barrier integrity). While previous research has shown that IECs are themselves able to release cytokines such as IL-6, IL-10 and TNF-α, it is not clear exactly how epithelial TLR4 signalling differs from immune TLR4 signalling in the gut (Andrews et al., 2018).

Determining the predominant initiator or contributor to mucosal injury would ensure a rational development of any final therapy (e.g. oral versus intravenous administration of a TLR4 antagonist). KO-targeted blockade of TLR4 on specific cell types will enable new knowledge of the role of TLR4 in a complex system such as the gastrointestinal tract. Therefore, a conditional KO mouse model, where TLR4 is specifically knocked out in IECs, may be a useful research tool. Sodhi et al. (2012) previously generated a similar model for use in necrotising enterocolitis research and found alterations in goblet cell development. However, this model used predominantly neonate mice, and there is evidence suggesting that TLR4 expression significantly alters in mice during their development (Inoue et al., 2017). Additionally, this paper was not so focussed on the microbial interactions with TLR4. Here, I proposed that IEC-specific KO of TLR4 may cause changes in microbiome composition that are relevant in the development of CIGT.

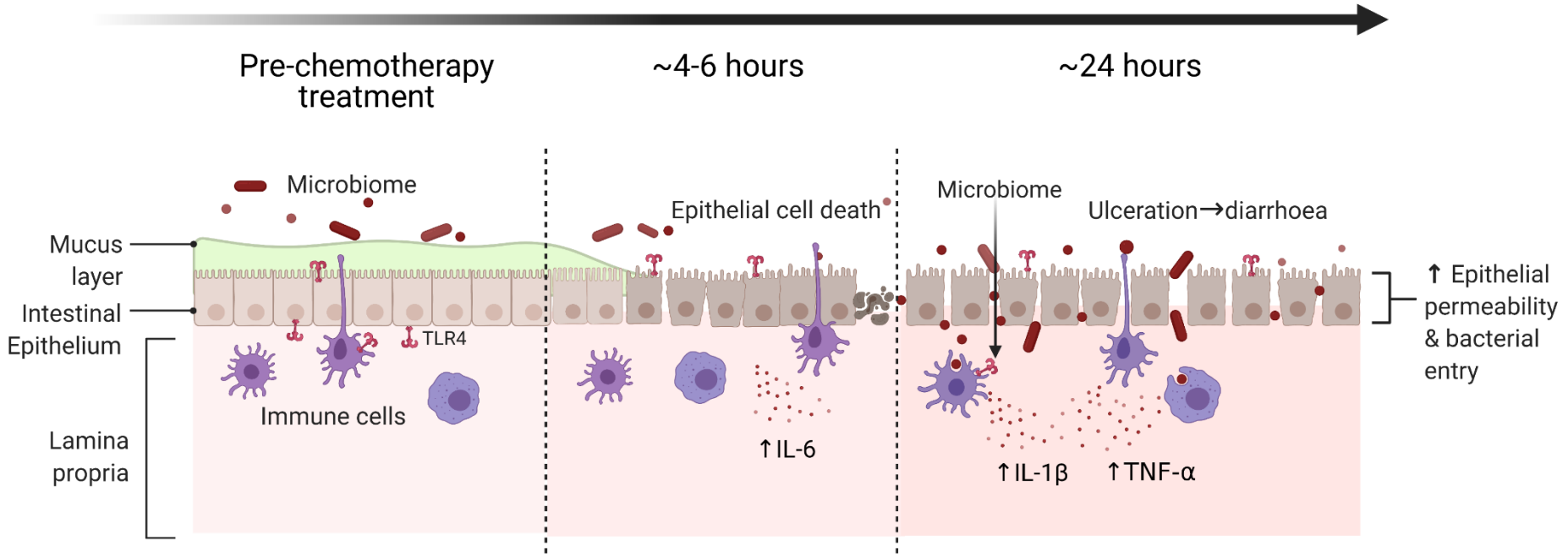

Figure 2.2: Proposed mechanism of epithelial and immune TLR4 activation during CIGT. TLR4 on epithelial cells could be activated by early epithelial damage occurring 4 - 6 hours post-treatment, or could be activated by impaired barrier integrity and associated bacterial translocation occurring 24 hours post-treatment. It is currently unknown whether TLR4 signalling from epithelial cells is the first event causing an inflammatory cascade. Created with Biorender.com.

2.2.5 Aims

Here, I aimed to firstly develop a C57BL/6 mouse model where TLR4 is conditionally knocked out in IECs (*Tlr4*$^{\Delta IEC}$) using a Vil-cre/floxed tissue technique, and then secondly to characterise the gut microbiome of the model.

My specific aims were therefore to:

1. Determine if intestinal-specific deletion of TLR4 leads to altered intestinal cell distribution.

2. Determine the effect of intestinal-specific TLR4 deletion on microbial composition in mice housed individually versus cohoused with WT mice.

3. Determine if the intestinal-specific TLR4 deletion alters how the gut microbiome changes following irinotecan administration compared to WT mice.

2.3.0 Methods

2.3.1 TLR4 KO mice lines

2.3.1.1 Global TLR4 KO (Tlr4$^{-/-}$) mouse model

In order to compare current results to previous research, archived caecal samples (aseptically collected during dissection into a sterile tube and stored at -80 ˚C) and data from female global TLR4 KO (*Tlr4*$^{-/-}$, n = 8) and WT (n = 4) BALB/c mice were utilised. This study was approved by the Animal Ethics Committee of the University of Adelaide (M-2015-017A), and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training (2013). *Tlr4*$^{-/-}$ mice, backcrossed onto BALB/c for more than 10 generations, were kindly sourced from Professor Paul Foster from the University of Newcastle (Newcastle, Australia).

2.3.1.2 Generation of Tlr4$^{\Delta IEC}$ mouse model

This study was approved by the Animal Ethics Committee of the University of Adelaide (M-2017-114), and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training (2013).

C57BL/6 mice with KO of the *Tlr4* gene in IECs specifically were generated (*Tlr4*$^{\Delta IEC}$, figure 2.3). This was achieved through breeding *Tlr4* floxed mice (‘*Tlr4* loxP’, Jackson Laboratories, B6(Cg)-Tlr4tm1.1Karp/J, #024872) with transgenic mice expressing cre recombinase in IECs under the control of the villin 1

promoter (‘Vil-cre’, Jackson Laboratories, B6.Cg-Tg(Vil1-cre)997Gum/J, #004586) (figure 2.3 A) (Madison et al., 2002). The *Tlr4* floxed mice had loxP sites on either side of exon 3 of the targeted *Tlr4* gene. Exon 3, the largest exon of mouse Tlr4, encodes part of the extracellular domain, as well as the transmembrane and cytoplasmic domains of TLR4 (Vaure et al., 2014). Mice were initially maintained as a line of homozygous *Tlr4* floxed mice, as well as a line of hemizygous Vil-cre mice that express normal *Tlr4*. These were cross-bred over two generations (figure 2.3 B and C) to eventually breed mice that were either 1. *Tlr4* homozygous/Vil-cre negative or 2. TLR4 homozygous/Vil-cre hemizygous (litters had approximately 50% each genotype, figure 2.3 D). Cre recombinase excised DNA sequences between two loxP sites. Therefore, this allowed the generation of mice with the *Tlr4* gene excised in all IECs. Mice containing the loxP site but lacking cre recombinase still had an intact *Tlr4* gene and therefore produced functional TLR4. The first group were the $Tlr4^{\Delta IEC}$ and the second group were used as littermate WT controls. All mice were maintained under standard conditions in ventilated cages with *ad libitum* access to food and water, with a 12 hour light/dark cycle.

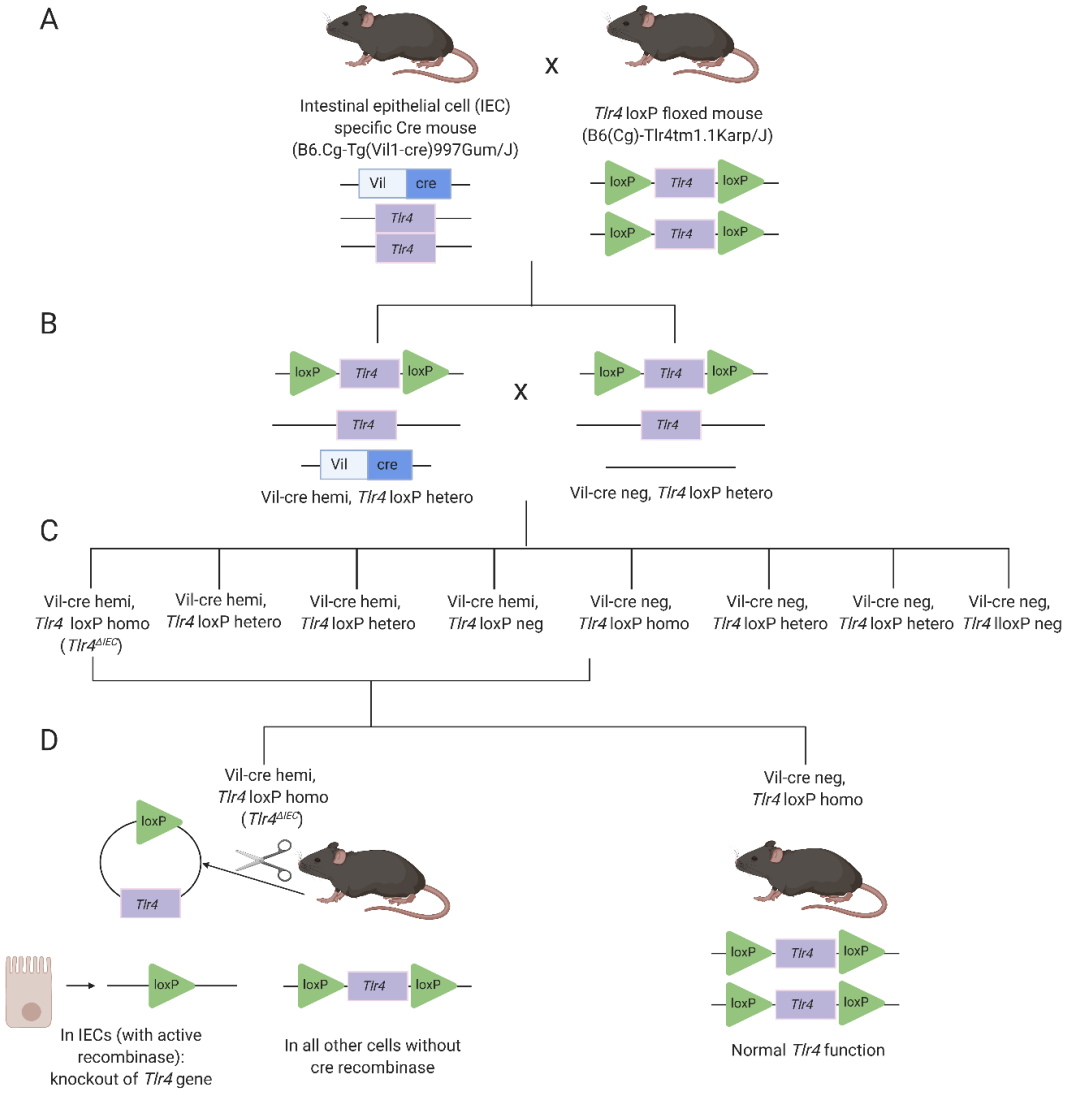

Figure 2.3: Schematic of *Tlr4*$^{\Delta IEC}$ model generation. Hemi = hemizygous, hetero = heterozygous, homo = homozygous. Initial cross between B6.Cg-Tg(Vil1-cre)997Gum/J and B6(Cg)-Tlr4tm1.1Karp/J (The Jackson Laboratory, A) generates mice heterozygous for the *Tlr4* floxed gene and either hemizygous or negative or Vil-cre (B). A subsequent cross of these two genotypes results in 8 possibilities, one being the desired *Tlr4*$^{\Delta IEC}$ (C). The *Tlr4*$^{\Delta IEC}$ mouse and Vil-cre negative, *Tlr4* loxP homozygous mouse can then be bred together to generate further litters consisting of approximately 50% *Tlr4*$^{\Delta IEC}$ mice and 50% Vil-cre negative, *Tlr4* loxP homozygous mice (used as WT controls) (D). The *Tlr4*$^{\Delta IEC}$ mice will have *Tlr4* knocked out in IECs, but active *Tlr4 in* all other cell types(figure created using BioRender.com).

2.3.1.3 Development of genotyping assay

Mouse genotypes (i.e. figure 2.3 C) were determined using genomic DNA isolated from mouse ear notches taken upon weaning. Briefly, the NucleoSpin Tissue DNA extraction kit was used according to manufacturer protocols (740952.250, Macherey-Nagel, Dűren, Germany). DNA was quantified using a Take3 plate and Gen5 analysis program (Biotek, Winooski, USA).

All genotyping was confirmed by specific polymerase chain reactions (PCRs) carried out in a 30 µL volume containing 100 ng of purified genomic DNA. Separate PCRs was undertaken to determine Vil-cre and *Tlr4* floxed status for each mouse using primer sequences (from The Jackson Laboratory) shown in table 2.1. Vil-cre PCRs also contained 40 µM deoxyribonucleoside triphosphates, 1 µM of each primer (using common forward and WT reverse or variant reverse in 2 separate reactions) , 1 x ThermoPol Reaction Buffer (20 mM Tris-hydrochloric acid, 10 mM ammonium sulphate, 10 mM potassium chloride, 2 mM magnesium sulphate and 0.1% Triton X-100), 6.5% glycerol and 2.5 U of Taq DNA Polymerase (M0267L, New England Bio-Labs, Ipswich, USA). *Tlr4* floxed PCRs contained 260 µM deoxyribonucleoside triphosphates, 0.5 µM of each primer, 1 x ThermoPol Reaction Buffer (as above), 6.5% glycerol and 2.5 U of Taq DNA Polymerase (as above).

All PCRs were run in a PTC-200 Peltier Thermal Cycler (MJ Research, Hercules, USA). Cycling conditions for the Vil-cre PCR were initial denaturation at 95 °C for 3 minutes, then 40 cycles of 95 °C for 5 seconds, followed by 60 °C for 30 seconds. Cycling conditions for the *Tlr4* floxed PCR were: initial

denaturation at 94 °C for 2 minutes; 10 cycles of 94 °C for 20 seconds, 65 °C for 15 seconds and 68 °C for 10 seconds, with the 65 °C step decreasing by 0.5 °C each cycle; 28 cycles of 94 °C for 16 seconds, 60 °C for 15 seconds and 72 °C for 10 seconds; and final elongation step at 72 °C for 2 minutes.

Samples were run on a 4% agarose gel (Sigma Aldrich, St Louis, USA) with a pUC19 DNA/MspI (HpaII) ladder (26 – 501 base pair (bp) fragments, ThermoFisher, Waltham, USA) and visualised using Midori Green Advance DNA stain (Nippon Genetics, Tokyo, Japan) to determine length of PCR products. For the Vil-cre PCRs, presence of the WT was indicated by a band of 119 bp and the presence of the hemizygous Vil-cre was indicated by a band of 85 bp. For the *Tlr4* floxed PCRs, presence of: homozygous WT was indicated by a band of 234 bp; heterozygous WT/*Tlr4* floxed was indicated by bands of 234 and 285 bp; and homozygous *Tlr4* floxed was indicated by a band of 285 bp.

The knockout of TLR4 in this model was also confirmed at the tissue level using quantitative RT-PCR on intestinal mucosal scrapings. This data is presented in the following, currently in press, manuscript:
Crame EE, Bowen JM, Secombe KR, Coller JK, François M, Leifert W, Wardill HR (2021) Epithelial-specific TLR4 knockout challenges current evidence of TLR4 homeostatic control of gut permeability, Inflammatory Intestinal Diseases.

Table 2.1: Primer sequences for Vil-cre/*Tlr4* floxed genotyping

Target	**Primer**	**Sequence**
Vil-cre	Common forward	5’-GCTTTCAAGTTTCATCCATGTTG-3’
	WT reverse	5’-TTCATGATAGACAGATGAACACAGT-3’
	Variant reverse	5’-GTCTTTGGGTAAAGCCAAGC-3’
Tlr4 floxed	Forward	5’-TGACCACCCATATTGCCTATAC-3’
	Reverse	5’-TGATGGTGTGAGCAGGAGAG-3’

2.3.2 Interventional studies

These studies was approved by the Animal Ethics Committee of the University of Adelaide (M-2017-115 and M-2020-028), and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training (2013).

2.3.2.1 Co-housing study

Once genotyped, WT and *Tlr4*$^{\Delta IEC}$ mice were separated from littermates into genotype- and sex-specific cages as soon as possible following weaning at 3 weeks of age (total n = 16, figure 2.4). The experiment ran for 8 weeks in total after weaning. Four weeks after weaning, faecal samples were collected to demonstrate initial microbial composition (baseline). Female WT and *Tlr4*$^{\Delta IEC}$ mice were co-housed. Male mice remained separated (due to fighting issues when co-housed) however total faecal contents of the male cages were transferred daily to simulate co-housing. Four weeks after the initial co-housing, mice were culled via CO_2 inhalation and cervical dislocation. Faecal pellets were aseptically collected following death from the distal portion of the excised colon and stored at -80 ˚C. Histology and 16S sequencing (see below) were conducted in this study.

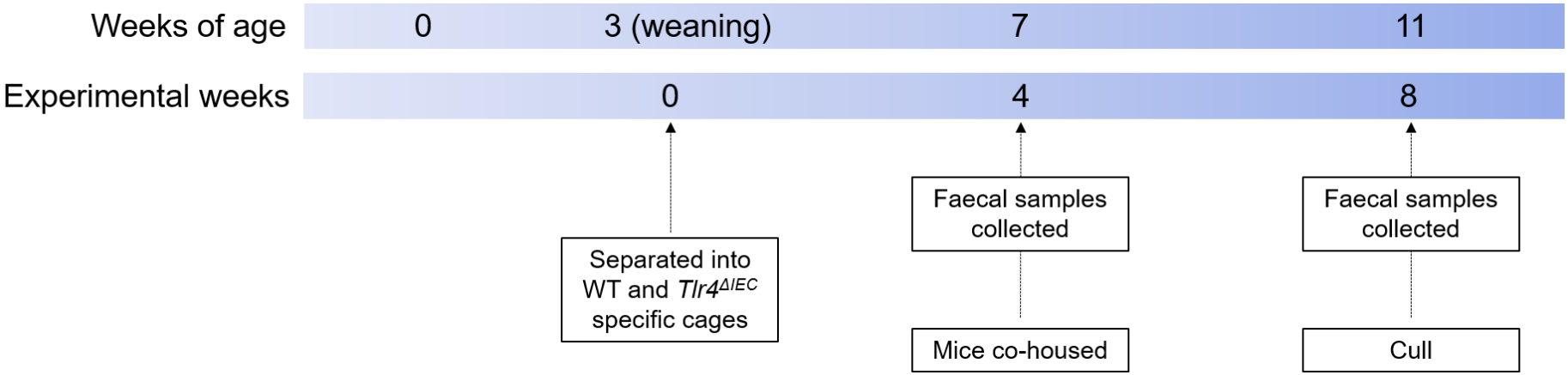

Figure 2.4: Co-housing study timeline. Mice were separated into genotype- and sex-specific cages at weaning (approximately 3 weeks of age). After 4 weeks of genotype- and sex-specific caging, mice were co-housed for a further 4 weeks before being culled.

2.3.2.2 Colorectal tumour model

MC-38 colorectal cancer cells (Grasselly et al., 2018) were cultured, and 2 x 10^6 cells were subcutaneously injected into the right flank of WT (n = 16) and *Tlr4*$^{\Delta IEC}$ mice (n = 15, aged 12 ± 4 weeks), that had been co-housed since weaning. When the tumour reached approximately 0.2 cm^3 (approximately 7 ± 1 days), mice were intraperitoneally injected with a 270 mg/kg dose of irinotecan hydrochloride (Pfizer, Kalamazoo, USA) (20 mg/mL) or vehicle (sorbitol-lactic acid buffer, 45 mg/mL sorbitol, 0.9 mg/mL lactic acid, pH = 3.4) (Wardill et al., 2016). Mice were culled 72 hours after irinotecan via CO_2 inhalation and cervical dislocation (figure 2.5). Faecal samples (total n = 31) were collected directly from the mouse at the time of tumour inoculation (n = 8) and irinotecan/vehicle injection (n = 8). Further faecal contents from the distal colon of mice treated with irinotecan or vehicle were collected at cull (n = 15). 16S sequencing (below) was then conducted on these samples.

Mice were weighed once daily (in the morning), with a 3 x daily comprehensive clinical symptom recording. Diarrhoea was graded according to an established grading system with four grades: 0 = no diarrhoea; 1 = mild (soft unformed stools); 2 = moderate (perianal staining and loose stools); and 3 = severe (watery stools and staining over legs and abdomen) (Wardill et al., 2016). Mice were killed if displaying a 15% or greater weight loss from baseline or significant distress and clinical deterioration.

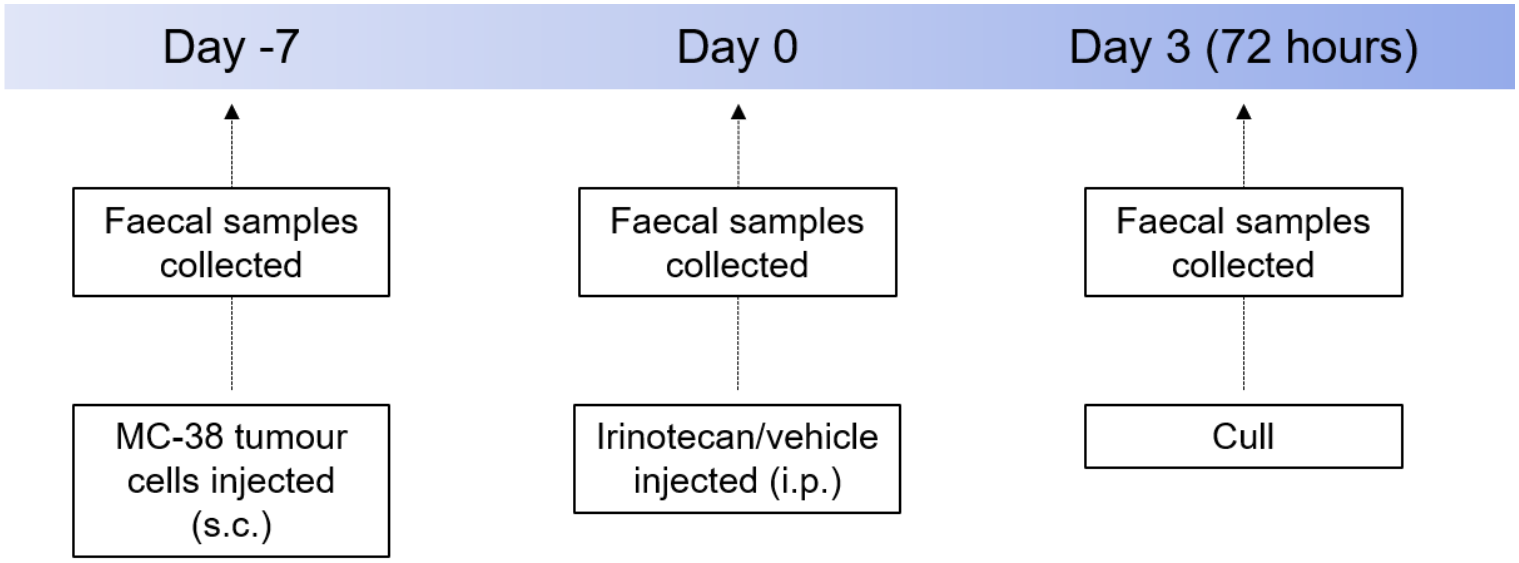

Figure 2.5: Colorectal tumour model timeline. Approximately 7 days after MC-38 tumour cells were injected, irinotecan or vehicle was given. Mice were culled 72 hours after irinotecan/vehicle. Faecal samples were collected at each time point.

2.3.3 Analysis techniques

2.3.3.1 Tissue collection and preparation

At cull, the gastrointestinal tract was removed from the pyloric sphincter to the rectum. The small and large intestine were flushed with chilled, sterile 1 x phosphate buffered saline (PBS, pH = 7.4) and weighed. Samples of duodenum, jejunum, ileum and distal and proximal colon were collected and either snap frozen in liquid nitrogen and stored at -80 ˚C or drop-fixed in 10% formalin (ChemSupply, Gillman, Australia) before embedding in paraffin (Sigma-Aldrich, St Louis, USA).

2.3.3.2. Histology

Histology was conducted on paraffin embedded intestinal samples, which were cut using a rotary microtome (RM2235, Leica, Wetzlar, Germany) and 4 µm sections mounted onto Superfrost glass slides (Menzel-Glaser, Braunschweig, Germany). All analysis was conducted in a blinded fashion. Sections of the lower intestinal tract (ileum and proximal and distal colon) were used here, due to the more significant number of bacteria in these regions (Donaldson et al., 2016).

Alcian blue-periodic acid-Schiff's reagent (AB-PAS) staining was conducted to assess numbers of goblet cells. The AB-PAS stain was completed as per previous literature (Stringer et al., 2009b). Slides had images taken using a NanoZoomer digital slide scanner (Hamamatsu Photonics, Hamamatsu, Japan) and viewed using the Nanozoomer digital pathology software (NDP View v1.2)

(Histalim, Montpellier, France). Fifteen well-oriented crypts or villi were then used to count cell numbers in the ileum and proximal and distal colon.

Immunofluorescence (IF) staining was performed for cluster of differentiation molecule 11B (CD11b) (#ab133357, Abcam, Cambridge, United Kingdom). This was undertaken in order to assess changes in macrophage numbers. IF was performed on an automated machine following standard protocols (Autostainer Plus, #AS480, Dako, Næstved, Denmark). Briefly, sections were deparaffinised using histolene and rehydrated in graded ethanols, before heat-mediated antigen retrieval in an EDTA/Trizma® base buffer (1.26 mM EDTA, 10 mM Trizma® base, pH = 9.0). The buffer was firstly preheated to 65 °C using the Dako PT LINK (#PT101, Dako), before the slides were added and the temperature of the buffer further increased to 97 °C for 20 minutes. Once the buffer had cooled to 65 °C, the slides were moved to the Autostainer Plus and moved through the following steps at room temperature: blocking step with 4% bovine serum albumin; 1 hour primary antibody incubation (CD11b) at a concentration of 1.35 µg/mL; and 1 hour incubation with the secondary antibody AlexaFluor 488 (A11034, Invitrogen, Carlsbad, United States) at a concentration of 2 µg/mL. Finally nuclei were stained with 1 µg/mL concentration 4′,6-diamidino-2-phenylindole (DAPI) for 15 minutes. Slides were imaged using the Nikon A1 confocal microscope with 40 x objective (Nikon, Tokyo, Japan). Sections were analysed using ImageJ v 1.48 (Schindelin et al., 2012) via calculation of percentage area stained by the primary antibody at 40 x magnification.

2.3.3.3 16S sequencing

A range of samples were analysed using 16S sequencing techniques. Caecal samples were analysed from the global TLR4 KO model (in BALB/c mice). Faecal samples were analysed from the *Tlr4*$^{\Delta IEC}$ and WT mice.

DNA was extracted from caecal and faecal samples using the Qiagen DNeasy PowerLyzer PowerSoil Kit with the semi-automated QIAcube Connect (Qiagen, Hilden, Germany) and eluted in nuclease free water (Invitrogen, Carlsbad, USA). Samples were sent to the Australian Genome Research Facility (AGRF, Melbourne, Australia) for 16S ribosomal RNA (rRNA) gene region analysis. The V3-V4 region of the 16S gene (300 bp) was targeted using the following primers, (using standard International Union of Pure and Applied Chemistry (IUPAC) nucleotide nomenclature, supplementary material 1):

Forward sequence: 5’-CCTAYGGGRBGCASCAG-3’

Reverse Sequence: 5’-GGACTACNNGGGTATCTAAT-3’

Sequencing analysis was performed in real time by the MiSeq Control Software v3.1.0.13 and Real Time Analysis v1.18.54.4, running on the instrument computer. The Illumina bcl2fastq 2.20.0.422 pipeline was used to generate the sequence data. CLC Genomics Workbench 21.0.3 (https://www.qiagenbioinformatics.com/) was used to complete bacterial diversity profiling. Paired-ends reads were assembled by aligning forward and reverse reads. Primers were identified and trimmed. Trimmed sequences were quality filtered, duplicate sequences removed and sorted by abundance. Reads

were assigned to taxonomic identities using the Greengenes 97% similarity database version 13.8. Alpha diversity was calculated using the Shannon diversity index. Beta diversity was calculated using Principal Coordinate Analysis (PCoA) based on generalised UniFrac distances (Chen et al., 2012a).

2.3.4 Statistical analysis

Data were compared using Prism version 9.0 (GraphPad Software, San Diego, USA). The assumptions of equality of variance for each group and normally distributed data were tested using Bartlett's test and D'Agostino & Pearson omnibus normality test, respectively. If data was normally distributed, 2-way analysis of variance (ANOVA) tests were used, and data were represented as means ± standard error of the means (SEMs). If these assumptions were violated, non-parametric equivalent tests were performed, including Kruskal-Wallis tests for independent data and Freidman's tests for repeated measures, and data were represented as medians. Diarrhoea proportions were analysed by a Chi-squared test. P-values less than 0.05 were considered statistically significant. Permutational multivariate analysis of variance (PERMANOVA) was used to compare PCoA clustering with generalised UniFrac distances.

2.4.0 Results

2.4.1 Baseline model characterisation

2.4.1.1 Global Tlr4$^{-/-}$ 16S analysis

16S sequencing analysis found global genetic KO of TLR4 was associated with a significantly altered caecal microbiome (figure 2.6). Alpha diversity, as assessed by Shannon's index, was higher in the *Tlr4*$^{-/-}$ group (n = 8) compared to WT (n = 4, P = 0.041, figure 2.6 A). Another measure of alpha diversity, phylogenetic diversity, was also higher in the *Tlr4*$^{-/-}$ mice (P = 0.03, figure 2.6 B). Next, PCoA clustering showed clustering differences between the groups, confirmed by PERMANOVA analysis (P = 0.03, figure 2.6 C). Finally, the relative abundance of the genus *Odoribacter* was higher in *Tlr4*$^{-/-}$ compared to WT mice (P = 0.0061, figure 2.6 D, E).

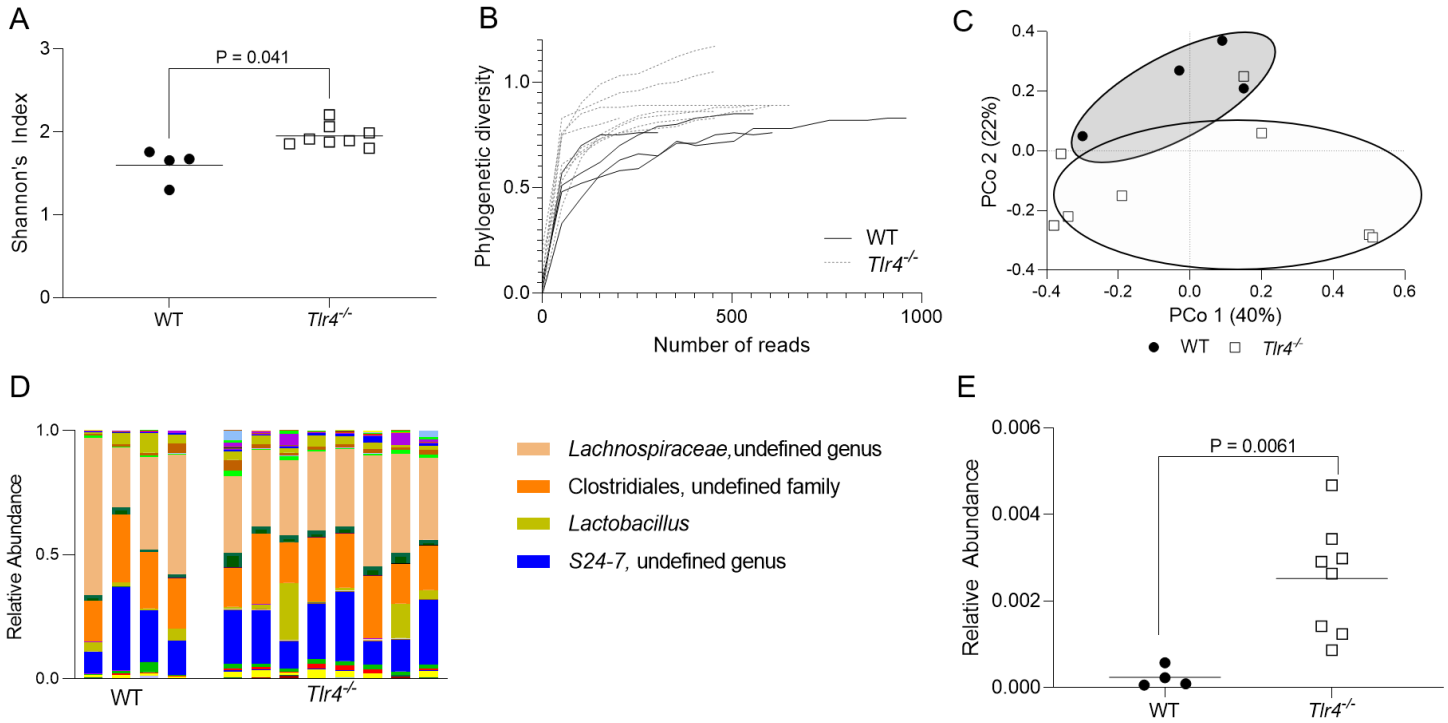

Figure 2.6: Global TLR4 KO (*Tlr4*$^{-/-}$) caused changes in microbial diversity. (A) *Tlr4*$^{-/-}$ mice had a higher Shannon's index at the genus level than WT mice (P = 0.041), indicating increased microbial diversity. (B) Phylogenetic diversity was also higher in *Tlr4*$^{-/-}$ mice compared to WT mice (P = 0.03). (C) PCoA showed altered clustering patterns between groups that was significant as confirmed by PERMANOVA, P = 0.03. (D) Relative abundance at the genus level. (E) At the genus level, there was a difference in *Odoribacter* levels (P = 0.0061), with higher levels in *Tlr4*$^{-/-}$ mice.

2.4.1.2 Tlr4$^{\Delta IEC}$ genotyping confirmation

Tlr4$^{\Delta IEC}$ mice were confirmed with PCR products representing homozygous floxed TLR4 and hemizygous Vil-cre genotype (figure 2.7 A and B). Each genotype within litters was born in approximately the expected Mendelian ratios. As mentioned previously, the knockout was also confirmed at the tissue level using RT-PCR (data not shown). *Tlr4$^{\Delta IEC}$* mice had a gross phenotype comparable with WT mice, with no differences in weight development, tight junction expression or intestinal barrier function as measured with Ussing chambers (data not shown, as part of another project).

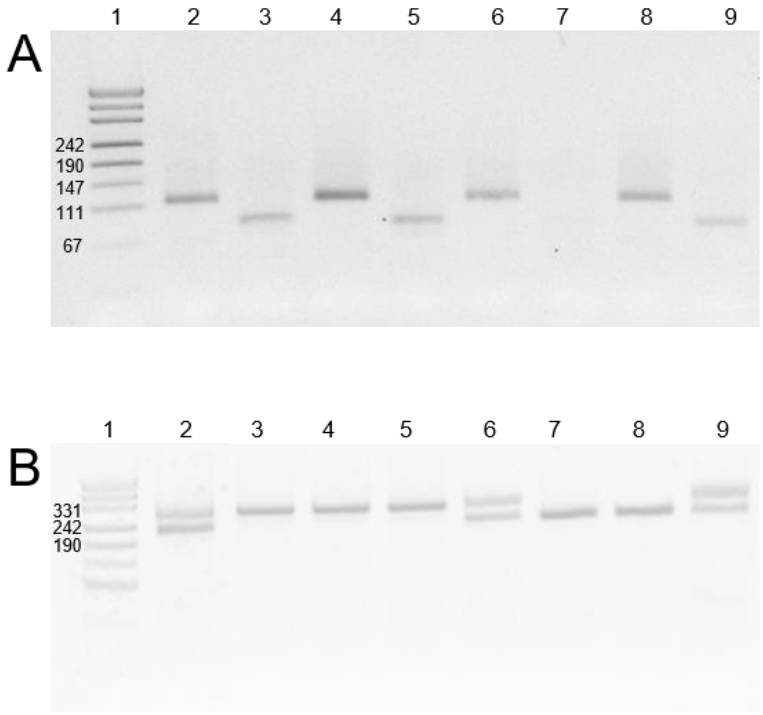

Figure 2.7: Example genotyping results for generation of *Tlr4*$^{\Delta IEC}$ mice visualised using Midori Green, following electrophoresis in 4% agarose gel. In both (A) and (B), lane 1 represents the pUC19 DNA/MspI (HpaII) marker (ThermoFisher). (A) Genotyping results for Vil-cre genotype WT and variant PCRs, respectively in adjacent lane; lanes 2 and 3 from mouse 1; lane 4 and 5 from mouse 2; lane 6 and 7 from mouse 3; and lane 8 and 9 from mouse 4. All Vil-cre hemizygous or Vil-cre negative mice had a band from the WT PCR (lanes 2, 4, 6 and 8) of 119 bp. Mice who are hemizygous for Vil-cre also had a band of 85 bp from the variant PCR (lanes 3, 5 and 9). Mouse 1, 2 and 4 were hemizygous for Vil-cre, mouse 3 was WT for Vil-cre. (B) Genotyping results for *Tlr4* floxed genotype. Each lane represents a different mouse. Mice who were heterozygous for the *Tlr4* floxed gene had two bands of 234 and 285 bp. Mice who were homozygous WT had one band of 234 bp, and mice who were homozygous *Tlr4* floxed had one band of 285 bp. Therefore, lane 2, 6 and 9 = heterozygous; lane 3, 4 and 5 = homozygous *Tlr4*; and lane 7 and 8 = homozygous WT. *Tlr4*$^{\Delta IEC}$ mice were homozygous *Tlr4* floxed and hemizygous for Vil-cre.

2.4.2 Co-housing study

2.4.2.1. Microbial analysis

Faecal samples were analysed to assess differences between WT and *Tlr4$^{\Delta IEC}$* mice, between baseline and cull and between male and female mice (figure 2.8). The main bacterial taxa observed were *S24-7*, *Clostridiales* and *Bacteriodales* (figure 2.8 A). There were no differences in alpha diversity (as measured by Shannon's index) between any two comparators (figure 2.8 B). The only PCoA comparison that was different using PERMANOVA analysis was comparing all males to females, regardless of genotype or study time point (PERMANOVA, P = 0.025, figure 2.8 C). A high level of *Akkermansia* was observed in two females at baseline (one WT and one *Tlr4$^{\Delta IEC}$*). A heatmap of OTUs showed some clustering between samples taken at the same time point (i.e. baseline or at cull), rather than between mice of the same genotype (i.e. WT or *Tlr4$^{\Delta IEC}$,* figure 2.9).

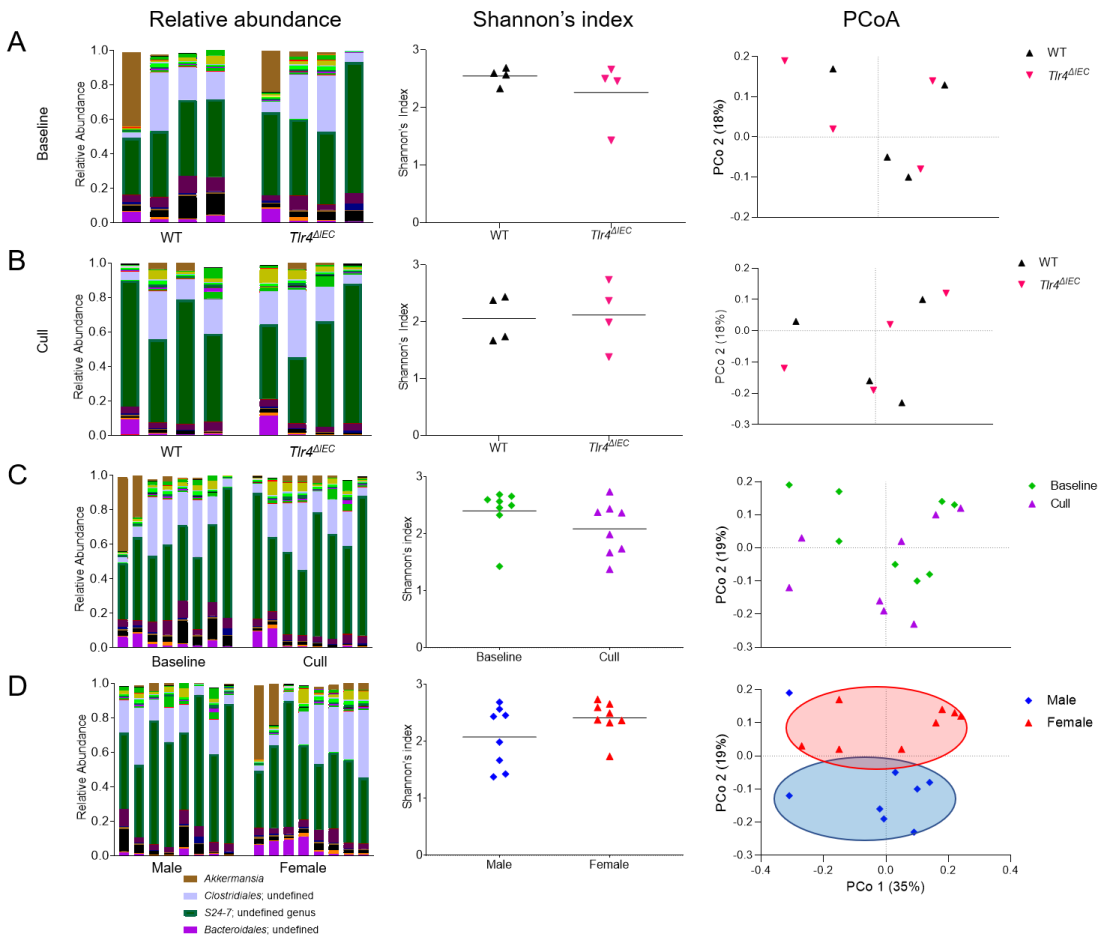

Figure 2.8: Microbial analysis of co-housed mice. Left to right: relative abundance at genus level. Highly abundant taxa represented in figure legend. Alpha diversity measurement using Shannon's index. Beta diversity measurement using PCoA plots. (A) Comparison between WT and *Tlr4*$^{\Delta IEC}$ mice at baseline. (B) WT and *Tlr4*$^{\Delta IEC}$ mice at cull. (C) Baseline compared to cull, regardless of genotype. (D) Male and female mice, regardless of genotype. PERMANOVA analysis showed different clustering between male and female mice (P = 0.025) only.

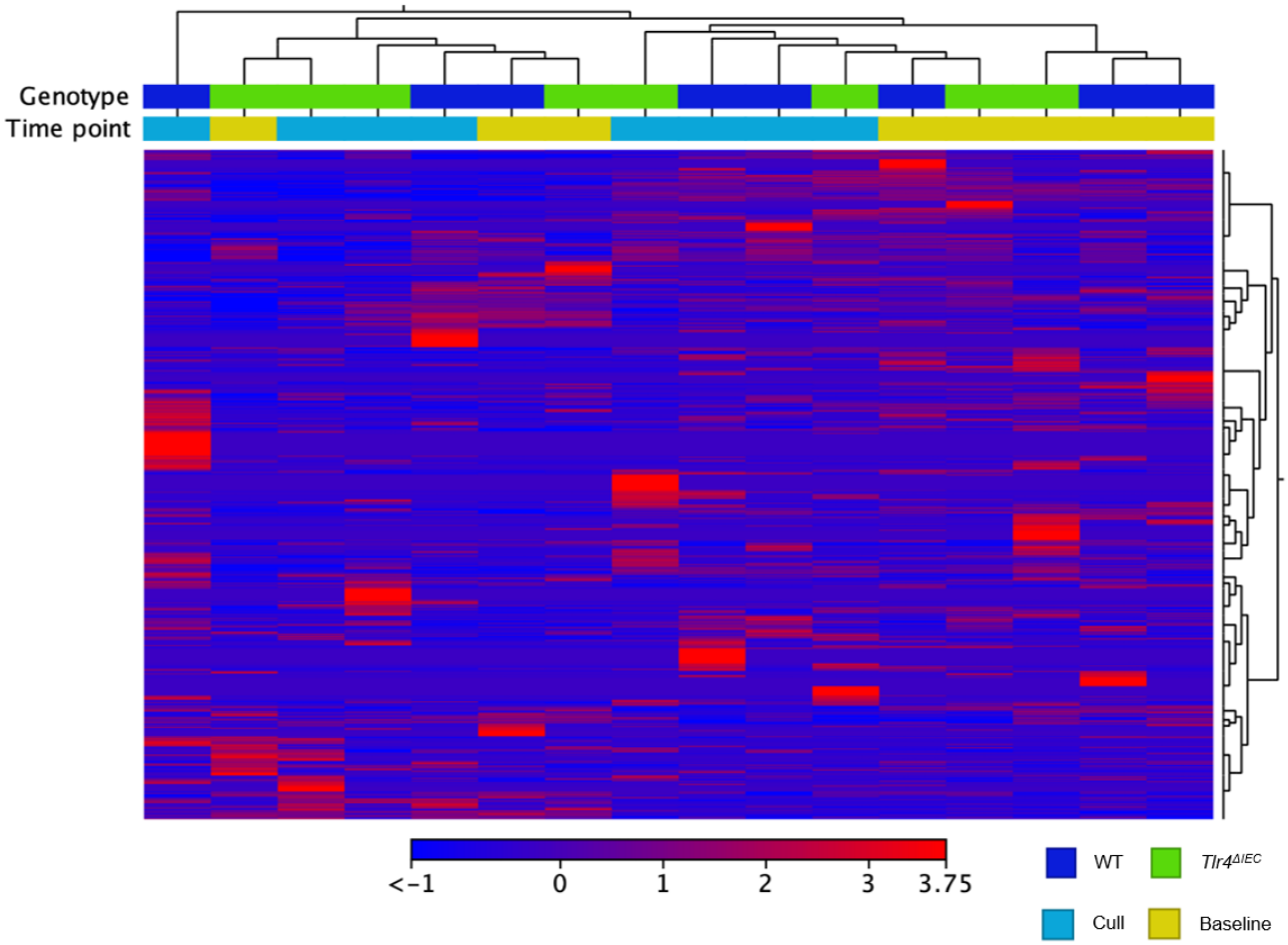

Figure 2.9: Heatmap of OTU clustering. No significant clustering patterns between genotypes or study time points.

2.4.2.2 Histological staining revealed no differences in goblet cell numbers between groups

No differences were found in number of goblet cells (indicated by AB-PAS staining) in the ileum villi or crypts, and proximal or distal colon between male or female mice, or between *Tlr4*$^{\Delta IEC}$ or WT mice (figure 2.10 A and B). There was clear CD11b staining in the ileum and colon however there were no differences between any groups ($P > 0.05$, figure 2.11 A and B).

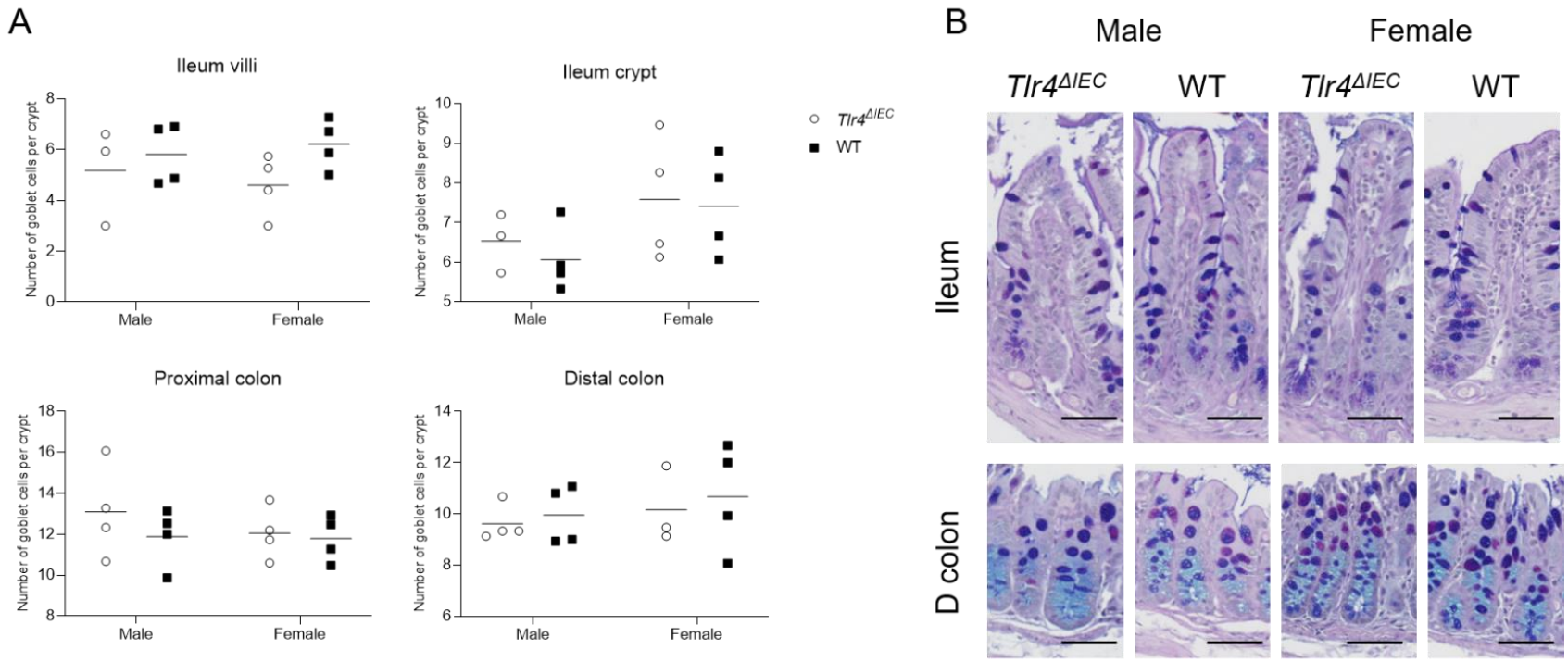

Figure 2.10: AB-PAS staining results. (A) Goblet cell counts in the ileum villi and crypt, and proximal and distal colon. Lines signify medians. There were no differences in goblet cell numbers between males and females or between WT and *Tlr4*$^{\Delta IEC}$ mice (Kruskal-Wallis test, P > 0.05) (B) Representative images of AB-PAS staining in the ileum and distal colon: original magnification is 400 ×; scale bars represent 50 µm in images. In the ileum, male *Tlr4*$^{\Delta IEC}$ group has n = 3 due to one tissue block being unavailable.

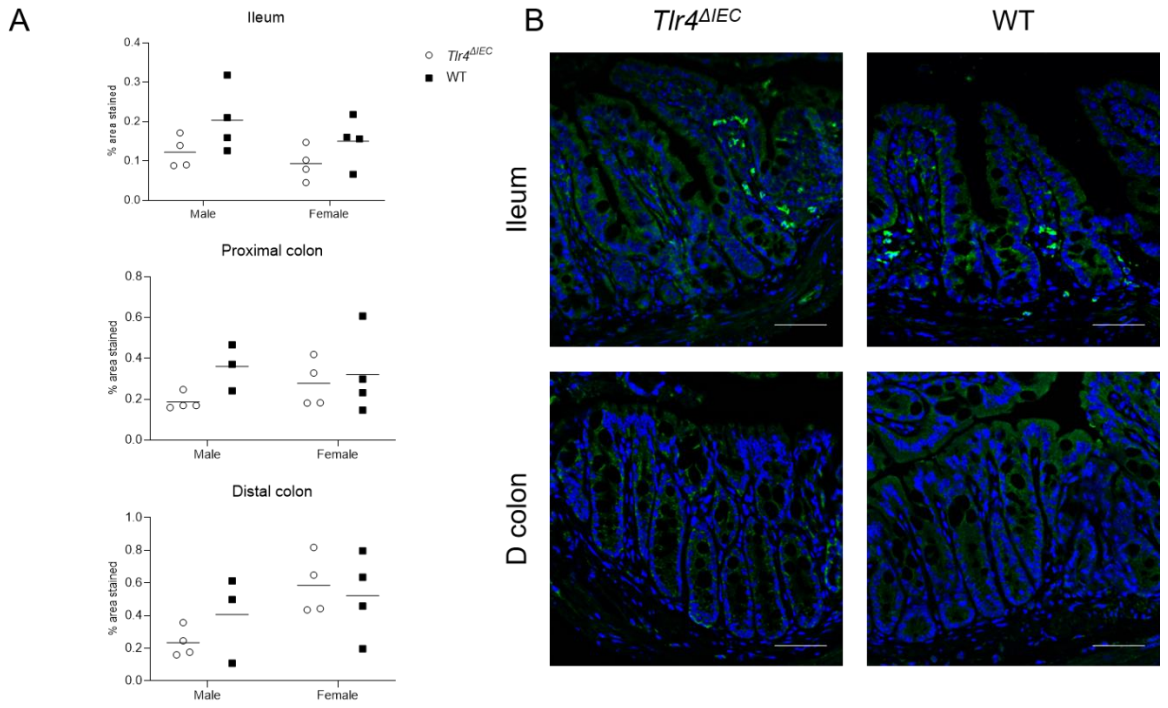

Figure 2.11: CD11b staining results. (A) Percentage area stained in the ileum, and proximal and distal colon. Lines signify means. There were no differences in staining between males and females or between WT and *Tlr4*$^{\Delta IEC}$ mice (Two-way ANOVA, P > 0.05). (B) Representative images of CD11b staining in the ileum and distal colon from male mice. Blue staining is DAPI, green staining is CD11b. Magnification is 40 ×; scale bars represent 50 µm in images.

2.4.3 Colorectal tumour model

Faecal microbiome relative abundance, alpha and beta diversity were longitudinally assessed within genotypes at: 1.baseline (prior to injection of MC-38 cells); 2. irinotecan/vehicle administration (after MC-38 tumour had grown to 0.2 cm^3); and 3. cull (following irinotecan or vehicle administration). Undefined *Lachnospiraceae, Clostridiales, S24-7* and *Akkermansia* were the most abundant taxa across all samples (figure 2.12 A and B). There were no differences between any group in alpha or beta diversity ($P > 0.05$, figure 2.13). Additionally, WT and *Tlr4*$^{\Delta IEC}$ mice were compared at each time point, however there were no differences observed (data not shown).

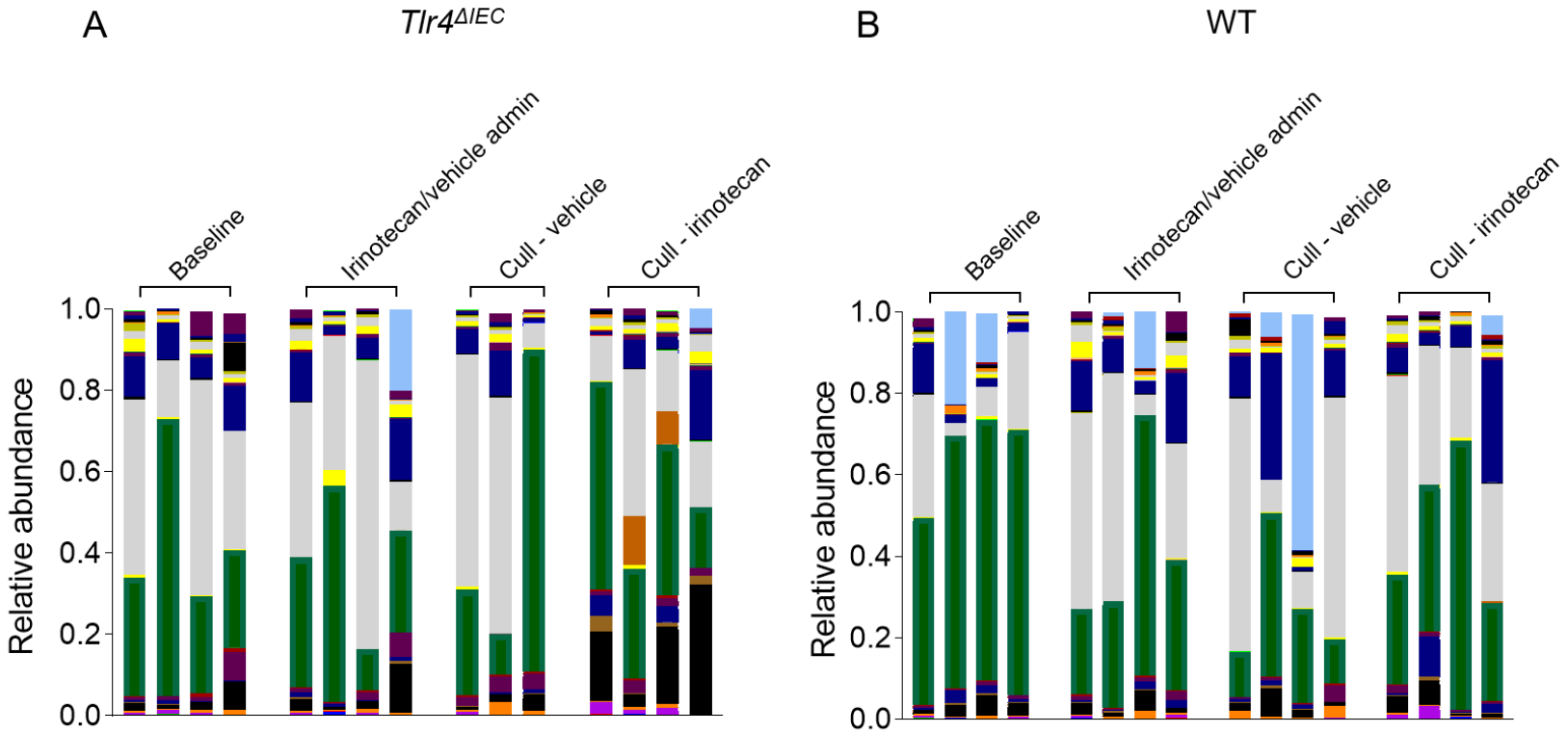

Figure 2.12: Relative abundance at the genus level for (A) *Tlr4*$^{\Delta IEC}$ and (B) WT mice. Baseline, at irinotecan/vehicle administration (following MC-38 tumour cell injection) and at cull time points presented. Highly abundant bacteria represented in figure legend. *S24-7* and *Clostridiales* most abundant across all samples.

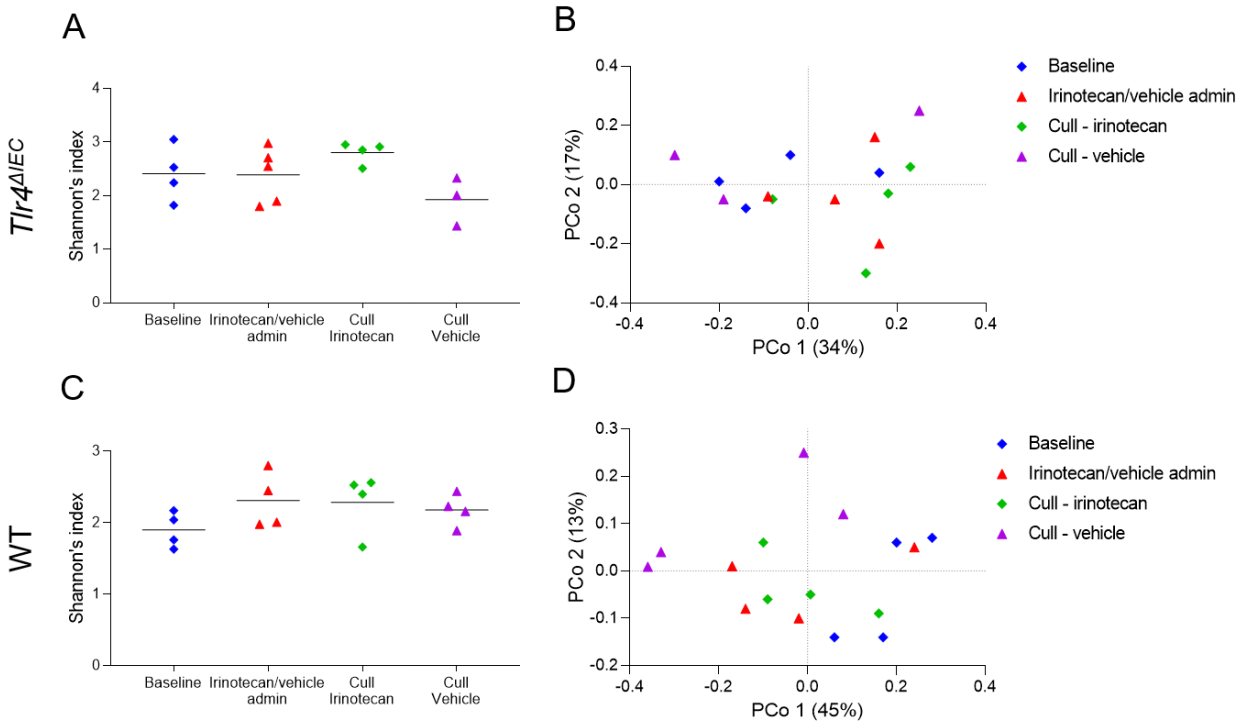

Figure 2.13: Alpha and beta diversity measures for *Tlr4*$^{\Delta IEC}$ and WT mice at each study time point. (A) No differences between groups of *Tlr4*$^{\Delta IEC}$ mice in Shannon's index. Lines represent mean. (B) PCoA plot of *Tlr4*$^{\Delta IEC}$ mice, with no differences in clustering (PERMANOVA, P > 0.05). (C) No differences between groups of WT mice in Shannon's index. Lines represent mean. (D) PCoA plot of WT mice, with no differences in clustering (PERMANOVA, P > 0.05).

2.5 Discussion

This series of experiments aimed to develop a *Tlr4*$^{\Delta IEC}$ mouse model to understand the links between the gut microbiome, epithelial TLR4 expression and the development of CIGT. I successfully developed this *Tlr4*$^{\Delta IEC}$ model, and determined no clear differences in the faecal microbiome or intestinal cell distribution of *Tlr4*$^{\Delta IEC}$ mice compared to WT mice. There were also no changes in faecal microbiome composition in these mice following MC-38 tumour or irinotecan administration.

I firstly profiled the caecal microbiome of a global *Tlr4*$^{-/-}$ in BALB/c mice. There were differences in alpha and beta diversity in the global KO. There was also a difference in *Odoribacter* levels. *Odoribacter* is known to be downregulated in IBD, and as a producer of various short-chain fatty acids, lower levels of *Odoribacter* have been suggested to affect host inflammation via this reduction in short-chain fatty acids (Morgan et al., 2012). These differences were not maintained in the *Tlr4*$^{\Delta IEC}$ model, although the composition of caecal and faecal microbiomes is likely different (Tanca et al., 2017). Additionally there is likely some baseline differences between BALB/c and C57BL/6 mice (Korach-Rechtman et al., 2019).

These results may suggest TLR4 expressed on immune cells is more important for the maintenance of microbiome composition compared to IEC TLR4, and that IEC TLR4 is more passive in microbial regulation, which may be logical to suggest as apical IEC TLR4 are generally immunotolerant in healthy states (Vamadevan et al., 2010). In this study, I assessed microbiome from caecal and

faecal samples to assess changes in luminal bacterial populations, however this sampling method did not take into account changes in the mucosal-associated microbiome (Wang et al., 2010; Xiao et al., 2019). Therefore, while it could be concluded that IEC TLR4 does not have major role in the regulation of the luminal-associated microbiome, further research would be required to understand its role in regulating the mucosal-associated microbiome.

Previous research has used a constitutive epithelial TLR4 overexpression model to show alterations to faecal microbiome expression and gut function throughout the intestinal tract (Dheer et al., 2016). This, together with the results shown here, suggest that overexpression of IEC TLR4 causes changes in the microbiome, but underexpression (i.e. KO) does not. The microbial changes in the overexpression model exacerbated DSS colitis symptoms and inflammation (Dheer et al., 2016), which could suggest immune activation of pro-inflammatory signalling pathways were a key driver of these microbial changes, rather than specific IEC TLR4 signalling.

The first interventional experiment undertaken in the present study was to co-house WT and *Tlr4*$^{\Delta IEC}$ mice for 4 weeks with the only significant difference in microbiome composition occurring between male and female mice, regardless of study time point. However, a high level of *Akkermansia* was observed in a small proportion of female mice at baseline, not seen in other samples. This is unlikely to be due to sample contamination, as *Akkermansia* is predominantly found in the gastrointestinal tract (Geerlings et al., 2018). This may therefore have been due to a short-term rack or cage effect, as the experiment was carried out over a period of approximately 6 months. Apart from this, these

results suggest no major microbial differences existed between the genotypes. As the mice in this study were initially littermates, it may be that maternal transfer effects and co-housing before weaning has dampened any genotype-specific effect on microbiome composition. Indeed, previous findings have suggested the murine microbiome has a biphasic development, meaning that following weaning, there is limited scope for the microbiome to alter (Pantoja-Feliciano et al., 2013). Two recent studies have aimed to assess how to best normalise the microbiome in different mice strains prior to a study beginning, to remove it as a variable (Caruso et al., 2019; Robertson et al., 2019). While Caruso et al. (2019) suggested co-housing was sufficient to normalise the microbiome, Robertson et al. (2019) demonstrated that littermate controls were most effective. Thus, as I separated the mice at weaning in this study, before co-housing again 4 weeks later, the microbiome was unlikely to significantly alter in this time. This limitation could potentially be overcome in a future experiments by genotyping prior to weaning to separate sooner after birth, and singly house mice for a longer period.

Gut microbiome composition was also longitudinally assessed in a tumour-bearing model following injection of irinotecan-sensitive MC-38 cells and subsequent treatment with irinotecan. Relative abundance showed some changes but small sample sizes meant no clear direction could be observed. There were no differences in alpha or beta diversity across the timeline of the study. This does not follow previous research which has shown chemotherapy-induced changes in the microbiome, as summarised in chapter 1. As there is a growing body of research describing microbiome-related colorectal

carcinogenesis (Cheng et al., 2020), an orthotopic MC-38 tumour model compared to the subcutaneous injection model of the present study could be useful to gain more clinically relevant results in this model.

Different background strains in this study may have had an important role in the development of toxicity following irinotecan treatment, as well as in baseline gut microbiome development (Qian et al., 2017). For example, C57BL/6 mice are known to have Th1-dominated immune response, whereas BALB/c mice have a Th-2 dominated response to immune challenge (Watanabe et al., 2004). This would have effects on the innate immune response, with previous research suggesting C57BL/6 mice produce higher levels of TNF-α and IL-12 after immune challenge compared to BALB/c mice (Watanabe et al., 2004). TNF-α in particular has been strongly implicated in irinotecan-induced toxicity (Melo et al., 2008).

Aside from microbiome composition, I assessed goblet cell populations, as well as presence of CD11b in the intestine of the WT and *Tlr4*$^{\Delta IEC}$ mice with previous work suggesting that secretory and other cell levels are altered (Sodhi et al., 2012). Both were unchanged between genotypes. A previous *Tlr4*$^{\Delta IEC}$ model in neonate mice (Sodhi et al., 2012) showed higher numbers of goblet cells along the entire small intestine (and most significantly in the ileum) of C57BL/6 *Tlr4*$^{\Delta IEC}$ mice compared to WT mice, a finding not replicated here. Sodhi et al. (2012) suggested the change in goblet cells was related to changes in bile acid levels, and not related to microbial flora. Future research in my *Tlr4*$^{\Delta IEC}$ model could therefore investigate bile acid levels, and expression of the apical ileal sodium-dependent bile acid transporter (ASBT). This may be especially

important in the context of irinotecan-induced gastrointestinal toxicity, as a previous study has shown that bile acid malabsorption stemming from irinotecan treatment is associated with the down-regulation of ASBT in the ileum (Shi et al., 2017). Future research into the baseline characterisation of the *Tlr4*$^{\Delta IEC}$ model could also assess changes to Paneth cells and the antimicrobial alpha and beta defensins they release. Sodhi et al. (2012) previously showed increased Paneth cell levels in both of their global *Tlr4*$^{-/-}$ and intestinal epithelial cell *Tlr4*$^{\Delta IEC}$ KO mice strains.

2.6 Conclusion

In conclusion, this study showed epithelial TLR4 expression does not mediate changes in the gut microbiome. This may suggest IEC TLR4 is a passive regulator of the microbiome. Specific immune profiling techniques could also be used in future to understand the key downstream effects of IEC TLR4 KO to determine specific targets for intervention in irinotecan-induced gastrointestinal toxicity.

2.7 Supplementary Material

Supplementary Material 1: International Union of Pure and Applied Chemistry (IUPAC) nuclei acid notation.

Code	Description
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil
R	Purine (A or G)
Y	Pyrimidine (C, T, or U)
M	C or A
K	T, U, or G
W	T, U, or A
S	C or G
B	C, T, U, or G (not A)
D	A, T, U, or G (not C)
H	A, T, U, or C (not G)
V	A, C, or G (not T, not U)
N	Any base (A, C, G, T, or U)

Chapter 3: FMT for prevention of chemotherapy-induced gastrointestinal toxicity: a systematic review and pilot study

This chapter is my second original research chapter and is presented as two parts aimed at developing an optimal methodology for faecal microbiota transplant (FMT) in pre-clinical models. Part 1 is a systematic review examining the methods currently used in FMT models of gastrointestinal (GI) disease in mice. This information was then synthesised to establish a set of minimum reporting requirements for pre-clinical FMT studies. Part 2 details a pilot FMT study using the *Tlr4*$^{\Delta IEC}$ model developed in chapter 2 that was specifically developed using these reporting requirements. This chapter is in the form of a traditional thesis chapter.

3.1 Abstract

Faecal microbiota transplant (FMT) is growing in use in pre-clinical models to connect changes in gut microbial composition with a variety of disease states and pathologies. While FMT enables causal relationships to potentially be identified, the field has much inconsistency, a high rate of false positives and poor reproducibility reflecting limited guidance on pre-clinical FMT protocols and reporting. Therefore, I conducted a systematic review of the methods currently employed for pre-clinical (mouse) FMT across three databases (PubMed, EMBASE and Ovid Medline) until June 30, 2020. Data related to donor attributes, stool collection, processing/storage, recipient preparation, administration and quality control were extracted. The reproducibility of each

protocol was assessed relative to ten criteria. A total of 1753 papers were identified, and after consideration of exclusion criteria, 87 were identified for data extraction and analysis. 51% of papers used FMT as a therapeutic intervention to reduce a pathology, 32% used FMT to determine fundamental host-microbiome interactions, with the remaining 17% using FMT as a prophylactic intervention to change the composition of the microbiome prior to disease induction. The majority of papers used oral FMT administration, with a mean of 4.5 doses of FMT over 1 - 87 day treatment periods. Very few papers reported FMT protocols deemed reproducible, containing an average of 6 of 10 key criteria required for efficient reproducibility. In response to these reporting limitations, I established the *Guidelines for Reporting Animal Faecal Transplant (GRAFT),* a minimum set of reporting requirements for animal FMT studies, which aimed to improve reproducibility and clinical translation. These guidelines were used to inform a pilot study of a pre-clinical FMT model in TLR4 conditional intestinal epithelial cell knockout (KO, $Tlr4^{\Delta IEC}$) mice treated with irinotecan chemotherapy.

3.2 Introduction

The collection of microorganisms in the gastrointestinal tract, termed the gut microbiome, is well known, with a growing appreciation for its dynamic regulation of host function and disease. While large scale sequencing studies have provided unprecedented insight into the range of conditions associated with the microbiome (Kho et al., 2018), they are unable to provide conclusive evidence for how the microbiome causally contributes to disease and how it can be exploited to modify disease risk or progression.

Faecal microbiota transplantation (FMT) is a powerful technique in which a microbial community is transferred from a donor to a host; transferring a unique microbial enterotype to prevent/treat disease or modulate host physiology. Clinically, FMT is now recommended for recurrent or refractory *Clostridioides difficile* infection (CDI) (Mullish et al., 2018) and has a growing list of emerging indications under investigation in a variety of pre-clinical models and pilot cohorts (for review see Allegretti et al., 2019; Wortelboer et al., 2019). In addition to its therapeutic application, pre-clinical FMT is increasingly used to dissect causal microbiome-dependent mechanisms and understand how unique microbial enterotypes dictate disease risk.

Although a powerful technique, the regulatory landscape for FMT in humans is challenging, and differs between countries and jurisdictions, largely due to the ambiguities regarding its classification (e.g. as a ‘biological’ treatment or as a drug treatment) (Hoffmann et al., 2017). Despite this, there are clear recommendations and guidelines for FMT preparation, administration and quality control (e.g. donor health screening and storage guidelines) when used for human recipients (Burz et al., 2019). In contrast, pre-clinical FMT protocols vary enormously, as highlighted by Le Roy et al. (2018), with little to no recommendations on best practice and reporting requirements. This profoundly hinders the reproducibility of pre-clinical FMT studies and introduces inconsistency in pre-clinical datasets compromising clinical translation.

The need for better guidance of pre-clinical FMT protocols is underscored by the additional layers of complexity introduced in such a setting. For example, experimental design, preparation and administration are complicated by the

coprophagic tendencies of rodents. While some studies have exploited this behaviour (co-housing to induce microbial transfer) (Ridaura et al., 2013), the reproducibility of this approach is limited, or at the least, unclear, due to difficulties in quantifying the amount of microbial transfer occurring. Similarly, while bowel preparation prior to FMT is recommended for colonoscopically-administered FMT in humans, the need for preparation of recipient rodents in pre-clinical models remains unclear.

Germ-free (GF, i.e. those without any resident microorganisms) rodents have often been used as recipients in FMT models, as their lack of existing gut microbiome represents a highly effective ‘take-up’ of the donor FMT. However, as discussed by Lundberg et al. (2016), they are expensive and labour-intensive to maintain and in addition, these GF animals have inherent differences related to social behaviour and immune tolerance due to their development without a gut microbiome (Lundberg et al., 2016). Therefore, antibiotic depletion of the microbiome has become common practice to ablate the microbial community of the rodent gut (Ji et al., 2017). However, there are vast differences in antibiotic treatment specifications, including type, dose and duration (e.g. Faivre et al., 2019; Freitag et al., 2019), which can introduce significant variability in ablative capacity, with persisting pathogens confounding results.

While antibiotic treatment represents a particularly common area of variability, in reality, each step of pre-clinical FMT protocols can introduce bias. This was recently highlighted by Walter et al. (2020), who identified that 95% of pre-clinical FMT studies reported successful transfer of the clinical phenotype to the

recipient rodent. This figure was deemed implausible by the authors as most reported “dysbioses” in the human microbiome are not disease specific (Walter et al., 2020). These data highlight the need to advocate for stronger scientific governance for pre-clinical FMT when inferring causality to prevent unrealistic expectations undermining the credibility of microbiome science and delay its translation.

A key element of this enhanced rigor must be clarity in the methodological reporting to improve consistency and transparency within the field, both of which will strengthen the reproducibility of findings. As such, I systematically reviewed published literature on pre-clinical FMT used to investigate gastrointestinal-based conditions (i.e. inflammatory bowel disease (IBD), enteric infection, metabolic disorders and gastrointestinal cancers) in mice. This enabled me to provide a snapshot of current reporting patterns and develop a set of minimum reporting guidelines for future pre-clinical FMT studies.

These guidelines were then used to develop a pre-clinical FMT model using the TLR4 conditional intestinal epithelial cell knockout (KO, *Tlr4*$^{\Delta IEC}$) mice described in chapter 2. While no clear differences were shown between WT and *Tlr4*$^{\Delta IEC}$ microbiome composition in chapter 2 using 16S techniques, microbial metabolites or secreted factors may be altered. As this may have an impact on the development of gastrointestinal toxicity following chemotherapy, FMT was used to transfer faecal contents from wild-type (WT) mice to *Tlr4*$^{\Delta IEC}$ mice and vice-versa to determine whether the specific *Tlr4*$^{\Delta IEC}$ KO conferred any effect in reducing gastrointestinal toxicity following irinotecan treatment.

3.3 Part 1: Systematic Review

3.3.1 Methods

3.3.1.1 Focus question

This systematic review aimed to answer the question: *what FMT protocols are being used in pre-clinical mouse models to study gastrointestinal function and disease?* FMT protocols were then used to define core aspects of pre-clinical FMT methodology and develop a set of minimum reporting requirements.

3.3.1.2 Study design

The protocol for this systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines (Moher et al., 2009).

3.3.1.3 Search strategy

I completed a comprehensive search using the electronic databases PubMed, Ovid Medline and EMBASE on June 30, 2020. The search parameters were tailored to each database, and the full search string for each database can be found in supplementary material 1. I searched for papers including faecal or caecal material transplant.

3.3.1.4 Selection criteria

The initial literature search was conducted, and duplicate articles removed. Subsequently, entries from prior to 2010 were removed to ensure only FMT

protocols currently in use were included. Initially, abstracts and titles were screened using the Covidence systematic review software web program to assess eligibility (Veritas Health Innovation, Melbourne, Australia. Available at www.covidence.org). After this abstract screen, full-text articles were again assessed by the same reviewers.

I aimed to retrieve only full-text, peer-reviewed, original experimental studies performed in mice (in order to remove inconsistencies regarding FMT volume etc). Studies must have been published in English. To be included in the review, studies must have completed a faecal or caecal microbiota transplant where conventionally housed mice were both the donor and the recipient. The study outcome must have been linked to gastrointestinal health, function or disease.

Studies were excluded if they: used humans or animals other than mice as donors or recipients; utilised GF mice; tested a non-gastrointestinal outcome; or utilised a co-housing only approach to FMT. Secondary studies such as review papers, methodological protocols and conference abstracts were also excluded.

3.3.1.5 Data extraction and analysis

Three independent reviewers and I extracted relevant information from the selected papers using standard data collection templates. All available methodological data on FMT was extracted from the main paper or supplementary information. Key information included: donor and recipient characteristics (age, strain, antibiotic use); FMT preparation and storage

methods; FMT administration (dosage, number of treatments, administration route); and use of quality control methods (i.e. analysis of FMT product or assessment of microbial changes in recipient). To quantify the reproducibility of pre-clinical FMT protocols included in the analysis, I developed a reproducibility index based on 10 variables of pre-clinical FMT, irrespective of the model or study goals. The criteria were as follows: buffer/vehicle; method of homogenisation; filtration steps; storage (if applicable); concentration of final FMT product; pre-conditioning of the recipient; route of administration; volume administered; frequency of administration; and the inclusion of anaerobic conditions. The same 3 independent reviewers and I marked each criterion as: 0 = not reported; 0.5 = mentioned; or 1 = mentioned with appropriate detail (to be able to effectively replicate the study). Importantly, studies were assessed based on whether these parameters were *reported*, not for *how* they were performed. While this index provides an objective assessment of the level of detail in reporting, the results should be interpreted with caution as the index was not developed to provide a statistically comprehensive measure of reproducibility, and as such there was not necessarily a linear relationship between the score and overall reproducibility of the study. Thorough review of the literature yielded no appropriate method for assessing methodological reporting in this way, and as such, the reproducibility index was developed specifically for this study.

3.3.2 Results and Discussion

3.3.2.1 Study selection

Of 1753 screened studies, a total of 87 were included (supplementary material 2). 1196 were screened via title and abstract, with 728 excluded as not relevant. 468 full-text articles were assessed, with 381 excluded at the full-text stage, leaving 87 papers for data extraction (figure 3.1).

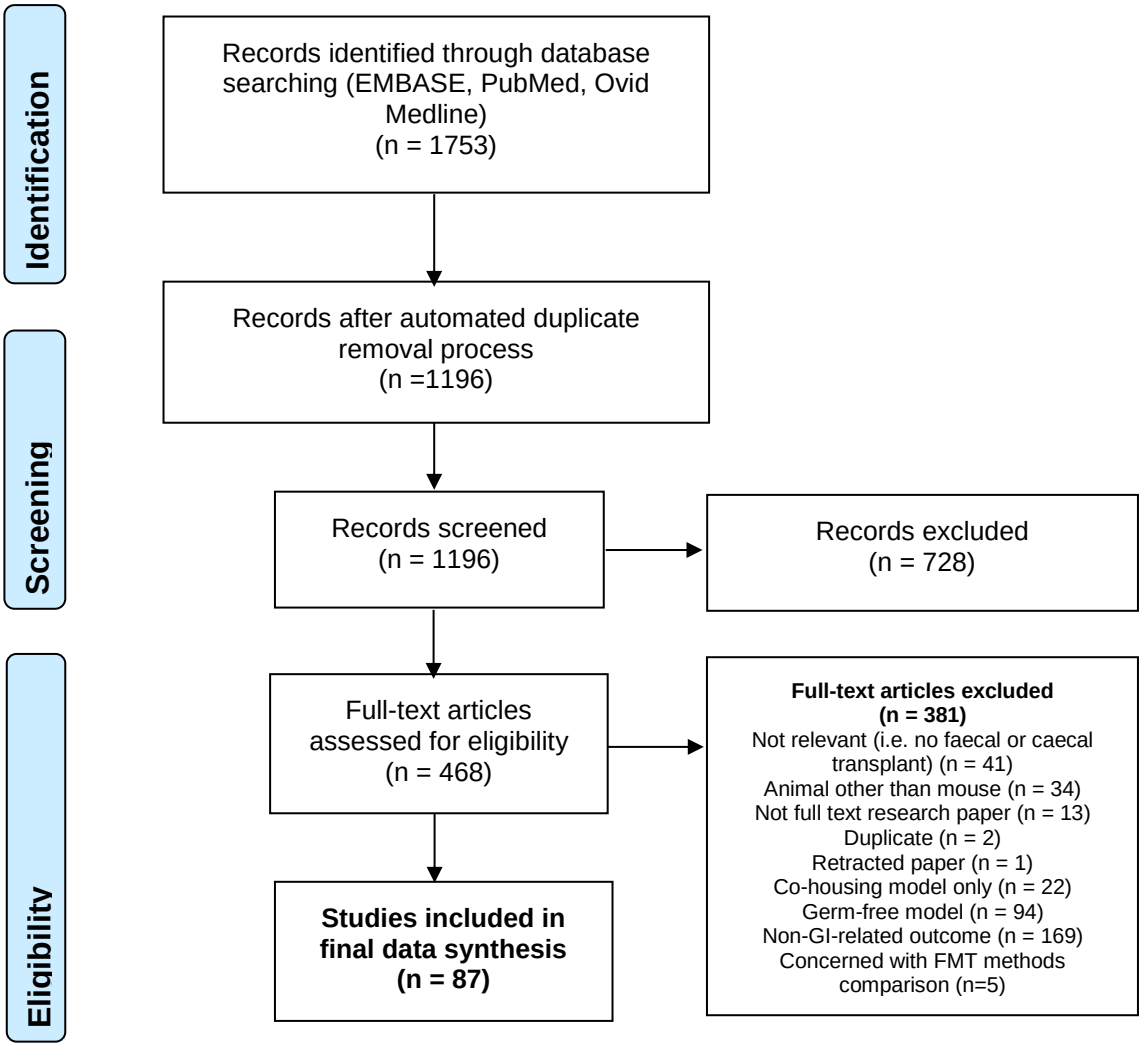

Figure 3.1: PRISMA flow chart for identification and selection of eligible studies. In screening, 728 papers were excluded where clearly fitting exclusion criteria based on title and abstract screening. Two additional duplicates, not found in the initial automated duplicate screening, were excluded manually at the full-text eligibility review stage.

3.3.2.2 Study characteristics of papers in final data synthesis

Papers with gastrointestinal specific outcomes were included. Of these, the largest group was IBD-related conditions (IBD/ inflammatory bowel syndrome/ necrotising enterocolitis) at 39% (figure 3.2). Other conditions included enteric infection (20.7%), metabolic and immune disorders (13.8%), drug-induced conditions (12.6%), cancer (4.6%), and other (9.3%).

Papers were sorted depending on the overall goal of the FMT within their experimental design. As expected, the papers had a wide range of experimental designs (figure 3.2); the categories being; 1. pre-conditioning (P, FMT administered prior to disease establishment, i.e. determined if microbial manipulation or unique enterotypes influenced disease dynamics); 2. fundamental (F, i.e. determined host-microbe interactions related to gastrointestinal function); and 3. interventional (I, FMT administered following disease establishment, i.e. determined therapeutic efficacy). Interventional papers were most common (50.6%), followed by fundamental (32.2%) and pre-conditioning (17.2%).

Studies ranged in the sample size used per experimental group (mean = 8.6, range = 3 - 27), reflecting varying power requirements for specific models, while 24% of the eligible studies did not clearly state the sample size of the recipient group. While the sample size for the donor FMT group was not extracted in the analysis (due to the low level of papers that reported this), it is also important to acknowledge that this should also be clearly reported.

The age of the recipient mice was reported in 90.8% of papers, with adult mice between the ages of 6 - 14 weeks were most common, used in 78% of papers.

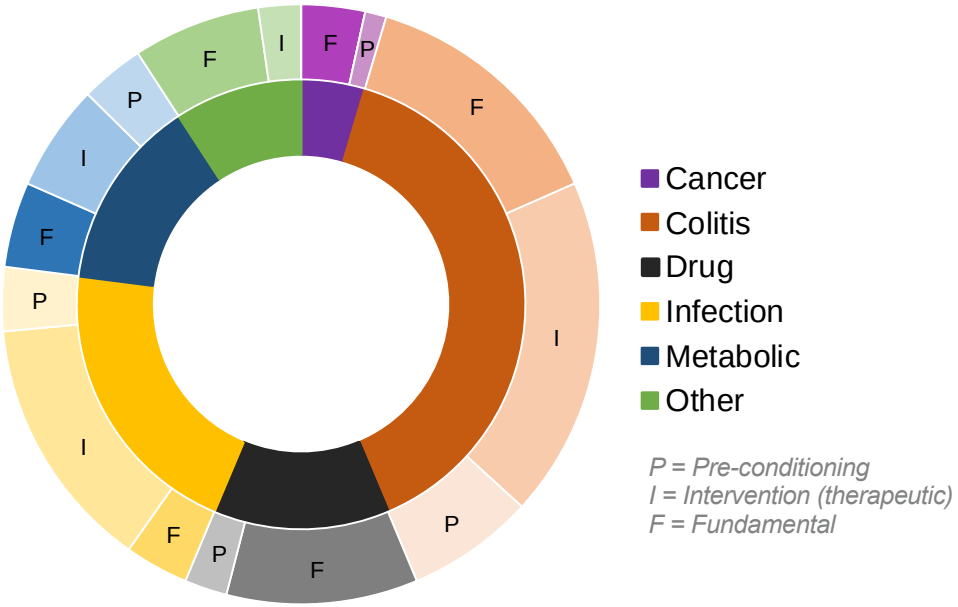

Figure 3.2: Summary of studies included for final data synthesis categorised by type of disease investigated and FMT intervention type (pre-conditioning (P), interventional (I, therapeutic) or fundamental (F)).

3.3.2.3 Data extracted from papers in final data synthesis

3.3.2.3.1 FMT preparation

There are several aspects of FMT preparation that must be acknowledged and highly protocolised for rigorous results: collection of donor stool; homogenisation; filtration; and storage. The vast majority of papers used faecal pellets to prepare the FMT product (79.4%), however 10.3% of papers used caecal content and a further 10.3% used a combination of various gastrointestinal products (e.g. duodenal aspirates and faeces, mucosal scrapings, small and large intestinal contents). In addition to the source of FMT, a range of preparation techniques were used to produce the FMT, including filtrates, supernatants and slurries. Of the papers reviewed, a faecal slurry was most commonly used (60%), with supernatants and filtered products used in 24.1% and 11.5% of papers, respectively (figure 3.3 A). The remaining 4.4% of papers did not report this. Faecal slurries are likely the most popular FMT form as this retains both the microbial community and their metabolome. However, in recent clinical studies, both highly filtered slurries and capsulated FMTs were capable of treating CDI, suggesting a more refined FMT product may be a viable solution (Kao et al., 2017; Ott et al., 2017). This is yet to be explored in pre-clinical models. Nonetheless, the vehicle buffer must be carefully considered. In the studies included for analysis, phosphate-buffered saline (PBS) alone was most commonly used (75.8%), with additives (e.g. glycerol or cysteine-HCl) to PBS in 6% of papers. Water was used in 4.5% of papers, other buffers in 3.5% of papers, with 10.2% of papers did not report what was used.

Regulated storage conditions have previously been reported to impact microbial preservation, i.e. maintenance of microbiome composition between donor faeces and the final FMT product, in both pre-clinical and clinical studies (Takahashi et al., 2019; Dorsaz et al., 2020). Of the papers analysed, 39.3% administered freshly prepared (i.e. not stored) FMT. A small number of papers (5.7%) noted the FMT product was stored at -80 °C, however the majority of papers (55%) did not report any methodological detail on storage conditions (figure 3.3 B). Proper reporting of storage methods, and indeed time at room temperature, would be useful information in understanding whether microbial preservation is a key factor in FMT success.

Clinically, FMT is often prepared under anaerobic conditions to protect both obligate and facultative anaerobes (Costello et al., 2019; Ng et al., 2020). It has been reported that human FMT prepared under ambient conditions profoundly decreased microbial viability, and altered microbial metabolite synthesis and abundance of many anaerobic commensals (Papanicolas et al., 2019). In this current analysis, only 5.7% of papers explicitly stated their use of anaerobic conditions. Additionally, over 35% of analysed pre-clinical studies did not report the final concentration of the FMT product major omission from pre-clinical FMT protocols included in the analysis was the final concentration of the FMT product. A wide range of concentration units were used; most common was grams or milligrams per mL of vehicle (34% of total papers), following this was pellets per mL of vehicle (13% of total papers, figure 3.3 C). The pellets/mL unit of concentration is difficult to reproduce as it can be highly variable on a mouse strain, day-to-day or animal facility basis.

There are various methods available to generate the FMT product. In the studies analysed here, a wide range of processes were used to homogenise and filter the solutions. Homogenisation method was not referred to in 39% of papers, and a further 28% of papers simply referred to homogenisation without further detail. Of the papers reporting a method, the most common was vortexing (48.3%), with inversion (10.3%), mixing (10.3%) and steeping (6.9%) also being used in small numbers of papers (other methods collectively made up 24.2% of papers, figure 3.3 D). Following this homogenisation step, some studies filter particulate matter out from the final product. Filtration method was not reported or unclear in 55% of papers. Of those which reported this, centrifugation was the most common filtration method (69.3%), followed by various forms of membrane/mesh filtration (30.7%).

3.3.2.3.2 Host preparation and FMT administration

Once the FMT has been prepared, there are many considerations in its administration related to both the product itself and the recipient. Of the studies included, 35% failed to provide any detail on animal housing conditions (e.g. single or co-housed). Where husbandry details were reported, co-housing was most common (71%) with individually housed mice reported in 29% studies.

I also investigated how, if at all, recipient mice were prepared for FMT. As suggested previously (Freitag et al., 2019), there is some evidence that a sufficiently efficient bowel lavage or cleansing may improve pre-clinical FMT efficacy. As such it will be important to collate this data in future studies. In the studies included in the present analysis, there was a lack of use or reporting of

these procedures, with only one study fasting the mice overnight and giving polyethylene glycol 3350 as a laxative beforehand (Perry et al., 2015). The other main pre-treatment used in the studies I assessed was antibiotics, with 48% of studies using antibiotic-depletion techniques to prepare the recipient prior to transplantation (figure 3.3 F, supplementary material 3). The most common way these antibiotics was administered was via drinking water (64%) reflecting the feasibility of this approach. Antibiotics were given for a median of 14 days (range = 1 - 90 days), and the most common combination was a cocktail of ampicillin, neomycin, vancomycin and metronidazole (42.5% of papers using antibiotics). A complete list of antibiotics used across the studies can be found in supplementary material 3.

While antibiotic-induced depletion is certainly a critical consideration for studies aiming to change the composition of the microbiome, increasing evidence suggests this may not be necessary for FMT uptake; albeit the evidence from pre-clinical models is conflicting. While Ji et al. (2017) reported good FMT durability with antibiotic depletion compared to either a MoviPrep bowel cleanse or no pre-treatment (Ji et al., 2017), others have shown no difference. For example, Freitag et al. (2019) showed pre-treatment with antibiotics did not improve the overall engraftment of the donor microbiome, and only improved the engraftment of a small number of taxa. One potential reason for this discrepancy could be due to different species of the donor microbiome. While Ji et al. (2017) utilised a human donor microbiome (not included in this analysis), Freitag et al. (2019) used a mouse donor microbiome, suggesting antibiotic administration may be useful in improving FMT engraftment across the species

barrier, but is not so pivotal where the donor and recipient are the same species.

When assessing methods of FMT administration, the majority of studies included in the analysis administered FMT orally (84%), with only a small amount using rectal administration (5%) and 1% of papers using both oral and rectal administration (figure 3.3 E). The final 10% of papers either did not report, or did not clearly state, how their FMT product was administered. It has previously been suggested in a pig model that as oral gavage inoculum needs to pass through the acidic stomach environment, rectal administration may be more efficient (Hu et al., 2018). However, a previous study of FMT in mice showed that specific-pathogen-free mice treated with antibiotics and then orally or rectally inoculated with donor mice gut microbiome had no differences in microbial community after inoculation (Lutzhoft et al., 2019). While rectal administration has correlated with better transplant outcomes in clinical settings, as well as reducing occurrence of sepsis (Park et al., 2020); in pre-clinical models, oral gavage is potentially easier and more convenient.

Similarly to concentration, volume of FMT and frequency of administration were not reported in enough detail to be able to easily reproduce these studies. The volume was not reported in 20 studies (23%). The median volume used in studies that did report was 216 (range 25 - 1000) µL (figure 3.3 G). The number of FMT treatments administered was clearly reported in 95% of studies. There was a large range (1 - 16 treatments), with a median of 3 and mean of 4.5 treatments (figure 3.3 H). These treatments were given over a treatment period of between 1 - 87 days. These wide ranges (both volume and number / duration

of treatments) may sometimes be required for a particular experimental design, however could also introduce variables in terms of quality control that would need to be investigated.

The time of day the FMT was administered was not reported in the final papers. Due to circadian rhythm-induced changes in the microbiome (Bishehsari et al., 2020), this may be a useful piece of information in future studies.

3.3.2.3.3 Quality control and uptake confirmation/durability

Studies were examined for data or information regarding confirmation of uptake, quality control and control FMT procedures. Le Roy et al. (2018) defined the efficacy of pre-clinical FMT procedures as: 1. establishment of high levels of bacterial taxa from the inoculum in recipients; 2. relative abundance of bacterial taxa as similar as possible in the inoculum and recipients; and 3. removal of a high amount of endogenous bacterial taxa in non-GF recipients. This can be determined by microbial analysis of the FMT inoculum, and gut microbial contents of the recipient both before and after FMT occurs. Overall, information in these areas was lacking. Approximately 28% of papers did not report or did not confirm uptake of the FMT. Of the papers reporting on this, 16S sequencing of faecal samples post-FMT was the primary method by which this was determined (92%), with bacterial cultures (4.6%), polymerase chain reaction (PCR, 2.3%) and short-chain fatty acid (SCFA, 1.1%) tests also used.

I defined quality control (of the FMT product) as analysis of the FMT product before administration to the recipient mice, i.e. identification of the presence of potential pathogens and confirmation of the viability of the FMT product. Using

this definition, there was no information regarding quality control in 88.8% of papers. This finding emphasises the flaws in experimental approaches used in FMT studies, as without appropriate quality controls, the viability and composition of the FMT is unknown. The remaining papers completing quality control processes utilised 16S sequencing (4.5% of total papers), bacterial culturing (4.5% of total) and optical density analysis (2.2%).

Use of an FMT control was determined by looking for studies that included a control (vehicle) group for the FMT protocol itself. A wide range of controls were used, including autologous transfer, vehicle solution, probiotics, and other study-specific conditions, however in over 50% of the papers there was no control procedure identified.

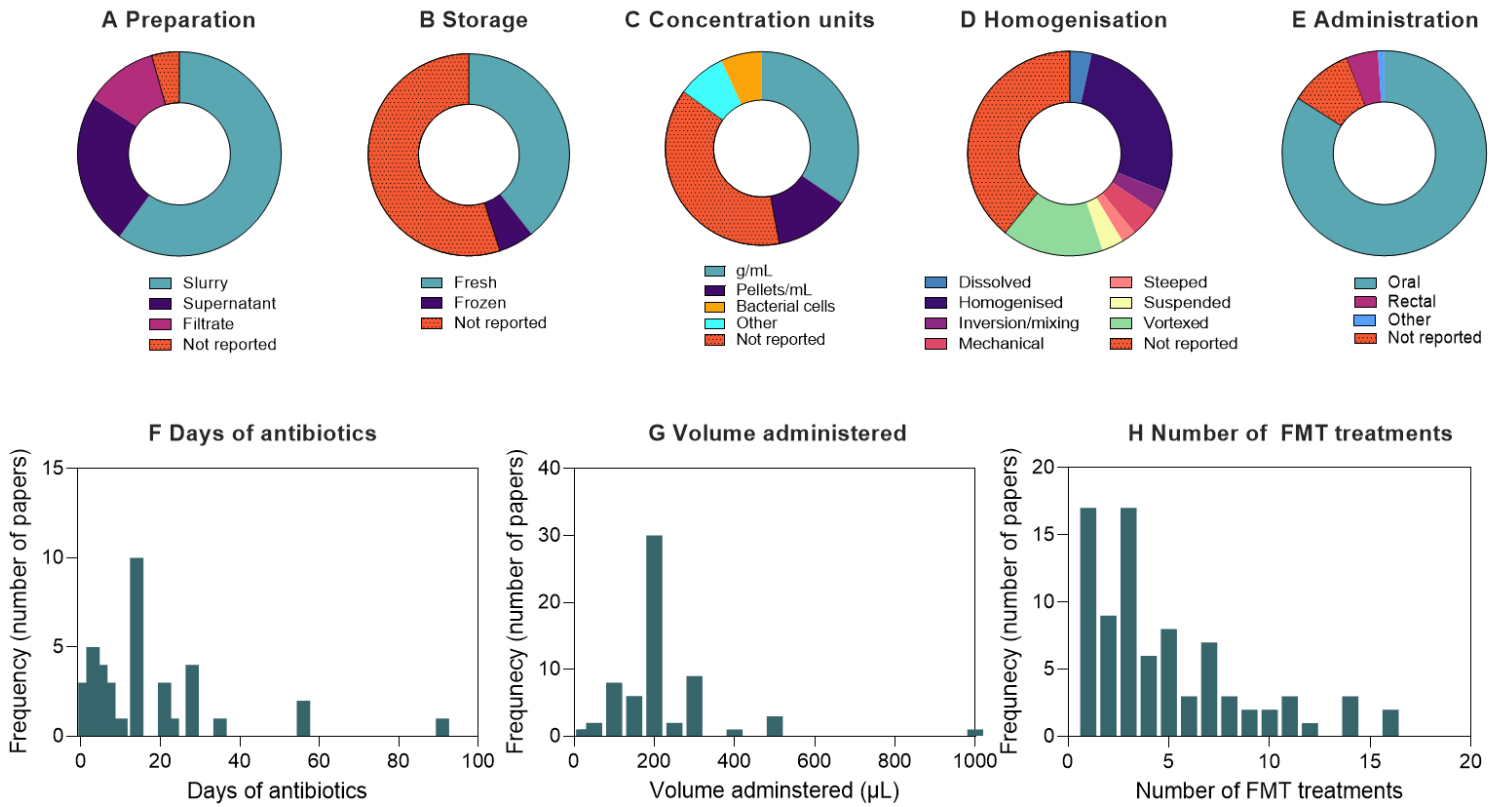

Figure 3.3: Key FMT methodologies used in studies eligible for analysis. (A) The most common preparation method was a faecal slurry. (B) Over 50% of papers did not report whether/how the FMT product was stored. In papers that did report this, fresh was more common than frozen. (C) A variety of units were used to define the final FMT product, with g/mL most commonly used. (D) There was a low level of detail included about homogenisation methods. 39% of papers did not report any method. (E) Oral administration was the most used method (84%). (F) Mean days of antibiotic prophylaxis was 16.4. (G) Mean volume of FMT administered was 216 µL. (H) Amount of treatments ranged from 1 - 16, with a median of 3 treatments.

3.3.2.3.4 Reproducibility and rigour of papers in final analysis

A recent systematic review suggested a causal relationship between an altered human microbiome and disease or physiological condition (Walter et al., 2020). Of the papers meeting their inclusion criteria, all but two (95%) suggested faecal transfer from diseased donors resulted in a disease phenotype. However, due to the wide range and complexity of pathologies studied in these papers, the authors suggested the causal claims seem unlikely across this wide range. Similarly, in the present analysis I found 93% of papers reported FMT had an effect. While this may reflect publication bias, a tendency to favour positive findings for publication; as suggested by Walter et al., microbiome science would benefit from increased rigour and critique (Walter et al., 2020). A key part of this scientific rigour is transparent and reproducible methodology (Brussow, 2020; Walter et al., 2020).

Through the present analysis, I documented methods, as described in the papers, did not have sufficient detail to be completely reproducible (table 3.1). Therefore, I developed a reproducibility index that scored each paper on 10 variables of pre-clinical FMT methodology. The median value of the papers, out of a total possible maximum of 10, was 6.5, with 21.9% of papers satisfying less than 50% of the criteria (figure 3.4). The criteria surrounding use of anaerobic conditions had the lowest mean reproducibility grade (0.045/1), whereas the criteria ‘recipient pre-conditioning’ had the highest mean reproducibility grade (0.95/1).

Table 3.1: Key findings from data extraction

Key findings:
• 93% of studies included reported a positive outcome of FMT intervention
• Faecal slurries, containing both the microbiome and their metabolome, were the most common form of FMT product
• Only 5.7% of studies reported on the use of anaerobic conditions during FMT product preparation
• Method of homogenisation was not referred to in 67% of studies
• 55% did not report storage conditions for FMT product
• 24% of studies did not report on the sample size of the recipient group
• Antibiotic-depletion was the most common form of recipient preparation
• 10% of studies did not describe how the FMT was administered
• 23% did not report the volume of FMT administered
• FMT durability/uptake was not confirmed in 27% of studies
• 88% of studies did not perform any quality control
• Over half of the studies included did not report on control FMT conditions

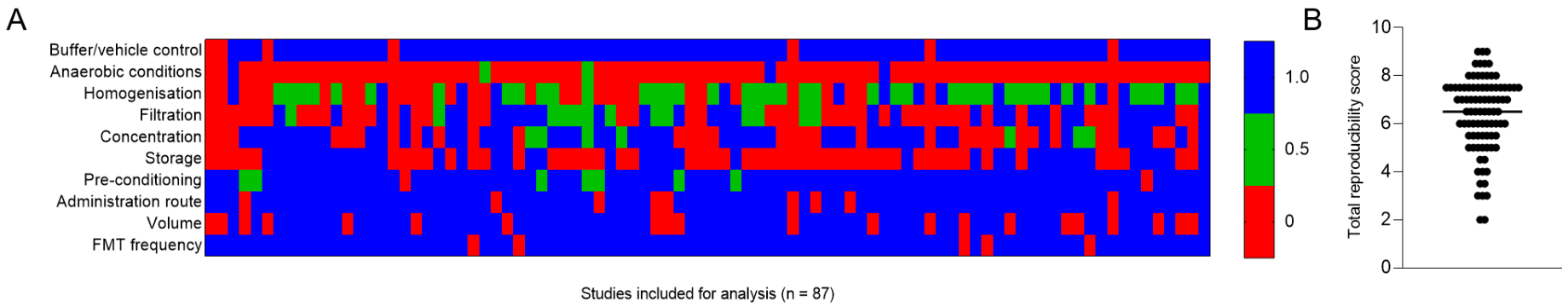

Figure 3.4: (A) Heatmap of the reproducibility index assessment. Each study (across the x-axis) was scored in 10 criteria (y-axis). Each criterion was scored as: 0 = not reported (red); 0.5 = mentioned (green); or 1 = mentioned with appropriate detail (to be able to effectively replicate the study, blue). For the 87 studies included for analysis, 21.9% of papers described less than half of the 10 criteria in enough detail to be reproduced. The use of anaerobic preparation conditions was only described in 5% of studies. Homogenisation, filtration and storage methods were also poorly reported. (B) Total reproducibility score of each paper, out of a maximum of 10. The median value (represented by the line) was 6.5.

3.3.3 The Guidelines for Reporting Animal Faecal Transplant (GRAFT) recommendations and future steps

This systematic review revealed an overall lack of clarity in the reporting of FMT methods. In almost all variables I investigated, there was not only a lack of consistency in FMT protocols, but also a lack of clarity and detail in methodological reporting. For example, for FMT concentration, as well as the actual concentration ranging widely, seven different units were used to report this. These findings indicate a lack of authoritative guidance on pre-clinical FMT studies for both authors and reviewers.

Due to the lack of clarity found in many papers and the low mean score from the reproducibility index scoring, I suggest a minimum set of reporting requirements for pre-clinical FMT studies are needed. As such, I present here the GRAFT recommendations (table 3.2). These guidelines were developed starting with the 10 criteria of the reproducibility index, with a further 7 guidelines added based on feedback from the Australia/New Zealand Human Microbiome Network, to encourage best practice reporting. I aimed for the recommendations to provide methodological guidance for pre-clinical FMT preparation/administration, reporting and peer-review of studies prior to publication. By providing these recommendations, I hope to increase the transparency and reproducibility of pre-clinical FMT procedures, thus elevating their translational strength. While the systematic review only included studies with mice in the setting of gastrointestinal disease to identify a feasible number of papers for analysis, I propose these guidelines can also be used in experiments using FMT generated from human donors, or stool of other

species. If generating a human-microbiome associated animal models, these guidelines should be followed jointly with the recommendations presented by Walter et al. (2020), in particular the need to confirm the successful engraftment of donor microbiome via multi-omics analysis.

While these guidelines provide the much needed structure for pre-clinical FMT protocols, it is critical to emphasise it is not aimed to recommend what methods should be used, as different experimental endpoints and research questions will need different methodological design. However, by consistently reporting the following set of guidelines, future studies will collect more consistent evidence and thus generate clinically relevant outcomes. Similarly, these guidelines should facilitate and structure the peer review process for pre-clinical FMT studies, which based on the analyses is currently lacking. I envisage future pre-clinical FMT studies using the GRAFT recommendations will provide easily reproducible and classifiable methodology, allowing better systematic reviews and meta-analysis of existing data.

Table 3.2: GRAFT recommendations for reporting of animal faecal transplant studies.

1.0	Preparation and storage	
1.1	Housing/husbandry for donor and recipient (as per ARRIVE Guidelines (Percie du Sert et al., 2020))	Co-housing vs individually-housed with detail regarding experimental group distribution. Arrangement of cages across racks (if relevant). Number of animals per group (including number of donors). Details of bedding and chow. Facility specifications (with respect to specific-pathogen-free (SPF), GF). If GF, specifications of animal unit/isolator.
1.2	Collection of donor faeces	Animal handling during collection. Time of collection. Method of faecal collection (i.e. placing animal into clean cage until defecation, direct collection from anus or post-cull collection). Aseptic procedures and protocols adopted.

		Methods to minimise oxidative stress (i.e. use of transport medium e.g. brain and heart infusion or carboxymethylcellulose buffer). Methods to maximise viability (i.e. time from collection to anaerobic conditions in minutes). Details on immediate storage if relevant (e.g. snap frozen in liquid nitrogen or stored in reduced oxygen medium).
1.3	Buffer / vehicle for FMT preparation and administration	Solution, pH, temperature, volume. Any additives used to support microbial viability. If de-oxygenated solution is used, specify method of de-oxygenation.
1.4	Concentration	Report using standard units (mg/mL). Avoid inaccurate units (e.g. pellets/mL).
1.5	Homogenisation	Equipment used (e.g. vortex, Stomacher, autoclaved spatula). Intensity (using standardised unit where possible). Homogenisation time and temperature.

1.6	Filtration	Method of filtration (e.g. gravity, centrifuge, strainer, stomacher bag). If centrifuge, specify time, speed (*x g*) and temperature. If gravity, specify time and conditions (i.e. ambient, anaerobic, temperature). If physical strainer/membrane, specify pore size or equivalent detail and filtration method.
1.7	Final product	Define administered product as: - Faecal slurry (i.e. faecal contents with minimal filtration). - Faecal supernatant/filtrate (i.e. microbial free). - Microbial preparation (i.e. lyophilised or other).
1.8	Quality control	Report method used to assess FMT quality and composition (prior to administration), e.g. colony forming units (CFU/mL) or diversity index (for genomic analyses).

1.9	Storage and thawing	For long-term storage between preparation and administration, report volume, temperature and duration. Provide details on thawing faecal product prior to administration, as well as any freeze-thaw cycles. If the faecal product is used fresh, this must be clearly stated with details on short-term storage conditions (i.e. stored on ice, in the fridge, at room temperature, anaerobic chamber) and the duration between preparation and administration.
1.10	Anaerobic conditions	Clearly state if/when anaerobic conditions were used. Include details of anaerobic conditions.
2.0	Administration	
2.1	Pre-conditioning	Details of antibiotic-depletion or other methods of host preparation prior to transplantation. Include relevant detail (i.e. duration, frequency of water change, specific antibiotics used and concentrations).

		Ideally, confirm depletion through faecal analysis prior to FMT. Report on pre-conditioning of control group.
2.2	Route and method of administration	Oral, rectal or both. Include relevant information on method of administration (e.g. oral gavage, lavage, enema). Include relevant detail on use of anaesthesia or fasting prior to administration (particularly rectal) and co-housing/coprophagic approaches (i.e. was additional FMT smeared on coat to improve uptake).
2.3	Volume	Define in standard unit for each individual FMT; specify if absolute unit or relative to body weight of recipient.
2.4	Frequency	Define as total number of faecal transplant procedures and daily frequency (i.e. a total of 3 FMT by oral gavage at a frequency of 1 per day).
2.5	Time	Time of day FMT was administered. Duration between administration and assessment (i.e. of disease status or microbiome composition).

2.6	Control formulation	Define what control animals received, e.g. vehicle solution, autologous transplant, heat-killed FMT.
3.0	Confirmation of uptake	
3.1	Durability/tractability confirmation	Define how the uptake/durability of the FMT procedure was confirmed, e.g. 16S rRNA/shotgun sequencing or faecal bacterial culture, and the time after FMT that it was performed. It is recommended that the same analysis be applied to the FMT administered to compare composition of donor and host.

3.4 Part 2: Pilot FMT study in *Tlr4$^{\Delta IEC}$* mice

Following the development of the GRAFT recommendations, I then used them to develop a pre-clinical FMT model in TLR4 conditional intestinal epithelial cell KO (*Tlr4$^{\Delta IEC}$*) mice. This model allowed assessment of the efficacy of FMT to reduce gastrointestinal damage following irinotecan, and assessed whether pre-treatment alterations of the gut microbiome environment reduced gastrointestinal toxicity. In this study, I prepared an FMT from faecal samples of WT mice to administer to *Tlr4$^{\Delta IEC}$* mice, and an FMT from faecal samples of *Tlr4$^{\Delta IEC}$* mice to administer to WT mice to assess whether changing the microbial environment of these mice altered development of irinotecan-induced gastrointestinal toxicity.

3.4.1 Methods

3.4.1.1 Animals

This study was approved (M-2020-026) by the Animal Ethics Committee of the University of Adelaide, and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training (2013). All mice were maintained under standard SPF conditions at the Laboratory Animal Services Facility at the University of Adelaide, in ventilated cages with *ad libitum* access to food and water, and a 12 hour light/dark cycle.

3.4.1.2 FMT

These FMT methods are set out as per the GRAFT recommendations (section 3.3.3).

3.4.1.2.1 Preparation and storage (GRAFT 1.0)

1.1 Housing/husbandry for donor and recipient

WT and *Tlr4*$^{\Delta IEC}$ mice (total n = 29, table 3.3) were separated into genotype- and sex-specific cages upon weaning (3 weeks of age, 1-5 mice per cage). Mice were kept on the same rack in the same holding room consistently throughout the experiment. Bedding (Corn-cob-ology, Mount Kuring-Gai, Australia) and chow (Teklad Global Soy Protein-Free Extruded Rodent Diet, irradiated, Envigo, Indianapolis, USA) were kept consistent throughout the study. Mice were individually housed from the first FMT treatment onwards.

1.2 Collection of donor faeces

Faecal samples were collected for FMT preparation the day prior to the first FMT administration, when mice were 8 weeks old. Animals were handled using clean gloves, and faecal samples were collected directly from the anus of the mice into sterile containers. Faecal samples were collected 2 hours after the light cycle began and samples from cage-mates (1 - 3 mice) were pooled together. Male mice only received FMT from other males and female mice only received FMT from other females.

1.3 Buffer / vehicle for FMT preparation and administration

Containers (1.5 mL sterile microcentrifuge tubes, Eppendorf, Hamburg, Germany) were kept closed to air except when adding faecal samples to reduce oxidative stress. Sterile, room temperature 1 x PBS, pH = 7.4, was added to the faecal samples within 20 minutes of collection.

1.4 Concentration

Room temperature, sterile PBS (1 x, pH = 7.4) was added to samples at a concentration of 100 mg of faeces per 1 mL of PBS.

1.5 Homogenisation

Immediately following addition of PBS, tubes were vortexed using the BR-2000 Vortexer (Bio-Rad, Hercules, USA) at top speed (3000 rpm) for 60 seconds at room temperature.

1.6 Filtration

Following vortexing, samples were left to settle by gravity for 5 minutes in the closed containers at room temperature. The faecal supernatant was then collected.

1.7 Final product

Finally, sterile glycerol was added to the supernatant at 10% v/v to ensure optimal conditions for -80 ˚C frozen storage and best clinical translation of results (Cammarota et al., 2017).

1.8 Quality control

As this was a pilot study, I did not assess FMT quality and composition however excess FMT product was stored at -80 ˚C for future assessment.

1.9 Storage and thawing

This mixture was aliquoted into 150 µL aliquots and frozen at -80 ˚C for approximately 28 hours. The FMT product was thawed at ambient temperature just prior to use and allowed to reach room temperature.

1.10 Anaerobic conditions

Anaerobic conditions were not used at any point in this protocol due to limited equipment availability.

Table 3.3: Group allocations for FMT study.

Genotype	**FMT treatment**	**Irinotecan treatment**	**Males**	**Females**	**Total**
WT	vehicle	vehicle	3	0	3
WT	vehicle	irinotecan	3	1	4
WT	FMT	vehicle	3	1	4
WT	FMT	irinotecan	2	2	4
Tlr4$^{\Delta IEC}$	vehicle	vehicle	2	1	3
Tlr4$^{\Delta IEC}$	vehicle	irinotecan	2	1	3
Tlr4$^{\Delta IEC}$	FMT	vehicle	2	2	4
Tlr4$^{\Delta IEC}$	FMT	irinotecan	2	2	4
		Total	19	10	29

3.4.1.2.2 Administration (GRAFT 2.0)

2.1 Pre-conditioning

No pre-conditioning methods (e.g. antibiotics or fasting) were used in this study.

2.2 Route and method of administration

Under isoflurane anaesthesia, FMT was rectally administered via a flexible 20 gauge gavage needle (Instech, Plymouth Meeting, USA) inserted 35 mm into the colon.

2.3 Volume

100 µL was administered at each treatment.

2.4 Frequency

FMT was administered once daily for 3 consecutive days (total of 3 FMT).

2.5 Time

FMT was administered approximately 4 hours prior to the commencement of the lights off cycle.

2.6 Control formulation

Vehicle groups received an equivalent dose of 1 x PBS (sterile, pH = 7.4) + 10% v/v glycerol. Mice were put into a clean cage following each FMT administration.

3.4.1.2.3 Confirmation of uptake (GRAFT 3.0)

3.1 Durability / tractability confirmation

Faecal samples were collected at baseline and at the time of irinotecan administration to assess changes in the microbiome. However, as this was a pilot study, these samples have been stored at -80 ˚C for future analysis.

3.4.1.3 Irinotecan administration

Six days following the final FMT, mice were intraperitoneally injected with a 270 mg/kg dose (as per previous research (Wardill et al., 2016)) of irinotecan hydrochloride (20 mg/mL, Pfizer, Kalamazoo, USA) or vehicle (sorbitol-lactic acid buffer, 45 mg/mL sorbitol, 0.9 mg/mL lactic acid, pH = 3.4). Mice were culled 72 hours after irinotecan via CO_2 inhalation and cervical dislocation. At necropsy, pellets from the distal colon were collected as well as mucosal samples of the mid-colon, for future analysis of the mucosa-associated microbiome (figure 3.5).

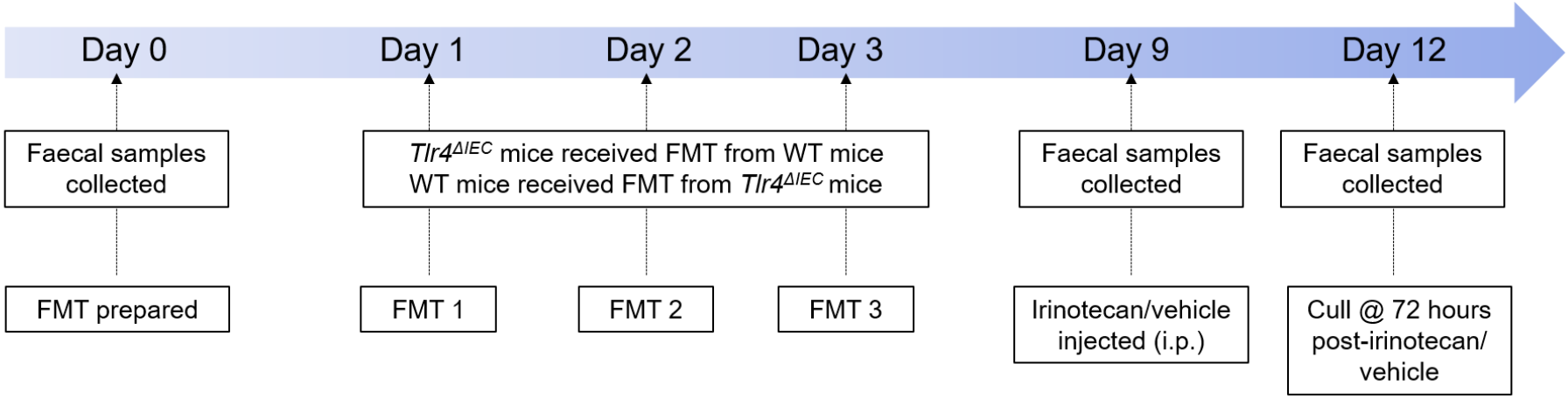

Figure 3.5: FMT study timeline. Mice were monitored twice daily between Day 0 and Day 9, and then at least three times daily from irinotecan/vehicle administration until cull.

3.4.1.4 Clinical symptom monitoring

Mice were weighed once daily (in the morning), with a 3 x daily comprehensive clinical symptom recording. Diarrhoea was graded according to an established grading system with four grades: 0 = no diarrhoea; 1 = mild (soft unformed stools); 2 = moderate (perianal staining and loose stools); and 3 = severe (watery stools and staining over legs and abdomen) (Wardill et al., 2016). Mice were euthanised if they recorded a 15% or greater weight loss from baseline or had significant distress and clinical deterioration.

Chemotherapy-induced pain was measured 3 x daily in a blinded manner, using a validated rodent facial grimace scoring system (Langford et al., 2010; Wardill et al., 2016). Each criteria (orbital tightening, cheek bulge, nose bulge, ear, and whisker position) was scored as: 0 = absent; 1 = present; or 2 = severe. The maximum total score was 10.

3.4.1.5 Tissue collection and preparation

In all studies, at necropsy, the gastrointestinal tract was removed from the pyloric sphincter to the rectum. The small and large intestine were flushed with chilled, sterile 1 x PBS (pH = 7.4) and weighed. Samples of each region of the gastrointestinal tract were collected and drop-fixed in 10% formalin (ChemSupply, Gillman, Australia) before embedding in paraffin (Sigma-Aldrich, St Louis, USA).

3.4.1.6 Tissue analysis

Haematoxylin and Eosin (H&E) stain and analysis was completed as per previous literature to assess irinotecan-induced gastrointestinal damage in the distal colon only (Wardill et al., 2016). There were 6 criteria: disruption of surface enterocytes; crypt loss/architectural disruption; disruption of crypt cells; infiltration of polymorphonuclear cells and lymphocytes; dilation of lymphatics and capillaries; and oedema; with a 0-2 scale, where 0 = absent, 1 = present and 2 = severe. The maximum total score was 12.

3.4.1.7 Statistical analysis

Data were compared using Prism version 9.0 (GraphPad Software, San Diego, USA). The assumptions of equality of variance for each group and normally distributed data were tested using Bartlett's test and D'Agostino & Pearson omnibus normality test, respectively. If data was normally distributed, 2-way analysis of variance (ANOVA) tests were used, and data were represented as means ± standard error of the means (SEMs). If these assumptions were violated, non-parametric equivalent tests were performed, including Kruskal-Wallis tests for independent data and data were represented as medians. Diarrhoea proportions were analysed by a Chi-squared test. P-values less than 0.05 were considered statistically significant.

3.4.2 Results

3.4.2.1 FMT tolerance

FMT was tolerated well, with no adverse events e.g. bowel perforation or bleeding. There was no weight change from baseline, diarrhoea or facial pain scoring in the period between the final FMT treatment and irinotecan administration (data not shown).

3.4.2.2 Diarrhoea levels

All mice receiving the vehicle only, or FMT and vehicle, did not develop diarrhoea (figure 3.6). All mice who received irinotecan developed diarrhoea by 36 hours post-treatment (figure 3.6). In WT mice receiving FMT and irinotecan, one of four mice developed grade 3 diarrhoea. Three of four WT mice who received irinotecan alone developed grade 3 diarrhoea. *Tlr4*$^{\Delta IEC}$ mice did not develop grade 3 diarrhoea at any time point. There was a difference in diarrhoea profiles between WT mice who received irinotecan (with or without FMT) compared to those who received vehicle (Chi-square, P = 0.03), and *Tlr4*$^{\Delta IEC}$ mice who received irinotecan (with or without FMT) compared to those who received vehicle (Chi-square, P = 0.01). There were no differences between any groups that received FMT (P > 0.05).

3.4.2.3 Pain levels

All mice treated with irinotecan had a peak in facial pain by 24 hours post-irinotecan treatment, after which scores decreased (figure 3.7 A). There were no differences between groups. Mice treated with irinotecan only recorded a

second increase in pain scoring at the 72 hours post-treatment time point. Mice treated with FMT and irinotecan had a median facial pain score of 1.5 or less (WT = 1, *Tlr4*$^{\Delta IEC}$ = 1.5) at 72 hours post-treatment. Mice treated with irinotecan alone had a median facial pain score of 4 or above(WT = 5, *Tlr4*$^{\Delta IEC}$ = 4) at 72 hours post treatment. Data in figure 3.7 A is presented as medians, as data was non-parametric.

3.4.2.4 Weight loss

All mice treated with irinotecan lost weight following irinotecan treatment (figure 3.7 B). At 72 hours post-treatment, WT mice treated with irinotecan only had lost 19.1 (± 0.5) % weight from baseline, whereas their *Tlr4*$^{\Delta IEC}$ counterparts, lost 13.4 (± 3.2) % weight from baseline. WT mice who received FMT and irinotecan lost 11.0 (± 2.4) % weight, and *Tlr4*$^{\Delta IEC}$ mice who received FMT and irinotecan lost 6.3 (± 1.1) % from baseline at 72 hours. However, none of these differences were significantly different ($P > 0.05$).

3.4.2.5 Organ weights

FMT or irinotecan did not cause any changes in the weight of small intestine, large intestine, liver, kidneys, spleen or stomach between groups (data not shown).

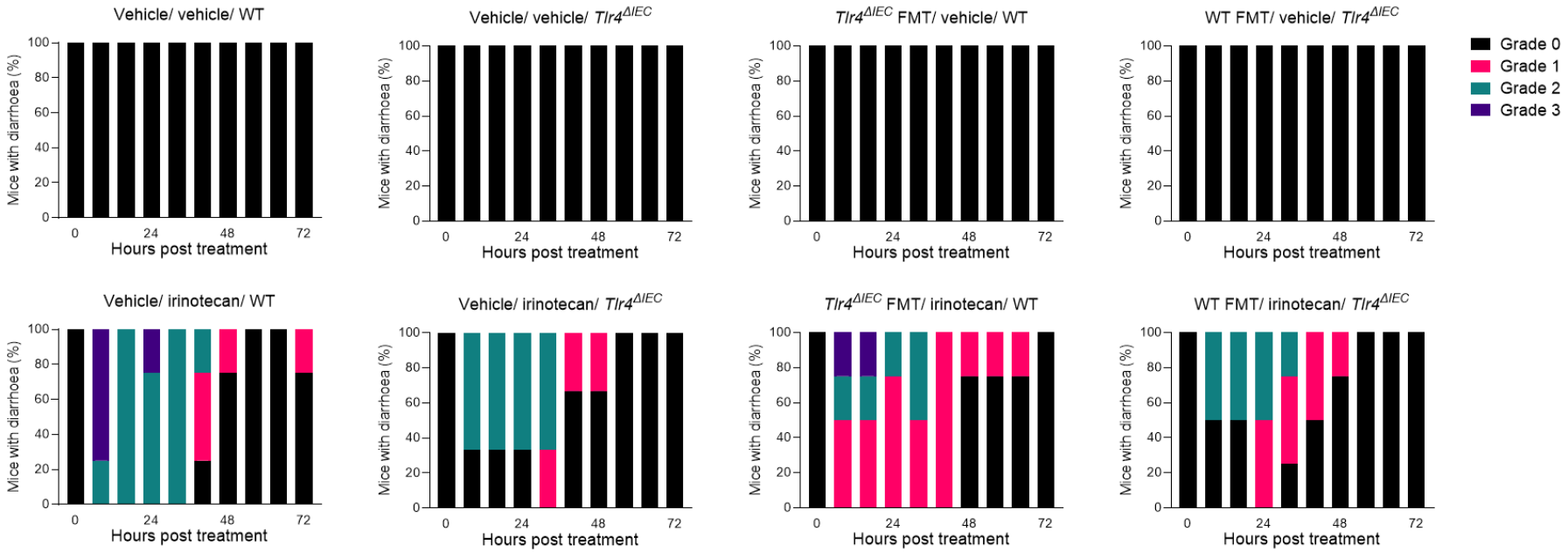

Figure 3.6: Diarrhoea profiles from FMT and irinotecan study. Diarrhoea profiles for mice treated with irinotecan or vehicle, 0 – 72 hours post treatment. *Tlr4*$^{\Delta IEC}$ mice did not develop grade 3 diarrhoea. Groups labelled as FMT treatment / irinotecan treatment / mouse genotype.

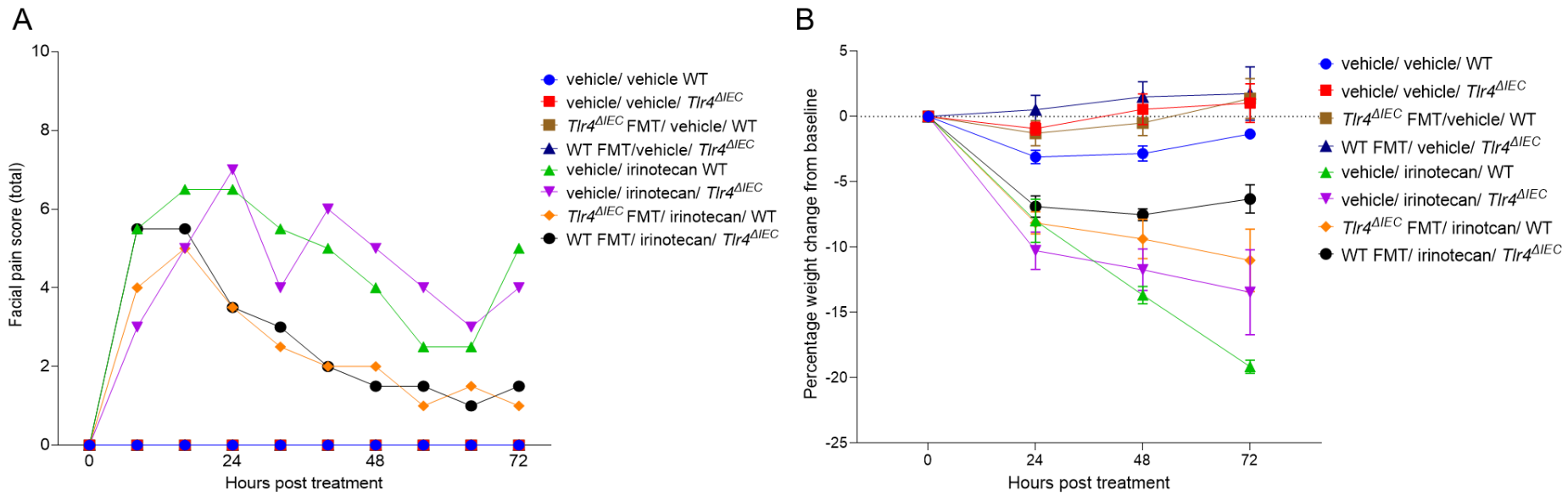

Figure 3.7: Clinical symptoms from FMT and irinotecan study. (A) Facial pain scores 0 – 72 hours following irinotecan / vehicle treatment, median scores shown. (B) Weight loss over the 72 hour time course. Data displayed as a percentage of weight change from baseline (at irinotecan / vehicle administration), mean ± SEM shown.

3.4.2.6 Colonic histopathology

Histopathology scoring of the distal colon showed an increase in damage score in the WT vehicle/irinotecan group compared to the WT vehicle/vehicle group ($P = 0.013$, figure 3.8). This difference was not observed in *Tlr4*$^{\Delta IEC}$ mice treated with irinotecan only ($P > 0.05$) or with any mice who received FMT ($P > 0.05$). Crypt cell loss and architectural derangement were among the characteristics observed in the WT vehicle/irinotecan group.

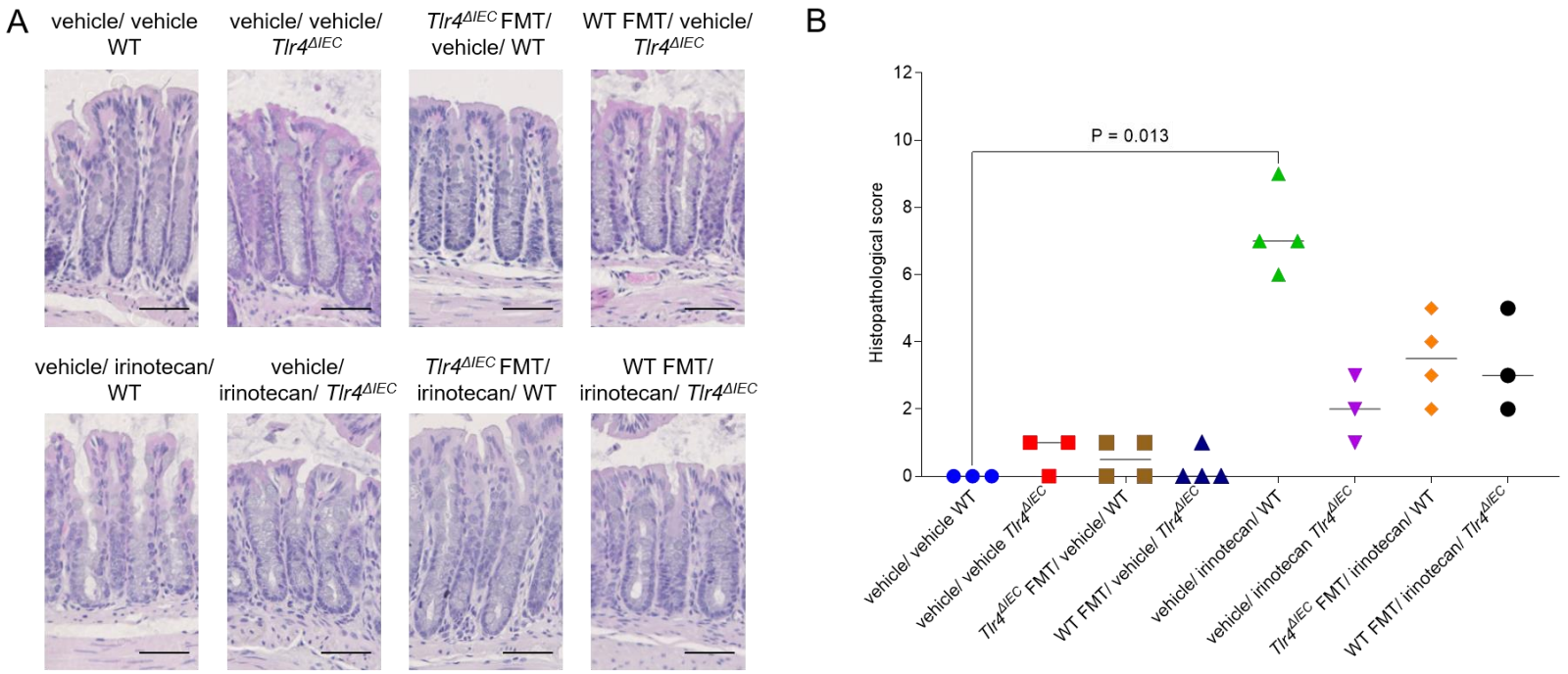

Figure 3.8: Intestinal histopathological injury. (A) Representative images of haematoxylin and eosin staining in the distal colon: original magnification is 400 ×; scale bars represent 50 μm as shown in images. (B) Histological damage scoring in the distal colon, lines are medians. There was an increase in damage due to irinotecan only in the WT group compared to vehicle / vehicle WT group (P = 0.013). This difference was not seen in the *Tlr4*$^{\Delta IEC}$ mice or in any mice treated with FMT. Statistical significance determined using a Kruskal–Wallis test. Damage scored on a scale of 0-12. One tissue block was not available in the FMT/irinotecan *Tlr4*$^{\Delta IEC}$ group.

3.4.3 Discussion

Here, I used the GRAFT recommendations to develop a pilot model of FMT in WT and *Tlr4*$^{\Delta IEC}$ mice given irinotecan treatment. In this small study, FMT was tolerated well and did not by itself, cause any diarrhoea, weight loss, facial pain or histological damage. Following irinotecan, there were differences in diarrhoea profile between mice who received irinotecan compared to those who received vehicle only. In addition, there were less WT mice who received FMT and irinotecan who then subsequently developed grade 2 and grade 3 diarrhoea than WT mice who received only irinotecan. Histopathology scoring showed a significant increase in damage in WT mice that received irinotecan only compared to vehicle. This increase was not seen in *Tlr4*$^{\Delta IEC}$ mice or mice who additionally received FMT.

This pilot study clearly demonstrated the methodology was feasible, and could be conducted on a larger scale to confirm these results. As reported in chapter 2, there were no clear differences in microbial composition between WT and *Tlr4*$^{\Delta IEC}$ mice. However, the FMT procedure could potentially transfer beneficial metabolites, or a higher overall bacterial load (Nusbaum et al., 2018) that could be protective against irinotecan-induced gastrointestinal toxicity. Alternatively, the FMT may cause a protective immune response that primes the gastrointestinal microenvironment (Pamer, 2014; Burrello et al., 2018) prior to irinotecan treatment.

Microbe-immune co-development and signalling may be important for mediating a response to injury. Therefore, the actual composition of the microbiome itself

may not be as important, but rather the fine-tuning with the mucosal immune system. Although *Tlr4*$^{\Delta IEC}$ mice have no measurable differences in microbiome (chapter 2), they had reduced levels of symptoms following irinotecan treatment compared to WT mice. Additionally, so far, only bacteria, via 16S sequencing, has been assessed. Other components of the microbiome e.g. viruses or phages have not been assessed in the area of CIGT to date, and may play an important role in toxicity development.

Future studies could additionally assess the use of autologous FMT to reduce irinotecan-induced diarrhoea, a technique that has previously been successfully used both pre-clinically and clinically to improve gastrointestinal function (Taur et al., 2018; Perez-Matute et al., 2020). As irinotecan has been shown to disrupt the microbiome (Stringer et al., 2009b), an autologous FMT could be prepared from faecal samples taken prior to irinotecan treatment, and then administered shortly before peak diarrhoea occurs. In this study peak diarrhoea occurred in FMT groups between 8-16 hours post-irinotecan administration.

There were some limitations in methodology of this pilot FMT study that need to be investigated before continuing on to a larger study. Firstly, as this was a small study, a larger study would have more statistical power to detect differences between groups. In this study, I did not use an anaerobic chamber to prepare the FMT solution due to equipment availability. In addition, due to time constraints I did not do bacterial sequencing to confirm whether the microbiome of the recipient changed due to the FMT procedure (however these samples were collected for future analysis). There is also conflicting evidence as to whether it is beneficial to remove microbial load from recipient mice via

antibiotic treatment prior to FMT. I followed the research of Freitag et al. (2019), who's findings suggested that pre-treatment with broad-spectrum antibiotics was unable to improve overall engraftment of the donor FMT, however, future studies may be useful in determining if this result is reproducible in other models.

3.5 Final conclusions

This systematic review aimed to determine the most common protocols for FMT experiments in pre-clinical mice models. The key overarching finding of the systematic review was that many of the details required to reproduce these protocols were missing from the majority of papers. In the future, researchers should clearly outline their protocols in order to provide transparency, increase reproducibility and ultimately improve the chances of clinically relevant and translatable knowledge. In the pilot FMT study, developed using the GRAFT recommendations, I clearly demonstrated FMT was well tolerated in WT and *Tlr4*$^{\Delta IEC}$ mice. I also demonstrated FMT had no impact on the development of gastrointestinal toxicity following irinotecan. A larger study in this model would be useful to confirm these results, as well as to determine the content of the FMT product and any changes that occur to the recipient's microbial composition.

3.6 Supplementary Material

Supplementary Material 1: Systematic review search strategies

Note: Rat studies were initially included in the search strategy but were removed at a later date due to a refinement of the original research topic.

Ovid Medline:

(Fecal OR faecal OR cecal OR caecal OR microbi*).ti,ab adj5 (Transplant* OR transfer* OR engraftment OR innocul*).ti,ab AND (Mice OR mouse OR rat OR rats OR murine).ti,ab

PubMed:

"Fecal Microbiota Transplantation"[mh] OR Fecal microbiota transplant[tiab] OR Fecal microbiota transfer[tiab] OR Fecal microbiota engraftment[tiab] OR Faecal microbiota transplant[tiab] OR Faecal microbiota transfer[tiab] OR Faecal microbiota engraftment[tiab] OR fecal microbiota inoculum[tiab] OR faecal microbiota inoculum[tiab] OR microbiome transplant[tiab] OR microbiome transfer[tiab] OR microbiome engraftment[tiab] OR microbiome transplant[tiab] OR microbiome transfer[tiab] OR microbiome engraftment[tiab] OR microbiome inoculum[tiab] OR microbiome inoculum[tiab] OR cecal microbiota transplant[tiab] OR cecal microbiota transfer[tiab] OR cecal microbiota engraftment[tiab] OR caecal microbiota transplant[tiab] OR caecal microbiota transfer[tiab] OR caecal microbiota engraftment[tiab] OR cecal microbiota

inoculum[tiab] OR caecal microbiota inoculum[tiab] AND Mice[mh] OR mice[tiab] OR mouse[tiab] OR murine[tiab] OR rats[mh] OR rat[tiab]

EMBASE:

“Fecal Microbiota Transplantation”/exp OR ‘Fecal microbiota transplant’:ti,ab OR ‘Fecal microbiota transfer’:ti,ab OR ‘Fecal microbiota engraftment’:ti,ab OR ‘Faecal microbiota transplant’:ti,ab OR ‘Faecal microbiota transfer’:ti,ab OR ‘Faecal microbiota engraftment’:ti,ab OR ‘fecal microbiota inoculum’:ti,ab OR ‘faecal microbiota inoculum’:ti,ab OR ‘microbiome transplant’:ti,ab OR ‘microbiome transfer’:ti,ab OR ‘microbiome engraftment’:ti,ab OR ‘microbiome transplant’:ti,ab OR ‘microbiome transfer’:ti,ab OR ‘microbiome engraftment’:ti,ab OR ‘microbiome inoculum’:ti,ab OR ‘microbiome inoculum’:ti,ab OR ‘cecal microbiota transplant’:ti,ab OR ‘cecal microbiota transfer’:ti,ab OR ‘cecal microbiota engraftment’:ti,ab OR ‘caecal microbiota transplant’:ti,ab OR ‘caecal microbiota transfer’:ti,ab OR ‘caecal microbiota engraftment’:ti,ab OR ‘cecal microbiota inoculum’:ti,ab OR ‘caecal microbiota inoculum’:ti,ab AND “Mouse”/exp OR mice:ti,ab OR mouse:ti,ab OR murine:ti,ab OR “rat”/exp OR rat:ti,ab

Supplementary Material 2: Studies included in final systematic review

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Becker, L., Spear, E. T., Sinha, S. R., Haileselassie, Y. and Habtezion, A. (2019). Age-Related Changes in Gut Microbiota Alter Phenotype of Muscularis Macrophages and Disrupt Gastrointestinal Motility. *Cell Mol Gastroenterol Hepatol* 7(1): 243-245 e2.

Beller, A., Kruglov, A., Durek, P., von Goetze, V., Werner, K., Heinz, G. A., et al. (2020). Specific microbiota enhances intestinal IgA levels by inducing TGF-beta in T follicular helper cells of Peyer's patches in mice. *Eur J Immunol* 50(6): 783-794.

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Cui, M., Xiao, H., Li, Y., Zhou, L., Zhao, S., Luo, D., et al. (2017). Faecal microbiota transplantation protects against radiation-induced toxicity. *EMBO Mol Med* 9(4): 448-461.

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Supplementary Material 3: Characteristics of antibiotics used in studies included for analysis

Parameter investigated	**Categories**	**# studies**
Antibiotics used	Yes	42
	No	45
Cocktail vs single	Cocktail	33
	Single	8
	Not reported	1
Most common combination	Ampicillin/Neomycin/Vancomycin/Metronidazole	22
Antibiotics used	Total types of antibiotics used	12
	Ampicillin	31
	Vancomycin	30
	Metronidazole	30
	Neomycin	26
	Streptomycin	7
	Ciprofloxacin	3
	Gentamicin	3
	Penicillin	2
	Amphotericin	2
	Imipenem	2
	Colistin	1
	Amoxicillin	1
	Mean number of antibiotics (single or cocktail) used per study	3.29

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Chapter 4: Diarrhoea induced by small molecule tyrosine kinase inhibitors compared to chemotherapy: potential role of the microbiome

This chapter is my second literature review, which provides a new hypothesis on the similarities between diarrhoea induced by tyrosine kinase inhibitors and chemotherapy. This was an invited review published in Integrative Cancer Therapies (*Secombe, K. R., Van Sebille, Y. Z. A., Mayo, B. J., Coller, J. K., Gibson, R. J. and Bowen, J. M. (2020). Diarrhea Induced by Small Molecule Tyrosine Kinase Inhibitors Compared With Chemotherapy: Potential Role of the Microbiome. Integr Cancer Ther 19: 1-12.*). This chapter is presented in its original publication format. The referencing style, spelling and figure/table numbers have been modified to maintain consistency throughout this thesis.

4.1 Abstract

Small molecule receptor tyrosine kinase inhibitors (SM-TKIs) are among a group of targeted cancer therapies, intended to be more specific to cancer cells compared to treatments such as chemotherapy, hence reducing adverse events. Unfortunately, many patients report high levels of diarrhoea, the pathogenesis of which remains under investigation. Here we compare the current state of knowledge of the pathogenesis of chemotherapy-induced diarrhoea (CID) in comparison to SM-TKI-induced diarrhoea, and investigate how a similar research approach in both areas may be beneficial. To this end, we review evidence that both treatment modalities may interact with the gut

microbiome, and as such the microbiome should be investigated for its ability to reduce the risk of diarrhoea.

4.2 Introduction

4.1 Abstract

Small molecule receptor tyrosine kinase inhibitors (SM-TKIs) are among a group of targeted cancer therapies, intended to be more specific to cancer cells compared to treatments such as chemotherapy, hence reducing adverse events. Unfortunately, many patients report high levels of diarrhoea, the pathogenesis of which remains under investigation. Here we compare the current state of knowledge of the pathogenesis of chemotherapy-induced diarrhoea (CID) in comparison to SM-TKI-induced diarrhoea, and investigate how a similar research approach in both areas may be beneficial. To this end, we review evidence that both treatment modalities may interact with the gut microbiome, and as such the microbiome should be investigated for its ability to reduce the risk of diarrhoea.

4.2 Introduction

Gastrointestinal toxicity, commonly manifesting as diarrhoea, is a common side effect of a range of cancer treatments including chemotherapy and tyrosine kinase inhibitor targeted therapy. This toxicity is currently without a specific prevention or treatment strategy, and can affect between 50-80% of patients, depending on their treatment protocol (Gibson et al., 2009). Diarrhoea, and associated intestinal ulceration, can lead to a host of severe issues including

dehydration, malnutrition, fatigue, renal insufficiency and increased risk of systemic infection (Stein et al., 2010). In addition to the severe compromise in quality of life, for people experiencing one or more of these side effects, treatment breaks or dose reductions are likely, leading to less effective cancer care and compromised remission rate (Di Fiore et al., 2009). Available economic data suggests each episode of severe diarrhoea requiring hospitalisation may cost up to $6616 USD, however this may be accompanied by broader costs related to emergency medical procedures or loss of productivity from time out of work (Elting et al., 2004; Carlotto et al., 2013; Vouk et al., 2016). As such, effective preventative and treatment strategies for cancer treatment-induced diarrhoea are critically needed. By understanding the pathogenesis of this diarrhoea, identification of appropriate treatment targets may be expedited.

Small molecule tyrosine kinase inhibitors (SM-TKIs) are used to treat a variety of solid tumour types including lung, breast and head and neck cancers (Rugo et al., 2019). They are used in combination or as a monotherapy, increasingly as first-line treatment (Hojjat-Farsangi, 2014). Most act by binding to the intracellular adenosine triphosphate (ATP) domain of the tyrosine kinase, preventing downstream signaling and subsequent cell division and growth(Jiao et al., 2018). While there are a large variety of targets, the most common are the Epidermal Growth Factor Receptors (EGFR) and Vascular Endothelial Growth Factor Receptors (VEGFR) (Hojjat-Farsangi, 2014). SM-TKI diarrhoea usually occurs in the first week of the treatment course and is typically managed with anti-diarrhoeal agents such as loperamide (Takeda et al., 2015;

McQuade et al., 2016). However for many patients, loperamide does not sufficiently reduce diarrhoea, and/or causes side effects including fatigue, constipation and abdominal pain (McQuade et al., 2016; Upadhyay et al., 2016). Thus there is merit in investigating new targets for this diarrhoea.

Comparatively, the mechanism of diarrhoea stemming from chemotherapy has had far more research than mechanisms of diarrhoea from SM-TKI treatments (Loriot et al., 2008). This is in part likely due to the length of time these treatments have been available. Chemotherapy-related diarrhoea occurs due to direct damage to the intestine, initiating a host of inflammatory pathways eventually leading to ulceration and potential bacterial translocation (Bowen et al., 2019). It was previously assumed that SM-TKI and chemotherapy toxicity had the same pathogeneses, however recent research has posited that they have different initiating events and mechanisms, and are therefore specific, separate toxicities (Loriot et al., 2008; Bowen, 2014). While it is now clear that there are treatment-specific mechanisms that differ between SM-TKI induced-diarrhoea and CID (similarly to CID and radiation-induced diarrhoea), there is a strong evidence base, explored here, to suggest they share core mechanisms relating to direct mucosal damage, changes to chloride secretion and upregulation of inflammatory processes (Van Sebille et al., 2015; Secombe et al., 2019a). Given the variance in the literature surrounding the mechanisms of cancer treatment-induced diarrhoea, we propose that taking a similar research approach to elucidate the causes of both chemotherapy- and SM-TKI-induced diarrhoea may be beneficial in further building our understanding.

The gut microbiome is gaining momentum as a key research and therapeutic target in cancer treatment -induced toxicities (Bowen et al., 2019). Changes in the microbiome following chemotherapy have been clearly shown, however we do not yet fully understand how to manipulate the microbiome, or determine the microbial-mucosal interactions that accelerate gut toxicity and diarrhoea (Bowen et al., 2019). In SM-TKI treatments, initial indications suggest altered microbial composition in people experiencing more severe diarrhoea (Pal et al., 2015). While there appears to be differences in the mechanisms of SM-TKI induced-diarrhoea and CID, these results suggest that both are underpinned by changes in the microbiome. Additionally, when the microbiome is compromised via antibiotics in both treatment modalities, diarrhoea and treatment efficacy outcomes are altered (van Vliet et al., 2009; Viaud et al., 2013; Liu et al., 2019). Given the suggestion that the microbiome may be a common and targetable mechanism of both SM-TKI induced-diarrhoea and CID, we propose that similar to the research into the microbiome in CID, a similar research approach is taken with SM-TKI-induced diarrhoea.

This review will focus on comparing the pathogenesis of diarrhoea stemming from highly mucotoxic chemotherapies (5-fluorouracil (5-FU) and irinotecan) with diarrhoea induced by EGFR and VEGFR inhibitors used for the treatment of solid tumours. The benefits and drawbacks of taking a similar research approach to determining the mechanisms of both of these types of diarrhoea will be explored. Additionally, we will examine the potential of the gut microbiome to play a key role in both of these treatment modalities.

4.3 Diarrhoea incidence in chemotherapy and SM-TKI cancer treatments

4.3.1 Chemotherapy

Cytotoxic chemotherapies irinotecan and 5-FU are two agents known to cause high levels of gastrointestinal toxicity, with up to 80% of patients developing at least some level of diarrhoea (Stein et al., 2010). The National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) classifies a diarrhoea grading over 3 (increase of ≥7 stools per day over baseline; hospitalisation indicated; severe increase in ostomy output compared to baseline; limiting self-care activities of daily life) as severe (2006). As single agents, 5-FU (bolus) has rates of up to 32% severe diarrhoea, and weekly irinotecan has rates of up to 22% severe diarrhoea (Stein et al., 2010). Combination therapy, often in the form of FOLFIRI (5-FU, folinic acid and irinotecan) is commonly used in a variety of cancer types (table 4.1) and is also associated with high risk of gastrointestinal damage (severe diarrhoea = 12 - 20%) (Falcone et al., 2007; Ribeiro et al., 2016). Adverse effects of these highly mucotoxic chemotherapies are often managed with loperamide, octreotide or non-pharmacological interventions such as oral rehydration, but still commonly require treatment breaks and dose reductions (Lalla et al., 2014; Ribeiro et al., 2016).

4.3.2 SM-TKIs

SM-TKIs are growing in use as cost-effective, orally administered agents known to inhibit extremely important oncological targets in a range of cancers (table 4.1) (Linger et al., 2008; Gaumann et al., 2016). After dermatologic toxicity, diarrhoea is the most common side effect of SM-TKI treatment, and is often severe enough to require a break in treatment or a dose reduction (Rugo et al., 2019). Unlike chemotherapy, it is important to consider that SM-TKI treatments are often taken daily for long periods (months - years), meaning that even low grade diarrhoea can have significant impacts on patient quality of life. Therefore, proactive and clinically effective management of side effects is required to enable treatment to continue successfully, and reduce the risk of chronic side effects that are less common in the relatively acute chemotherapy setting (Bowen, 2013).

EGFR TKIs, a common group of SM-TKIs, are associated with diarrhoea that typically occurs early in a treatment course (often within the first week), similar to chemotherapy (Rugo et al., 2019). Severe diarrhoea is common, occurring in up to 25% of cases (table 4.1), requiring individualised management (lowered dosing, diet modification and intravenous fluids and electrolytes) to reduce the risk of hospitalisation and treatment delays (Rugo et al., 2019). Clinical data has shown patients receiving second generation multi-EGFR SM-TKIs, such as afatinib and dacomitinib, have a higher incidence of severe, grade 3 diarrhoea compared to first generation agents (e.g. gefitinib), which are more target specific and less likely to inhibit other tyrosine kinase groups (Takeda et al., 2015). Diarrhoea associated with VEGFR-TKIs in monotherapy is

predominantly mild to moderate (table 4.1), but needs to be managed as early as possible (with anti-diarrhoeal agents including loperamide) in order to avoid symptoms progressing to more severe, higher grade diarrhoea (Liu et al., 2018). VEGFR-TKIs are also often given in combination with chemotherapy, which can cause a compound effect of more severe diarrhoea (Liu et al., 2018). For example, in the ICON6 trial of combination platinum-based chemotherapy and VEGF inhibitor cediranib, 39% of people in the intervention arm had to stop the trial early with highly toxic diarrhoea and fatigue (Ledermann et al., 2016). Anecdotal evidence has also suggested that diarrhoea following cediranib treatment leads to a sustained reduction in health-related quality of life (Liu et al., 2018).

4.4 Chemotherapy toxicity in comparison to SM-TKI toxicity

4.4.1 Chemotherapy

Currently, diarrhoea from chemotherapy treatment is largely understood to follow the 5-phase model proposed in 2004, and updated in 2010 and 2019 (Sonis, 2004b; Sonis, 2010; Bowen et al., 2019). Briefly, initiating events such as reactive oxygen species generation and DNA damage lead directly to transcription factor activation (e.g. nuclear factor kappa B (NF-κB)) and pro-inflammatory cytokine release, leading to apoptosis and mucosal ulceration (Sonis, 2004a; Bowen et al., 2019). This eventual thinning of the mucosal layer from activation of these inflammatory pathways can lead to bacterial translocation and an inability to properly absorb fluids from the intestine, eventuating in diarrhoea. In this vein, indirect biological signalling is the basis of

much of the damage, as opposed to direct tissue damage by chemotherapy itself. Current research is attempting to understand the connection of the microbiome to the host immune response underlying this signalling, specifically targeting the role of inflammation and the enteric nervous system (Bowen et al., 2019).

4.4.2 SM-TKI: direct target inhibition

SM-TKI-induced diarrhoea is likely to be multi-factorial, and there are multiple hypotheses currently under investigation. One such hypothesis surrounds the inhibition of the specific kinase targets in the intestinal epithelium. EGFR and VEGFR are both highly expressed in the gut, and inhibition in the intestine leads to lowered cell proliferation, and reduced capillary networks in the intestinal villi respectively (Fan et al., 1998; O'Brien et al., 2002; Kamba et al., 2006; Echavarria et al., 2017). EGFR pathways also have stimulatory effects on enterocyte proliferation and nutrient and electrolyte transport, causing structural and functional changes when inhibited (Van Sebille et al., 2015). These changes in the intestinal architecture may lead to mucosal atrophy and thereby a reduction in the absorptive capacity of the gut. However, many SM-TKI agents have multi-targeted activity. For example, sorafenib is able to block tyrosine kinases in the VEGFR, PDGFR, BRAF, KIT, FLT3 and RET families. It is therefore challenging to determine how inhibition of which particular kinase targets affects diarrhoea levels and how this could be used to reduce diarrhoea without affecting treatment efficacy (Kitagawa et al., 2013; Rugo et al., 2019). In EGFR SM-TKIs; second generation agents, which have a broader inhibitory profile, have more diarrhoea of any level, and also more severe diarrhoea

compared to first generation agents (Rugo et al., 2019). Diarrhoea is also the most frequent adverse event for the FDA approved third-generation agent osimertinib, which targets the T790M mutation of EGFR, but has a low affinity for wild-type EGFR (Gao et al., 2016). In a phase I escalation trial, diarrhoea increased with escalating doses of osimertinib, suggesting that direct target inhibition by osimertinib may be involved in the development of diarrhoea (Gao et al., 2016). In a larger phase III clinical trial of 253 patients with non-small cell lung cancer, 47% of patients developed diarrhoea (Janne et al., 2015; Tan et al., 2018). Similar to multi-kinase EGFR SM-TKIS, in VEGFR SM-TKIs, diarrhoea is observed at higher levels in the more common multi-kinase inhibitors such as sorafenib compared to pure VEGFR inhibitors (Schmidinger, 2013; Liu et al., 2018).

4.4.3 SM-TKI: chloride secretion

Despite evidence that direct inhibition of EGFR and VEGFR may drive diarrhoea following treatment, pre-clinical SM-TKIs have shown differing results around intestinal histopathological damage. Multiple SM-TKI studies have demonstrated a lack of this histopathological damage (Bowen et al., 2014; Van Sebille et al., 2017; Secombe et al., 2019a). As this damage is a hallmark of chemotherapy-induced gastrointestinal toxicity, this vast histopathological difference has been a driving factor for the hypothesis that SM-TKI-induced diarrhoea has a distinctly different mechanism than CID. However, in a recent pre-clinical study, the EGFR-targeting SM-TKI dacomitinib caused significant blunting and fusion of the villi in the ileum (Van Sebille et al., 2017), challenging the notion that chemotherapy and SM-TKI-induced toxicities are unrelated. One

recent hypothesis has theorised that diarrhoea from SM-TKIs (in particular EGFR TKIs) had a secretory phenotype (Loriot et al., 2008; Van Sebille et al., 2015). In secretory diarrhoea, activation of chloride channels including the cystic fibrosis transmembrane conductance regulator (CFTR) and calcium activated channels increases fluid secretion into the lumen, and inhibition of intestinal sodium transport lowers fluid absorption (Thiagarajah et al., 2015). In the intestine, EGFR has an inhibitory effect on chloride secretion, and it has therefore been hypothesised that SM-TKI inhibition of EGFR allows excessive chloride secretion into the gut lumen (Van Sebille et al., 2015). Studies have additionally shown that in *ex vivo* models, potassium channels as well as CFTR chloride channels are directly activated by EGFR TKI treatments including afatinib, gefitinib and lapatinib (Duan et al., 2019). Additionally, pre-clinical rat studies have suggested that EGFR inhibitor dacomitinib-induced diarrhoea was of a secretory form, and clinical studies of neratinib show a faecal osmotic gap consistent with secretory diarrhoea (Abbas et al., 2012; Van Sebille et al., 2017). Crofelemer is an anti-chloride secretory medication currently being trialled clinically to reduce diarrhoea in HER2+ breast cancer being treated with EGFR targeting monoclonal antibodies (NCT02910219) (Gao et al., 2017; Pohlmann, 2018). However, targeting secretory changes in a dacomitinib rat model using crofelemer actually worsened diarrhoea levels (Van Sebille et al., 2015; Van Sebille et al., 2017). In addition, pre-clinical studies of neratinib and lapatinib have both shown no changes in serum chloride levels (Bowen, 2013; Secombe et al., 2019a). Collectively, this leaves the role of chloride secretion unclear and requiring further investigation.

4.4.4 SM-TKI: Inflammation

Inflammation is known to have a key role in the development of CID (Secombe et al., 2019a), however has only more recently been considered as a factor in SM-TKI-induced diarrhoea. In chemotherapy, upregulation of inflammatory pathways is a key part of the 5-phase model. Inflammasome activation and pro-inflammatory cytokine release has been shown to mediate irinotecan-induced gastrointestinal damage and diarrhoea (Arifa et al., 2014; Lima-Junior et al., 2014). In addition, 5-FU treatment causes upregulation of a host of pro-inflammatory cytokines including tumour necrosis factor alpha, interleukin-1 beta, interleukin-6, interleukin-17A and interleukin-22 (Sakai et al., 2013). Activation of the Toll-like receptor (TLR) signalling pathways leads to upregulation of many of these inflammatory mediators, and has recently been implicated in irinotecan-induced diarrhoea (Wardill et al., 2016). A mouse model demonstrated TLR4 knockout mice had reduced diarrhoea and other clinical indicators of gastrointestinal toxicity (Wardill et al., 2016). Similarly, a pre-clinical study of diarrhoea from neratinib also showed potential for inflammation to be involved by demonstrating that budesonide, a locally acting corticosteroid, reduced levels of diarrhoea and colonic injury, as well as increasing levels of anti-inflammatory interleukin-4 levels (Secombe et al., 2019a). This result has been reflected in data reported by Liu and Kurzrock showing a marked alleviation in EGFR-TKI-induced diarrhoea following budesonide administration (Liu et al., 2014). Further, the STEPP trial of panitumumab in colorectal cancer showed that patients receiving prophylactic doxycycline for skin toxicity developed less diarrhoea than patients on a reactive skin treatment regimen

(15% vs 32%, n=95) (Lacouture et al., 2010). While panitumumab is an EGFR targeting monoclonal antibody, its side effects display many similar features of SM-TKI treatment (Fakih et al., 2010). It has since been suggested that as doxycycline may have acted as an anti-inflammatory, the diarrhoea may have had an inflammatory component (Hirsh et al., 2014). Alternatively, it could be speculated that the antibiotic nature of doxycycline altered the gut microbiota to influence diarrhoea development through other pathways. Together, these findings have suggested that while SM-TKIs and chemotherapies induce diarrhoea via different preliminary mechanisms, initiation of inflammatory processes may be a key contributing factor to its development following both treatment types.

Table 4.1: Comparison of diarrhoea incidence in highly mucotoxic chemotherapies and VEGFR and EGFR TKIs. VEGFR and EGFR TKIs listed are FDA approved for cancer treatment with diarrhoea incidence results from Phase III trials or clinical use studies.

Treatment type	**Agent**	**Target**	**Indication**	**Diarrhoea incidence**		**Reference**
				All grades (%)	**Severe (Grade 3+) (%)**	
Chemotherapy	Irinotecan	Topoisomerase 1	Primarily colorectal cancer	60-90%	20-40 % (single agent)	Mego et al. (2015), Michael et al. (2004); Stein et al. (2010)
	5-fluorouracil	Thymidylate synthase	Primarily colorectal cancer	Up to 50%	32% (bolus) 6-13% (CI)	Stein et al. (2010)
VEGFR TKI	Sorafenib	VEGFRs, PDGFRs, BRAF, KIT, FLT3, RET	Renal, hepatocellular and prostate cancers	30-50%	>10%	Stein et al. (2010); Que et al. (2018)
	Sunitinib	VEGFRS, PDGFRs, FLT3, TIE-2, RET, KIT, CSF1-R	Gastrointestinal stromal tumour and renal cancers	30-50%	>10%	Stein et al. (2010); Que et al. (2018)
	Regorafenib	VEGFRs, PDGFRs, RAF, KIT, TIE-2, FGFR, PlGF, RET and BRAF	Colorectal cancers and gastrointestinal stromal tumours	33%	4-8.5%	Carrato et al. (2019), Yin et al. (2018)
	Pazopanib	VEFGRs, PDGFRs, KIT, FGFR, TIE-2	Renal cancers and soft tissue sarcomas	33-52%	2-4%	Santoni et al. (2014), Que et al.

						(2018), Sternberg et al. (2010)
	Axitinib	VEGFR, eNOS, AKT, ERK1/2, PDGFR, KIT	Renal cell carcinoma	55%	11%	Rini et al. (2011)
	Vandetanib	VEGFR, MET, RET, EGFR	Advanced carcinomas (e.g. medullary thyroid cancers and non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), breast cancer, malignant glioma, hepatocellular cancer)	52.1%	5.6%	Miller et al. (2013), Huo et al. (2016), Pozo et al. (2018), Yin et al. (2019)
	Lenvatinib	VEGFR, EGFR, RET	Thyroid cancer, Renal cell carcinoma	45-67%	4-9%	Berdelou et al. (2018), Haddad et al. (2017)
	Cabozantinib	VEGFRs, EGFR MET, KIT, RET, AXL, TIE2, TRKB and FLT3	Medullary thyroid cancers	72-75%	10-13%	Schmidinger et al. (2018), Fazio et al. (2019)
EGFR TKI	Gefitinib	HER1/EGFR	NSCLC	27-69%	1-25%	Rugo et al. (2019)
	Erlotinib	HER1/EGFR	NSCLC and pancreatic cancer	18-68%	1-12%	Becker et al. (2010), Rugo et al. (2019), Hirsh et al. (2014)
	Lapatinib	HER1/2	Breast cancer	65%	14%	Rugo et al. (2019), Azim et al. (2013)
	Afatinib	HER 1, 2, 3 and 4	NSCLC	87-95%	5-17%	Tagliamento et al. (2018)
	Neratinib	HER 1, 2 and 4	HER2+ breast cancer	95%	40%	Rugo et al. (2019), Chan et al. (2016)
	Osimertinib	T790M EGFR mutation	NSCLC	41%	1%	Rugo et al. (2019)
	Dacomitinib	HER 1, 2 and 4	NSCLC	72-78%	12-13%	Van Sebille et al. (2016)

4.5 Potential role of the microbiome in chemotherapy and SM-TKI treatment diarrhoea

While it has been widely posited that chemotherapy and SM-TKI treatments induce diarrhoea via different mechanisms, interventions targeting these hypotheses have so far been unable to definitively reduce diarrhoea. Therefore, using similar research methods to that used to elucidate CID may be useful to rule out or more fully understand mechanisms underlying SM-TKI-induced diarrhoea. The gut microbiome could potentially play a key role in both treatments due to its relation to inflammatory responses and chloride secretion (figure 4.1).

4.5.1 Intestinal inflammation

The gut microbiome has been shown to play an integral role in mediating intestinal inflammation. This has been demonstrated in Inflammatory Bowel Disease, where there is a marked decrease in microbial diversity and richness compared to healthy controls (Knox et al., 2019). Similarly, in Crohn's Disease, the treatment-naïve microbiome is strongly correlated with disease status, with increased abundance of *Enterobacteriaceae*, *Pasteurellaceae*, *Veillonellaceae*, and *Fusobacteriaceae* occurring along with decreases in Erysipelotrichales, Bacteroidales, and Clostridiales in people with the disease (Gevers et al., 2014).

The gut microbiome is proposed to mediate these inflammatory responses via the innate immune system, and has a particularly important role in the

development of this system. For example, pre-clinical studies have demonstrated early life exposure to commensal bacteria is required to develop appropriate invariant Natural Killer T cell tolerance (Olszak et al., 2012). Additionally, dysbiosis of the microbiome can alter levels of metabolites from the microbiome such as butyrate. Butyrate is a short-chain fatty acid produced by colonic bacteria by fermenting elements from our dietary intake. It can induce Treg cell development to maintain immune tolerance and maintain the balance between Th17 and Treg cells (Zhou et al., 2018). This balance is highly important in modulating intestinal inflammation. Finally, the gut microbiome and innate immune system are intrinsically linked via many types of Pattern Recognition Receptors (PRRs). TLRs are important in sensing molecular patterns originating from the gut microbiome such as lipopolysaccharide (LPS), that cause activation of downstream signaling pathways of transcription factor (e.g. NF-κB) upregulation and pro-inflammatory cytokine release.

4.5.2 Chloride secretion

In addition, there is an emerging link between gut microbiome composition and intestinal chloride secretion, particularly via CFTR, which allows exit of chloride ions across the apical membrane. Two studies have investigated this link with lubiprostone, used clinically to treat constipation and known to stimulate electrogenic chloride secretion (Keely et al., 2012; Musch et al., 2013). Upregulation of chloride secretion with this agent caused large shifts in the stool microbiome, with an increased abundance of *Lactobacillus* spp. in the stool of lubiprostone-treated mice. It was concluded that epithelial chloride secretion may have a key role in influencing bacterial-epithelial interactions. In addition,

changes to the CFTR have also shown to cause significant gut microbial changes. In a mouse model, CFTR gene mutations were sufficient to alter the gut microbiome (Meeker et al., 2020), and in a clinical study of 31 patients aged 1 - 6 years with cystic fibrosis (who have mutations in the CFTR), it was suggested that gut microbiota enterophenotypes were direct expressions of altered intestinal function (Vernocchi et al., 2018). These studies show the close links between chloride secretion and the gut microbiome. As excess chloride secretion into the intestinal lumen may cause diarrhoea in some SM-TKI treatments, this provides further evidence for SM-TKI-induced diarrhoea to be influenced by gut microbial changes. However, while there is some evidence that probiotic bacteria or pathogenic bacteria can alter chloride secretion (Hecht et al., 1999; Heuvelin et al., 2010), there are low levels of evidence to suggest that the native gut microbiome changes are able to drive chloride channel dysfunction. Future work needs to be done to understand whether microbial dysbiosis is a direct driver of diarrhoea, or whether the diarrhoea itself causes dysbiosis as an outcome.

4.5.3 Microbiome changes due to cancer treatment

Pre-clinical studies have shown marked changes to overall microbiome composition in the gut following chemotherapy treatment, towards a dysbiotic state. The key finding has been a decrease in commensal bacterial species, along with a corresponding increase in pathogenic species (Von Bultzingslowen et al., 2003; Stringer et al., 2008; Stringer et al., 2009b; Lin et al., 2012; Stringer et al., 2013). These pathogenic species were usually gram negative species, which can release LPS known to initiate the inflammatory pathways that are key

mediators in development of diarrhoea (Riehl et al., 2000; Akira et al., 2004). Clinical studies have shown similar findings, with a decrease in total bacterial abundance and diversity, as well as decreases in commensals such as *Lactobacillus* and *Bifidobacteria*, with increases in *Bacteriodetes* and *Escherichia coli.* In addition to these dynamic changes during chemotherapy, the TIMER (translocation, immunomodulation, metabolism, enzymatic degradation, reduced diversity) model proposed by Alexander and colleagues has outlined how the functions of the microbiome may itself have a central role in determining the extent and intensity of diarrhoea (Alexander et al., 2017). Key to this model is the facilitation of inflammatory responses to chemotherapy by the microbiome (Secombe et al., 2019b).

4.5.4 Evidence of microbial changes in SM-TKI treatment

More recently, there has been some direct evidence suggesting links between gut microbial changes and diarrhoea following SM-TKI treatments. A pre-clinical study of the EGFR inhibitor lapatinib showed lapatinib-treated rats had significantly lowered microbial diversity (Mayo et al., 2020). In addition, decreases in *Betaproteobacteria* were seen following lapatinib treatment. In contrast, chemotherapy studies have shown changes in *Gammaproteobacteria*, and this difference was suggested to be a key difference between chemotherapy- and SM-TKI-induced diarrhoea outcomes (Blijlevens et al., 2007). A study of 20 patients receiving VEGF-TKI treatment for metastatic renal cell carcinoma (mRCC) assessed the microbiome via 16S sequencing of stool samples. Patients with diarrhoea had higher levels of *Bacteroides* spp. and lower levels of *Prevotella* spp. (Pal et al., 2015). However, it was inconclusive

whether these microbial changes were simply due to the occurrence of diarrhoea, or the drivers of this diarrhoea.

4.5.5 Probiotics and faecal microbiota transplant

Probiotics and dietary modification have also been suggested as a treatment or preventative measure for cancer treatment-induced diarrhoea. In chemotherapy, probiotics have had varying levels of success in reducing diarrhoea (Secombe et al., 2019b). While some studies have shown lowered gastrointestinal damage levels and less diarrhoea, others have shown no benefit. A meta-analysis recently found insufficient current evidence to support widespread implementation of probiotics after chemotherapy (Wardill et al., 2018). The authors noted the wide variety in probiotic types and dosing schedules, and stressed the need for rationally designed probiotic mixtures and trials. Probiotics are commonly used alongside some forms of SM-TKI treatment (Rossi et al., 2019). However, to date, there is no robust evidence for probiotic use during SM-TKI treatment (Schmidinger, 2013). One study of the EGFR inhibitor dacomitinib in 173 non-small cell lung cancer patients demonstrated that VSL#3 probiotics were unsuccessful in reducing diarrhoea or intestinal damage (Lacouture et al., 2016). Subsequent commentary on this paper suggested some issues with the study, further highlighting the need for consistently designed probiotic studies (Ceccarelli et al., 2016; Lacouture, 2016). Currently, a clinical trial is underway to assess the use of probiotic yoghurt in reducing diarrhoea following VEGF inhibitor treatment for kidney cancer (NCT02944617) (Pal, 2020). Another form of microbiome modulation under investigation for use in cancer treatment is faecal microbiota transplant

(FMT) (Wardill et al., 2019). Pre-clinically, FMT was able to reduce gut dysbiosis caused by 5-FU (Le Bastard et al., 2018). Clinically, a recent study of 21 patients having treatment with VEGFR inhibitors pazopanib and sunitinib for mRCC was completed (Rossi et al., 2019). Patients with diarrhoea received FMT via colonoscopy or L. casei DG probiotics as control. After 7 days, all patients in the FMT group had resolution of diarrhoea compared to 54.5% of patients in the probiotic group. At a longer term 15 and 30 day follow up, 90% of FMT patients had no diarrhoea compared to 0% of patients in the probiotic group, demonstrating the potential of the microbiome to be a key part of SM-TKI-induced diarrhoea.

While there is only a small amount of direct evidence suggesting gut microbiome changes occurs in SM-TKI treatment, the close relationship of a dysbiotic microbiome with both inflammatory activation and chloride secretion processes is an incentive to further elucidate the use of the microbiome in treating diarrhoea from SM-TKI treatments.

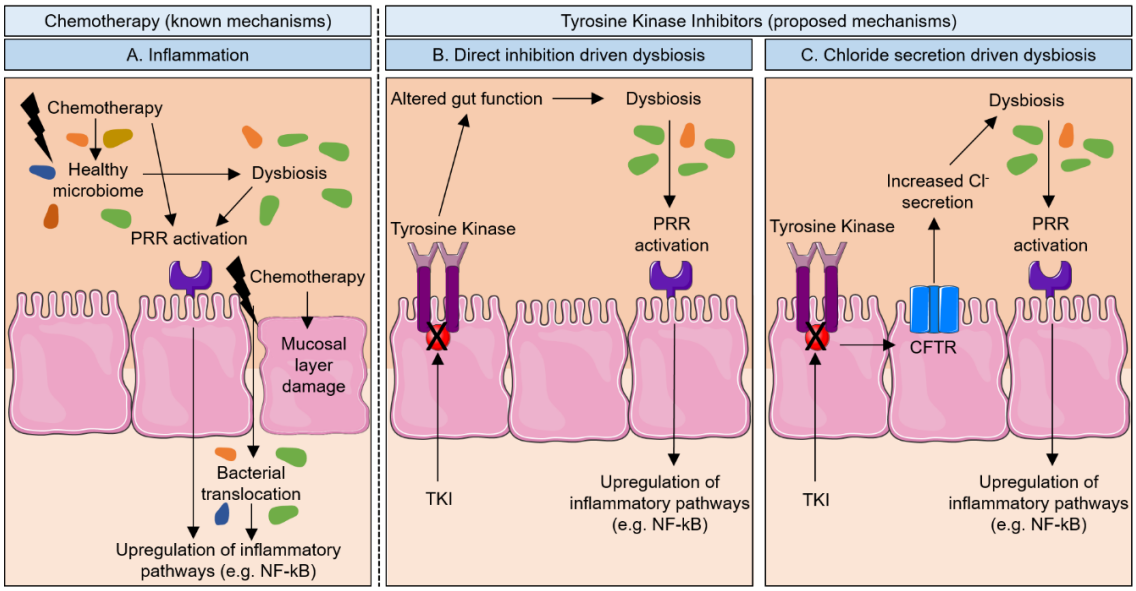

Figure 4.1: Potential interactions of the gut microbiome with TKI treatments leading to diarrhoea. (A) Chemotherapy causes vast changes to the gut microbiome (Stringer et al., 2009b; Stringer et al., 2013), as well as activation of inflammatory pathways via Pattern Recognition Receptors (PRR) such as Toll-like receptor 4 (Sonis, 2004b; Wardill et al., 2016), that lead to ulceration and eventual diarrhoea. Tyrosine kinase inhibitor treatment also leads to diarrhoea, but the mechanism is not well understood. We propose that the gut microbiome may play a key role. (B) Long term TKI treatment may lead to a dysbiotic microbiome (Pal et al., 2015). Additionally, direct inhibition of EGFRs or VEGFRs in the gut can lead to altered gut function (e.g. changes in cell proliferation and capillary networks) that can alter microbial composition (Fan et al., 1998; O'Brien et al., 2002; Kamba et al., 2006; Echavarria et al., 2017). This could lead to similar inflammatory outcomes as in chemotherapy (Secombe et al., 2019b). (C) Alternatively, increased chloride secretion in the gut (causing diarrhoea itself) (Van Sebille et al., 2015) could lead to a significant shift in the microbiome that may lead to additive effect on the diarrhoea (Pal et al., 2015; Vernocchi et al., 2018; Meeker et al., 2020).

4.6 Predicting toxicity and treatment response - role of the microbiome

In the future, individual microbial composition could be used as a predictor of risk of a range of gastrointestinal conditions. Recent reviews have summarised the initial work that has been done to characterise microbial profiles putting people at severe risk of diarrhoea following radiotherapy (Secombe et al., 2019b) and how this work is now being translated into the chemotherapy setting (Wardill et al., 2017). In the past, toxicities have previously been used as an indicator of SM-TKI treatment response (Liu et al., 2013), and moving forward, the microbiome may represent a unique opportunity to be able to predict both toxicity and response to treatment.

Aside from diarrhoea, the other main side effect from SM-TKI treatment is skin rash. Patients receiving EGFR TKI treatment are at particular risk, and may develop secondary skin infections. Multiple studies have suggested a correlation between incidence of rash and subsequent response to treatment, where occurrence of rash was associated with better response (Widakowich et al., 2007; Liu et al., 2014). Commonly, broad-spectrum antibiotics are used to treat this rash. These antibiotics have a deleterious effect on the gut microbiome, and importantly their use has been shown to be a negative predictor of efficacy and toxicity of EGFR-TKI treatment in non-small cell lung cancer populations (Liu et al., 2019). This study retrospectively grouped 102 patients into antibiotic and non-antibiotic-treated groups and found that people who took antibiotics had worse progression free survival and more instances of

severe diarrhoea (Liu et al., 2019). However, this retrospective study may not take into account the reasons for antibiotic use and whether the results may actually reflect that patients receiving antibiotics were a more vulnerable group overall. In immunotherapy settings, there is a growing link between antibiotic use and treatment response (Pinato et al., 2019). Similarly, antibiotic use during chemotherapy may also have detrimental effects on treatment efficacy (van Vliet et al., 2009). In addition, chemotherapies including oxaliplatin and cyclophosphamide are reliant on an immune response to induce tumour cell death. A study showed that the anti-tumour efficacy of cyclophosphamide was reduced in germ-free mice or mice treated with antibiotics (Viaud et al., 2013). These results suggest that the gut microbiome is integral in shaping an anti-tumour immune response during both chemotherapy and SM-TKI treatment.

Finally, it has also been shown that occurrence of diarrhoea relates to treatment success during SM-TKI treatment (Koschny et al., 2013). An analysis of four phase I trials (total 179 patients) showed that patients who had diarrhoea with VEGFR inhibitor sorafenib treatment had a significantly increased time to progression compared to patients who did not develop diarrhoea (Strumberg et al., 2006). Another study of 223 patients showed that diarrhoea from gefitinib (EGFR inhibitor) treatment was predictive of lowered risk of progression in multivariate analysis (Thomas et al., 2006), while increased progression free survival was seen when patients receiving first line VEGF-TKI treatment for mRCC were given antibiotics that gave protection from *Bacteroides* spp. (Hahn et al., 2018). This is contradictory to the results seen in EGFR targeted treatment discussed above, and hence these findings may be target, or

antibiotic-specific. Also in mRCC, a small US study of six patients showed significant differences in the gut microbiome compositions of responders and non-responders to sunitinib (Gong et al., 2018).

Taken together, these findings suggest levels of diarrhoea and antibiotic use could be predictive for a person's positive and negative responses, respectively, to chemotherapy and SM-TKI treatment. Due to the widespread effects of antibiotics on the gut microbiome, and the proposed role of the microbiome in causing diarrhoea in these treatments, future research should focus on connecting gut microbial composition with overall survival and treatment response. It should be noted that a potential drawback of taking a similar research approach, is that current research provides evidence for overlapping mechanisms in both toxicity and treatment efficacy. This also suggests that exploiting the gut microbiome to enhance efficacy may lead to increased levels of toxicity. Recent research into microbial changes relating to diarrhoea from Crohn's Disease has suggested that microbial dysbiosis drives clinical symptomology, despite a lack of mucosal injury (Boland et al., 2021). This suggests that the microbiome is likely to govern the duration of symptoms via mechanisms independent of mucosal injury. As some SM-TKIs do not cause overt tissue injury, this is of potential importance.

Future studies should be careful to determine methods to manipulate the microbiome in a way to minimise toxicity while simultaneously enhancing efficacy. This may be via specialised pre or probiotics that ensure the production of specific bacterial metabolites, or defined microbial modulation techniques including FMT that could be used prophylactically.

4.7 Conclusion

Diarrhoea from any cancer treatment can have negative effects on a person's ability to complete their treatment course, as well as impacting financial welfare and quality of life. Precision treatment, and ideally preventative strategies are required to reduce the burden of diarrhoea. Here, we have compared gastrointestinal toxicity stemming from SM-TKI treatment with the more well-understood toxicity seen with traditional, highly mucotoxic chemotherapy treatment, in order to understand key commonalities. Although past research has shown differences in the mechanism of pathogenesis, we hypothesise that the gut microbiome may play a key role in the gastrointestinal response to both treatments. A common mechanism between the two would allow for a more rapid development of targeted treatments and prophylactic medications. In addition, as SM-TKIs and chemotherapy are often given in combination, a common way to target this diarrhoea would be highly beneficial. Current research in SM-TKI toxicity has focused on the use of interventional treatments to reduce diarrhoea severity, however these interventions may be unable to reduce gut microbial changes and subsequent inflammatory responses. It may therefore be advantageous to alter this research direction to focus on how to predict toxicity and treatment efficacy using pre-treatment microbiome profiling techniques.

Statement of Authorship

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Name of Principal Author (Candidate)	Kate Secombe		
Contribution to the Paper	Analysed and synthesised data, completed histological staining, microbial sampling and analysis, manuscript preparation.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	27/04/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 5: Pathophysiology of neratinib-induced diarrhoea in male and female rats: microbial alterations a potential determinant

Chapter five is my first original research chapter on the toxicity of the tyrosine kinase inhibitor neratinib. This chapter was published in Breast Cancer (*Secombe, K. R., Ball, I. A., Shirren, J., Wignall, A. D., Keefe, D. M. and Bowen, J. M. (2021). Pathophysiology of neratinib-induced diarrhea in male and female rats: microbial alterations a potential determinant. Breast Cancer 28(1): 99-109*). The rat study presented in this chapter was completed before my PhD. However, during my candidature I analysed and synthesised the clinical data, completed histological staining and scoring of intestinal sections and completed all work related to 16S microbial sequencing. This chapter is presented in its original publication format. The referencing style, spelling and figure/table numbers have been modified to maintain consistency throughout this thesis.

5.1 Abstract

Background: Neratinib is a potent irreversible pan-ErbB tyrosine kinase inhibitor, approved by the FDA for extended adjuvant treatment of HER2-positive breast cancer. Diarrhoea is the most frequently observed adverse event with tyrosine kinase inhibitor therapy. In this study, we developed a reproducible model for neratinib-induced diarrhoea in male and female rats.

Methods: At first, male rats were treated with neratinib at 15, 30 or 50 mg/kg or vehicle control via oral gavage for 28 days (total n = 12). Secondly, we

compared outcomes of male (n = 7) and female (n = 8) rats, treated with 50 mg/kg neratinib.

Results: Rats treated with a 50 mg/kg daily dose of neratinib had a reproducible and clinically relevant level of diarrhoea and therefore was confirmed as an appropriate dose. Male rats treated with neratinib had significant changes to their gut microbiome. This included neratinib-induced increases in *Ruminococcaceae* (P = 0.0023) and *Oscillospira* (P = 0.026), and decreases in *Blautia* (P = 0.0002). On average, female rats experienced more significant neratinib-induced diarrhoea (mean grade = 1.526) compared with male rats (mean grade = 1.182) (P < 0.0001). Neratinib caused a reduction in percentage weight gain after 28 days of treatment in females (P = 0.0018) compared with vehicle controls. Females and males both showed instances of villus atrophy and fusion, most severely in the distal ileum. Serum neratinib concentration was higher in female rats compared to male rats (P = 0.043).

Conclusions: A reproducible diarrhoea model was developed in both female and male rats, which indicated that diarrhoea pathogenesis is multifactorial, including anatomical disruption particularly evident in the distal ileum, and alterations in microbial composition.

5.2 Introduction

Small molecule tyrosine kinase inhibitors (TKIs) are increasingly used for the treatment of cancers in blood and solid tissues. These drugs compete with the intracellular ATP binding site of oncogenic tyrosine kinases including the ErbB

group (Rugo et al., 2019). Over-expression of one ErbB member, HER2, is known to be an oncogenic driver in approximately 20% of breast cancers (Cherian et al., 2017). Neratinib (HKI-272) is an orally available, high affinity, irreversible small molecule pan-ErbB TKI. It has been FDA approved for extended adjuvant treatment of HER2-positive breast cancer and is now being assessed for other HER2 related cancers (NCT03289039, NCT02932280). It has been shown to be beneficial in metastatic and early settings, and also for patients who previously received trastuzumab (Kourie et al., 2017).

Diarrhoea has been a common adverse effect in multiple clinical trials for neratinib. In the ExteNET trial of 2840 patients randomised to 12 months of treatment with oral neratinib or placebo (Chan et al., 2016), 40% of patients developed severe, grade 3-4 diarrhoea. Fifty-five percent of patients developed grade 1 and 2 diarrhoea. Most grade 3 diarrhoea occurrences happened in the first month of treatment, with a reduction in frequency thereafter (Cherian et al., 2017). Diarrhoea management guidelines advise prophylactic loperamide, and in the I-SPY 2 trial of 115 patients receiving neoadjuvant neratinib plus chemotherapy, prophylactic loperamide reduced severe diarrhoea from 47% to 36% (Park et al., 2016).

While in some cases loperamide is able to somewhat decrease diarrhoea incidence, there may be other, more effective diarrhoea management strategies that are more specific to the mechanism of neratinib-induced diarrhoea. Models of diarrhoea have previously been developed with other TKIs such as the dual-HER inhibitor lapatinib (Bowen et al., 2012), however pan-HER inhibitors such as neratinib are becoming more commonly used to overcome resistance to

these other treatments. Therefore we attempted to develop a reproducible animal model of neratinib-induced diarrhoea in both male and female rats that could be used in future to target and test possible interventions.

5.3 Materials and Methods

5.3.1 Chemicals

Neratinib was kindly provided by Puma Biotechnology (USA). Neratinib was diluted in 0.5% (w/w) hydroxypropyl methylcellulose (HPMC) buffer (Sigma-Aldrich, USA).

5.3.2 Animals and ethics

All experiments were conducted on Albino Wistar (AW) rats obtained from the University of Adelaide Laboratory Animal Service, Waite Campus. Rats were housed in groups of up to 4 in ventilated cages. The environmental controls were set to maintain temperature within the range of 19 to 23 °C and relative humidity within the range of 45% to 65%; with a 12 hour light/dark cycle. Food and water were consumed *ad libitum*. If rats were experiencing moderate to severe treatment-related toxicity (e.g. diarrhoea, weight loss, stress marks) they were allowed soaked chow (normal feed softened in water to ease mastication) in addition. Rats were acclimatised to local housing conditions for a minimum of 7 days prior to the first day of dosing. On Day 1 of treatment, the rats were between 7 - 9 weeks old. This study was approved by the Animal Ethics Committee of the University of Adelaide (study number M-2015-006), and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training (2013).

5.3.3 Experimental design

All rats were randomly allocated to experimental groups and were identified by a unique animal number written with an indelible marker on their tail. During the 28 day treatment period, rats received daily oral gavages using a soft plastic feeding tube. Neratinib or vehicle controls were given at a constant dose volume of approximately 5 mL/kg. Individual dose volumes were adjusted daily according to most recent body weight records. The first day of dosing was designated Day 1. The final dose was given on the day before scheduled necropsy. The 28-day treatment schedule aimed to mimic long term clinical administration. All rats were deeply anaesthetised via isoflurane inhalation (Isothesia, Henry Schein, USA), and culled by cardiac exsanguination with death confirmed by cervical dislocation.

5.3.3.1 Dose finding study

Male rats were treated for 28 days with either vehicle control (0.5% (w/w) HPMC buffer) (n = 3) or neratinib at 15 mg/kg (n = 3), 30 mg/kg (n = 3) or 50 mg/kg (n = 3).

5.3.3.2 Outcome comparison in males and females

Comparisons were made between female rats, treated for 28 days with either vehicle control (0.5% (w/w) HPMC buffer) (n = 4) or 50 mg/kg neratinib (n = 8) and male rats treated for 28 days with vehicle control (n = 7) or 50 mg/kg neratinib (n = 7).

5.3.4 Clinical gut toxicity assessment

Rats were weighed daily, and there was a twice daily comprehensive clinical symptom recording. Diarrhoea was graded by two assessors according to a validated grading system (Bowen et al., 2012) with four grades: 0, no diarrhoea; 1, mild (soft unformed stools); 2, moderate (perianal staining and loose stools); 3, severe (watery stools and staining over legs and abdomen). Rats were euthanised if displaying 15% or over weight loss from baseline or significant distress and clinical deterioration.

5.3.5 Tissue collection and preparation

At necropsy, the gastrointestinal tract was removed from the pyloric sphincter to the rectum. The small and large intestine were flushed with chilled, sterile phosphate buffered saline and weighed. Samples of duodenum, jejunum, ileum and colon were collected and fixed in 10% formalin (ChemSupply, Australia) for embedding in paraffin (Sigma-Aldrich, USA). As ileal damage is common in other models of TKI-induced diarrhoea, samples were taken of both distal and proximal ileum. Other organs (stomach, spleen, liver, brain, kidney, lung and heart) were also collected, weighed, fixed in formalin and embedded in paraffin.

5.3.6 Histological examination

All histology was conducted on paraffin embedded intestinal samples, which were cut using a rotary microtome (RM2235, Leica, Germany) and 4 µm sections mounted onto Superfrost glass slides (Menzel-Glaser, Germany). All histology had images taken using a NanoZoomer digital slide scanner

(Hamamatsu Photonics, Japan) and viewed using the NanoZoomer Digital Pathology Software (NDP View v1.2) (Histalim, Montpellier, France). All analysis was done in a blinded fashion.

5.3.7 Serum analysis

Blood samples were collected by cardiac puncture using a 23 gauge needle (Becton-Dickinson, USA) in K3EDTA Vacuette tubes (Greiner Bio-One, Austria). Serum was separated by centrifugation at 300 *x g* for 5 minutes before being analysed via a multiple blood analysis (MBA-20). This analysis was completed by the Department of Clinical Pathology, SA Pathology, Adelaide, South Australia.

Serum neratinib concentration was measured by liquid chromatography tandem mass spectrometry / time of flight (LC-MS/TOF) (validated range 10 – 10,000 ng/ml). This work was conducted in the Good Laboratory Practice (GLP)-certified Pharmaceutical Science Sector Laboratory, School of Pharmacy and Medical Sciences, University of South Australia. Calibrator and quality control working control solutions were prepared by dissolving neratinib powder in methanol. Sample supernatant was aspirated and eluted through a Kinetex 1.7 µm C18 2.1 x 100 mm column (Phenomenex, USA) using a 30 series UHPLC system (Shimadzu, Japan). All rats were assessed for neratinib concentration 24 hours after the final dose (C_{min}).

5.3.8 Mucosal damage analysis

Routine haematoxylin and eosin (H&E) staining was completed and an injury score assigned using a well-established system of histological criteria (Howarth et al., 1996; Wardill et al., 2016). Criteria were villus fusion, villus atrophy, disruption of brush border and surface enterocytes, crypt losses/architectural disruption, disruption of crypt cells, infiltration of polymorphonuclear cells and lymphocytes, dilation of lymphatics and capillaries and oedema. The latter six criteria were examined in the colon. Each criterion was scored as present = 1 or absent = 0.

5.3.9 Microbial analysis

In a subset of male rats treated with 50 mg/kg neratinib (n=4) or vehicle control (n=4) for 28 days, caecal contents were aseptically collected during dissection into a sterile tube at each time point, and stored at -80˚C. Samples were sent to the Australian Genome Research Facility (AGRF) for DNA extraction and 16S ribosomal RNA (rRNA) gene region analysis. DNA was extracted from 250 mg of caecal sample using the Qiagen DNeasy PowerLyzer PowerSoil Kits with the PowerLyzer 24 Homogeniser. 16S analysis sequencing details are as follows:

Target: 16S: 341F (V3-V4) (V1-V3), read length = 300 bp.

Forward sequence: 5’- CCTAYGGGRBGCASCAG- 3’

Reverse Sequence: 5’- GGACTACNNGGGTATCTAAT- 3’

Image analysis was performed in real time by the MiSeq Control Software v2.6.2.1 and Real Time Analysis v1.18.54, running on the instrument computer. The Illumina bcl2fastq 2.20.0.422 pipeline was used to generate the sequence data. CLC Genomics Workbench 12.0 (https://www.qiagenbioinformatics.com/) was used to complete bacterial diversity profiling. Paired-ends reads were assembled by aligning forward and reverse reads. Primers were identified and trimmed. Trimmed sequences were quality filtered, duplicate sequences removed and sorted by abundance. Reads were assigned to taxonomic identities using the Greengenes 97% similarity database version 13.8. Alpha-diversity was calculated using the Shannon diversity index. Beta diversity was calculated using Principal Coordinate Analysis (PCoA) based on generalised UniFrac distances (Chen et al., 2012a). The program BURRITO (McNally et al., 2018) was used to visualise the links between taxonomic composition and function in the dataset. Statistical Analysis of Metagenomic Profiles (STAMP) (Parks et al., 2014) was used with the predicted metagenome using Welch's *t* test with the Benjamini-Hochberg correction for the false-discovery rate (FDR).

5.3.10 Statistical analysis

Data were compared using Prism version 7.0 (GraphPad Software, USA). The vehicle control and neratinib treated groups were compared separately in males and females. If data was normally distributed, bars on graphs are mean ± SEM. If not, median is displayed in graphs. The assumptions of equality of variance for each group and normally distributed data were tested using Bartlett's test and D'Agostino & Pearson omnibus normality test, respectively. If these

assumptions were violated, non-parametric equivalent tests were performed, including Kruskal-Wallis for independent data and Freidman's test for repeated measures. When assumptions held, ANOVA's were performed using the 2-way analysis of variance (ANOVA). Diarrhoea proportions were analysed by Chi Squared test. P-values less than 0.05 were considered statistically significant.

5.4 Results

5.4.1 Model and dose development in male rats

5.4.1.1 50 mg/kg neratinib causes reproducible diarrhoea and weight loss in male rats

From day 19 of treatment until the end of the experiment, rats treated with 30 mg/kg and 50 mg/kg neratinib had significantly less weight gain than vehicle controls (30 mg/kg: $P = 0.043$, 50 mg/kg: $P = 0.0183$) (figure 5.1). No rats treated with vehicle control developed diarrhoea at any time during the experiment. 15 and 30 mg/kg doses caused diarrhoea in one-third of animals, whereas 50 mg/kg neratinib caused diarrhoea in all animals, including one who developed grade 3, severe diarrhoea ($P < 0.0001$ compared to vehicle control) (figure 5.1).

5.4.1.2 Histopathological changes were most pronounced in the ileum

The groups treated with 15 mg/kg and 30 mg/kg neratinib had minimal histopathological changes, with minor evidence of lymphocyte infiltration in the ileum (data not shown). There were no statistically significant differences in histopathological scoring between the vehicle control group and the 50 mg/kg neratinib groups in any intestinal region, however there was evidence of polymorphonuclear cell and lymphocyte infiltration in the ileum and colon, as well as fusion of villi in the ileum of the 50 mg/kg neratinib group (figure 5.1). Due to low sample numbers in this pilot study, it may be possible that it was not sufficiently powered for statistical significance.

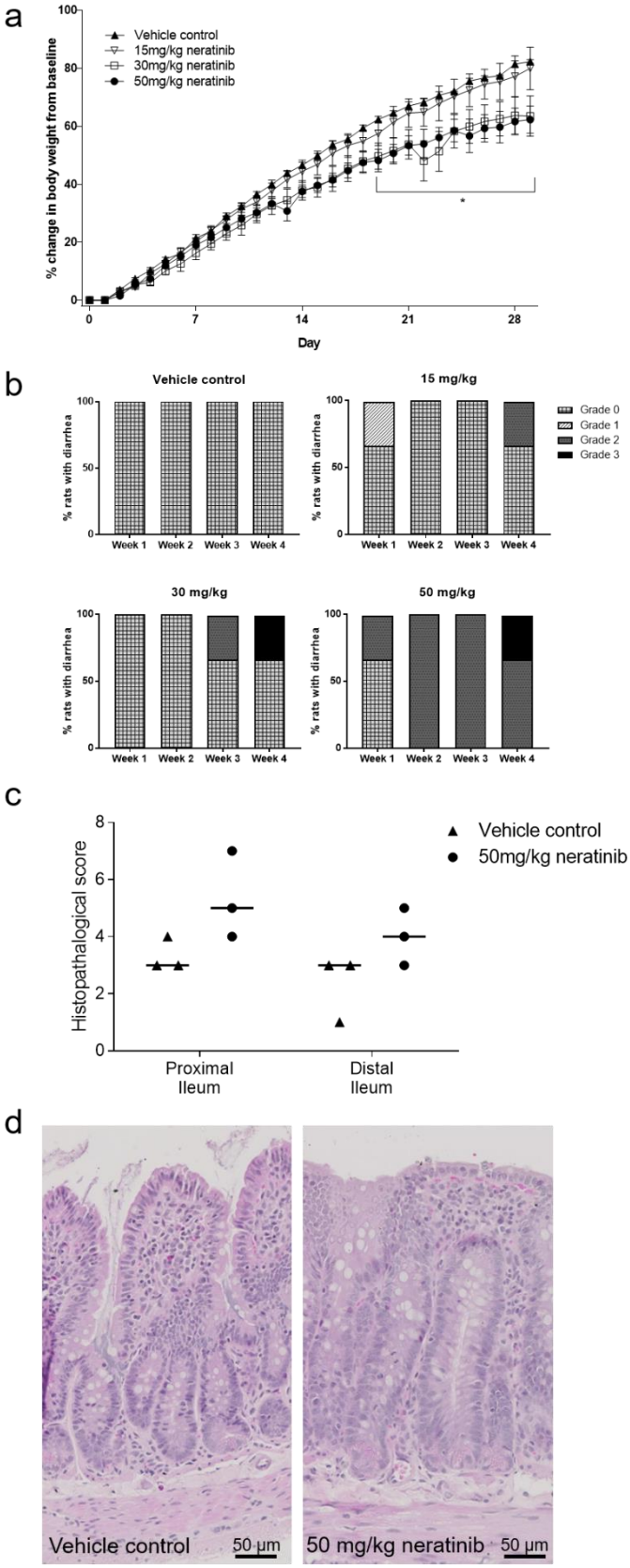
a
Vehicle control
15mg/kg neratinib
30mg/kg neratinib
50mg/kg neratinib
% change in body weight from baseline
Day
b
Vehicle control
15 mg/kg
30 mg/kg
50 mg/kg
% rats with diarrhea
Week 1
Week 2
Week 3
Week 4
Grade 0
Grade 1
Grade 2
Grade 3
c
Vehicle control
50mg/kg neratinib
Histopathalogical score
Proximal Ileum
Distal Ileum
d
Vehicle control
50 µm
50 mg/kg neratinib
50 µm

Figure 5.1: a) Graph showing the weight change from baseline in each treatment group. Rats in the 30 mg/kg and 50 mg/kg group had significantly less weight gain from day 18 onwards, compared to the vehicle control group (30 mg/kg: $P = 0.043$, 50 mg/kg: $P = 0.0183$, 2-way ANOVA). Data presented as mean ± SEM. b) Graph shows percentage of rats at their highest grade of diarrhoea after each week of treatment. 100% of rats receiving a 50 mg/kg dose of neratinib developed moderate diarrhoea throughout the treatment course. Rats treated with 50 mg/kg neratinib had significantly more severe diarrhoea than other dosages ($P < 0.0001$, Chi-squared test). c) There was no significant difference in histopathological scoring in the ileum between the vehicle control and 50 mg/kg neratinib group ($P > 0.05$, Kruskal-Wallis test). Non-parametric data, line represents median. d) Photomicrographs of distal ileum at 200x original magnification (stained with H&E) showing evidence of blunting and fusion of villi, flattening of surface enterocytes and crypt elongation. Scale bars shown in diagram.

5.4.1.3 Neratinib induced changes in serum biochemistry

All biochemical serum values were within normal ranges for Wistar rats (Tucker, 1997). There were no significant differences between doses for any parameter other than lactate dehydrogenase (LD). LD levels were significantly higher in the 30 mg/kg (P = 0.014) and 50 mg/kg (P = 0.0006) group compared to the 15 mg/kg group (table 5.1). As expected, there was a dose-dependent increase in neratinib serum levels (15 mg/kg: mean= 111 ± 13.1 ng/mL, 30 mg/kg: mean= 286 ± 59.0 ng/mL, P = 0.045 compared to 15 mg/kg, 50 mg/kg: mean= 407.3 ± 30.9 ng/mL, P =0.004 compared to 15 mg/kg) measured in serum 24 hours after final dose (figure 5.2).

Overall, 15 mg/kg and 30 mg/kg doses of neratinib had little effect on clinically relevant markers of neratinib-induced injury. A 50 mg/kg dose of neratinib caused diarrhoea in all animals and 23% less weight gained. 50 mg/kg was therefore confirmed as a robust and reproducible dose for use in a model for neratinib-induced diarrhoea.

Table 5.1: Blood biochemistry in male rats (n = 12) (mean ± SEM). One-way ANOVA used to determine statistical significance. No significance noted except for lactate dehydrogenase levels in the 30 and 50 mg/kg dosage.

	Vehicle		**15mg/kg**		**30mg/kg**		**50mg/kg**	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Sodium	141.67	0.33	141.33	0.67	141.67	0.33	143.33	1.20
Potassium	5.00	0.10	5.13	0.09	5.27	0.19	5.20	0.06
Chloride	100.33	0.88	100.67	0.33	100.33	0.33	100.33	0.33
Bicarb	1.60	0.77	1.66	0.81	1.73	0.82	1.73	0.84
Anion Gap	16.00	1.15	15.00	0.00	17.00	1.73	19.67	2.33
Glucose	8.47	0.03	8.90	0.36	8.37	0.28	8.23	0.71
Urea	6.67	0.07	6.50	0.25	6.40	0.21	6.63	0.39
Creatinine	21.00	1.15	20.00	0.58	20.00	0.58	22.67	1.86
Cholesterol	1.73	0.03	1.57	0.12	1.63	0.09	1.70	0.12
Urate	0.05	0.01	0.06	0.01	0.08	0.01	0.09	0.02
Phosphate	2.50	0.15	2.43	0.04	2.55	0.03	2.58	0.03
Tot. Ca.	2.57	0.06	2.52	0.03	2.50	0.02	2.50	0.06
Albumin	16.67	0.33	15.67	0.33	15.00	0.00	15.33	0.33
Glob.	41.67	0.88	38.67	0.88	38.67	0.88	39.67	1.76
Protein	58.33	1.20	54.33	1.20	53.67	0.88	55.00	2.08
Tot. Bili.	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
GGT	3.00	0.00	3.00	0.00	3.00	0.00	3.00	0.00
ALP	217.67	22.30	258.33	40.34	248.33	40.71	256.67	39.96
ALT	58.33	3.28	74.33	4.91	69.33	5.24	76.33	6.36
AST	96.67	7.31	107.00	4.16	107.00	3.21	113.67	7.75
LD	786.00	149.95	718.00	45.40	830.00*	81.21	865.00*	141.48

* denotes significance. LD: 15 mg/kg vs 30 mg/kg: P = 0.014, 15 mg/kg vs 50 mg/kg: P = 0.0006.

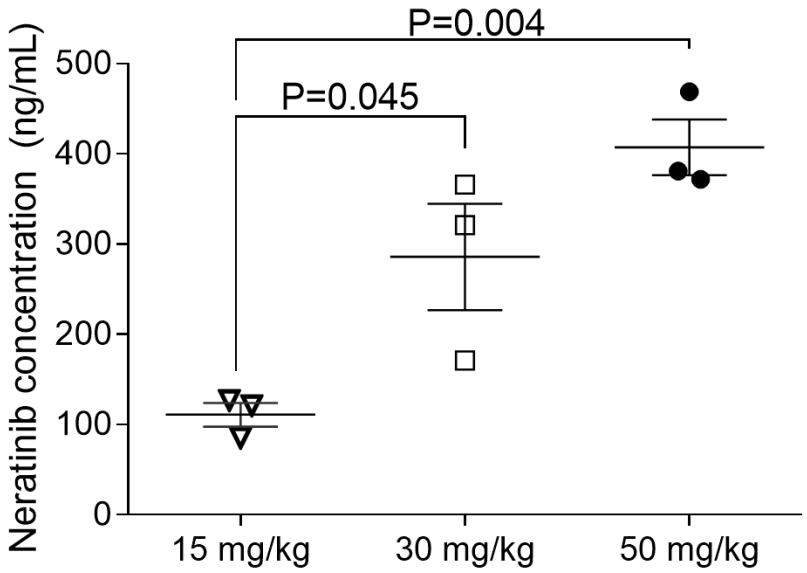

Figure 5.2: Rats treated with 30 mg/kg (P = 0.004) and 50 mg/kg (P = 0.045) neratinib had significantly higher levels of serum neratinib after 28 days compared to rats treated with 15 mg/kg neratinib (total n = 9). Parametric data, mean ± SEM shown, one-way ANOVA used.

5.4.1.4 Neratinib induced vast changes in microbiome composition

Microbial changes were assessed in a subset of male rats treated with vehicle control or 50 mg/kg neratinib (figure 5.3). Caecal microbial diversity, measured using Shannon's diversity index at the genus level, was increased in the neratinib treated group compared to vehicle control (P = 0.0066). However, there were no significant differences between the groups when the Simpson or Chao1 indexes were used. Principal coordinate analysis showed that neratinib treated rats and vehicle control treated rats clustered separately. This was confirmed with pairwise PERMANOVA testing showed significant differences between the groups (P = 0.0288).

Rats treated with neratinib had significantly higher relative abundance of the family *Ruminococcaceae* (P = 0.0023) and the genus *Oscillospira* (P = 0.026). The genus *Blautia* was highly significantly increased in vehicle control animals compared to neratinib treated (P = 0.0002).

Metabolic pathways predicted to alter significantly due to changes in microbial composition are shown in figure 5.3. The most significantly increased pathway in the control group compared to neratinib treated was arginine biosynthesis. In the neratinib treated group, the most significantly increased pathway compared to the control group was chaperones and folding catalysts.

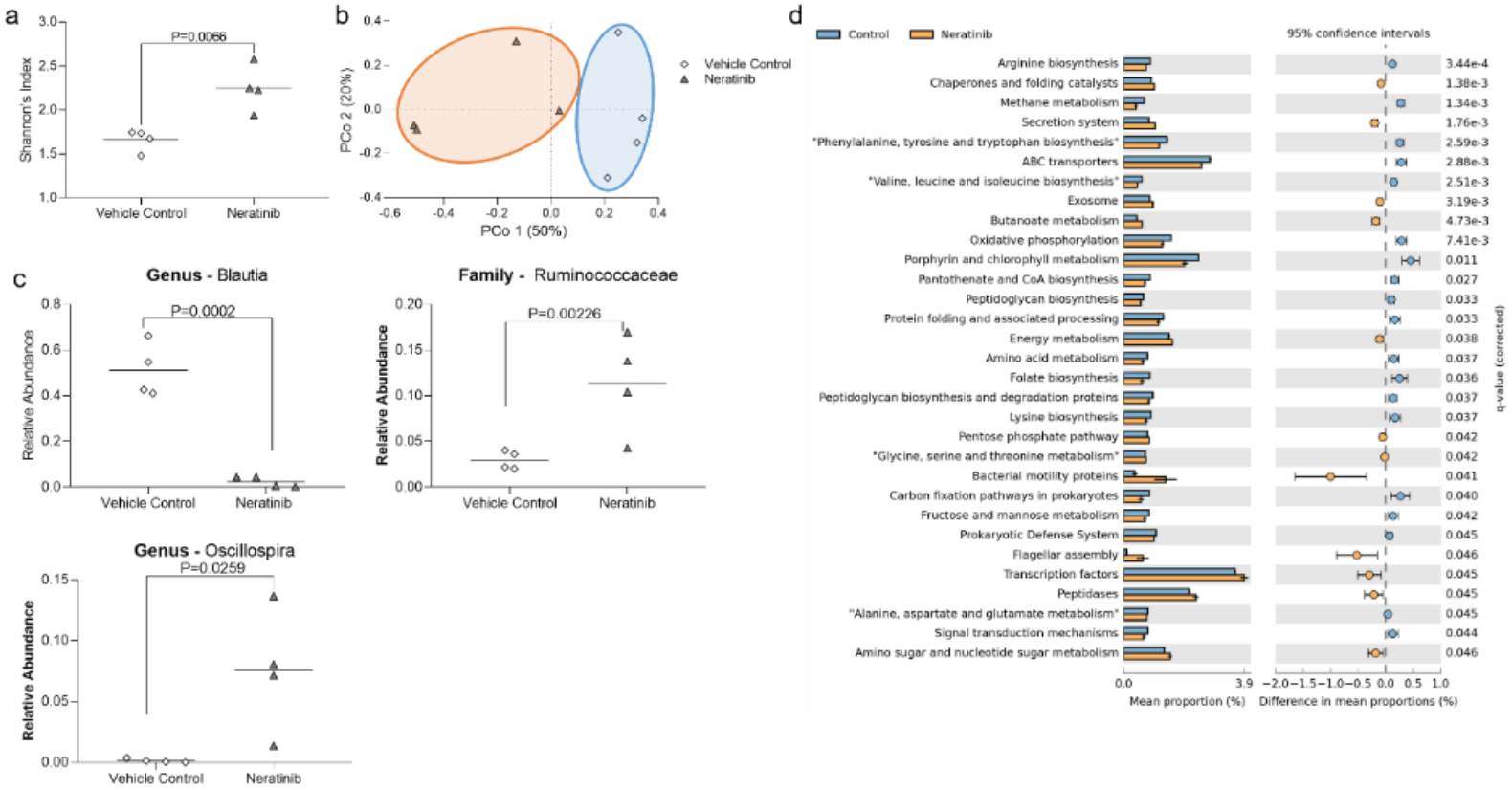

Figure 5.3: Caecal microbial analysis of male rats. a) Shannon's diversity index showed a significant increase in microbial alpha diversity in neratinib treated animals. Genus level shown in this graph. b) Principal coordinate analysis showed separate clustering between vehicle control and neratinib treated animals. c) Relative abundance of bacterial species. The genus *Blautia* was significantly higher in vehicle control animals, whereas *Ruminococcaceae* and *Oscillospira* were significantly higher in neratinib treated animals. d) Significantly different predicted changes in the caecal metabolome based on changes in the microbiome. Mean proportions of pathways and Welch's t test with Benjamini-Hochberg false discovery rate corrected significance are shown here.

5.4.2 Outcome comparison of male and female rats

5.4.2.1 Females suffered more severe diarrhoea and weight loss than males

Following the determination of 50 mg/kg as the optimal neratinib dose, the model was expanded to female rats. At 50 mg/kg, females suffered more severe diarrhoea and weight loss than male rats receiving the same dose. Female rats had a mean diarrhoea grade of 1.53 ± 0.37 compared to 1.18 ± 0.42 for males ($P < 0.0001$) (figure 5.4). In females, all rats had diarrhoea by day three, and there were a total of eight days where rats had severe diarrhoea. In males, only 30% of rats had diarrhoea by day three, and none developed severe diarrhoea.

Female rats treated with neratinib had significantly less weight gain than females treated with vehicle control at days 25 ($P = 0.0498$) and 29 ($P = 0.0490$). Neratinib did not cause any male rats to lose weight at any time throughout the experiment, however did in females in the first week of treatment. 88% of female rats were below their starting weight at day 7, compared to no male rats.

5.4.2.2 Moderate increase in systemic neratinib concentrations in females

LC-MS/TOF was used to assess serum neratinib level (female n = 8, male n = 7). There was an approximately two-fold increase in female (mean = 827.5 ng/mL) serum neratinib values compared to male rats (mean = 409.4 ng/mL) (P

= 0.0012) (figure 5.4). However, these samples were collected and assayed on different days, so inter-day/inter-assay variability cannot be ruled out.

5.4.2.3 Organ weights

Males had a significant increase, not seen in females, in small intestine (P = 0.013) weight (normalised to brain weight) in neratinib treated animals compared to vehicle control (figure 5.4). Neratinib treated males also had a significantly lowered liver weight compared to vehicle control (P = 0.049). Neither males nor females had a significant change in large intestine, spleen, kidney, stomach, heart or lung weight (data not shown).

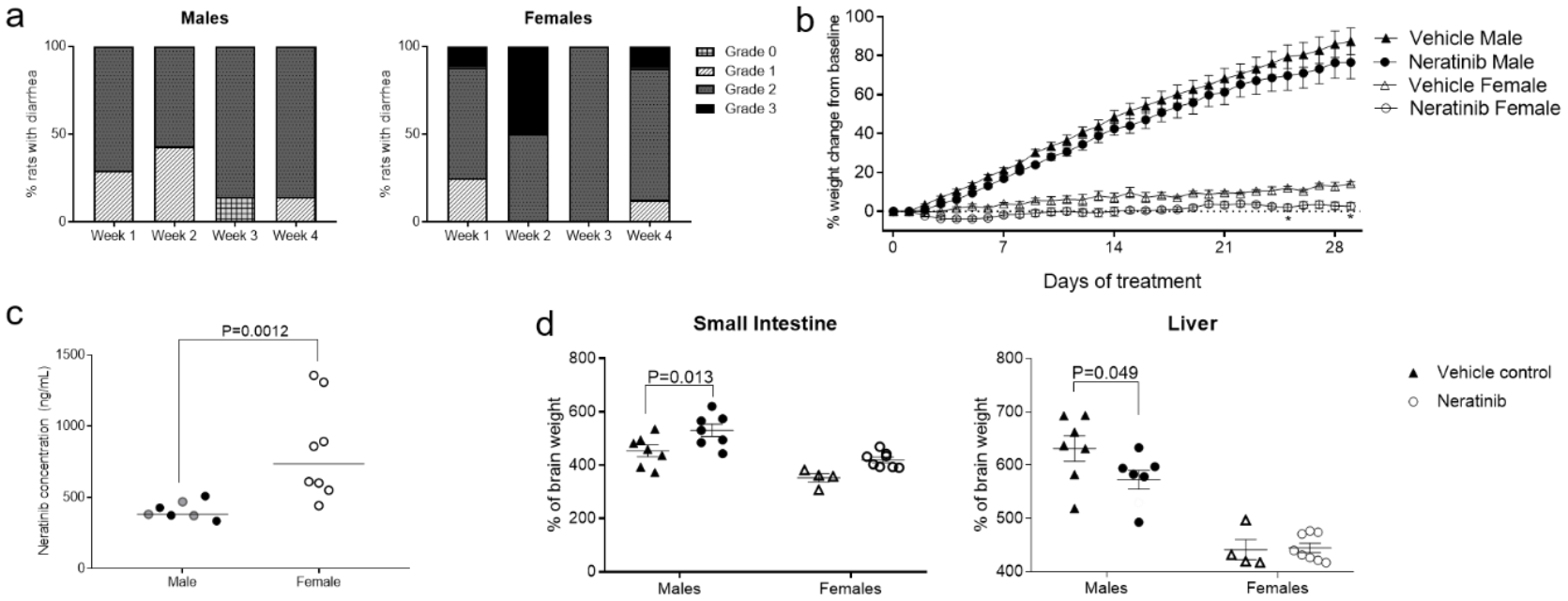

Figure 5.4: a) Comparison of diarrhoea levels in female and male rats after receiving 50 mg/kg neratinib. Figure represents highest grade of diarrhoea for each animal after each week of treatment. Female rats had an overall significantly higher average grade of diarrhoea ($P < 0.0001$, Chi-squared test). b) Percentage weight change from baseline after neratinib treatment in male and female rats. 88% of females lost weight in the first week of treatment, no male rats lost weight. Female neratinib treated rats had significantly less weight gain at day 25 (* $P = 0.0498$) and 29 (* $P = 0.0490$). Parametric data, data presented as mean ± SEM, two-way ANOVA used. c) Serum neratinib concentration was significantly higher in female rats compared to male rats ($P = 0.0012$). Data non-parametric, line shows median, Mann-Whitney test used. Grey data points in male column indicate data from initial dose finding study, these are included as 3 serum samples were missing from the second study dataset. d) Organ weights in male and female mice. Males had a significant change in small intestine ($P = 0.013$) and liver ($P = 0.049$) weight not seen in females. Parametric data, two-way ANOVA used. Data shown as mean ± SEM.

5.4.2.4 Neratinib caused multiple blood biochemistry changes in female rats

Female neratinib-treated rats had a number of changes in serum biochemistry between vehicle control and the neratinib treated group that were not seen in male rats. These include liver toxicity markers alanine transaminase (ALT) and urate, and metabolic markers such as bicarbonate and glucose (table 5.2). Males had significantly higher levels of alkaline phosphatase (ALP) in the neratinib treated group than vehicle control ($P = 0.039$). Both males ($P = 0.0023$) and females ($P = 0.0020$) treated with neratinib had higher levels of albumin than vehicle control animals. Although a significant difference in LD was seen in the dose-finding pilot study, this finding was not replicated here.

Table 5.2: Mean and SEM of blood serum biochemistry in male and female rats. Two-way ANOVA used for normally distributed data.

	Male: vehicle		**Male: neratinib**		**Female: vehicle**		**Female: neratinib**	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Sodium	141.57	0.69	141.43	0.57	140.75	1.03	139.63	0.60
Potassium	4.86	0.087	5.21	0.14	4.53	0.25	5.2	0.10
Chloride	100.57	0.48	102.29	0.57	102.75	0.48	103.88	0.85
Bicarb	29.57	0.84	28.71	0.57	28.00	1.23	24.89*	0.44
Anion Gap	16.29	0.81	15.43	0.61	14.50	0.65	15.88	0.67
Glucose	8.49	0.41	8.59	0.28	8.33	0.46	6.18#	0.27
Urea	6.60	0.20	6.21	0.26	5.15	0.30	6.49	0.61
Creatinine	21.57	1.41	19.43	2.02	28.75	1.03	30.50	1.93
Cholesterol	1.84	0.072	1.70	0.04	1.93	0.11	1.89	0.11
Urate	0.039	0.003	0.053	0.006	0.050	0.004	0.098^	0.007
Phosphate	2.60	0.052	2.44	0.10	2.03	0.057	2.10	0.045
Tot. Ca.	2.51	0.035	2.44	0.049	2.51	0.023	2.42	0.028
Albumin	15.86	0.14	14.43**	0.20	19.000	0.000	15.0##	0.27
Glob.	38.57	0.65	36.71	0.36	40.25	0.63	42.88	1.32
Protein	54.43	0.75	51.14	0.34	59.25	0.63	57.88	1.46
Tot. Bili.	1.00	0	1.00	0	1.00	0	1.00	0
GGT	3.00	0	3.00	0	3.00	0	3.13	0.13
ALP	229.43	12.65	337.14^^	34.02	139.25	11.14	156.88	5.65
ALT	50.00	2.43	61.86	2.98	47.00	1.87	115.75***	14.67
AST	97.71	6.68	133.86	28.35	94.25	4.33	177.00	21.09
LD	685.57	180.37	730.71	193.22	546.50	126.43	944.63	113.91

Symbol denotes significance between vehicle control and neratinib group in either male or females. *P = 0.046, #P = 0.029, ^P = 0.0002, **P = 0.0023, ##P = 0.002, ^^P = 0.039, ***P = 0.0006.

5.4.2.5 Female rats treated with neratinib had significant histological damage in the ileum and colon

After 28 days of neratinib treatment, female rats had significantly higher levels of gastrointestinal damage in the proximal ileum, distal ileum and colon than vehicle controls (figure 5.5). In males, there was only a significant difference between groups in the distal ileum. Similar to the dose finding study, observations included blunting and fusion of villi and infiltration of polymorphonuclear cells and lymphocytes.

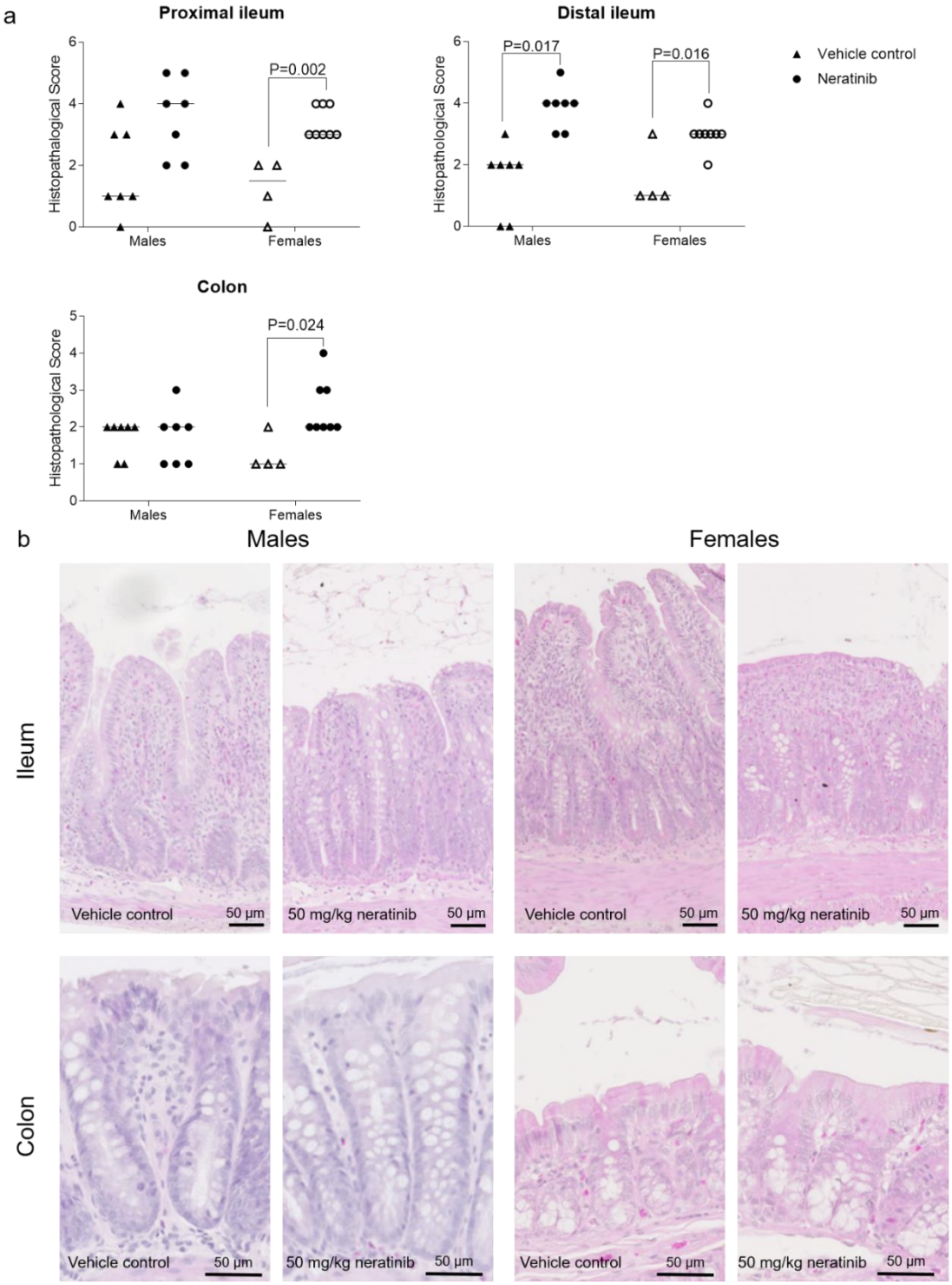

Figure 5.5: a) Female neratinib-treated rats had higher histopathological scores than vehicle controls in the ileum (proximal: P = 0.002, distal: P = 0.0016) and colon (P = 0.024). Male neratinib treated rats had higher scores than vehicle controls in the distal ileum (P = 0.017). Data non-parametric, line shows median. Mann-Whitney test used on male and female data, non-parametric data. b) Photomicrographs of distal ileum at 200 x original magnification and colon at 400 x original magnification (stained with H&E) showing evidence of blunting and fusion of villi and crypt elongation. Scale bars shown in diagram.

5.5 Discussion

This study aimed to develop a rat model of neratinib-induced diarrhoea that can in future be used to test potential interventions. Neratinib is used primarily for breast cancer, so we sought to carry out this model in a pilot study of female rats. However, as previously developed similar models are in male rats, we began a dose finding study in male rats. It was concluded that a 50 mg/kg daily dose of neratinib most successfully models effects in humans. As seen in other small molecule TKI models of diarrhoea, this is higher than the human equivalent dose (Reagan-Shaw et al., 2008; Bowen et al., 2012).

The present study showed that the changes observed in the gastrointestinal tract were mainly surrounding anatomical disruption of the ileum, and there was evidence of inflammatory infiltrate in the intestines. In male rats, significant microbial perturbations were observed in rats treated with neratinib.

This model was based on a previously developed model for targeted therapy-induced diarrhoea, where the drug lapatinib was used (Bowen et al., 2012). In that model, moderate diarrhoea was induced in 60% of rats, whereas we induced moderate diarrhoea in 100% of male rats (in the dose finding study). Interestingly however, there was no significant tissue pathology seen in that model. This is in contrast to another TKI animal model, of dacomitinib-induced diarrhoea, which caused severe tissue pathology localised in the ileum. The main factors of this pathology were severe villus atrophy with increased inflammatory infiltrate (Van Sebille et al., 2017), mirroring that seen in the development of this neratinib-induced diarrhoea model. Results from the initial

lapatinib model, where blood biochemistry showed a significant decrease in serum chloride, partly led to the hypothesis that TKIs may lead to a secretory type diarrhoea (Van Sebille et al., 2015). In addition, a study of neratinib in healthy volunteers showed a faecal osmotic gap that was consistent with that of secretory diarrhoea (Abbas et al., 2012). No evidence of this was seen in the current study when evaluating serum biochemistry.

Our results showed that neratinib caused more severe diarrhoea, weight change and biochemical parameters in female compared to male rats. This may be due to a variety of reasons. In this study we saw a statistically significant difference in systemic drug concentration between males and females. However, as previous work has shown no relationship to diarrhoea and serum concentration, it is unlikely to be responsible for the increase in diarrhoea in female rats. For example, a rat model of lapatinib showed no relationship between serum lapatinib levels and diarrhoea (Bowen et al., 2012). Additionally, our study of budesonide and neratinib in rats showed a decrease in diarrhoea in rats treated with budesonide and neratinib compared to neratinib alone, however these rats did not have a corresponding change in serum neratinib (Secombe et al., 2019a). Finally, a study of healthy volunteers having neratinib treatment failed to show a relationship between neratinib serum levels and diarrhoea occurrence (Abbas et al., 2012). We also saw that females had a higher level of variability than males in serum levels, and therefore cannot rule out inter-day variability in this study. Differential levels of ErbB expression in males and females may also cause variances in response. While there is little current research surrounding this, it is known that there is a significant

difference between males and females in liver expression levels of ErbB1 in mice (Wang et al., 2016a). A final possible mechanism is related to the female hormonal cycle. The hormonal cycles are well known to cause response changes in animal models of addiction and cardiovascular disease (Mahmoodzadeh et al., 2012; Becker et al., 2016). Progesterone and estrogen can also affect intestinal contractility and transit, gastric emptying time and higher gastrointestinal pH (Soldin et al., 2009). In this study, female rats had higher levels of histopathological damage in the colon compared to males, and this may cause less fluid absorption and therefore diarrhoea in the female rats.

One aim of developing this animal model was to be able to better understand the mechanisms of neratinib-induced diarrhoea in order to identify potential interventions. Many past studies have hypothesised how TKIs cause diarrhoea, and have included gut microbiome alterations, impaired water absorption or fluid transport and altered gut motility (Al-Dasooqi et al., 2009). The results of this dose-finding study showed blunting and fusion of the villi in the ileum. The ileum is the region of most pronounced histopathological changes, which impacts on surface area and loss of transporters. Additionally, it has shown that ErbB1 is expressed at a relatively higher level in the ileum compared to the rest of the gastrointestinal tract (Van Sebille et al., 2017). Moreover, histopathological analysis showed an increase in inflammatory infiltrate in the ileum. This may suggest that the neratinib-induced injury has an inflammatory basis, and could be targeted with anti-inflammatory agents. In this study, we did not see any statistically significant increase in histopathological damage in the colon of male rats, only females. However, in another study where we showed

the effects of neratinib in a male rat model (Secombe et al., 2019a), there were significant levels of damage in the colon. This may have been due to the use of a carboxymethylcellulose (CMC) buffer rather than a HPMC buffer. CMC has been shown to have negative effects on the colon in human studies (Chassaing et al., 2015).

In this study, we examined differences in the caecal microbiome of male rats treated with either vehicle control or neratinib. Examination of alpha diversity using Shannon's index showed a significant increase in diversity in rats treated with neratinib. This was an unexpected result, as a previous study in lapatinib showed decreases in alpha diversity as a result of treatment (Mayo et al., 2020). In addition, decreases in diversity have also been seen in chemotherapy studies (Touchefeu et al., 2014). The changes seen in chemotherapy may be attributed to the severe histological injury seen in these models, which was not seen in this neratinib model. In addition, the use of the caecal microbiome rather than the faecal microbiome may reflect these changes. Another potential hypothesis is that the large decrease in the *Blautia* genus in neratinib treated animals allowed a niche for a variety of other bacteria to multiply in. Despite the change in the Shannon's index, no significant differences between the groups were seen when using the Simpson and Chao1 index. This reflects a clinical study of the VEGF TKI sunitinib, where there was no significant difference in any diversity measure between patients with diarrhoea and patients who did not develop diarrhoea (Pal et al., 2015). The genus *Blautia* has previously been implicated in another side effect of cancer treatment, Graft-versus-Host Disease (GvHD). In that study, higher levels of intestinal *Blautia* led to reduced levels of

GvHD (Jenq et al., 2015). In addition, pilot work has shown that in patients having chemotherapy, higher levels of *Blautia* are related to less severe diarrhoea (Secombe et al., 2018). These results may form a hypothesis that changes in *Blautia* abundance during cancer treatment are a key component of diarrhoea development. Further research would be required to ascertain if the changes in *Ruminococcaceae* and *Oscillospira* are due to the diarrhoea or are direct drivers of the diarrhoea. In addition, this study did not examine the female microbiome, and therefore it is unknown if differences in the microbiome between male and female rats may explain the more severe toxicity seen in females. Additionally, as this study was not a tumour-bearing model, we have been unable to determine if the microbiome, or the diarrhoeal effect of neratinib, would be altered by the presence of a tumour. Future work could employ shotgun metagenomic sequencing or functional tests of the microbial metabolome to gain a further understanding of neratinib-induced changes to microbiome function. In any case, these results suggest that gut microbial changes may be a beneficial avenue of research in determining how neratinib-induced diarrhoea is exacerbated.

5.6 Conclusion

Severe neratinib-induced diarrhoea occurs in around 40% of patients and therefore an effective solution is greatly needed. This study has successfully developed a rat model for neratinib-induced diarrhoea, which can be used to test potential interventions for this diarrhoea. The model was reproducible and manageable in both female and male rats, with females suffering from more severe diarrhoea and colonic histopathological damage. The diarrhoea seen

was multi-factorial and focused on the distal small intestine, with elements of anatomical disruption and inflammation in the ileum. The vast microbial changes seen in rats treated with neratinib suggests that future research should interrogate manipulating the microbiome as a strategy to reduce diarrhoea levels.

Chapter 6: Antibiotic treatment targeting gram-negative bacteria prevents neratinib-induced diarrhoea in rats

Chapter six is my second original research chapter on the toxicity of the tyrosine kinase inhibitor neratinib. This chapter aimed to extend upon the findings of chapter five, by determining whether alteration of the gut microbiome prior to neratinib administration changed how diarrhoea developed. This chapter is presented as a traditional thesis chapter.

6.1 Abstract

Background: Neratinib is a pan-ErbB tyrosine kinase inhibitor approved by the FDA for extended adjuvant treatment of HER2-positive breast cancer. Diarrhoea is the main adverse event associated with neratinib treatment. Previous research has indicated that gut microbial changes and intestinal inflammation are involved in the pathogenesis of neratinib-induced diarrhoea. I aimed here to determine whether antibiotic-induced gut microbial shifts altered development of neratinib-induced diarrhoea.

Methods: Female Albino Wistar (AW) rats (total n=44) were given antibiotics (vancomycin, neomycin, or a cocktail of vancomycin, neomycin and ampicillin) in drinking water for four weeks, and then treated daily with neratinib (50 mg/kg) for 28 days. Diarrhoea, along with markers of gastrointestinal damage and microbial alterations were measured by histopathology and 16S sequencing, respectively.

Results: Rats treated with vancomycin or neomycin had significantly lower levels of diarrhoea than rats treated with neratinib alone. In the distal ileum, neratinib was associated with a statistically significant increase in histological damage in all treatment groups expect the antibiotic cocktail. Key features included villous blunting and fusion and some inflammatory infiltrate. Differences in microbial composition at necropsy in vehicle control, neratinib and neratinib + neomycin groups, were characterised by a neratinib-induced increase in gram-negative bacteria that was reversed by neomycin. Neomycin shifted bacterial composition so that *Blautia* became the dominant genus.

Conclusions: Narrow-spectrum antibiotics reduced neratinib-induced diarrhoea. This suggests that the microbiome may play a key role in the development and prolongation of diarrhoea following neratinib treatment, although further research is required to understand the key bacteria and mechanisms by which they reduce diarrhoea, as well as how this may impact presentation of diarrhoea in clinical cohorts.

6.2 Introduction

Neratinib is an orally available, irreversible small molecule pan-ErbB tyrosine kinase inhibitor (TKI). It is Food and Drug Administration (FDA) approved for the extended adjuvant treatment of early-stage Human epidermal growth factor receptor 2 (HER2) positive breast cancer, and in combination with capecitabine for advanced and metastatic HER2 positive breast cancer (Secombe et al., 2019a; FDA, 2020). However, diarrhoea has been a major adverse event in clinical trials conducted thus far. Two recent meta-analyses looking at the

safety and efficacy profile of neratinib (Tao et al., 2019) or the risk of gastrointestinal events during neratinib treatment (Chen, 2019b) found the most frequently occurring adverse event (all-grade) in neratinib monotherapy was diarrhoea. The study by Tao et al. (2019) found diarrhoea occurred in 83.9% of patients, while Chen (2019b) concluded that all-grade diarrhoea occurred in 78% of patients.

The phase III ExteNET trial of 2840 patients recruited patients to receive one year of neratinib treatment following one year of trastuzumab. In this trial, without diarrhoea prophylaxis, 40% of patients developed severe, grade 3 - 4 diarrhoea (Chan et al., 2016). Pre-clinical investigations suggested neratinib-induced diarrhoea may be reduced by the corticosteroid budesonide, or the bile acid sequestrant colesevelam (Secombe et al., 2019a). The phase II CONTROL study aimed to replicate this in a clinical setting (Barcenas et al., 2020). Again, patients received one year of neratinib treatment following completion of trastuzumab-based adjuvant therapy. Study groups consisted of loperamide prophylaxis (n = 137), loperamide + budesonide (n = 64), loperamide + colestipol (n = 136), colestipol + as needed loperamide (n = 104) and a neratinib dose escalation group (interim data, n = 60). Grade 3 diarrhoea rates were lower than in the ExteNET trial, ranging from 32% of patients in the colestipol + as needed loperamide group, to 15% in the neratinib dose escalation group. No grade 4 diarrhoea occurred throughout the trial. Despite this improvement, there remains a gap in finding the most effective way to mitigate neratinib-induced diarrhoea.

Previous work has shown a role for the gut microbiome in cancer treatment-related diarrhoea. In models of chemotherapy-induced diarrhoea, shifts in gut microbial composition are evident and believed to be associated with the pathogenesis of intestinal changes (Stringer et al., 2013; Pedroso et al., 2015). A recent hypothesis suggests the microbiome may have a similar role in diarrhoea from TKI treatments (Mayo et al., 2020; chapter 4). Notably, I have shown neratinib treatment in rats leads to, in the caecal microbiome, changes in abundance of the family *Ruminococcaceae* and the genera *Blautia* and *Oscillospira,* and altered principal coordinate analysis clustering between vehicle and neratinib treated rats (chapter 5). Further investigation is required to understand whether these changes are a key factor in diarrhoea development or a downstream effect of other mechanisms such as changes in the topography of the intestine or inflammatory signal milieu. Whilst some emerging evidence suggests a regulatory role for the microbiome in response to immunotherapy (Frankel et al., 2017; Roy et al., 2017), the mechanisms of how epidermal growth factor receptor (EGFR)-targeted therapies affect the microbiome is unknown, and whether there is any relationship to outcomes of therapy. Previous research has demonstrated that antibiotic use may negatively affect efficacy of EGFR-targeted treatment for non-small cell lung cancer (Liu et al., 2019), but the effect of antibiotics on diarrhoea from treatment has not been adequately researched.

As such, this study aimed to determine the impact of antibiotic treatment on gut microbial changes following neratinib and the effect on neratinib-induced diarrhoea. In this study, we tested a variety of antibiotics. Vancomycin is a

glycopeptide antibiotic that is predominantly active against gram-positive bacteria (Isaac et al., 2017). It is commonly used in patients with *Clostridioides difficile* infection or in multi drug-resistant *Staphylococcus aureus* infections (Isaac et al., 2017). Neomycin is an aminoglycoside antibiotic that works by causing irreversible binding of nuclear 30S ribosomal subunit (Sasseville, 2010). It is most effective against gram-negative organisms, and is used to sterilise the gut before digestive tract surgery (Sasseville, 2010). Both are poorly systemically absorbed into the blood from the intestinal tract. Finally, we tested a broad-spectrum antibiotic cocktail of vancomycin, neomycin and ampicillin (previously used in a variety of pre-clinical models (Rey et al., 2018)) that aimed to completely ablate the gut microbiota.

6.3 Methods

6.3.1 Chemicals and reagents

Neratinib was kindly provided by Puma Biotechnology (USA). Neratinib was diluted in 0.5% (w/w) hydroxypropyl methylcellulose (HPMC) buffer (Sigma-Aldrich, St. Louis, USA).

6.3.2 Animals and ethics

All experiments were conducted on female Albino Wistar (AW) rats obtained from the Animal Resource Centre, Perth, Australia. Rats were housed in groups of between 4 and 5 in individually ventilated cages. Temperature was maintained between 19 to 23 °C and relative humidity within the range of 45 to 65%; with a 12 hour light/dark cycle. Food and water were consumed *ad libitum*. If rats were experiencing moderate to severe treatment-related toxicity (e.g. diarrhoea, weight loss, stress marks) they were allowed soaked chow (normal feed softened in water to ease mastication). Where rats were experiencing severe treatment-related toxicity, a neratinib treatment was also halted until symptoms were graded as moderate or mild. Rats were acclimatised to local housing conditions for a minimum of 7 days prior to the first day of dosing. On Day 1 of treatment, rats were between 7 - 9 weeks old. This study was approved by the Animal Ethics Committee of the University of Adelaide (study number M-2019-025), and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training (2013).

6.3.3 Antibiotics

Antibiotics were added to sterile drinking water 4 weeks prior to the beginning of neratinib treatment. Vancomycin hydrochloride was diluted to 0.5 g/L (Cayman Chemicals, #15327, Ann Arbor, USA) and neomycin trisulphate salt hydrate was diluted to 1 g/L (Sigma-Aldrich, #N1876, St. Louis, USA). The antibiotic cocktail consisted of vancomycin and neomycin as above, in addition to ampicillin sodium salt (1 g/L, Sigma-Aldrich, #A9518). A maltodextrin and 1.2% sucralose mixture (Splenda®, Collingwood, Australia) was also added in the antibiotic cocktail at 0.75 g/L to ensure water consumption. Antibiotics were shielded from light and refreshed daily.

6.3.4 Experimental design

Rats were randomly assigned to study groups as follows: vehicle (0.5% (w/w) HPMC buffer) and no antibiotics (n = 8), neratinib (50 mg/kg) and no antibiotics (n = 10), neratinib (50 mg/kg) and antibiotic cocktail (n = 8), neratinib (50 mg/kg) and vancomycin (n = 8) and neratinib and neomycin (n = 10). During the 28 day neratinib treatment period, rats received daily oral gavages using a soft plastic feeding tube (18 gauge, Instech, Plymouth Meeting, USA) coated in 30% sucrose solution. Neratinib or vehicle treated rats were given at a constant dose volume of approximately 5 mL/kg. Individual dose volumes were adjusted daily according to the body weight of each rat on each treatment day. The first day of dosing was designated Day 1. The final dose was given on the day before scheduled necropsy. All rats were deeply anaesthetised via isoflurane

inhalation (Isothesia, Henry Schein, Melville, USA), and culled by cardiac exsanguination with death confirmed by cervical dislocation.

6.3.5 Clinical gut toxicity assessment

Rats were weighed once daily, and comprehensively monitored twice daily via a clinical symptom reporting system. Diarrhoea was graded by two assessors according to a well-established grading system (Bowen et al., 2012) with 4 grades: 0, no diarrhoea; 1, mild (soft unformed stools); 2, moderate (perianal staining and loose stools); or 3, severe (watery stools and staining over legs and abdomen). Rats were culled if displaying 15% or greater weight loss from baseline or significant distress and clinical deterioration (although no animals reached this endpoint).

6.3.6 Tissue collection and preparation

At necropsy, the gastrointestinal tract was removed from the pyloric sphincter to the rectum. The small and large intestine were flushed with chilled, sterile 1 x phosphate buffered saline (PBS), pH = 7.4 and weighed. Samples of duodenum, jejunum, proximal and distal ileum and proximal and distal colon were collected and fixed in 10% formalin (ChemSupply, Gillman, Australia) for embedding in paraffin (Sigma-Aldrich, St. Louis, USA).

6.3.7 Histological examination

Paraffin embedded intestinal samples were cut with a rotary microtome (RM2235, Leica, Wetzlar, Germany) and 4 µm sections were mounted onto Superfrost glass slides (Menzel-Glaser, Braunschweig, Germany). Images of all

slides were taken using a NanoZoomer digital slide scanner (Hamamatsu Photonics, Japan) and viewed using the NanoZoomer Digital Pathology Software (NDP View v1.2, Histalim, Montpellier, France). All analysis was conducted in a blinded fashion.

6.3.8 Mucosal damage analysis

Routine haematoxylin and eosin (H&E) staining was completed and an injury score assigned using a well-established system of histological criteria (Howarth et al., 1996; Wardill et al., 2016). Criteria were villus fusion, villus atrophy, disruption of brush border and surface enterocytes, crypt losses / architectural disruption, disruption of crypt cells, infiltration of polymorphonuclear cells and lymphocytes, dilation of lymphatics and capillaries and oedema. The latter six criteria were examined in the colon. Each criterion was scored as present = 1 or absent = 0.

6.3.9 Serum endotoxin analysis

Blood samples were collected at necropsy by cardiac puncture using a 23 gauge needle (Becton-Dickinson, Franklin Lakes, USA) in Z Serum (Sep) Clot Activator Vacuette tubes (Greiner Bio-One, Kremsmünster, Austria). Serum was separated by centrifugation at 931 *x g* for 15 minutes at room temperature. Serum was then aliquoted and stored at -80 °C until used.

A serum limulus amebocyte lysate (LAL) endotoxin assay was run on heat-treated serum samples. Serum was diluted 1:10 in endotoxin free water, and heat treated at 70 °C for 15 minutes. The PyroGene Recombinant Factor C

Endotoxin Detection Assay (Lonza, Basel, Switzerland; #50-658U) was then used to quantify serum endotoxin, as per manufacturer's guidelines. Endotoxin concentration was determined relative to a linear standard curve (range, 0.005 - 5 EU/mL).

6.3.10 Gut bacterial DNA extraction and diversity profiling

Faecal samples from vehicle control, neratinib only, and neratinib + neomycin groups were analysed using 16S sequencing techniques. Distal faecal contents were aseptically collected during dissection into a sterile tube at each time point, and stored at -80 ˚C. Samples were sent to the Australian Genome Research Facility (AGRF) for DNA extraction and 16S ribosomal RNA (rRNA) gene region analysis. DNA was extracted from 250 mg of faecal sample using the Qiagen DNeasy PowerLyzer PowerSoil Kits with the PowerLyzer 24 Homogeniser. 16S analysis sequencing details are as follows:

Target: 16S: 341F (V3-V4) (V1-V3), read length = 300 bp.

Forward sequence: 5’- CCTAYGGGRBGCASCAG -3’

Reverse Sequence: 5’- GGACTACNNGGGTATCTAAT -3’

Image analysis was performed in real time by the MiSeq Control Software v2.6.2.1 and Real Time Analysis v1.18.54, running on the instrument computer. The Illumina bcl2fastq 2.20.0.422 pipeline was used to generate the sequence data. CLC Genomics Workbench 12.0 (https://www.qiagenbioinformatics.com/) was used to complete bacterial diversity profiling. Paired-ends reads were assembled by aligning forward and reverse reads. Primers were identified and

trimmed. Trimmed sequences were quality filtered, duplicate sequences removed and sorted by abundance. Reads were assigned to taxonomic identities using the Greengenes 97% similarity database version 13.8. Alpha-diversity was calculated using the Shannon diversity index. Beta diversity of operational taxonomic units (OTUs) was calculated using Principal Coordinate Analysis (PCoA) based on generalised UniFrac distances (Chen et al., 2012a). The program BURRITO (McNally et al., 2018) was used to visualise the links between taxonomic composition and function in the dataset using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Principal Component Analysis (PCA) was used to assess differences in KEGG pathways.

6.3.11 Statistical analysis

Data were compared using Prism version 7.0 (GraphPad Software, USA). If data was normally distributed, bars on graphs are mean ± SEM. If not, median is displayed in graphs. The assumptions of equality of variance for each group and normally distributed data were tested using Bartlett's test and D'Agostino & Pearson omnibus normality test, respectively. If these assumptions were violated, non-parametric equivalent tests were performed, including Kruskal-Wallis for independent data. When assumptions held, ANOVA's were performed using the 2-way analysis of variance (ANOVA). A Mantel-Cox test was used to determine differences in the survival curves for diarrhoea levels. Statistical Analysis of Metagenomic Profiles (STAMP) (Parks et al., 2014) was used to assess the predicted metagenome using Welch's *t* test with the Benjamini-Hochberg correction for the false discovery rate (FDR). P-values less than 0.05 were considered statistically significant.

6.4 Results

6.4.1 Single narrow-spectrum antibiotics significantly reduced diarrhoea levels

Neratinib induced significantly longer duration of moderate (grade 2) diarrhoea (mean = 13.8, range = 9 – 21 days) compared to the vehicle group (mean=0, range = 0 – 0 days, P = 0.0003) (figure 6.1 A). The addition of vancomycin (mean = 0.25, range = 0 - 1 days, P = 0.0013) or neomycin (mean = 0, range = 0 – 0 days, P < 0.0001) significantly reduced days with grade 2 neratinib-induced diarrhoea compared to neratinib alone. There was no significant difference between grade 2 diarrhoea in the antibiotic cocktail (mean = 2.25, range = 0 – 6 days, P > 0.05) and neratinib alone. The antibiotic cocktail caused mild grade 1 diarrhoea which preceded the beginning of neratinib treatment (figure 6.1 B). Only two rats developed grade 2 or 3 diarrhoea in the vancomycin group, and no animal in the neomycin group developed grade 2 or 3 diarrhoea (figure 6.1 C).

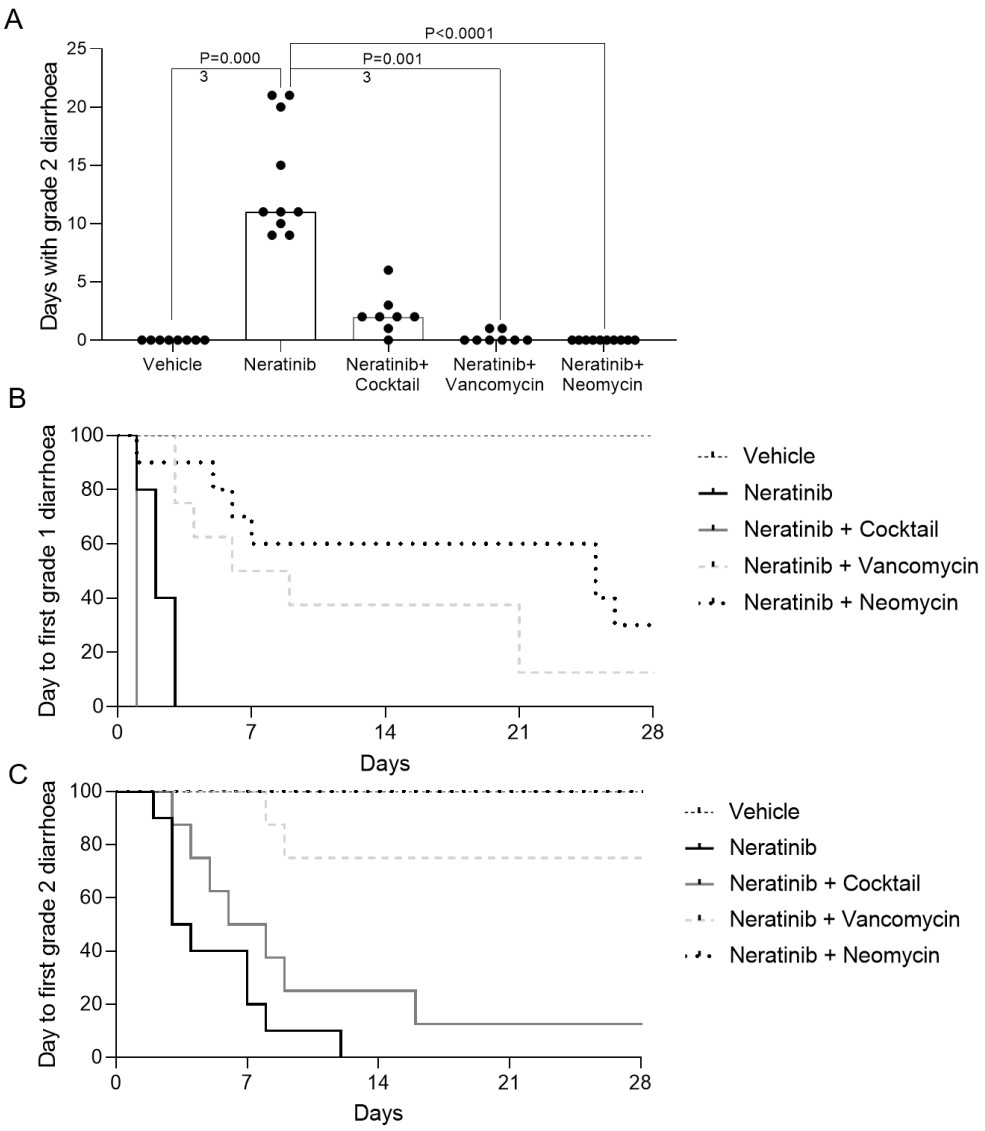

Figure 6.1: Diarrhoea development. A) Days in total with grade 2 (moderate) diarrhoea. Bar signifies median. Rats treated with vancomycin (P = 0.0013) or neomycin (P < 0.0001) had less days with diarrhoea compared to the neratinib alone group. Kruskal-Wallis test used to determine significance. B) Survival graph of days until first occurrence of grade 1 diarrhoea. C) Survival graph of days until first occurrence of grade 2 diarrhoea. Antibiotic treated groups had different survival curves to neratinib alone in both (B) and (C) (Mantel-Cox test, P < 0.0001).

6.4.2 Rats treated with antibiotic cocktail gained less weight than all other groups

All rats continuously gained weight over the course of the experiment. Neratinib only, and neratinib + antibiotic cocktail treated rats gained less weight over the time course than vehicle rats ($P < 0.0001$). The neratinib and cocktail group also gained less weight than the vancomycin group ($P = 0.0082$) and the neomycin group ($P = 0.014$) (figure 6.2 A).

6.4.3 Neratinib increased small intestinal weight in all groups

The wet weight of the small intestine was normalised to percentage of brain weight, based on previous research (Secombe et al., 2019a). All neratinib treated groups had increased compared to vehicle group (mean=4.69%) (figure 6.2 B). The neratinib + antibiotic cocktail group had the highest mean (7.42%, $P < 0.0001$ vs vehicle). The neratinib only group had a mean of 6.24% ($P = 0.0007$ vs vehicle), neratinib + vancomycin had a mean of 5.85% ($P = 0.026$ vs vehicle) and the neomycin group had a mean of 6.41% ($P = 0.0002$ vs vehicle). There were no differences in weight of the large intestine, liver, spleen, kidneys, stomach, heart or lungs (data not shown).

6.4.4 No change in serum endotoxin between groups

Endotoxin assay results were variable within groups, and there were no differences between groups (figure 6.2 C). Values were within previously published ranges of healthy female AW rats (Kosyreva et al., 2018).

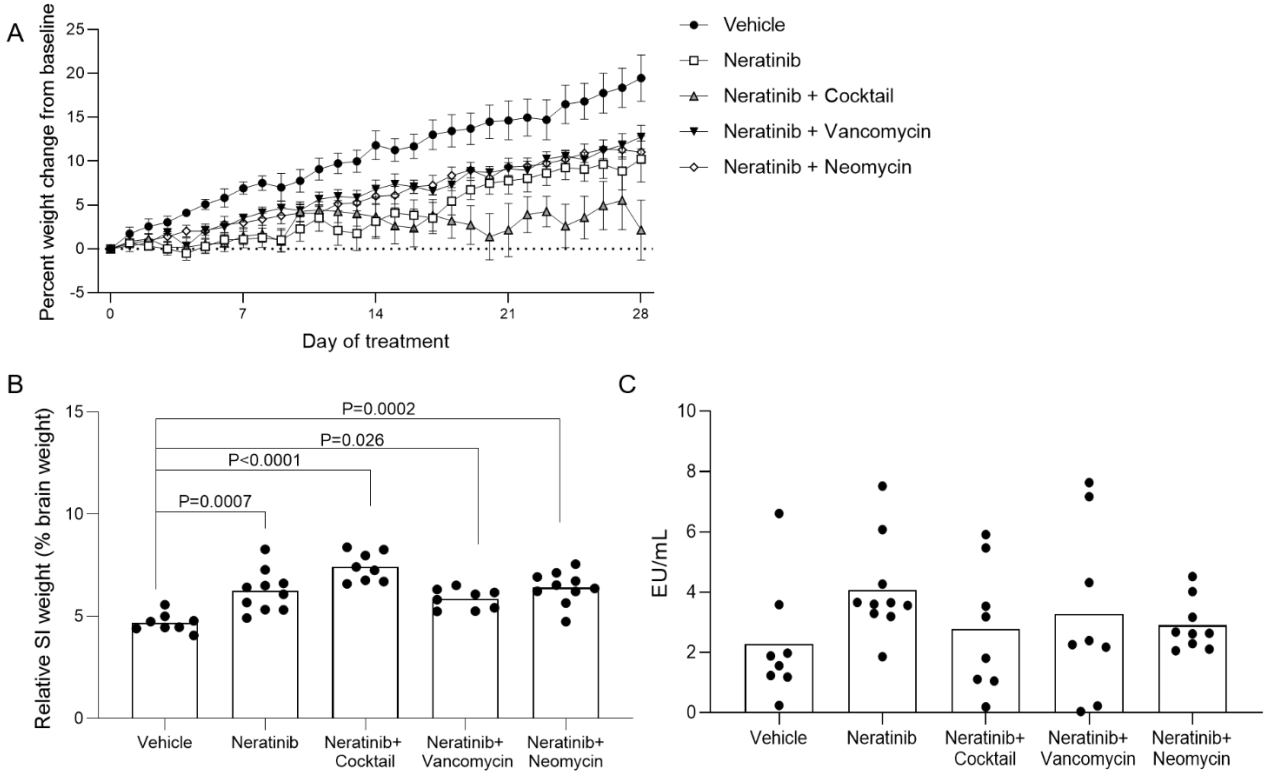

Figure 6.2: Body and organ weight and serum endotoxin investigations. A) Baseline-corrected weight gain across 28 days of treatment. Rats treated with neratinib + antibiotic cocktail had less weight gain than all other groups of rats ($P < 0.0001$). There was no difference between the vehicle group and the neratinib + vancomycin or neratinib + neomycin groups ($P > 0.05$). Data shown as mean ± SEM. Kruskal-Wallis test used. B) Small intestinal weight normalised to brain weight. All neratinib treated groups increased compared to vehicle group (one-way ANOVA with Tukey's multiple comparison test). C) Serum endotoxin was assessed using a serum limulus amebocyte lysate (LAL) endotoxin assay. Bar signifies mean. Samples were analysed using a one-way ANOVA with Tukey post-hoc testing. There were no differences between groups ($P > 0.05$). One outlier was removed from the neratinib + neomycin group as it was more than 3 standard deviations above the mean, likely due to being haemolysed. Bars signify mean.

6.4.5 Neratinib caused significant intestinal injury in the ileum

In the proximal ileum (figure 6.3 A,C), neratinib caused increased histopathological injury compared to vehicle ($P = 0.042$). Antibiotic treated groups did not have increased injury compared to the vehicle group ($P > 0.05$). Blunting and fusion of villi was observed in the neratinib only group. In the distal ileum (figure 6.3 B, D), neratinib only and single antibiotic treated groups had higher levels of damage than vehicle group (vehicle vs neratinib; $P = 0.042$, vehicle vs vancomycin, $P = 0.032$, vehicle vs neratinib + neomycin, $P = 0.013$). In the distal ileum, blunting and fusion of the villi was observed in all neratinib treated groups. No differences were observed between groups in the distal or proximal colon (figure 6.3 E, F).

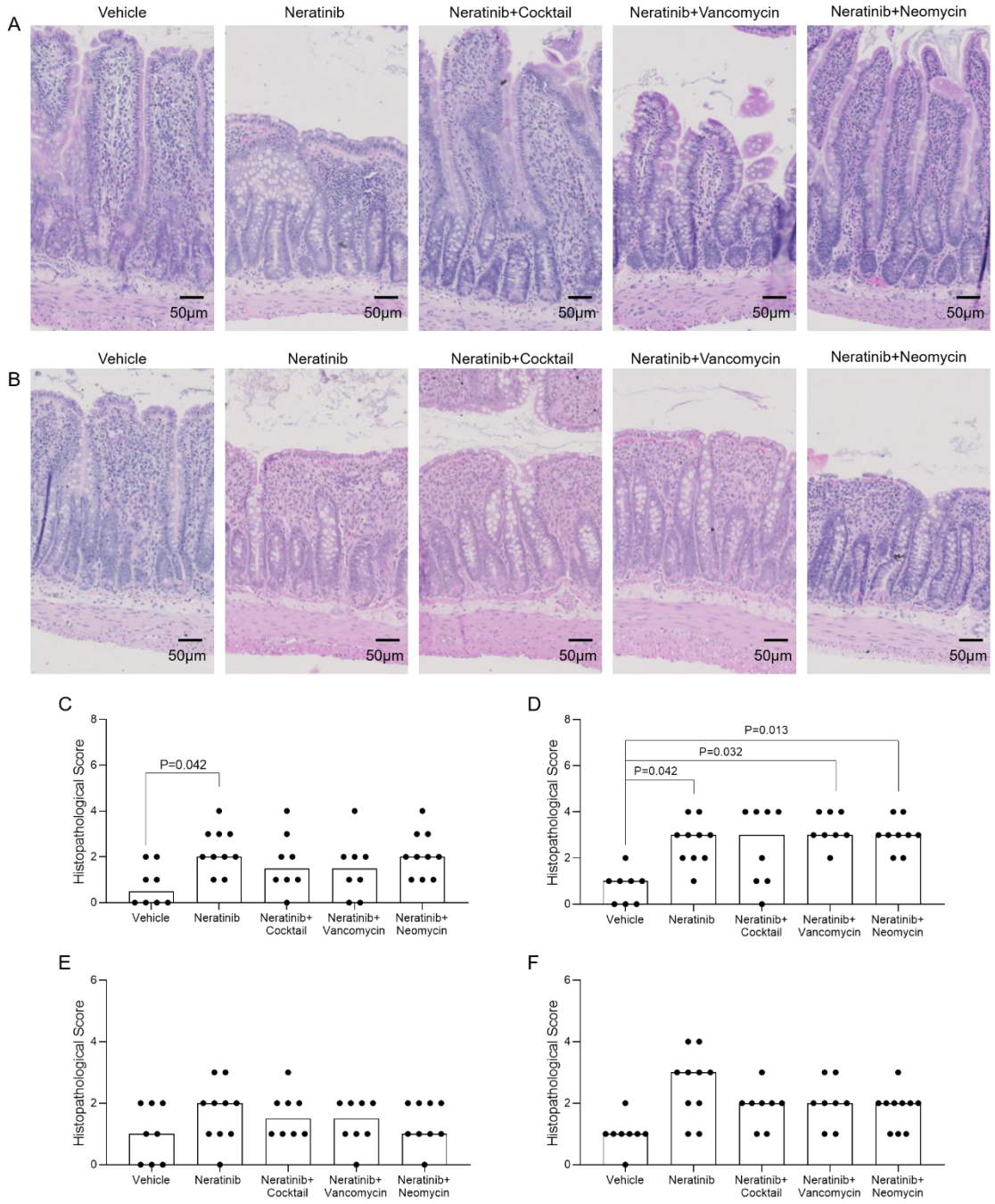

Figure 6.3: Intestinal histopathological injury. Representative images of haematoxylin and eosin staining in the proximal (A) and distal (B) ileum: original magnification is 200 ×; scale bars represent 50 μm as shown in images. Histological damage scoring in the proximal ileum (C), distal ileum (D), proximal colon (E) and distal colon (F). Bar signifies median. In the distal ileum, neratinib alone injury scores were higher than vehicle (P = 0.042). In the proximal ileum, neratinib alone (P = 0.042), neratinib + vancomycin (P = 0.032) and neratinib + neomycin (P = 0.013) had higher scores than vehicle control. There were no differences in the colon. Statistical significance determined using a Kruskal–Wallis test. Damage scoring on a scale of 0 - 8 for C and D and 0 - 6 for E and F.

6.4.6 Microbial changes

To assess the faecal microbiome, distal colonic pellets were taken at necropsy from a subset of 6 rats from each of the vehicle, neratinib only and neratinib + neomycin groups. The neomycin group was the focus of microbial analysis since these rats experienced no grade 2 diarrhoea. Samples were analysed using 16S sequencing and microbial analysis (figure 6.4). To ensure the targeting effect of neomycin on gram-negative bacteria, we analysed relative abundance of the phylum Proteobacteria. Proteobacteria are gram-negative, and are often present at low levels in healthy controls but increased in instances of inflammation or dysbiosis (Shin et al., 2015). Here, neratinib alone treated animals had higher levels of Proteobacteria than neratinib + neomycin treated rats ($P = 0.0029$), whereas there was no difference between vehicle rats and neratinib + neomycin treated rats ($P > 0.05$) (figure 6.4 A). At species level, the neomycin treated group had lowered alpha diversity (Shannon's index) than both the vehicle group ($P = 0.0002$) and the neratinib alone group ($P = 0.003$) as expected (figure 6.4 B). There were no differences between the vehicle and neratinib groups. The same pattern was seen at the genus level. PCoA demonstrated each group clustered differently, confirmed by pairwise PERMANOVA tests ($P = 0.0065$, figure 6.4 C).

At genus level, there were marked differences between each group in the faecal microbiome at necropsy (figure 6.4 D). There was an increase in the relative abundance of the genus *Blautia* in the neratinib + neomycin group ($P < 0.001$) compared to both vehicle and neratinib only (figure 6.4 E). The genus *Allobaculum* was also increased in the neratinib group compared to vehicle ($P =$

0.0031). This difference was not seen in the neratinib + neomycin group (figure 6.4 F).

Finally, analysis of metabolic pathways predicted to be altered due to microbial changes were investigated. The nearest sequenced taxon index (NSTI) scores of each sample varied from 0.073 to 0.17 (mean = 0.11, data not shown). These scores are moderate, suggesting somewhat accurate and reliable predictions (Langille et al., 2013). However without functional tests of the metabolome, it would not be prudent to completely rely on these findings. At the KEGG pathway level, results were filtered to those with a corrected p-value of < 0.01 and an effect size of > 0.85. The four functional groups with the highest effect size between treatment groups were all related to metabolism (methane metabolism, lysine biosynthesis, glyoxylate and dicarboxylate metabolism and cysteine and methionine metabolism) (table 6.1). Principal component analysis (PCA) demonstrated altered clustering between the treatment groups (figure 6.5 A). Also of particular note was levels of oxidative phosphorylation, which were lower in the neratinib alone treated group compared to vehicle and neratinib + neomycin groups ($P < 0.05$, figure 6.5 B).

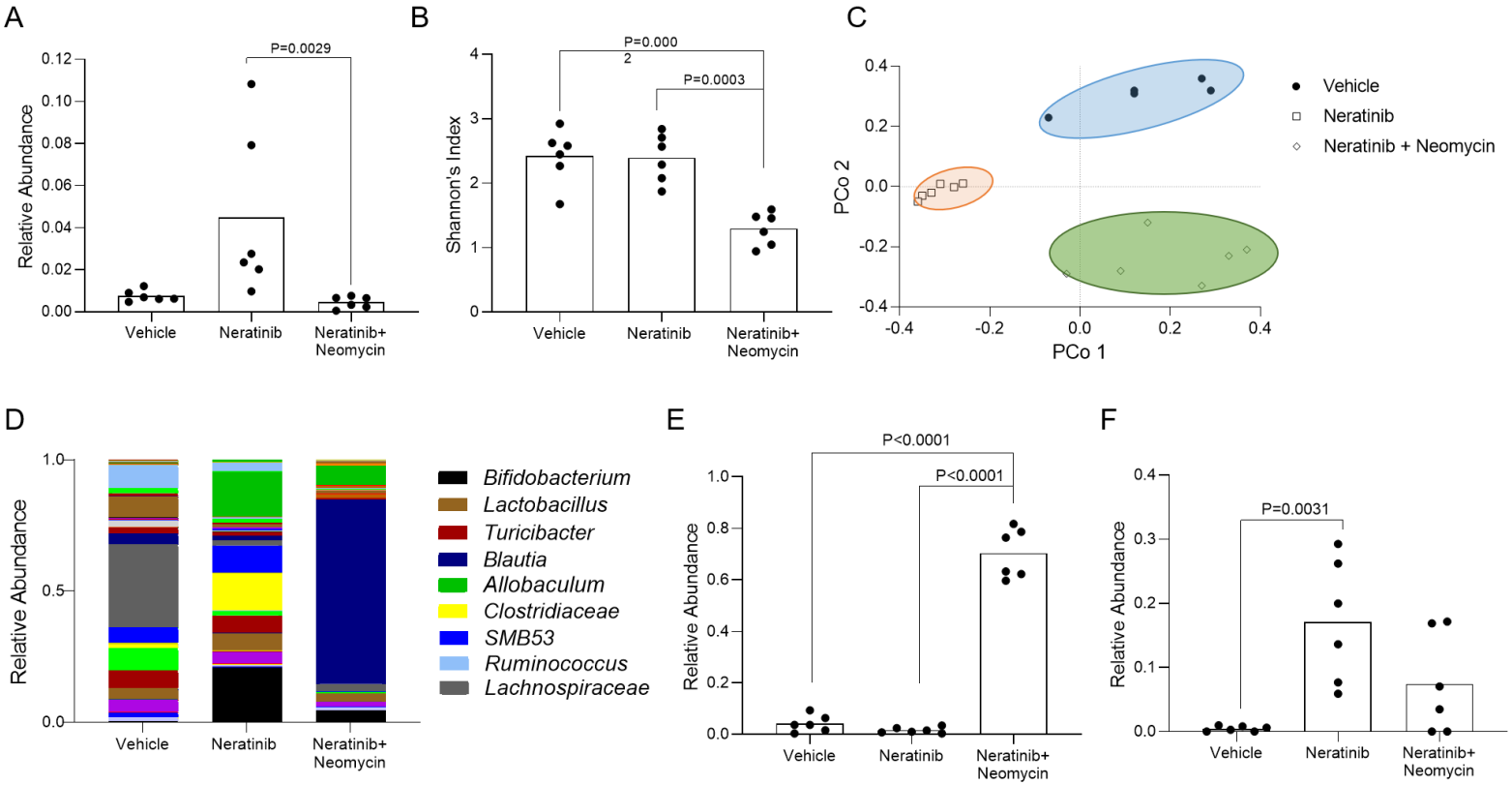

Figure 6.4: Microbial analysis. (A) Relative abundance of the gram-negative phylum Proteobacteria. The neratinib group had higher levels compared to neratinib + neomycin (P = 0.0029). (B) Alpha diversity measured using Shannon's index. Neomycin treated rats had lower diversity than both vehicle (P = 0.0002) and neratinib only (P = 0.0003). (C) PCoA demonstrated that each group clustered separately, confirmed by PERMANOVA (P = 0.0065). (D) Relative abundance at the genus level. Highly abundant genera shown in figure legend. (E) Relative abundance of *Blautia*. The neratinib + neomycin group had higher levels than vehicle (P < 0.0001) and neratinib only (P < 0.0001). (F) Relative abundance of *Allobaculum*. Neratinib only had higher levels than vehicle (P = 0.0031). No difference was seen in the neratinib + neomycin treated group. In (B), (C), (D) and (E), bar signifies mean and significance determined via 2-way ANOVA or Kruskal-Wallis test.

Table 6.1: Analysis of metabolic pathways predicted to be altered due to microbial changes. Results were filtered to the four functional groups with the highest effect size between treatments groups.

KEGG Level					Mean relative frequency (%)		
1	**2**	**3**	**P-values (corrected)**	**Effect size**	**Vehicle**	**Neratinib**	**Neratinib + Neomycin**
Metabolism	Energy metabolism	Methane metabolism	1.73×10^{-8}	0.94721	0.43589	0.410734	0.738497
Metabolism	Amino acid metabolism	Lysine biosynthesis	1.09×10^{-8}	0.945554	0.782927	0.74089	0.929823
Metabolism	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism	4.54×10^{-8}	0.930514	0.723007	0.674503	0.798065
Metabolism	Amino acid metabolism	Cysteine and methionine metabolism	8.81×10^{-8}	0.92111	0.958553	1.148907	0.833396

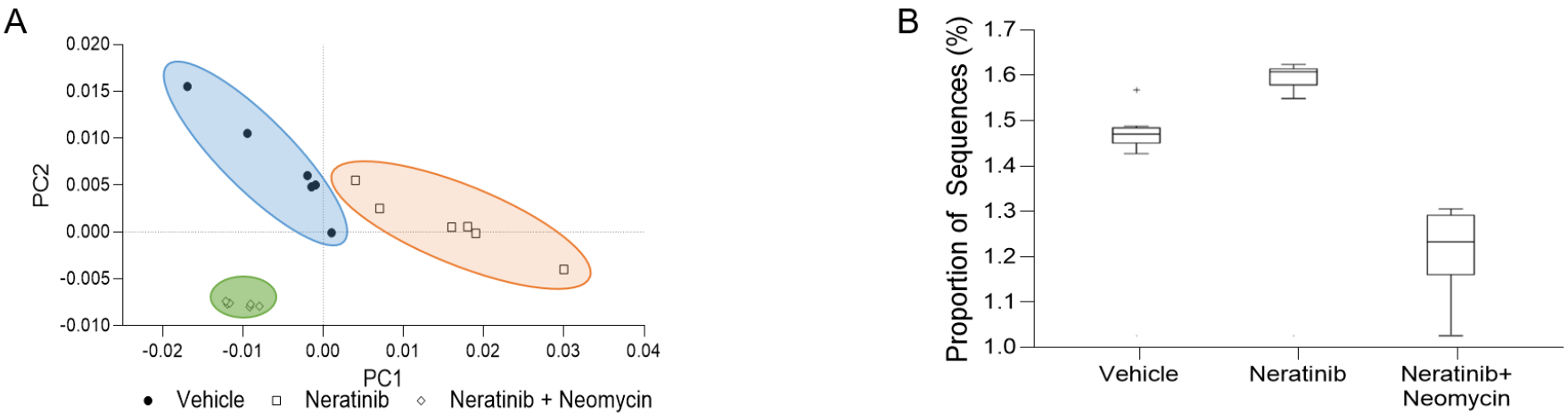

Figure 6.5: Inferred gut microbiome functions analysed using STAMP from 16S rRNA gene sequences. (A) PCA plot at the KEGG level showing separate clustering between each treatment group. (B) Alterations in oxidative phosphorylation function between groups. Box plots show the top quartile, median and bottom quartile, with '+' indicating outlier. Benjamini–Hochberg FDR method was used to correct for multiple comparisons.

6.5 Discussion

This study aimed to investigate how alterations in gut microbial composition affect the development of neratinib-induced diarrhoea. I found narrow-spectrum antibiotics targeting specifically gram-negative or positive bacteria (neomycin or vancomycin respectively) caused a highly significant decrease in diarrhoea levels, whereas a broad-spectrum antibiotic cocktail was less effective.

While neomycin and vancomycin treatment significantly reduced diarrhoea and caused a weight gain pattern more similar to vehicle than neratinib only treatment, antibiotic treatment did not cause improvement in the pathological markers measured in the distal ileum. In addition, no changes in serum endotoxin levels were noted for any group, indicating that the mucosal barrier remained intact. More research may be required to understand the exact way in which neomycin and vancomycin were able to cause such vast improvement in diarrhoea levels without modifying gross-histological changes to the tissue. It is unlikely to be due to direct inhibition of neratinib absorption, although serum concentration was not measured in the current study to definitely exclude this concern. One recent review (Ergun et al., 2019) has suggested that antibiotics in the macrolide groups may decrease TKI metabolism via inhibition of cytochrome P450 3A4 enzyme, however this does not cover the antibiotics used in this study. Anyhow, decreasing TKI metabolism may actually lead to increased systemic concentration and therefore increased toxicity. Additionally, while there is evidence to suggest that the gut microbiome assists in the metabolism of chemotherapies, there is no clear evidence as of yet to suggest a similar effect in neratinib or other TKIs (Alexander et al., 2017).

Neomycin and vancomycin are minimally absorbed from the gastrointestinal tract when administered orally and therefore is the basis of their use to specifically suppress intestinal bacteria (National Institute of Diabetes and Digestive and Kidney Diseases, 2012). There is however some evidence to suggest systemic uptake of vancomycin may be increased in instances of intestinal inflammation (Aradhyula et al., 2006). Neomycin is an aminoglycoside antibiotic with strong activity against gram-negative bacteria. I confirmed this, showing that rats treated with neomycin had lower levels of gram-negative Proteobacteria compared to rats treated with only neratinib. Previously, pre-clinical studies have shown extensive increases in pathogenic gram-negative bacteria following various cancer treatments corresponding with an increase in diarrhoea (Von Bultzingslowen et al., 2003; Stringer et al., 2008; Stringer et al., 2009b; Stringer et al., 2013). Additionally, these gram-negative species can often release lipopolysaccharide (LPS) known to initiate the key inflammatory mediators that are known to cause diarrhoea following cancer treatment (Akira et al., 2004). Furthermore, a small study showed complete amelioration of diarrhoea in 6 out of 7 patients receiving the chemotherapy agent irinotecan as well as neomycin (Kehrer et al., 2001), although this result was not replicated in a larger study (de Jong et al., 2006). Vancomycin, when orally administered, is commonly used to treat intestinal *Clostridioides difficile* infection. It is highly effective against gram-positive bacteria. As both vancomycin and neomycin had similar effectiveness in decreasing diarrhoea following neratinib, this diarrhoea does not appear to be due to a gram-positive or -negative specific effect.

The antibiotic cocktail used was not as successful in reducing diarrhoea compared to the single antibiotics. In fact, mild diarrhoea was noted in this group prior to the first neratinib treatment. This may be due to the sucralose artificial sweetener that was added causing further non-beneficial changes to the microbiome (Abou-Donia et al., 2008). However, the same antibiotic cocktail with sucralose at a higher concentration has been used in our laboratory (Al-Qadami et al., 2019) and by others (Shen et al., 2017) in Sprague-Dawley rats with no diarrhoea noted. Additional research has administered sucralose long-term via gavage or in feed, with no mention of diarrhoea or gastrointestinal issues (Soffritti M., 2016; Bornemann et al., 2018). Alternatively the broad-spectrum nature (vancomycin, neomycin and ampicillin) of the antibiotic cocktail used could have led to a form of antibiotic-induced diarrhoea, although no previous research using a similar antibiotic cocktail has reported such instances of diarrhoea (Rey et al., 2018).

A key unknown regarding the microbiome in TKI-induced diarrhoea is whether microbiome changes cause diarrhoea, or if these changes are simply a consequence of diarrhoea occurring via other mechanisms. Many studies, both pre-clinical and clinical, have shown changes to the gut microbiome following treatment with TKIs, or indeed that people with diarrhoea had a different microbial composition to those who did not develop diarrhoea (Pal et al., 2015; Mayo et al., 2020). However, this study is the first to show that alterations in the gut microbiome before treatment begins leads to changes in diarrhoea severity. This follows a recent hypothesis by Wardill and Tissing that the pre-treatment gut microbial composition could be used to predict risk of developing gut toxicity

from a range of cancer treatments (Wardill et al., 2017). However, future research may focus on whether the microbiome has a key role in the development of diarrhoea, or if it is more likely to be exacerbating and prolonging diarrhoeal symptoms that are due to other mechanisms such as chloride secretion or epithelial damage (Van Sebille et al., 2015). While our results support a causative role for microbiome composition in development of diarrhoea, the specific microbes most important have not been identified in this model. Studies of germ-free rats monocolonised with specific bacteria may be a useful model for future studies.

This experiment showed a clear relationship between antibiotic administration and reduction of neratinib-induced diarrhoea. Additionally, while neomycin and vancomycin are poorly absorbed via the gastrointestinal tract, use of antibiotics would not be clinically ideal in this patient cohort due to long treatment periods. Additionally, apart from diarrhoea, the other main side effect from EGFR targeting TKIs is skin rash. This rash is often treated with broad-spectrum antibiotics, however their use in previous studies was a negative predictor of efficacy and toxicity of EGFR-TKI treatment in a non-small cell lung cancer population (Liu et al., 2019). One clinical study of dacomitinib, a similar pan-EGFR-TKI to neratinib, found in a non-small cell lung cancer population, doxycycline was effective at prophylactically reducing dermatological adverse events, and also caused a non-significant decrease in grade 2 diarrhoea. Conversely, the use of the probiotic VSL#3 did not reduce incidence of diarrhoea or mucositis scores (Lacouture et al., 2016). In a study of patients receiving first line VEGF-TKI treatment for metastatic renal cell carcinoma

(mRCC), patients who received antibiotics with *Bacteroides* spp. Coverage had increased progression-free survival compared to patients not receiving antibiotics (Hahn et al., 2018). This study however, was a retrospective study that was unable to take into consideration the gut microbiome composition before or after treatment of these patients. To completely understand any changes to neratinib efficacy caused by antibiotics, a tumour-bearing model of neratinib-induced diarrhoea would be required.

In the present study, relative abundance of the *Blautia* genus was highly increased in neratinib + neomycin treated rats, who had less diarrhoea than neratinib alone treated rats. Research presented in chapter 5 showed vehicle treated rats had higher levels of *Blautia* compared to neratinib-treated rats. *Blautia* is a genus of obligate anerobic bacteria that is of increasing research interest in gut health. *Blautia* falls within the Lachnospiraceae family, which is important in breaking down polysaccharides consumed in the diet to short-chain fatty acids (SCFA) including acetate, butyrate and propionate. Therefore, the presence of *Blautia* may support a healthy microbiome composition, and its presence in the intestine has also been associated with reduced deaths from Graft-versus Host disease (Jenq et al., 2015). Additionally, the potentially mucin-degrading *Allobaculum* (Chen et al., 2019a) was higher in neratinib-alone treated rats than in treatments groups with less diarrhoea. My previous observation (unpublished) in ileal sections stained with Alcian-Blue Periodic Acid- Schiff's staining suggested that neratinib treatment decreases goblet cell numbers in the villi while increasing numbers in the crypts. Staining also revealed many goblet cells in the process of releasing mucin stores following

neratinib treatment. Goblet cell topographical changes may impact on mucin availability in the lumen which could subsequently affect bacterial composition. This has been previously shown in a colitis model, where melatonin treatment significantly increased goblet cell levels in the gut, leading to beneficial modulation of the gut microbiome that was suggested to be due to mucin regulation by goblet cell differentiation (Kim et al., 2020). However, some care should be taken when directly assessing particular bacterial taxa in pre-clinical studies, as one study has suggested that 85% of bacterial sequences seen in a mouse representing genera are not detectable in humans (Ley et al., 2005).

Previous studies in my laboratory has shown histological damage of the gut stemming from neratinib is mainly focused on the ileum, however here I studied the faecal microbiome. There is known to be high variation in the spatiotemporal organisation of the microbiome throughout the length of the intestinal tract, due to changing pH and oxygen levels. It is possible that the changes seen in the faecal microbiome may be different to the changes in the ileal or caecal microbiome. In addition to differences throughout the length of the intestine, further research could begin to assess the differences between the mucosal and luminal microbiomes following neratinib treatment. It has previously been shown that *Lachnospiraceae*, the family that *Blautia* falls within, is found in high volumes in the transverse folds of the proximal colon. These areas provide a protected area that is separated from the luminal flow of digesta. Subsequently, microbes localised to such safe havens likely have an advantage in recolonising the intestine after disruption by antibiotics and infection (Tropini et al., 2017). Additionally, bacterial composition has been

shown to affect the structure and function of the intestinal mucus layer (Jakobsson et al., 2015).

This study has opened new questions and research avenues in investigating the link between the microbiome and neratinib-induced diarrhoea. In particular, I have shown a potential opportunity to specifically target specific gut microbiome profiles in order to reduce diarrhoea from neratinib and other TKIs. However, a deeper understanding of the exact microbiome changes in patients is required to precisely target the desired microbial profile. One study has previously used network analysis to stratify renal cell carcinoma patients with prior exposure to TKIs and antibiotics using gut microbiome composition (Iebba et al., 2019). This approach could be broadened to determine microbial profiles that lead to development of toxicity.

6.6 Conclusion

High levels of diarrhoea occur in many patients being treated with neratinib, and current interventions both carry risks of side effects, and may not be specific to the pathogenesis of this diarrhoea. In this study I have shown that narrow-spectrum antibiotics are able to reduce the development of diarrhoea. An antibiotic cocktail was not as successful. Overall, these results suggest that the microbiome may play a key role in the development and prolongation of diarrhoea following neratinib treatment, although further research is required to understand the exact mechanisms that have reduced diarrhoea in this study.

Chapter 7: The PREDiCT study: Personalised risk evaluation for cancer treatment -induced diarrhoea, an interim analysis

Chapter 7 is my final original research chapter, and assesses two independent cohorts of participants with cancer to investigate the use of the pre-treatment gut microbiome in predicting development of cancer treatment-induced gastrointestinal toxicity. This chapter is written as a traditional thesis chapter.

7.1 Abstract

Background: Diarrhoea is a commonly occurring side effect of many cancer treatments. It is unclear why some people develop severe diarrhoea and others do not, independently of cancer or treatment type. A growing body of research suggests the gut microbiome may be used as a predictor of diarrhoea development. However, this has not previously been assessed in a prospective, longitudinal cohort of participants having chemotherapy-based treatment.

Methods: Two independent studies were undertaken; one using archival samples, and one longitudinal study. The first study was an analysis to determine the feasibility of associating microbial composition with toxicity outcomes. Stool samples were collected from 12 participants prior to the commencement of chemotherapy cycle 1 or above. Samples were subsequently analysed using 16S sequencing. Diarrhoea levels were assessed via clinical case note review. Following this, a second study, the PREDiCT (Personalised Risk Evaluation During Cancer Treatment) study, was developed and undertaken. In this study, 15 participants were recruited. Four stool

samples were collected prior to, and during, chemotherapy-based cancer treatment, for analysis via 16S sequencing. Patient-reported toxicity outcomes were assessed via surveys collected at 3 points during treatment.

Results: In study one, participants who subsequently developed diarrhoea had significantly lower amounts of the bacterial genus *Blautia* (P = 0.018), and higher amounts of *Collinsella* (P = 0.027) compared to those who did not develop diarrhoea. In the PREDiCT study, participants were stratified into groups who did, or did not, develop clinically relevant diarrhoeal symptoms over the course of the study. No clear microbial factors separated these two groups.

Conclusions: The two studies presented here suggest microbiome composition following initial cycles of cancer treatment may be more important in determining subsequent development of toxicity than the treatment-naïve microbiome. This suggests an understanding of longitudinal microbiome recovery from chemotherapy is an important area of future research.

7.2 Introduction

Diarrhoea and gastrointestinal (GI) toxicity are clinically significant complications of many cancer treatments, including chemotherapy. This common side effect reduces patient quality of life, as well as resulting in treatment breaks and dose reductions (Carlotto et al., 2013; Lalla et al., 2014; Elad et al., 2020), compromising chances of remission and increasing mortality. Effective treatment of GI toxicity and resulting diarrhoea is difficult due to the incompletely characterised mechanisms by which it occurs. In addition, there is

currently no efficient way of predicting the occurrence of GI symptoms in patients receiving chemotherapy (Miaskowski et al., 2014).

A wealth of research has been dedicated to determining the molecular mechanisms leading to the induction of GI toxicity following treatment with chemotherapy or targeted agents (Logan et al., 2008a; Gibson et al., 2009; Bowen et al., 2019). Much of this research has shown both direct cytotoxic and indirect inflammatory-based mechanisms lead to gut dysfunction (Sonis, 2004a; Bowen et al., 2019). Briefly, the innate immune system recognises pathogen (e.g. bacterial lipopolysaccharide (LPS)) or damage (e.g. heat shock proteins) associated molecular patterns released via direct cytotoxic mechanisms. This leads to activation of transcription factors including nuclear factor kappa B (NF-κB) and subsequent upregulation of pro-inflammatory cytokines, resulting in amplified apoptosis and epithelial damage, over time leading to ulceration, bacterial colonisation, diarrhoea and bleeding. In the clinic, loperamide or octreotide are commonly used to manage diarrhoea, however these medications do not target the specific mediators of this diarrhoea or effectively prevent the initial damage (Andreyev et al., 2014; Elad et al., 2020).

The understanding of the role the gut microbiome in GI toxicity and diarrhoea has substantially expanded over the past 25 years. Initially, bacteria in the gut were thought to only colonise ulcerated sites in the mouth and gut, leading to potential secondary complications including bacteraemia (Sonis, 1998; Sonis, 2004b). More recently, research has shown the microbiome composition of the gut undergoes many changes following chemotherapy and radiotherapy, summarised in Touchefeu et al. (2014) and Secombe et al. (2019b). Increases

in gram-negative taxa such as *Escherichia coli* (*E. coli*) and *Staphylococcus* have been reported (Stringer et al., 2009b). These species release LPS, which causes activation of various inflammatory downstream signalling pathways involving NF-κB and pro-inflammatory cytokine release. Decreases in 'good' bacterial taxa such as *Bifidobacterium* and *Lactobacillus* have also been shown to occur over a similar time frame (Stringer et al., 2009b). These bacteria have effects including improving digestive function and motility and carbohydrate metabolism, and have been researched for their use as probiotics (O'Callaghan et al., 2016). These findings then led to an intense interest in the use of probiotics to reduce the occurrence of diarrhoea (Osterlund et al., 2007; Wada et al., 2010; Lalla et al., 2014). However, results from clinical use have been highly variable, with a recent meta-analysis showing a lack of overall efficacy for probiotics in chemotherapy (Wardill et al., 2018). Key among the issues underlying this lack of efficacy were studies used a variety of probiotic formulations and dosages. Most importantly though, these studies lacked a targeted approach in determining the specific bacterial species, or metabolic benefits from these bacteria, used in the probiotic.

A more mechanistic role has recently been suggested for the microbiome. Alexander et al. (2017) outlined the TIMER model by which the gut microbiome may influence the development of toxicity from cancer treatments, highlighting how the following effects stemming from the microbiome can cause toxicity: translocation; immunomodulation; metabolism; enzymatic degradation; and reduced diversity. A recent position paper on cancer treatment-related GI toxicity development has further suggested a more active and dynamic role for

the gut microbiome in the development of toxicity (Bowen et al., 2019). This paper stressed the need to consider the role of the microbiome in all phases of mucositis development, instead of as a “passive contributor of the ulcerative phase”.

It is now hypothesised that the unique composition of an individual’s gut microbiome prior to treatment is critical in shaping their risk of developing GI side effects (diarrhoea) during cancer treatment, and could potentially be used as a predictive marker of mucositis risk (Wardill et al., 2017). When my PhD project was first envisaged in 2017, there was some initial evidence in small studies suggesting the microbiome could be used to predict toxicity from radiotherapy and ipilimumab treatment (Covington et al., 2012; Wang et al., 2015; Dubin et al., 2016; Zhu et al., 2017). However, there was no investigation of this following chemotherapy-based treatment, and therefore this study aimed to investigate this.

To support what has been observed in pre-clinical models, and to undertake initial discovery in people with cancer treated by chemotherapy, I completed the following investigations. This chapter describes two studies aimed at understanding the role of the pre-treatment microbiome in determining risk of diarrhoea during chemotherapy for cancer. The first study is a detailed analysis of previously collected stool samples collected from participants before a chemotherapy cycle (prior to treatment cycles ranging from 1 - 10) to investigate the feasibility of comparing microbial composition to GI toxicity outcomes. As much of the current research in this area has been retrospective or in comparison to healthy controls, this chapter subsequently developed a

prospective, longitudinal study of participants before and during chemotherapy. The primary outcome measure of the PREDiCT (Personalised Risk Evaluation During Cancer Treatment) study was change in patient reported diarrhoeal symptoms, with patient reported cognitive symptoms as a secondary outcome measure. Here I report on the development of the PREDiCT study and present an interim analysis.

7.3 Methods

7.3.1 Archival Study 1: Retrospective analysis

7.3.1.1 Ethical approval

This study was approved (in 2009) by the Ethics of Human Research Committee of the Royal Adelaide Hospital (RAH, RAH protocol number 080901) and was carried out in accordance with the Declaration of Helsinki in 2009, Informed consent was obtained from each participant prior to enrolment in the study.

7.3.1.2 Participant recruitment

As per Stringer et al. (2013), participants receiving chemotherapy for breast cancer or GI tumours were recruited through the Oncology Department at the RAH.

7.3.1.3 Sample collection

Stool samples were collected by participants directly into a sterile container before a chemotherapy cycle commenced. This was not necessarily the first cycle of chemotherapy treatment (cycles 1 - 10). Samples were given to investigators directly, transported to the laboratory and then frozen and stored at -80 °C.

7.3.1.4 Diarrhoea symptom classification

Case note review was completed to identify the level of diarrhoea as determined by the treating clinician. The National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE v 3.0) scale (table 7.1), in use at the time the original study was conducted, was used to classify participants diarrhoea levels into no/mild toxicity or high toxicity, with grade 0 and 1 representing no/mild toxicity and grades 3 and above representing high toxicity.

Table 7.1: NCI CTCAE v 3.0 classification of diarrhoea during chemotherapy treatment (2006).

Gastrointestinal Disorders					
Adverse Event	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**	**Grade 5**
Diarrhoea	Increase of < 4 stools per day over baseline. Mild increase in ostomy output compared to baseline.	Increase of 4-6 stools per day over baseline; moderate increase in ostomy output compared to baseline.	Increase of ≥ 7 stools per day over baseline; incontinence; hospitalisation indicated; severe increase in ostomy output compared to baseline; limiting self-care ADL	Life-threatening consequences. Urgent intervention indicated.	Death

ADL: Activities of Daily Living

7.3.1.5 Gut bacterial DNA extraction and diversity profiling

Samples were sent to the Australian Genome Research Facility (AGRF, Melbourne, Australia) for DNA extraction and 16S ribosomal RNA (rRNA) gene region analysis. DNA was extracted from 250 mg of faecal sample using the Qiagen DNeasy PowerLyzer PowerSoil Kits with the PowerLyzer 24 Homogeniser (Qiagen, Hilden, Germany). The V3-V4 variable region of the bacterial 16S rRNA gene was amplified using the following primers, (using standard International Union of Pure and Applied Chemistry (IUPAC) nucleotide nomenclature, see chapter 2, supplementary material 1):

Forward sequence: 5'-CCTAYGGGRBGCASCAG-3'

Reverse Sequence: 5'-GGACTACNNGGGTATCTAAT-3'

Sequencing was performed using the Illumina (San Diego, USA) MiSeq. The Illumina bcl2fastq pipeline was used to generate sequence data. CLC Genomics Workbench 12.0 (https://www.qiagenbioinformatics.com/) was used to complete bacterial diversity profiling. Paired-ends reads were assembled by aligning forward and reverse reads. Adapters were identified and trimmed. Trimmed sequences were quality filtered, duplicate sequences removed and sorted by abundance. Reads were assigned to taxonomic identities (operational taxonomic units (OTUs)) using the Greengenes 97% similarity database version 13.8. Alpha-diversity was calculated using the Shannon diversity index. Beta diversity was calculated using principal coordinate analysis (PCoA) based on generalised UniFrac distances (Chen et al., 2012a). PCoA results were displayed graphically as the two principal coordinates that explained the highest

percentages of variation (PCo 1 and PCo 2). The exact percentage of variation explained by these axes is noted on the axis labels.

Linear discriminant analysis effect size (LEfSe) analysis was used to determine the genera most likely to explain differences between no/mild and high toxicity groups (Segata et al., 2011).

7.3.2 Longitudinal Study 2: PREDiCT study

7.3.2.1 Ethical approval

This study was approved (in 2018) by the Human Research Ethics Committee of the Central Adelaide Local Health Network (HREC/17/RAH/531) and Southern Adelaide Local Health Network (SSA/18/SAC/52), and was carried out in accordance with the Declaration of Helsinki. Informed consent was obtained from each participant prior to enrolment in the study.

7.3.2.2 Participant recruitment

Participants were recruited from the Oncology Department of the RAH and Flinders Medical Centre (FMC), Adelaide, South Australia between October 2018 and December 2020. Participants were excluded if they were under the age of 18, were pregnant, had a previous history of severe bowel issues such as inflammatory bowel disease or had received systemic cancer treatment in the preceding 10 years.

7.3.2.3 Sample collection

Four stool samples were collected from each participant. The first sample was collected before the first chemotherapy treatment, with the 3 subsequent samples collected in accordance with each participant's treatment plan (figure 7.1). Stool samples were collected by participants into Zymo DNA/RNA Shield Faecal Collection Tubes (R1101, Zymo, Irvine, USA), posted to investigators at ambient temperature and frozen at -80 °C upon receipt.

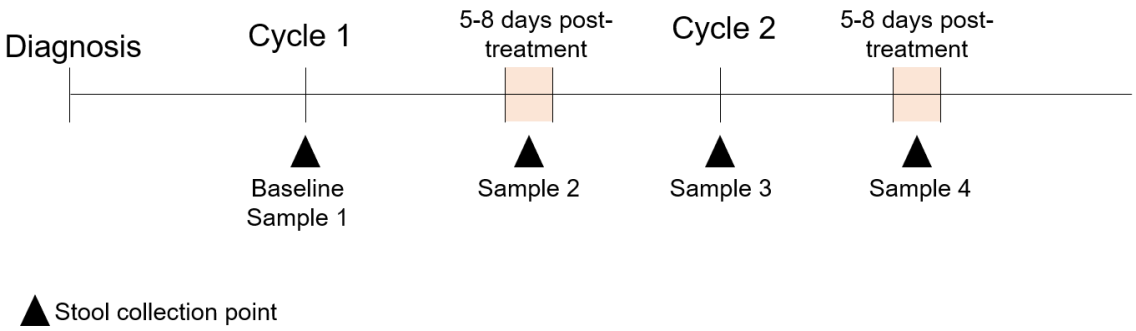

Figure 7.1: Longitudinal stool collection time points. Samples were collected by participants and sent back to investigators at ambient temperatures and frozen at -80 °C upon receipt.

7.3.2.4 Patient reported outcomes (PROs)

Prior to sample 1 collection, participants completed an initial survey for details of demographics, previous GI or cancer-related issues, as well as acute GI issues or antibiotic use over the previous 2 weeks. With each stool sample, participants completed a 3 day basic food diary and patient reported outcome (PRO) symptom questionnaire. The food diary has not been analysed in this interim analysis due to small sample size, however will be analysed in future. The questionnaire was based on the chemotherapy-symptom assessment scale (C-SAS) (Brown et al., 2001) and was refined in consultation with a local patient advocacy group (Cancer Voices SA). The symptom questionnaire included general questions on well-being and side effects assessed via a yes/no question, and an additional 5 point Likert scale assessed how much the symptom bothered the participants at its worst where: 0 = not at all; 1 = a little; 2 = somewhat; 3 = quite a bit; and 4 = very much. In addition, the questionnaire included validated surveys assessing diarrhoea concerns (Functional Assessment of Chronic Illness Therapy – Diarrhoea subscale (FACIT - DS)) (Webster et al., 2003; Harder et al., 2020) and cognitive issues (a modified version of the Functional Assessment of Cancer Therapy – Cognitive (FACT-COG)) (Bell et al., 2018; Dyk et al., 2020). The modified FACT - COG used had a total maximum score (i.e. no cognitive symptoms) of 100. In the FACT - COG section, where participants failed to complete sections of the survey, a percentage of the total possible score was calculated. For further information on the contents of the survey, please see supplementary material 1.

7.3.2.5 Diarrhoea symptom classification

Results of the FACIT - DS (total possible score = 44) were used to classify participants based on diarrhoea levels. A distribution-based approach was used to determine the clinically important difference in diarrhoea scoring, as per previous literature (Norman et al., 2003; Coon et al., 2016; Harder et al., 2020). The threshold for this difference was set at 0.5 standard deviations from the mean baseline (pre-cancer treatment) score. Participants were subsequently classified as having experienced no deterioration (ND) or a deterioration (D) in diarrhoea symptoms at any survey time point.

7.3.2.6 Gut bacterial DNA extraction and diversity profiling

The first (baseline) stool sample of each participant was analysed. Subsequent samples were stored for future longitudinal analysis. Samples stored in Zymo DNA/RNA Shield tubes at -80 °C were thawed to room temperature. Tubes were vortexed, and 2 mL of the contents transferred to a sterile microcentrifuge tube and centrifuged for 20 minutes at 4 °C at 16000 *x g*. The supernatant and pellet were separated. The pellet was processed in the Qiagen PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany), with the TissueLyzer LT. The supernatant was also run through the column prior to DNA elution. Following elution, samples were precipitated to increase purity using 100% ethanol and 1 M sodium chloride and resuspended in nuclease free water (Invitrogen, Carlsbad, USA). DNA was quantified using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, United States), and sent to the South Australian Genomics Centre (SAGC) for 16S ribosomal RNA (rRNA) gene region analysis.

The V3-V4 variable region of the bacterial 16S rRNA gene was amplified using the following primers, (using standard IUPAC nucleotide nomenclature, see chapter 2, supplementary material 1):

Forward sequence:

5’-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG - 3’

Reverse sequence:

5’- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC - 3’

Sequence data was generated with Illumina (San Diego, USA) MiSeq and bioinformatic analysis was conducted as per archival study 1. In addition, the program BURRITO (McNally et al., 2018) was used to visualise the links between taxonomic composition and function in the dataset. BURRITO uses Kyoto encyclopedia of genes and genomes (KEGG) pathways to assess alterations in metabolic pathways. Additionally, a nearest sequenced taxon index (NSTI) was calculated, which is a measure of phylogenic distance between each OTU and the matched reference genome. The functional predictions decrease as the NSTI score increases, however previous research has suggested an NSTI score up to 0.17 will give an accurate prediction (Langille et al., 2013; Mushinski et al., 2018). Microbial analysis results gained through 16S sequencing were firstly compared using relative abundance at the genus level for all samples before being compared between the diarrhoea

deterioration (D) and no deterioration (ND) groups and subsequently by cancer type.

7.3.2.7 Statistical analysis

Data were compared using Prism version 9.0 (GraphPad Software, San Diego, USA). The assumptions of equality of variance for each group and normally distributed data were tested using Bartlett's test and D'Agostino & Pearson omnibus normality test, respectively. If data was normally distributed, data on graphs are mean ± SEM and 2-way analysis of variance (ANOVA) tests were performed. If data was not normally distributed, median is displayed in graphs and non-parametric equivalent tests were performed, including Kruskal-Wallis tests for independent data. Statistical analysis of metagenomic profiles (STAMP) (Parks et al., 2014) was used to assess the predicted metagenome using Welch's t-test with the Benjamini-Hochberg correction for false discovery rate (FDR). Corrected P-values less than 0.05 were considered significant.

7.4 Results

7.4.1 Archival Study 1: Toxicity classification

7.4.1.1 Participant cohort/demographics

Twelve participants were included in the study with demographics shown as absolute values in table 7.2. Four were classified as having high levels of toxicity, and 8 had no/mild toxicity. There were no differences between the two groups in sex, cancer type, treatment protocol or age at treatment ($P > 0.3$). Additionally, the median treatment cycle the stool sample was collected in was not different ($P = 0.5$). Given no differences in demographics, and small sample size, the data in the table is presented in terms of total participants.

Table 7.2: Archival study participant demographics. There were no differences between the groups in sex, cancer type, treatment protocol or sample treatment cycle.

Demographic	
Age	
Median (range)	58.5 (38 - 72)
Sex	
Female	5
Male	7
Cancer type	
Breast	2
Colorectal	10
Treatment protocol	
FOLFOX	8
FOLFIRI	1
FEC-D/ adjuvant FEC	2
Unknown	1
Sample treatment cycle	
Median (range)	3.5 (1 - 10)

FOLFOX= folinic acid, fluorouracil and oxaliplatin; FOLFIRI = folinic acid, fluorouracil and irinotecan; FEC= fluorouracil, epirubicin and cyclophosphamide; FEC-D= fluorouracil, epirubicin, cyclophosphamide and docetaxel.

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7.4.1.2 Toxicity level determined pre-treatment microbial samples clustering

16S microbial sequencing was used to assess the gut microbiome composition of participants prior to their next cycle of chemotherapy. First, the alpha diversity of the microbiome was assessed using Shannon's diversity index. No differences were noted (P = 0.27, figure 7.2). PCoA was used to assess beta diversity. The analysis showed separated groupings for the high and no / mild toxicity groups, with PERMANOVA analysis showing differences in the PCoA between the two groups (P = 0.018).

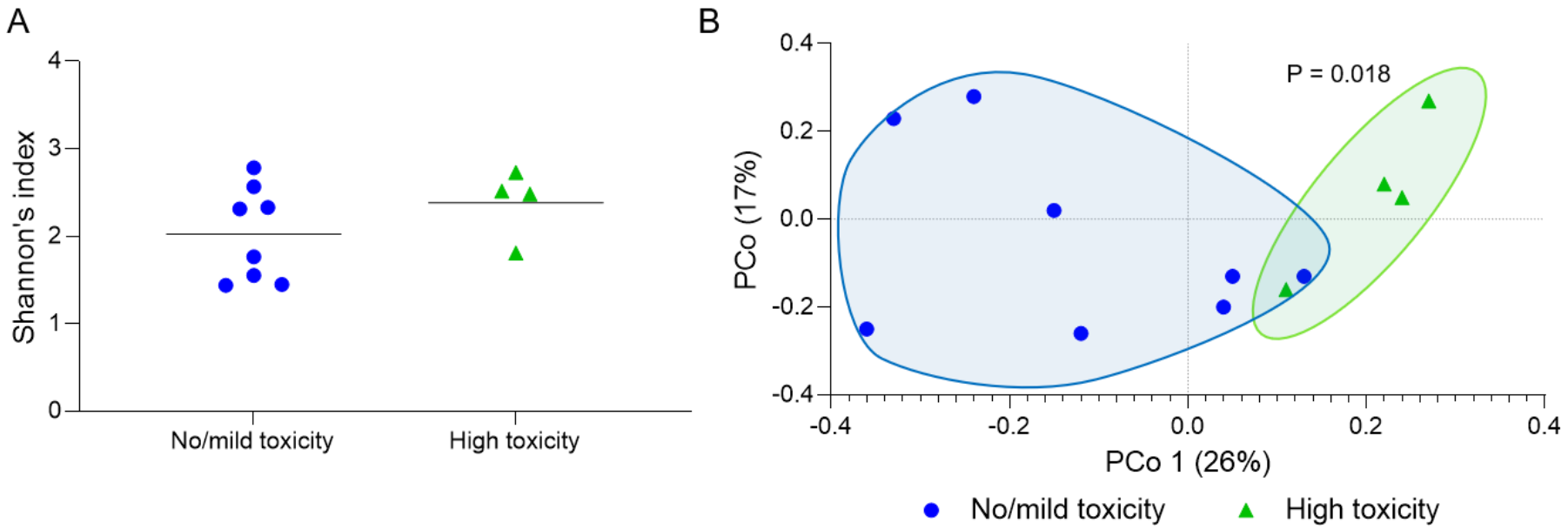


Figure 7.2: Microbial analysis of pre-treatment cycle stool sample. (A) Shannon's diversity index at the genus level showed no difference between the no / mild and high toxicity groups (P = 0.27), lines indicate means. (B) PCoA analysis showed altered clustering between the no / mild and high toxicity groups (PERMANOVA P = 0.018).

7.4.1.3 The genera Blautia and Collinsella were key distinguishing factors between toxicity groups

At genus level, there were marked differences between each group in the faecal microbiome (figure 7.3). Linear discriminant analysis (LDA) scores were calculated, and linear discriminant analysis effect size (LEfSe) was used to determine the genera that were significantly different between the toxicity groups. At the genus level, 3 genera were determined to be of increased abundance in the no / mild toxicity group, and 7 genera were increased in the high toxicity group. The most increased genus in the no / mild toxicity group was *Blautia* (P = 0.018) and the most increased in the high toxicity group was *Collinsella* (P = 0.027).

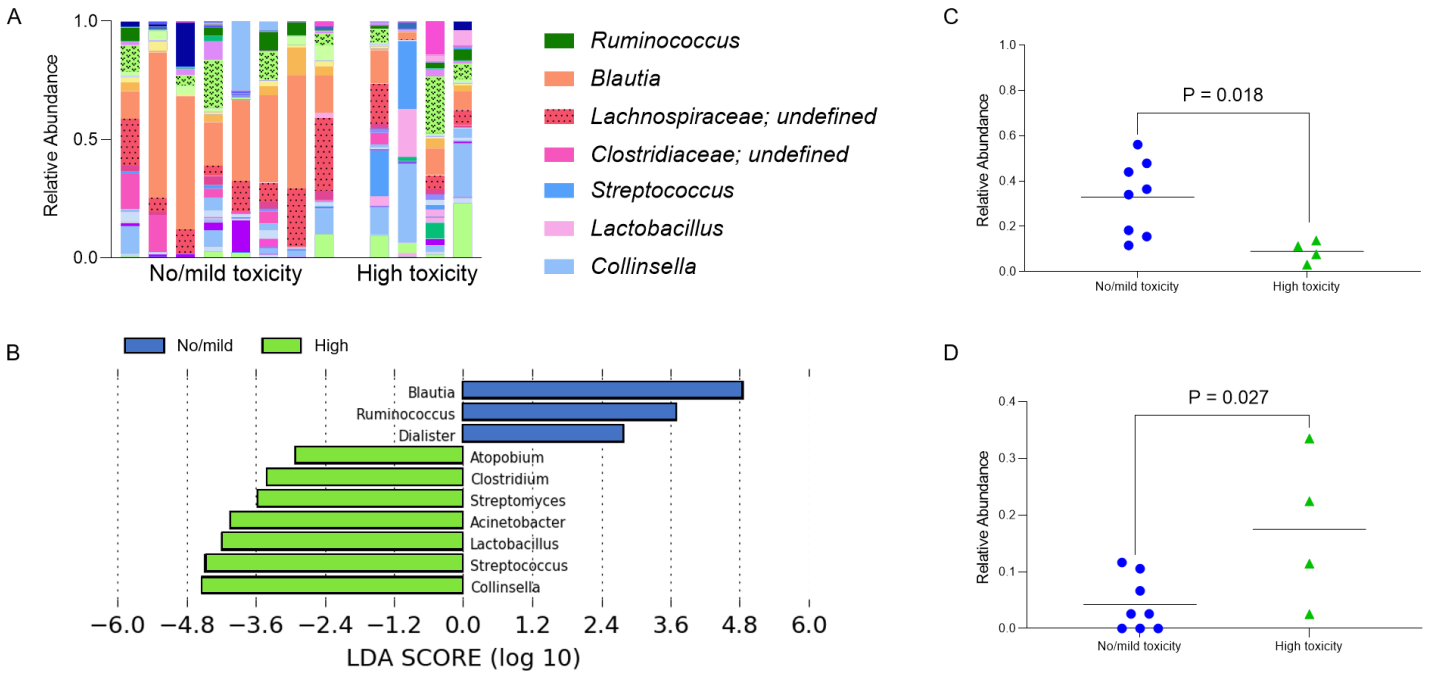


Figure 7.3: Relative abundance and LEfSe analysis of pre-treatment cycle stool sample. (A) Relative abundance at the species level, highly abundant genera represented in legend. (B) LDA scores determined for differentially abundant taxa in the gut microbiome of participants who developed no/mild toxicity (blue) or high toxicity (green). (C) Relative abundance of *Blautia*, the no/mild toxicity group had higher levels than the high toxicity group (P = 0.018). (D) Relative abundance of *Collinsella*, the high toxicity group had higher levels than the no/mild toxicity group (P = 0.027). Lines in C and D represent means.

7.4.2 Longitudinal Study 2: PREDiCT study, interim analysis

7.4.2.1 Participant cohort/ demographics

Of the 15 participants recruited, 3 did not return subsequent PROs surveys, and therefore were excluded from further analysis. Participant demographics are shown in table 7.3. Absolute values are shown here, due to the small number of participants. All participants were born vaginally, and there were no instances of emphysema or chronic obstructive pulmonary disease in any participant. Due to the short amount of time between diagnosis and start of treatment, pre-treatment symptom questionnaires were only received from 6 participants. However, a pre-treatment stool sample was received from all participants. Of a total possible score of 44, the threshold representing a clinically important difference in FACIT - DS scores was calculated to be 41.0. Six participants were determined to have had deteriorating diarrhoea symptoms (D group) and 6 participants did not deteriorate in terms of diarrhoea (ND group, table 7.4, supplementary material 2). Participants were also divided into cancer type groups: colorectal, upper GI and other (encompassing breast and cervical cancer). There were no differences in any of the demographics reported in table 7.3 between the D and ND groups, or between cancer type groups. Given no differences in demographics, and small sample size, the data is presented in terms of total participants. Of the 12 participants included for analysis, all provided at least 2 stool samples and 2 PROs surveys (table 7.5).

Table 7.3: PREDiCT study participant demographics, reported as number of participants out of a total of 12.

Demographic	
Age	
Median (range)	62.5 (37 - 70)
Sex (n)	
Female	7
Male	5
Body Mass Index	
Mean	25.6
Alcohol usage (standard drinks/week, n)	
0-5	9
6-14	0
15-20	2
20+	1
Smoking (n)	
Current	3
Ex-smoker	3
Never	6
Self-reported ethnicity (n)	
Caucasian	8
Other	4
Co-morbidities (n)	
Diabetes	3
Arthritis	3
Hypertension	3
Asthma	2
Medications (n)	
Mental health conditions with medication use	2
Polypharmacy (5 or more regular medications)	2
Reflux medications	3
GI conditions (2 weeks prior to study, n)	
Antibiotic use	2
Diarrhoea	3
Constipation	2
Nausea / vomiting	2

Table 7.4: Diarrhoea scores and grouping as per FACIT - DS scoring over the study. Participants were subsequently grouped as no deterioration (ND) or deterioration (D) based on their FACIT-DS score (total possible score = 44).

Diarrhoea scores	**Baseline Sample 1 (n=6)** Mean ± SEM (range) [n]	**Sample 2 (n=12)** Mean ± SEM (range) [n (%)]	**Sample 3 (n=11)** Mean ± SEM (range) [n (%)]	**Sample 4 (n=11)** Mean ± SEM (range) [n (%)]
Overall	42.6 ± 1.4 (37 – 44) [6]	40.5 ± 5.49 (28 - 44) [12]	37.5 ± 9.47 (18 - 44) [11]	36.09 ±10.96 (8 - 44) [11]
No deterioration (ND) group	N/A	43.3 ± 0.33 (41 - 44) [9 (75%)]	43.6 ± 0.30 (42 - 44) [7 (64%)]	43.8± 0.2 (43 - 44) [5 (45)]
Deterioration (D) group	N/A	32.0 ± 2.31 (28 - 36) [3 (25%)]	27.0 ± 4.02 (18 - 37) [4 (36)]	28 ± 4.67 (8 - 39) [6 (55)]
P-value, ND vs D	N/A	0.0045	0.0030	0.0043

Table 7.5: Summary of participant cancer type and grouping, treatment type, previous treatment for cancer and diarrhoea classification (D=deterioration, ND= no deterioration). Stool sample and survey numbering as per figure 7.1.

Pt #	Stool samples	Surveys	Sex	Age	Cancer type	Cancer grouping	Treatment type (during study period)	Previous systemic cancer treatment (more than 10 years prior)	Diarrhoea grouping
1	1,2,3,4	1,2,3,4	M	64	Rectal carcinoma	Colorectal	Chemoradiotherapy	N	D
2	1,2,3,4	2,3,4	F	66	Anal squamous cell carcinoma	Colorectal	Chemoradiotherapy	N	D
3	1,2,3,4	2,3,4	F	70	Ascending adenocarcinoma	Colorectal	Chemotherapy	N	ND
4	1,2,3,4	1,2,3,4	M	46	Metastatic colorectal cancer	Colorectal	Chemotherapy	N	D
5	1,2,3,4	1,2,3,4	M	61	Gastroesophageal junction adenocarcinoma	Upper GI	Chemoradiotherapy	N	ND
6	1,2,3,4	2,3,4	M	56	Oesophageal adenocarcinoma	Upper GI	Chemoradiotherapy	N	ND
7	1,2,3,4	1,2,3,4	F	62	Oesophageal squamous cell carcinoma	Upper GI	Chemotherapy	N	ND
8	1,2,3,4	2,3,4	F	66	Pancreatic head adenocarcinoma	Upper GI	Chemotherapy	Y	D
9	1,2	1,2	F	58	Cholangiocarcinoma	Upper GI	Chemotherapy	Y	ND
10	1,2,3,4	2,3,4	F	63	Cervical adenocarcinoma	Other	Chemoradiotherapy	N	D
11	1,2,3,4	1,2,3,4	M	37	Invasive ductal carcinoma	Other	Chemotherapy	N	ND
12	1,2,3,4	2,3,4	F	69	Invasive ductal adenocarcinoma	Other	Chemotherapy	N	D

7.4.2.2 Other treatment-related symptoms were reported regardless of diarrhoea status

Treatment related symptoms reported as part of the C-SAS were also assessed. Symptom occurrence were grouped based on the previously developed D and ND groups (table 7.6). Diarrhoea is also one of the symptoms assessed by the C-SAS, and in this cohort, no participant in the ND diarrhoea group reported diarrhoea in the C-SAS at any time point.

Table 7.6: Prevalence of other symptoms reported by participants in each PRO survey (S1 – S4) who had deterioration (D) and no deterioration (ND) of diarrhoea scores during the study.

Symptoms	**Deterioration (D)**								**No Deterioration (ND)**							
	S1 (n=2)	**S1 high**	**S2 (n=6)**	**S2 high**	**S3 (n=6)**	**S3 high**	**S4 (n=6)**	**S4 high**	**S1 (n=4)**	**S1 high**	**S2 (n=6)**	**S2 high**	**S3 (n=5)**	**S3 high**	**S4 (n=5)**	**S4 high**
	Y	***2+***	***Y***	***2+***	***Y***	***+2***	***Y***	***2+***	***Y***	***2+***	***Y***	***2+***	***Y***	***+2***	***Y***	***2+***
Nausea or vomiting in anticipation	0	0	0	0	1	0	0	0	0	0	1	1	2	1	1	0
Nausea following treatment	0	0	2	1	2	1	2	1	0	0	6	2^	4	0	4	2
Vomiting following treatment	0	0	0	0	1	0	0	0	0	0	1	2	0	0	0	0
Constipation	0	0	0	0	0	0	1	1	0	0	4	2^	2	2	2	2
Diarrhoea	1	0	3	2	5	3	5	3*	0	0	0	0	0	0	0	0
Pain	2	0	3	1	1	0	2	2	1	0	2	1*	2	1*	2	1*
Shortness of breath	0	0	1	0	2	1	2	0	1	0*	2	1*	3	0	2	0
Sign of infection	0	0	0	0	2	1	0	0	0	0	0	0	0	0	1	0
Bleeding or bruising	1	0	1	0	1	0	2	0	0	0	1	0*	1	0	1	0
Pins and needles or numbness/pain in hands or feet	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0
Problems with skin or nails	0	0	1	1	2	0	1	0	0	0	1	0*	1	0	2	0
Hair loss	0	0	0	0	0	0	1	1	0	0	1	0*	1	1	1	1
Problems with throat or mouth	0	0	2	1	1	1	1	1	0	0	2	0*	1	0	2	1

A change in taste or appetite	0	0	4	2	5	3*	3	3	1	0*	4	1*	3	1	3	1
Weight loss or gain	0	0	3	1	1	1	1	1	1	0*	3	1*	2	0	3	0*
Problems with chewing or swallowing	0	0	1	1	0	0	1	0	1	0*	2	1*	3	2	2	1
Feeling weak or muscles shaking	0	0	2	0	4	3	2	2	0	0	2	1*	3	1	3	1
Feeling unusually tired	1	0	4	3	5	3*	5	3*	1	0*	4	1	5	4	5	2
Difficulty sleeping. Frequently waking or restlessness	2	0	5	3	5	1*	3	2*	1	1	3	2*	3	3	2	1*
Headaches or dizziness	0	0	2	2	2	2	2	1	0	0	3	1*	2	0	3	0
Feeling anxious or worried	0	0	3	0	1	1	2	1	2	2	2	0*	1	1	1	1
Feeling low or depressed	0	0	1	0	1	1	4	2*	1	1	2	0*	4	1	4	1
Fluid retention, swelling in hands or feet	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0
Problems with eyes	0	0	1	0	1	0	1	0	0	0	1	1	0	0	0	0

S1 – S4: survey 1 - survey 4; Y indicated number of participants who reported the symptom; 2+ indicated symptom bothered participant “somewhat” to “very much” on the Likert scale; * = 1 participant did not complete severity score; ^ = 2 participants did not complete severity score.

7.4.2.3 Cognitive scores declined over the study period

While the mean FACT-COG score was lower at each survey stage in the deterioration (D) group compared to the no deterioration (ND) group, these differences were not significant (figure 7.4, mixed-effects analysis, $P > 0.05$).

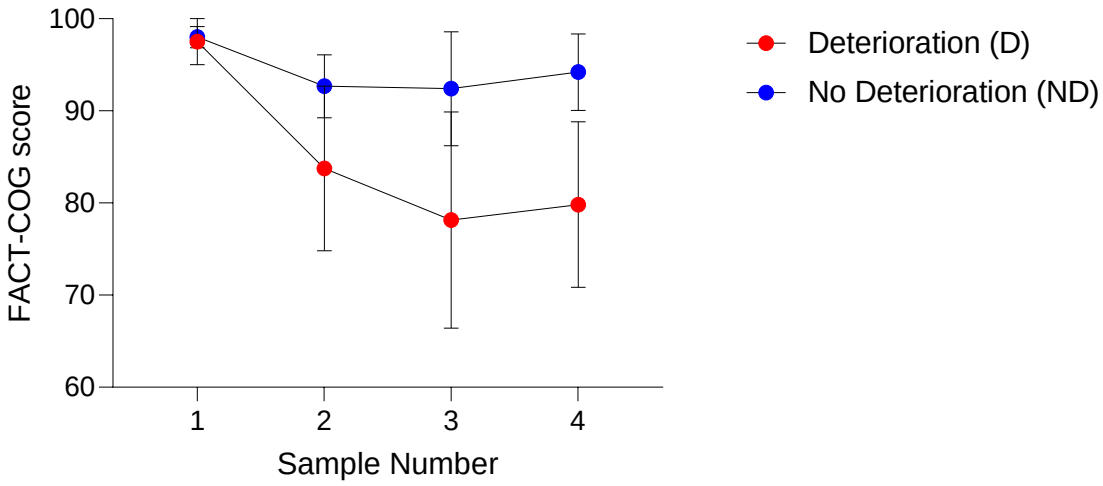


Figure 7.4: FACT - COG scores from each survey period. Maximum possible score, that represented no cognitive symptoms was 100. Mean ± SEM indicated on graph. At baseline, mean score for ND group was 98 and 97.5 for D group. At sample 4, mean score for ND group was 94.2 compared to 79.8 for D group.

7.4.2.4 Microbial analysis

Microbial analysis results were firstly compared using relative abundance at the genus level for all samples (figure 7.5). Some variation was noted in the most abundant genera between participants in both the D and ND group. There was no clear associations with cancer type.

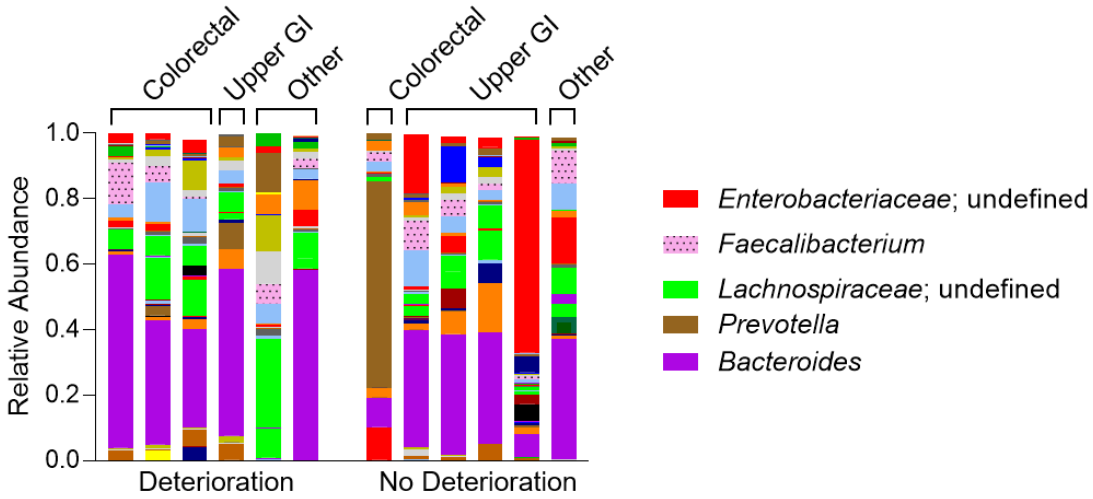


Figure 7.5: Relative abundance at the genus level of pre-treatment baseline samples (Sample 1) collected in the PREDiCT study in participants with different cancer types who had deterioration (D) and no deterioration (ND) in diarrhoea scores over the course of the study. Highly abundant genera represented in legend.

7.4.2.4.1 Diarrhoea stratification into D and ND groups did not show any clear microbial differences

There were no differences in absolute abundance between the D and ND groups (figure 7.6 A and B) (P = 0.27). Alpha diversity indicated by Shannon's index (figure 7.6 C), similarly revealed no differences between the groups (P = 0.73). Other measures of alpha diversity were assessed, including Simpson's and phylogenetic diversity, also showed no differences between groups (data not shown). PCoA analysis determined that the groups had somewhat overlapping clustering; and pairwise PERMANOVA resulted in no difference in clustering between the diarrhoea groups (P = 0.90).

Linear discriminant analysis effect size (LEfSe, using LDA scores) demonstrated the genera *Oscillospira* and *Bifidobacterium* were of increased abundance in the D group (figure 7.6 D). However, upon further statistical analysis (using t-tests of the relative abundance between groups), there were no differences between the relative abundance of these groups alone (figure 7.6 E, 7.6 F, outliers in each graph are different participants). Finally, a heatmap of the 60 most differentially abundant OTUs across the samples revealed some clustering within the D and ND groups (figure 7.7).

The program BURRITO was used to estimate functional abundances from the 16S OTU data. The nearest sequenced taxon index (NSTI) scores of the sequenced samples ranged from 0.045 to 0.135 (mean = 0.083, data not shown). No functional groups were shown to have different effects between the two groups (data not shown).

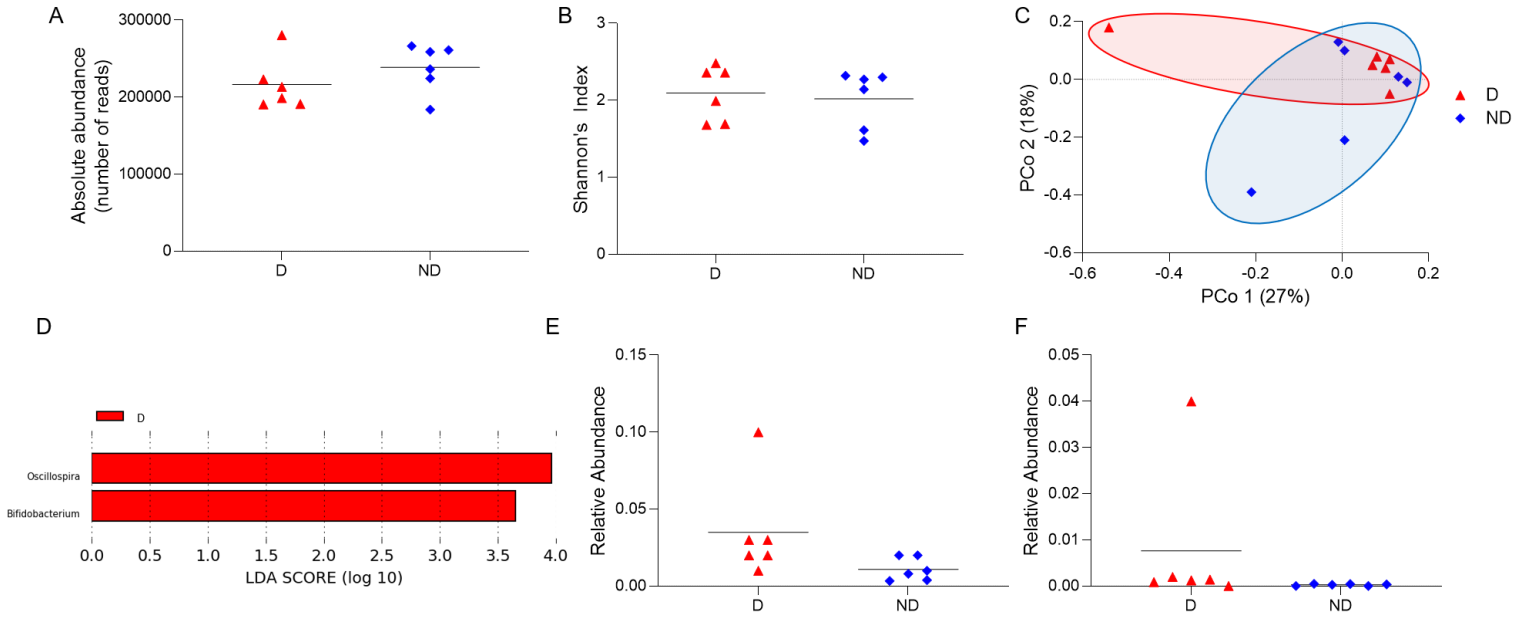


Figure 7.6: Microbial analysis from PREDiCT study, stratified based on diarrhoea grouping (D = deterioration, ND = no deterioration). (A) Absolute abundance, no differences between groups. (B) Shannon's index measuring alpha diversity, no difference between groups. (C) PCoA graph, PERMANOVA analysis showed no differences between groups (P = 0.90). (D) LDA scores determined for differentially abundant taxa in the gut microbiome of participants in the D group. (E) Relative abundance of *Oscillospira*, no differences between groups (P > 0.05). (F) Relative abundance of *Bifidobacterium*, no differences between groups (P > 0.05, outlier in F and G are different participants). Lines in A, B, E and F represent mean.

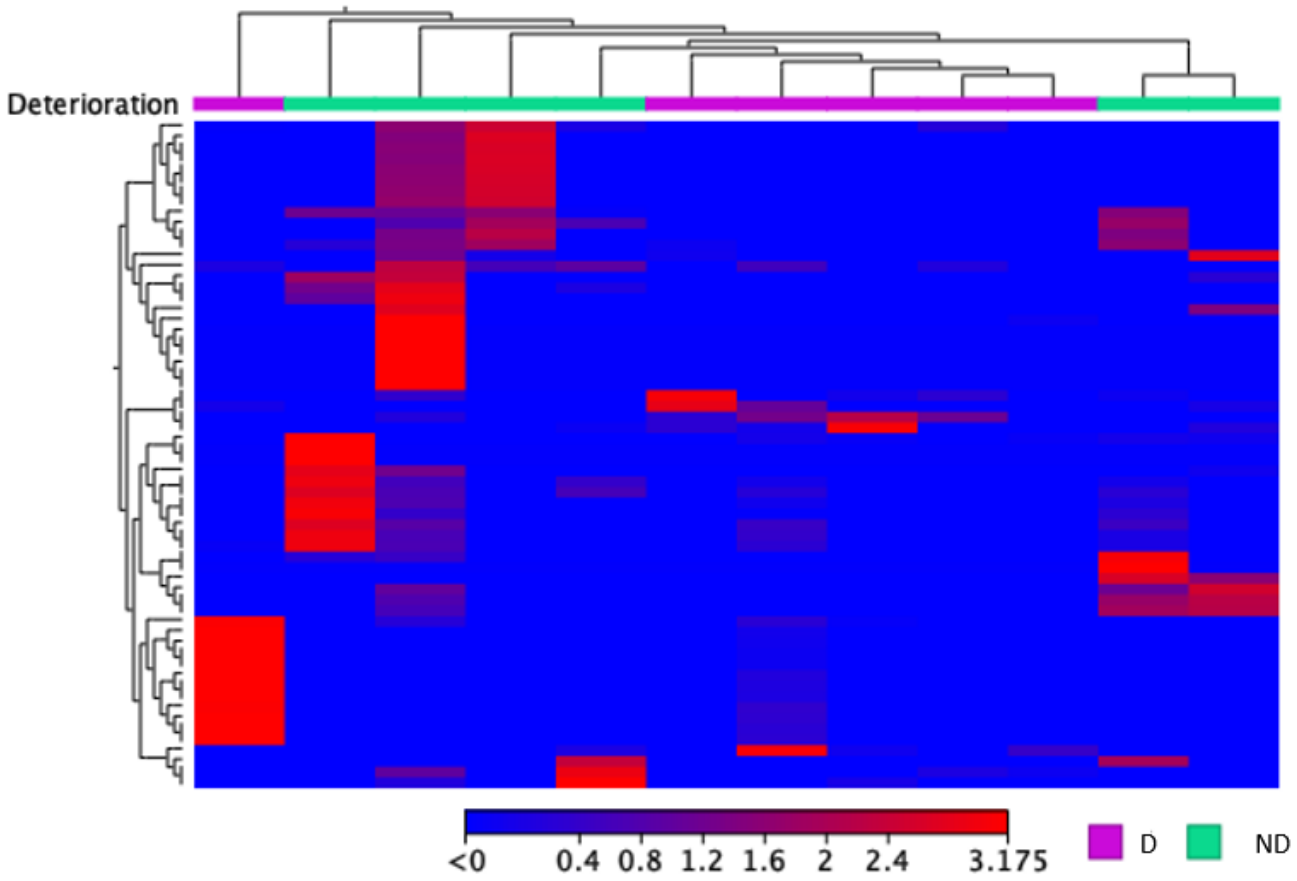


Figure 7.7: Heatmap of the 60 most differentially expressed OTUs across all samples.

7.4.2.4.2 Cancer type led to alterations in microbial function

All measures of alpha diversity showed no differences between any groups (data not shown). PCoA analysis for beta diversity showed some clustering between groups, however PERMANOVA analysis indicated this was not significant (figure 7.8 A). The corrected (Bonferroni) P-value for the colorectal group compared to upper GI was 1.00, for colorectal compared to other, P = 0.86 and for upper GI compared to other, P = 0.43. LEfSe analysis between groups additionally showed no differences. Finally, functional abundances were assessed. While there were no differences between the colorectal and upper GI groups, or colorectal and other, 8 functional groups were significantly different between the upper GI and other group (figure 7.8 B). These were porphyrin and chlorophyll metabolism, oxidative phosphorylation, ubiquinone and other terpenoid-quinone biosynthesis, lipopolysaccharide biosynthesis proteins, lipopolysaccharide biosynthesis, chaperones and folding catalysts, folate biosynthesis and pyruvate metabolism.

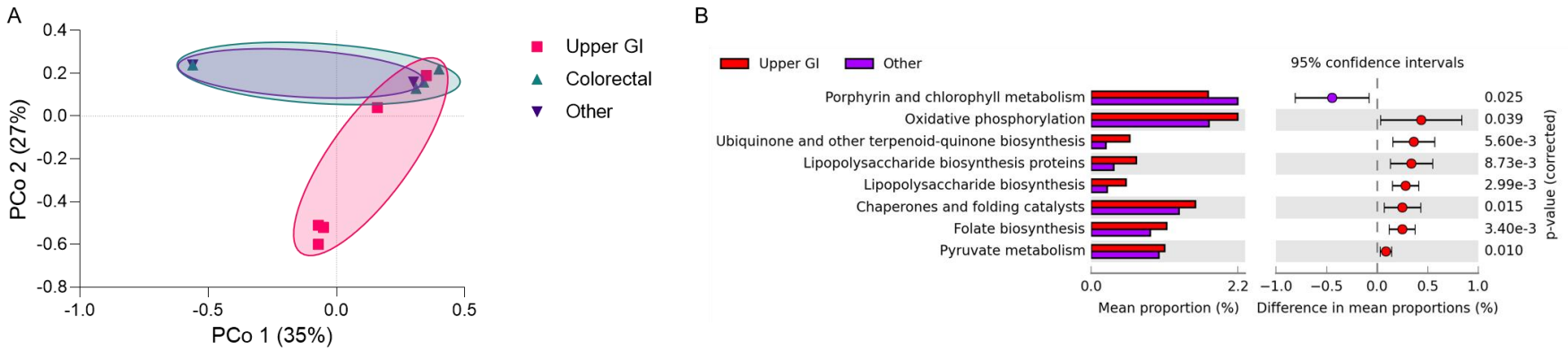


Figure 7.8: Microbial analysis stratified based on cancer type. (A) PCoA graph revealed clustering between cancer type groups, however PERMANOVA analysis showed no differences between groupings ($P > 0.05$). (B) Predicted changes in the metabolome based on changes in the microbiome of the upper GI and other cancer types group, with mean proportions of pathways and Welch's t-test with Benjamini-Hochberg FDR-corrected P-values.

7.5 Discussion

The two studies presented here aimed to provide a meaningful insight into the link between pre-treatment gut microbiome and development of diarrhoea following cancer treatment. In the first study, archived stool samples from participants who were part-way through chemotherapy treatment were analysed. Participants who developed high levels of diarrhoea during their next cycle of chemotherapy had lower pre-cycle levels of the bacteria *Blautia* compared to those who did not develop diarrhoea. As these participants had already undergone some chemotherapy treatment, I then aimed to develop a prospective longitudinal study incorporating analysis of the cancer treatment-naïve microbiome (the PREDiCT study). Presented here is an interim analysis of the PREDiCT study with a discussion of learnings from the development of the study so far.

Due to differences in methodology, it would not be prudent to make direct comparisons between the two studies. However, the first study demonstrated the feasibility of assessing microbial composition in tandem with toxicity outcomes. These results then informed the approach moving forward.

In the first study, I did not have access to PROs. Therefore, clinician-based toxicity reporting was used to determine diarrhoea status. Previous research has shown clinicians may under-report diarrhoea and other GI toxicities compared to patients (Flores et al., 2012; Di Maio et al., 2015). As such, only participants that scored either 0 - 1 or 3 and above on the NCI CTCAE were included in the study, to better stratify the groups. Despite this, I determined

that *Blautia* was present at higher levels in the no/mild toxicity group, and *Collinsella* was present at higher levels in the high toxicity group. Myself and others have previously identified *Blautia* to be an important genus in cancer treatment response in pre-clinical models (Jenq et al., 2015; chapter 5). For example, my research has suggested that in a rat model of tyrosine kinase inhibitor diarrhoea, rats treated with neratinib (who developed diarrhoea), had significantly lower amounts of *Blautia* than vehicle rats (chapter 5 of this thesis). Similarly, clinical studies have reported increased abundance of *Blautia* led to reduced mortality following Graft-versus-Host Disease (Jenq et al., 2015), and multiple studies as summarised by Aarnoutse et al. (2019) suggested *Blautia* decreased following chemotherapy. Patient groups with better progression-free survival had higher levels of *Blautia* in the gut (Routy et al., 2018). Conversely, there is less research linking *Collinsella* to cancer treatment toxicities. One study suggested the species *Collinsella aerofaciens* is more abundant in people who respond well to anti-programmed cell death protein 1 immunotherapy (Matson et al., 2018). However this study was not linked to toxicity outcomes.

In the longitudinal PREDiCT study, when stratifying participants based on diarrhoea development (as measured by PROs) or cancer type, there were no clear, group-based differences in pre-treatment microbiome composition. It is possible this is due to a low sample size. When comparing microbial abundance between cancer type groups, I identified some significant predicted functional differences in the metabolome between the upper GI group and the other (non-GI cancers) group. Most participants in this study were diagnosed with a GI tract-related cancer, and previous research has shown alterations in

microbial composition of people with GI tract cancers depending on stage and also tumour location (Bhatt et al., 2017). These effects may be obfuscating any potential microbiome-mediated effects, and hence why it will be important to validate this work in a larger, more defined cohort, and re-examine this impact once the PREDiCT study is complete. A recent study has suggested four distinct profiles of diarrhoea occurrence in people having outpatient chemotherapy (Diaz et al., 2021). These were none, decreasing (over time), increasing, and high (throughout treatment). Due to the small sample size of this study, participants were not separated into these profiles, instead they were separated into two groups of D and ND. Future analysis may allow this further separation, which may reveal specific microbial changes. In addition, the PREDiCT study focussed on diarrhoea events during the first 2 cycles of chemotherapy. However, it is possible participants may have had an improvement or deterioration in diarrhoeal symptoms after I stopped collecting PROs.

One of the key differences between these studies was analysis of pre-treatment stool collection in the longitudinal PREDiCT study compared to mid-treatment in archival Study 1. Therefore, it could be hypothesised pre-treatment microbial composition does not broadly predict for diarrhoea development, and instead is more closely linked to cancer type. This may suggest recovery of the microbiome following early cycles of treatment is a more important contributing factor in determining whether diarrhoea will develop. While there is no long-term research profiling dynamic long-term changes and recovery of the microbiome following cancer treatment using current best practice sequencing

techniques, previous research looking into microbial recovery following antibiotic treatment has shown recovery depends on factors as wide-ranging as probiotic use, diet and environment (Suez et al., 2018; Ng et al., 2019). This association provides motivation to sequence and analyse the longitudinal stool samples collected in this study once the study is complete. This will allow more in-depth assessment of whether the microbiome's capacity for recovery (or alternatively resistance to change in the first place) is comparatively more important in the development of diarrhoea than the treatment-naïve pre-treatment baseline microbiome.

While no clear microbial differences were shown based on diarrhoea outcomes in the longitudinal PREDiCT study, some difference in cognitive symptoms were demonstrated between the two groups. While the difference was not significant, which is not unexpected given the study was not powered for this secondary outcome, the mean FACT - COG score was lower at each survey point in the deterioration group. Future research should continue to examine this potential evidence of a microbiome-gut-brain axis link being active following cancer treatment, which has been outlined previously by Subramaniam et al. (2020).

Here, I have reported on the development of the PREDiCT study. As the study has developed, several changes to the protocol have been made from that reported here in order to obtain the most meaningful and relevant data possible. These changes included: the inclusion of the full FACT-COG study in order to gain data more comparable to other studies; the inclusion of a follow-up survey and stool sample six months following the conclusion of active treatment, to assess longer-term symptoms and microbial alterations; and the inclusion of the

Rome IV diagnostic criteria in the participant induction survey, to gain a deeper understanding of any pre-existing intestinal issues (Hellstrom et al., 2019). In addition, I found completion rates of the baseline (pre-treatment) symptom questionnaire were low, and could be improved by implementing changes in recruitment materials and instructions to ensure it is consistently completed. In future, online PROs surveys and reminder systems could also be considered, as these have been previously shown to increase completion rates (Basch et al., 2020; Nielsen et al., 2020). Finally, the work described here has not analysed the content of the 3 day food diaries completed with each stool sample. This will be completed in future, as not only is it known diet is a key modulator of the gut microbiome (Shanahan et al., 2021), but diet can have an impact on diarrhoea development following chemotherapy (Mardas et al., 2017; Mallick et al., 2018; Holma et al., 2020).

Treatment type has historically been a poor predictor of toxicity, as previous research has shown that within relatively homogenous patient populations having the same cancer treatment, some people will develop far more severe toxicity than others (Miaskowski et al., 2014). Additionally, treatment type can be based on a range of factors including tumour type, cancer stage, and patient preference. Here, I aimed to identify new ways, independent of treatment type, of identifying risk of diarrhoea development. In future models, with a larger cohort, treatment type may need to be included to ensure that a comprehensive model is used to support conclusions. Sophisticated bioinformatics and machine learning would be required to capture the complex potential for interactions between treatment type and the microbiome. In addition, treatment

type would be an important variable when assessing longitudinal stool samples collected after treatment has begun.

Finally, future studies may find it useful to move beyond associations between particular species or genera, and examine changes in the functional capacity of the microbiome. Although this study estimated the metabolomic capacity of the microbiome using bioinformatic tools, there were no significant differences between the groups based on diarrhoea status. However, this could be confounded by cancer type effects, and laboratory functional tests would be required to confirm this result. A recently developed workflow has demonstrated the ability to gain metaproteomic visualisations from faecal samples in 24 hours, a turnaround that would be required for any useful future predictive model of diarrhoea risk (Heyer et al., 2019). Alternatively, minION technology has recently been successfully used in a clinical setting to rapidly generate metagenomic datasets (Leggett et al., 2020) that were used to identify longitudinal microbiome profiles and associations with clinically relevant outcomes.

7.6 Conclusion

Diarrhoea can have a marked effect on quality of life and treatment adherence during cancer treatment. Currently, clinicians are unable to predict who will develop diarrhoea and who will not. This study has demonstrated that in this cohort, participants were unable to be stratified based on diarrhoea occurrence using stool samples taken prior to treatment. This result may be due to a small, heterogenous cohort so far in the PREDiCT study. These results do however

show the importance of longitudinally collecting microbial samples during and beyond cancer treatment, to more fully understand the microbial changes on an individual level and how they are connected to treatment regimen and occurrence of toxicities. For example, whether the microbiome's resistance to change in response to chemotherapy represent an important factor in addition to the initial composition. Additionally, the results demonstrate a sound rationale for future development of the PREDiCT study, with an increased sample size and logical stratification based on cancer and treatment type.

7.7 Supplementary Material

Supplementary Material 1: Patient reported outcome (PRO) symptom questionnaire given to participants to fill in with each stool sample.

How to complete this questionnaire

The list below describes symptoms that may be associated with cancer treatment. It is unlikely that you will have all the symptoms listed, however it is important that you tell us which symptoms you are experiencing so we can help you manage them.

Please look at the list of symptoms in column A and note which symptoms you have experienced in the past week by circling **Yes or No**. If you have circled **Yes** for a symptom please tell us how much the symptom bothered you at its worst by circling a number in column B.

Please complete this questionnaire on the same day as each stool donation.

Date: _____

Column A			**Column B**				
Have you experienced any of the following symptoms in the past **7 days**?			How much does the symptom **bother you** at its worst?				
			Not at all	A little	Some-what	Quite a bit	Very much
Nausea or vomiting in anticipation	Yes	No	0	1	2	3	4
Nausea following treatment	Yes	No	0	1	2	3	4
Vomiting following treatment	Yes	No	0	1	2	3	4
Constipation	Yes	No	0	1	2	3	4
Diarrhoea	Yes	No	0	1	2	3	4
Pain	Yes	No	0	1	2	3	4
Please state where you experienced the pain and the medication taken for it							
Shortness of breath	Yes	No	0	1	2	3	4
Signs of infection *e.g. feeling unusually hot or cold, flu-like feeling, high temperature, pain when urinating.*	Yes	No	0	1	2	3	4
Bleeding or bruising *e.g. nose bleeds, rectal bleeding, blood in urine, bruises*	Yes	No	0	1	2	3	4
Pins and needles or numbness/pain in hands and feet	Yes	No	0	1	2	3	4
Problems with your skin or nails *e.g. dry, itchy or inflamed skin, sun sensitivity, changes in your nails, vein markings*	Yes	No	0	1	2	3	4
Hair loss	Yes	No	0	1	2	3	4
Problems with your throat or mouth *e.g. sore or dry mouth/throat, mouth ulcers*	Yes	No	0	1	2	3	4

A change in taste or appetite	Yes	No	0	1	2	3	4
Problems with chewing or swallowing	Yes	No	0	1	2	3	4
Feeling weak or muscles shaking	Yes	No	0	1	2	3	4
Feeling unusually tired	Yes	No	0	1	2	3	4
Difficulty sleeping, frequently waking/restless	Yes	No	0	1	2	3	4
Headaches or dizziness	Yes	No	0	1	2	3	4
Feeling anxious or worried	Yes	No	0	1	2	3	4
Feeling low or depressed	Yes	No	0	1	2	3	4
Fluid retention, swelling in hands or feet	Yes	No	0	1	2	3	4
Problems with your eyes *e.g. sore, scratchy, dry or watery eyes*	Yes	No	0	1	2	3	4

These questions relate to diarrhoea more specifically. Please mark your response for the past **7 days.**

Specific gastrointestinal concerns	Not at all	A little bit	Some-what	Quite a bit	Very Much
I have control of my bowels	0	1	2	3	4
I move my bowels more frequently than usual	0	1	2	3	4
I am afraid to be far from a toilet	0	1	2	3	4
I have to limit my social activity because of diarrhoea	0	1	2	3	4
I have to limit my physical activity because of diarrhoea	0	1	2	3	4
I have to limit my sexual activity because of diarrhoea	0	1	2	3	4
I am embarrassed by having diarrhoea	0	1	2	3	4
I have abdominal cramps or discomfort due to my diarrhoea	0	1	2	3	4
My problem with diarrhoea keeps/wakes me up at night	0	1	2	3	4
I must move my bowels frequently to avoid accidents	0	1	2	3	4
I wear pads or protection to prevent soiling my underwear	0	1	2	3	4

Please mark your response for the past **7 days.**

Specific neurocognitive concerns	Never	About once a week	2-3 times / week	Nearly every day	Several times day
I have had trouble forming thoughts	0	1	2	3	4
My thinking has been slow	0	1	2	3	4
I have had trouble concentrating	0	1	2	3	4
I have had trouble finding my way to a familiar place	0	1	2	3	4
I have had trouble remembering where I put things, like my keys or wallet	0	1	2	3	4
I have had trouble recalling the name of an object when talking to someone	0	1	2	3	4
I have used the wrong word when I referred to an object	0	1	2	3	4
I have had trouble finding the right word(s) to express myself	0	1	2	3	4
I have walked into a room and forgotten what I meant to get or do there	0	1	2	3	4
I have had to work really hard to pay attention or I would make a mistake	0	1	2	3	4
I have forgotten names of people soon after being introduced	0	1	2	3	4
My reactions in everyday situations have been slow	0	1	2	3	4
I have had to work harder than usual to keep track of what I was doing	0	1	2	3	4

My thinking has been slower than usual	0	1	2	3	4
I have had to use written lists more often than usual so I would not forget things	0	1	2	3	4
I have trouble keeping track of what I am doing if I am interrupted	0	1	2	3	4
I have trouble shifting back and forth between different activities that require thinking	0	1	2	3	4
Other people have told me I seemed to have trouble remembering information	0	1	2	3	4
Other people have told me I seemed to have trouble speaking clearly	0	1	2	3	4
Other people have told me I seemed to have trouble thinking clearly	0	1	2	3	4
Other people have told me I seemed confused	0	1	2	3	4

If you have had any of concerns raised in the table above, please complete the following four (4) questions. Please mark your response for the past **7 days**.

Impact on quality of life	Not at all	A little bit	Some-what	Quite a bit	Very much
I am upset about these problems	0	1	2	3	4
These problems have interfered with my ability to work	0	1	2	3	4
These problems have interfered with my ability to do things I enjoy	0	1	2	3	4
These problems have interfered with the quality of my life	0	1	2	3	4

Please answer the following questions in as much detail as possible regarding any complementary medicines or therapies you are doing to support your treatment.

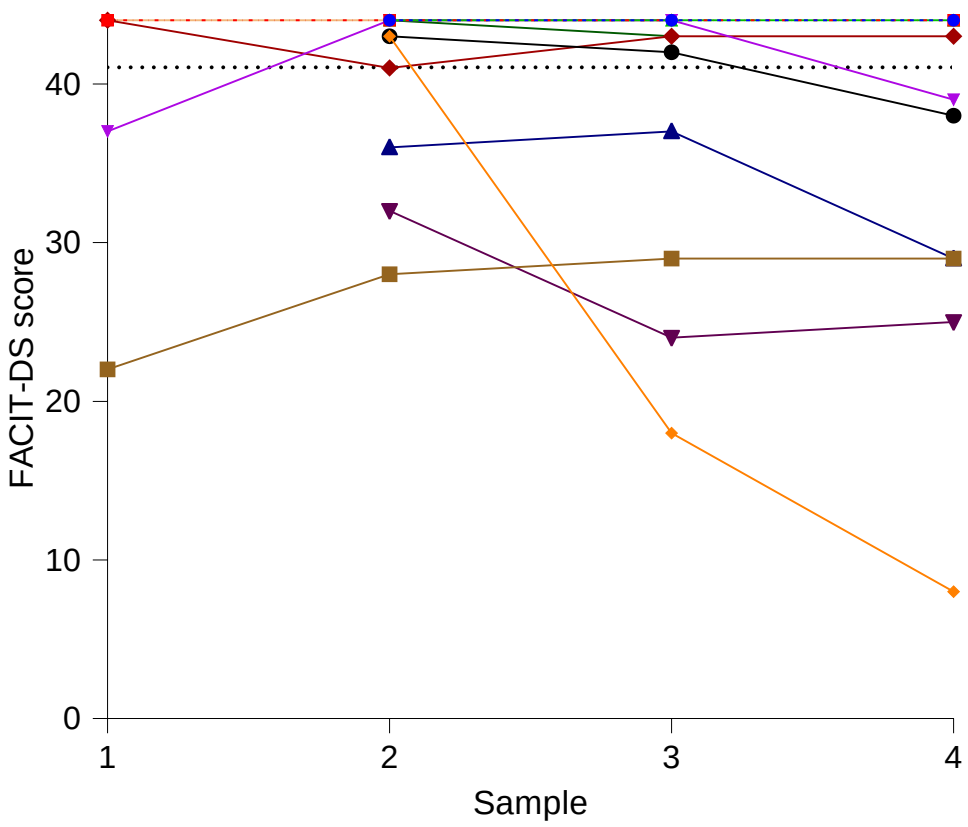
Have you experienced any other symptoms or problems not included in this questionnaire?

Have you taken probiotics or antibiotics in the past week? If yes, please include the name of each.

In the past week, have you had any medicinal herbs, vitamins, acupuncture, supplements or dietary restrictions?

In the past week, have you been involved in any additional health activities, e.g. yoga, exercise or meditation?

Supplementary Material 2: FACIT-DS scores, with each line representing an individual participant. Maximum score possible is 44, representing no diarrhoeal symptoms. Dotted line (at 41.0) indicates the calculated threshold of a clinically important difference in diarrhoeal symptoms. Four participants scored 44 for all surveys.



Chapter 8: General discussion

8.1 Prior knowledge in the field

Gastrointestinal toxicity is caused by many types of cancer treatment. While the gut microbiome has been implicated in its development (Wardill et al., 2017; Bowen et al., 2019), it has previously been unclear whether the pre-treatment gut microbiome composition is important in determining the severity of toxicity, and whether its interaction with the innate immune system is necessary to induce damage.

This thesis aimed to understand how microbial composition could be used as a targetable mechanism of cancer treatment-induced toxicity. This was investigated in chemotherapy and small molecular tyrosine kinase inhibitor (SM-TKI) settings. These two settings were chosen as: 1. they are commonly used in a range of tumour types; 2. they can be used in isolation or often in combination with each other; and 3. it is unclear whether the resulting toxicity develops by the same or different cellular mechanisms.

When the studies in this thesis began, it was well known the balance of gut bacteria significantly changes due to chemotherapy (Von Bultzingslowen et al., 2003; Stringer et al., 2009b; Touchefeu et al., 2014; Forsgard et al., 2017), and there was initial emerging evidence of a similar effect due to SM-TKI treatment (Pal et al., 2015; Liu et al., 2019) . However, there was less understanding of how the gut microbiome may itself have an active role in the development of side effects from cancer treatment, and indeed in the efficacy of cancer

treatment itself. During my candidature, interest in the links between the microbiome and cancer treatment toxicity and efficacy has significantly increased, with over 300 papers in this area indexed in PubMed in 2020 alone. Recent key findings in the field have illustrated that patients who developed diarrhoea following chemotherapy for colorectal cancer had gut microbial alterations compared to those who did not develop diarrhoea (Fei et al., 2019). Additionally, it has been shown that the gut microbiome modulates efficacy of particular immunotherapy treatments (Gopalakrishnan et al., 2018, Vétizou et al., 2015). This PhD project has additionally contributed to knowledge in this field by demonstrating that the microbiome has, in some situations, a key role in actively determining the development of diarrhoea following cancer treatment.

The body of work described in this thesis:

- Characterised the microbiome of a conditional intestinal epithelial cell knockout model of TLR4 (*Tlr4*$^{\Delta IEC}$) for use in cancer treatment toxicities research;
- Developed a faecal microbiota transplant model for use in cancer treatment toxicities research;
- Determined the gut microbiome changes due to neratinib treatment, and that microbial composition has an effect on neratinib-induced diarrhoea development; and
- Demonstrated in a clinical cohort that mid-treatment gut microbiome composition can be linked to diarrhoea in subsequent cycles.

During my PhD, I have published a body of work examining the mechanisms by which neratinib causes diarrhoea following treatment. I have determined that both inflammatory and microbial changes are important. These findings have now been translated into clinical practice, with these findings being tested in a large ongoing multi-national clinical trial (the CONTROL trial, NCT02400476), with similarly positive results (Barcenas et al., 2020).

Here I will discuss the findings of this thesis in light of other research in the field, and suggest future research that may be required to further move this work towards further clinically translatable outcomes.

8.2 Comparing the role of the microbiome in diarrhoea from chemotherapy and SM-TKI treatments

These studies investigated the role of the microbiome in the development of diarrhoea following SM-TKI treatment. Chapter 4 generated the hypothesis that the microbiome has a similar role in the development of diarrhoea from chemotherapy and SM-TKI treatment. This differs from previous hypotheses suggesting diarrhoea from many SM-TKIs has a secretory basis (Van Sebille et al., 2015). If this new hypothesis is correct, it would suggest targeting the microbiome may be a useful mechanism of reducing diarrhoea from both chemotherapy and SM-TKIs. Chapters 5 and 6 provided further evidence for this, demonstrating neratinib treatment alters faecal microbiome composition. Further, it also demonstrated altering the microbiome via antibiotics significantly reduces diarrhoea levels.

Since beginning this PhD, more evidence has emerged showing changes in the gut microbiome following SM-TKI treatment. For example, osimertinib, a third-generation epidermal growth factor receptor (EGFR) TKI, has recently been shown to cause changes to the beta diversity of the gut microbiome throughout treatment, as well as alterations in the taxa *Sutterella*, *Peptoniphilus*, and *Anaeroglobus* (Cong et al., 2020). Additionally, Ianiro et al. (2020) recently described a study demonstrating in a metastatic renal cell carcinoma (mRCC) cohort, faecal microbiota transplant (FMT) was useful in reducing diarrhoea induced by TKIs to grade 0 or 1 in 80% of people after 8 weeks of treatment. Most recently, the microbial composition of a small cohort of people having neratinib treatment for human epidermal growth factor receptor 2 positive (HER2+) breast cancer was used to generate a predictive machine learning model of diarrhoea occurrence (Wong et al., 2021). These overlaps further strengthen our understanding of the role of the microbiome in toxicity from SM-TKIs, and do suggest a similar role to that in chemotherapy-induced diarrhoea.

Future research into the impact of microbiome changes on SM-TKI-induced diarrhoea would now be valuable. SM-TKIs are taken over a long period of time, however peak diarrhoea often occurs early in treatment, so longitudinal microbial analysis may be of use to determine whether the microbiota is able to recover following an initial change.

Another area that was not investigated in this thesis was the impact of combined chemotherapy and SM-TKI therapy. Previous work in my group has shown that diarrhoea is more severe in rats treated with combined chemotherapy and SM-TKI in the form of paclitaxel and lapatinib, compared to

lapatinib alone (Bowen et al., 2012). A further understanding of the microbial changes that occur in this combination therapy may be useful in the management of side-effects developing from this treatment.

8.3 Epithelial and immune TLR4 in the development of gastrointestinal toxicity

It has been previously summarised how the microbiome can interact with innate immune receptors like Toll-like receptor 4 (TLR4, chapter 1), and also how the cellular location of TLR4 can be closely linked to its function. Therefore, chapter 2 demonstrated the development of a *Tlr4*$^{\Delta IEC}$ model, where TLR4 was conditionally knocked out in intestinal epithelial cells.

In this thesis, I showed differences in the faecal microbiota of WT and the global KO (*Tlr4*$^{-/-}$) mice, but not between WT and *Tlr4*$^{\Delta IEC}$ mice. This may have been due to the WT and *Tlr4*$^{\Delta IEC}$ mice being littermates, and therefore genotype-specific effects may have been overcome by maternal transfer effects. Alternatively, it may suggest intestinal epithelial cell TLR4 does not have a key role in maintaining microbiome composition, and TLR4 on immune cells (absent in the global KO) may be more important. This hypothesis is supported by a previous *Tlr4*$^{\Delta IEC}$ model, which also showed similar microbiome profiles between WT and *Tlr4*$^{\Delta IEC}$ mice (Sodhi et al., 2012). Future research could also assess caecal microbial composition. As TLR4 expression is more dynamic in the distal small intestine compared to the large intestine, caecal microbiome composition may display more profound changes relative to faecal microbiome composition.

However, it is important to note, the global TLR4 KO (*Tlr4*$^{-/-}$) and WT mice utilised in chapter 2 were not littermates at any point. This may suggest that the microbial differences were simply due to individual housing. This issue represents a current limitation in the microbiome field, with little clarity in much of the published literature surrounding whether mice have been individually or co-housed. This clearly has implications for correct interpretation of results. A potential alternative way of assessing genotype–based alterations to microbial composition may be to assess recolonisation of the microbiome of different mice strains following antibiotic treatment.

In the *Tlr4*$^{\Delta IEC}$ model, no differences were seen in goblet cell numbers between WT and *Tlr4*$^{\Delta IEC}$ mice. However, goblet cell and mucus layer function in the gastrointestinal tract may be a sensible future research target to further determining the role of TLR4 in cancer treatment-induced diarrhoea. Kim et al. (2020) recently showed melatonin supplementation reduced colitis severity via increasing goblet cell numbers, and changing microbial composition via TLR4 signalling, suggesting that goblet cell number and function, TLR4 and the microbiome are all closely linked.

Additionally, in the irinotecan/FMT model (chapter 3), *Tlr4*$^{\Delta IEC}$ mice who received irinotecan alone developed less grade 2 and 3 diarrhoea, and less severe histopathological damage in the colon compared to WT mice. This finding aligns with previous work demonstrating global KO *Tlr4*$^{-/-}$ mice had less severe gastrointestinal toxicity than WT mice (Wardill et al., 2016). Therefore, while immune TLR4 signalling seems to be most important for maintaining

microbiome composition, gastrointestinal toxicity from irinotecan may be mediated by any form (i.e. immune or epithelial) of TLR4 signalling.

As discussed in chapter 2, the global knockout TLR4 model referred to was in BALB/c mice, whereas the *Tlr4*$^{\Delta IEC}$ model is in C57BL/6 mice. While there are some differences between these mice in terms of Th1/Th2 response, much of the research in this field has used lipopolysaccharide (LPS) to assess immune response (Watanabe et al., 2004). Immune response, and therefore subsequent involvement of the microbiome is likely much more complex following chemotherapy treatment, involving a multi-factorial response encompassing both metabolic and multi-organ system effects

In chapter 3, FMT treatment did not cause any significant changes in gastrointestinal toxicity symptoms following irinotecan in either mouse strain. This is likely due to the lack of microbial differences between WT and *Tlr4*$^{\Delta IEC}$ mice. However a larger study, where the FMT product, and recipient microbial composition post-FMT are analysed would be required to confirm this.

8.4 Abundance of specific microbial genera corresponded to changes in diarrhoea development following chemotherapy and SM-TKI treatment

I have shown evidence that diarrhoea stemming from chemotherapy and SM-TKI treatments are both affected by specific gut microbiome composition. In chapter 6, a pre-clinical model suggested alteration of the gut microbiome by narrow-spectrum antibiotic administration caused a marked decrease in

neratinib-induced diarrhoea development. However, this approach is unlikely to be clinically translated, given antibiotic stewardship is increasingly important in preventing antibiotic resistance. Additionally, data of antibiotics in chemotherapy settings has shown variable results. While some small clinical trials of irinotecan and neomycin led to reduced diarrhoea severity (Kehrer et al., 2001; Alimonti et al., 2003), a larger study was unable to replicate this (de Jong et al., 2006). Therefore, a closer investigation of the specific taxa that have been altered throughout the studies presented here would be useful.

In this thesis, following neratinib in a pre-clinical model and chemotherapy-based treatment in a clinical model, the genera *Blautia* was implicated, with higher levels of *Blautia* associating with lower levels of diarrhoea. *Blautia* is a genus of obligate anaerobic bacteria within the *Lachnospiraceae* family, important in the breakdown of polysaccharides into short-chain fatty acids, an important energy source for intestinal epithelial cells (Wong et al., 2006). A recent review also summarised the potential probiotic properties of *Blautia*, describing its ability to prevent inflammation by upregulating regulatory T cells, and bio-transforming bioactive substances (Liu et al., 2021). However, the authors suggested assessing *Blautia* solely at genus level may lead to overgeneralising, and assessing *Blautia* at species or even strain level may be required to completely understand its effects (Liu et al., 2021). Care also needs to be taken when assessing *Blautia* in people with cancer, as it has been shown to be down-regulated in people with colorectal cancer compared to healthy controls, and therefore comparing to healthy controls may not provide useful results (Chen et al., 2012b).

Given my findings, as well as others, it may be rational to design further studies to more deeply assess the role of *Blautia* and its subtypes in the development of toxicity following cancer treatment. For example, pre-clinical studies could use a cultured colony of a *Blautia* species to give as an interventional treatment in different schedules (before/during cancer treatment), or an assessment of diarrhoea development in *Blautia* monocolonised compared to germ-free mice. Research may also need to investigate whether Blautia itself is responsible for these beneficial effects, or whether the metabolites produced by Blautia (e.g. short chain fatty acids including acetate) are able to support the epithelial layer and positively modulate the mucosal immune system (Liu et al., 2021). Finally,

In addition to *Blautia*, my study also suggested changes in other key genera. In chapter 5, following neratinib treatment, *Ruminococcaceae*, *Oscillospira* and *Allobaculum* were all increased in abundance, whilst in chapter 7 in a clinical chemotherapy-based cohort, increased levels of *Collinsella* was indicative of increased diarrhoea. There is no research linking any of these particular taxa to diarrhoea or in relation to cancer treatment as a whole. However, in a rheumatoid arthritis study, *Collinsella* has been suggested to alter intestinal permeability via reduced expression of the tight junctions ZO-1 and occludin (Chen et al., 2016). There is also some suggestion that *Allobaculum* has a role in the development of the mucus barrier and which bacteria can subsequently penetrate it (Johansson et al., 2015). Despite these links, some caution should be taken with directly associating specific bacterial types between rodents and humans. As discussed in chapter 6, Ley and colleagues suggested that 85% of bacterial sequences seen in a mouse representing genera are not detectable in

humans (Ley et al., 2005). Consequently, human-associated microbiota rodent models could be used in future to overcome these issues in pre-clinical models (Walter et al., 2020).

8.5 Gut microbiome composition prior, or during cancer treatment may be used as a predictive factor for diarrhoea

In chapter 7, interim analysis of the longitudinal PREDiCT study did not show clear stratification of pre-treatment microbiome composition in comparison to diarrhoea levels. Clearer separation was seen in the archival study, where stool samples were taken mid-treatment. Although the small sample size in the interim longitudinal study analysis may have influenced this, it could be possible the gut microbiome composition before treatment is not the determining factor in whether someone will develop diarrhoea.

Two studies investigating FMT as a treatment for cancer treatment-induced diarrhoea have suggested that how the microbiome recovers from cancer treatment may be important in the development of diarrhoea. In a clinical study of patients with mRCC given SM-TKI treatment, FMT was given after diarrhoea had already developed – thus 'restoring' the microbiome. This had beneficial effects, with a significant proportion of patients reporting no diarrhoea up to 4weeks post-treatment, and reduced diarrhoea up to 8 weeks post-treatment (Ianiro et al., 2020). In a pre-clinical mouse model, FMT given during and after 5-fluorouracil (5-FU) also alleviated diarrhoea and gastrointestinal toxicity symptoms compared to 5-FU alone (Chang et al., 2020). Additionally, while TLR4, myeloid differentiation primary response 88 (MyD88) and serum

interleukin-6 (IL-6) were all upregulated following 5-FU treatment, FMT was able to significantly reduce expression (Chang et al., 2020). However, preparatory methods for FMT such as bowel prep or colonoscopy are invasive, and potentially not preferred by people already undergoing chemotherapy or other extensive systemic cancer treatment due to perceived risks of perforation and bacteraemia (Wardill et al., 2019). Presence of oral mucositis may also limit uptake of oral FMT capsules. Therefore, significantly more exploration of the feasibility of FMT-type treatments in these patient populations is still required.

Differences in microbiota recovery capacity has been investigated following antibiotic treatment, and indicates that assessment of microbiota recovery may have merit in cancer treatment toxicity. For example, one study found mouse microbiome recovery following antibiotics was strongly associated with factors such as diet, environment and housing (Ng et al., 2019). Particular strains of bacteria have also been shown to recover differently from antibiotics (Koo et al., 2019). Any predictive model would therefore need to take into consideration the capacity for the microbiota to recover from treatment, whether that be from chemotherapy or SM-TKIs. However, the possible association between diarrhoea and the recovery of the microbiome after the initial phases of treatment, does not follow the recent work published by Wong et al. (2021), who was able to clinically predict diarrhoea occurrence following neratinib using pre-treatment stool microbial composition. Due to small study size in Wong et al. (2021) and the PREDiCT study, future work would need to be done to confirm these findings. Regardless, my studies do suggest there is some element of predictability in the microbiota in development of diarrhoea from

chemotherapy or SM-TKI treatment. Future studies could assess the importance of microbial recovery in the pre-clinical models developed in this thesis (chapter 2 and 5) by either giving FMT following the development of irinotecan-induced diarrhoea, or giving antibiotics only until neratinib began, to assess this effect.

8.6 Future research targets

I suggest that bile acids may be a good additional future research target, as they have been linked to the function of TLR4 and the gut microbiome as well as having previously been implicated in the pathogenesis of neratinib- and irinotecan-induced diarrhoea (Shi et al., 2017; Secombe et al., 2019a). Previous research has suggested bile acid malabsorption is a key contributor to cancer treatment-induced diarrhoea (Phillips et al., 2015). I did not assess bile acid levels in the *Tlr4*$^{\Delta IEC}$ model, however a similar model showed TLR4 KO (global or partial) reduced levels of luminal bile acids (Sodhi et al., 2012). This could suggest global TLR4 KO reduced irinotecan-induced gastrointestinal toxicity as shown by Wardill et al. (2016), was in part mediated by lowered luminal bile acids. Bile acids are additionally a key regulator of the microbiota, with the bile acid-microbiota axis suggested to play a role in not only the regulation of the microbiome, but also in the development and progression of cancer (Ridlon et al., 2014; Phelan et al., 2017).

Future studies could also examine the role of the mucosal microbiome in comparison to the luminal microbiome. This may be most important in further investigating the role of TLR4 in cancer treatment-induced gastrointestinal

toxicity, as TLR4 samples bacteria residing in the mucosal microbiome (chiefly via dendritic cells). As luminal-facing TLR4 is predominantly located in the small intestine, this could also be highly important in correctly determining small intestinal changes in gut function. Indeed, specific differences have been seen in the mucosal microbiome of patients with colorectal cancer compared to healthy controls (Chen et al., 2012b). Finally, potential changes to goblet cell function that would have downstream effects on the function of the mucus layer and its microbes could be investigated.

Mucosal microbiome samples were collected from the *Tlr4*$^{\Delta IEC}$ FMT study however due to time constraints were not analysed. It may be difficult to clinically translate any results gained in differentiating the mucosal and luminal microbiome, as a faecal sample would not be suitable. Instead, a biopsy or another, more invasive technique would be required (Tang et al., 2020). Additionally, previous research has shown that the mucosal microbiome may be important in understanding colonisation resistance, and why some people 'take up' probiotics while others do not (Zmora et al., 2018). This would be important in translating work with *Blautia,* as discussed above, into humans.

8.7 Clinical translation and use of predictive models

There is increasing interest in and work determining the use of the microbiome in precision medicine (Ryu et al., 2021). In future, the work generated in this thesis, especially within chapter 7, could be expanded to generate a more specific predictive model of gastrointestinal toxicity development. In the future development of a predictive model for gastrointestinal toxicity, thought would

need to go into how this information would be used by clinicians and patients. If the gut microbiome (or its capacity to recover from treatment) could be used as a predictive factor, the next step would be to determine how this information would be best used to avoid gastrointestinal toxicity symptoms.

Dietary changes may be difficult to put into place quickly enough to effect proper change prior to treatment, with additional difficulties in effectively studying any changes (Shanahan et al., 2021). If, as I hypothesise, recovery (or resistance to change) of the microbiome is more clinically important than pre-treatment composition, then the issue of speed may be less of a problem. A particular dietary type (e.g. high fibre) could be maintained to aid in optimal microbial recovery prior to future treatment cycles. Directly targeting the microbiome using a probiotic-type product remains a long-term goal, however, as described throughout this thesis, the findings of current probiotic studies in reducing cancer treatment-induced diarrhoea remains unclear (Lalla et al., 2014; Wardill et al., 2018; Elad et al., 2020).

There is also a growing research field determining how complex predictive models can best be accepted and effectively used in the clinic (Stetson et al., 2020), and how patients would use any information coming from this modelling (Waters et al., 2020). A large number of predictive models already exist in the oncology field, so the format of the model, and how it is presented to clinicians and patients, would need to be carefully designed (Kappen et al., 2018) in order to ensure correct use.

8.8 Conclusion

Chemotherapy and SM-TKIs induce diarrhoea in many patients, with no current way of determining who will be most affected prior to treatment. Microbiome composition analysis represents a novel way to identify patients who may need effective intervention to prevent severe diarrhoea, and associated dose reductions and discontinuations.

This thesis suggests alterations in gut microbiome do affect the development of diarrhoeal symptoms, however this may be related to whether the microbiome recovers from initial treatment rather than the composition of the treatment naïve microbiome. Future progress in this area would benefit from large scale, longitudinal clinical assessments of the gut microbiome during chemotherapy and SM-TKI treatment, in order to further assess this microbiome recovery hypothesis.

Chapter 9: References

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Appendix 1: Publications arising from this thesis

Chapters 1, 4 and 5 have been published in peer-reviewed journals. These chapters are presented in this thesis in their original format, with the exception of spelling and table/figure number changes to ensure consistency, and referencing style. Here, these chapters are included in their original published format.

IJC
International Journal of Cancer

The bidirectional interaction of the gut microbiome and the innate immune system: Implications for chemotherapy-induced gastrointestinal toxicity

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Mini Review

Chemotherapy-induced gastrointestinal toxicity (CIGT) occurs in up to 80% of all patients undergoing cancer treatment, and leads to symptoms such as diarrhoea, abdominal bleeding and pain. There is currently limited understanding of how to predict an individual patient's risk of CIGT. It is believed the gut microbiome and its interactions with the host's innate immune system plays a key role in the development of this toxicity and potentially other toxicities, however comprehensive bioinformatics modelling has not been rigorously performed. The innate immune system is strongly influenced by the microbial environment and *vice-versa*. Ways this may occur include the immune system controlling composition and compartmentalisation of the microbiome, the microbiome affecting development of antigen-presenting cells, and finally, the NLRP6 inflammasome orchestrating the colonic host-microbiome interface. This evidence calls into question the role of pre-treatment risk factors in the development of gastrointestinal toxicity after chemotherapy. This review aims to examine evidence of a bidirectional interaction between the gut microbiome and innate immunity, and how these interactions occur in CIGT. In the future, knowledge of these interactions may lead to improved personalised cancer medicine, predictive risk stratification methods and the development of targeted interventions to reduce, or even prevent, CIGT severity.

Introduction

Gastrointestinal toxicity is a significant and often dose-limiting adverse event of many chemotherapeutic agents used in cancer treatment, and is currently without a widely effective preventative or treatment strategy. Chemotherapy-induced gastrointestinal toxicity (CIGT) covers a constellation of cancer treatment-related adverse events often referred to as mucositis, as inflammation of the mucosa is a key aspect of tissue injury.[1] Characterised by painful ulcerative lesions along the entire gastrointestinal tract from mouth to anus, CIGT affects up to 80% of patients, depending on treatment regimen.[1] This leads to a heightened risk of adverse events such as infection and diarrhoea.[2] Symptoms such as abdominal bleeding and abdominal pain are common, and result in increased hospital stays and the need for parenteral nutrition.[1] These interventions, as well as use of pain management medication, results in a significantly increased economic cost, with Medicare data from Australia suggesting a cost of $1,500 per episode of severe diarrhoea.[3] Available economic evidence substantially underestimates the larger societal burden and loss of quality of life of CIGT, caused by loss of productivity, need for informal care arrangements and increases in anxiety and depression levels.[4] Severe complications of CIGT such as bacteraemia and sepsis cause chemotherapy dose reductions and in profound cases, treatment cessation, compromising remission and increasing mortality.[5] Subsequently, CIGT presents as a major clinical and economic burden.[2]

Activation of transcription factors and upregulation and release of pro-inflammatory cytokines, in response to initiating events, are integral in the pathobiology of CIGT. More recent reports have focussed on an altered gut microbiome and damaged epithelial cells that produce cellular damage signals, causing activation of the innate immune system.[6,7] These types of damage signals are recognised by receptors in the innate immune system, present on gastrointestinal tract cells.[8]

It is now known that cancer treatments cause a raft of changes to the microbiome and host innate immune system.[9] There is significant heterogeneity in the microbiome and in immune function, and as such, this emergent data represents a chance to personalise cancer treatment, and allow the identification of patients at risk of severe symptoms.[10,11] However

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there is a clear gap in knowledge in translating these results from studies in radiotherapy- and immunotherapy-based patients into those with chemotherapy. The symptoms of CIGT and other cancer-treatment induced toxicities (including radiotherapy) are similar, and while occur from different initiating events, have a similar timeline of pathogenesis.[12] These radiotherapy studies are beyond the scope of this review, however chemotherapy and radiotherapy are often concurrently used and these studies have been included in this review, in order to best assess available evidence. In addition to translating results into chemotherapy studies, it is not yet known if there is a specific gut microbiome profile that establishes risk of acute CIGT, and the role of the host immune system in maintaining or changing that profile. This review will provide evidence for an updated mechanistic hypothesis of CIGT wherein the bidirectional interaction of the host innate immune system and native microbiome may predict the severity of gastrointestinal toxicity a patient will suffer after chemotherapy. A semi-structured search of PubMed for full-text articles in English found more than 1,000 studies investigating the microbiome and/or innate immune system in chemotherapy-induced gastrointestinal toxicity. Search terms included: 'microbiome', 'chemotherapy-induced gastrointestinal toxicity', 'chemotherapy-induced mucositis', 'cancer treatment diarrhoea' and 'chemotherapy microbiome'. Findings of key studies are summarised in Tables 1–3.

Pathobiology of CIGT

Although CIGT is a significant concern in the treatment of cancer, the underlying mechanisms remain unclear. Sonis introduced a model in 2004, consisting of five continuous and overlapping phases. This model elegantly showed the integral role of transcription factor activation and subsequent upregulation and release of pro-inflammatory cytokines, in response to initiating events.[12] These initiating events may be the innate immune system's recognition of a Pathogen-Associated Molecular Pattern (PAMP) (e.g. lipopolysaccharide (LPS)), Damage-Associated Molecular Pattern (DAMP) (e.g. high mobility group box chromosomal protein 1, heat shock proteins) or ChemoRadiotherapy-Associated Molecular Pattern (CRAMP).[13] There are several cellular mediators critical in these developmental events. These include nuclear factor kappa B (NF-κB), which causes the upregulation of up to 200 genes possibly involved in CIGT development.[12] Pro-inflammatory cytokines (primarily tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, IL-18 and IL-33[14–16]) are significantly elevated, leading to an amplification of apoptosis and epithelial damage.[12]

Since this model was introduced, more recent research suggests that the large population of bacteria resident in the small and large intestines plays a fundamental role in CIGT development (Fig. 1). With recent advances in 'omics' technology, our ability to understand the unique idiosyncrasies of the microbiome is vastly improving. This onslaught of information highlights the need for improved translational integration of the role of gut bacteria in the pathobiological model.

The Gut Microbiome

The gut microbiome, defined here as the collection of bacteria and other microorganisms present in and around tissues from the mouth through to the anus, is made up of almost one hundred trillion microorganisms.[17] Functions include protective and immunological actions, supporting energy metabolism and triggering mucous barrier formation.[8,18] The importance of the microbiome in normal development and functioning can be observed in germ-free mice, which are completely devoid of a microbiome raised under special conditions so they have no bacterial population. As a consequence of this, they have a variety of developmental differences including immature immune systems and altered digestive enzyme development, and are thus extremely susceptible to infection.[19]

Although the composition of the human gut microbiome is dependent on many factors such as diet, sex and ethnicity, much of its composition is determined in birth and infancy.[19] New findings also suggest that microbiome composition is not significantly associated with genetics, and is often more associated with environment.[20] While each individual's gut microbiome is unique; the overall framework is often similar with the *Firmicutes* and *Bacteroidetes* phyla comprising over 90% of the gut microbiome.[21] Most bacteria belong to the *Clostridium* and *Bacteroides* genera, with major commensal (species which cohabitate with mutual benefit) species being *Lactobacillus* spp. and *Bifidobacterium* spp.[18] Additionally, there are marked differences between the oral, gastric, small and large intestinal bacterial populations,[22] reflecting physiological differences such as oxygen gradient, pH and presence of antimicrobial peptides. The oral microbiome is dominated by *Streptococcus* spp., the small intestine by *Lactobacillaceae* and *Enterbacteriaceae*, and the colon by species such as *Bacteriodaceae* and *Prevotellaceae*.[22,23]

The gastrointestinal epithelium is constantly in contact with adherent bacteria. A sensitive balance exists, with continual cross talk between the microbiome, immune cells and the mucosal barrier to maintain homeostasis.[24] A disruption in this balance, known as dysbiosis, has been shown to have a role in multiple autoimmune diseases such as multiple sclerosis.[25] Additionally, the inter-individual differences in the gut microbiome are now considered to be one of the key contributors to immune response in humans. This is thought to be *via* influence of an individual's cytokine response (with strongest effects on interferon gamma (IFN-γ) and TNF-α production), and therefore disease susceptibility and overall immune function.[26]

The Innate Immune System

The innate immune system is the first line of response to bacterial invasion or an aseptic tissue injury. It responds to danger signals by recruiting immune cells to the injury site, inducing inflammation and activating the adaptive immune system. The innate immune system is vital in the gastrointestinal tract, with

the luminal gastrointestinal surface being one of the largest common surface areas between host and environment. With a diverse range of microbes living so close to the host, the innate immune system is critical in maintaining immune tolerance to commensal microbes, whilst ensuring the rapid initiation of an immune response after invading pathogens.

The innate immune system utilises many systems in the gastrointestinal tract in order to maintain homeostasis, including separation mechanisms including epithelial and mucosal layers, and compounds such as antimicrobial peptides and antibodies. Additionally, the innate immune system utilises a system of pattern recognition receptors that recognise microbial molecular patterns.

One type of pattern recognition receptor, Toll-Like Receptors (TLRs), are particularly important in sensing molecular patterns from gut microbes. TLRs are highly conserved transmembrane receptors and are members of the Toll-Interleukin 1 Receptor signalling pathway.[27] TLRs are present in the gastrointestinal mucosa on basolateral and apical surfaces of epithelial cells, professional immune cells, enteric neurons and glia.[28,29] There are eleven TLRs found in humans, recognising diverse ligands including RNA, DNA and LPS.[27] TLR4 is a particular focus of recent research due to its known expression changes after chemotherapy, and its ability to recognise patterns released by chemotherapy-damaged cells and therefore will be the focus here.[6] TLR4 can be activated by exogenous and endogenous danger signals such as LPS, high mobility group box chromosomal protein 1 and heat shock proteins,[27] causing a downstream signalling pathway of transcription factor (e.g. NF-κB) upregulation and pro-inflammatory cytokine release.

The role of TLR4 in CIGT development is now well known, with TLR4 knockout mice having less diarrhoea, weight loss and histological damage in response to irinotecan treatment.[6] However, there is some disparity surrounding the role of TLR4. One study where TLR4 was blocked using naloxone was unable to reduce irinotecan-induced gastrointestinal damage,[30] whereas other research has shown that TLR4 agonist LPS can protect intestinal crypts from other insults such as radiation.[31] Studies have shown protection and exacerbation, possibly due to differences between acute and chronic injury[32] or chemotherapeutic agents, and therefore the role of TLR4 appears to be complicated and context-specific. Immunologic cell death, a form of cell death where dendritic cell activation leads to a specific T cell response, is caused by some anti-cancer agents such as oxaliplatin and is characterised by release of DAMPs. As TLR4 is of vital importance in binding DAMPs, it is possible that immunogenic cell death may contribute to modulating the role of TLR4 in CIGT.

A Bidirectional Interaction: Innate Immunity and the Gut Microbiome

The innate immune system is strongly influenced by the microbial environment.[26] However, there is growing evidence that the reverse is also true, and that the microbial environment is similarly influenced by the innate immune system.[8,33] This bidirectional interaction between microbiome and immune system has been described by Hooper *et al.*[34] as 'inside-out' and 'outside-in' interactions, and is observed in a number of ways.

The immune system controls composition and compartmentalisation of the microbiome.[34] There is ample evidence to suggest that the host immune system is integral in selecting mucosal and luminal bacterial populations, ensuring there is minimal direct contact between the gastrointestinal surface and bacteria, keeping penetrant bacteria constrained to the lumen.[8,21] Van den Abbeele *et al.*[21] hypothesised that a distinct mucosal-associated microbial community has many immune-regulating effects with large potential biological outcome. Conversely, bacteria likely to be targeted by host defences are restricted to the lumen. This paper also suggested an outer colonic mucus layer, situated between the inner mucus layer and the lumen, may contain a 'backup' of microorganisms, which could act as an inoculum to restore the initial microbial balance after a perturbation, ensuring continual stability. In contrast, the colonic inner mucus layer is effectively devoid of bacteria.[35] The small intestine does not have these two distinct layers, and instead relies on antimicrobial peptides/receptors such as RegIIIγ and TLRs to minimise bacterial penetration of the mucus layer.[22]

An alternative mechanism explaining the interaction between the microbiome and immune system is through the development of antigen-presenting cells (APCs) such as dendritic cells and macrophages. Gastrointestinal dysbiosis has been shown to reduce infiltrating mature APCs.[36] Additionally, commensal bacteria can regulate dendritic cell activity. When investigating the role of the microbiome in APC development, it was found there were less gastrointestinal, but not systemic, dendritic cells in germ-free animals, and subsequent monocolonisation of the gastrointestinal tract with *E. coli* caused gastrointestinal dendritic cell recruitment.[37,38] Additionally, microbe-derived adenosine triphosphate has been shown to stimulate CD70 and CX3CR1 expressing dendritic cells, which can then go on to induce differentiation of Th17 cells.[39]

The NLRP6 inflammasome is key in orchestrating the colonic host-microbiome interface,[40] and is important in production of pro-inflammatory IL-18. Mice deficient in NLRP6 have been shown to be more susceptible to enteric infection,[40] have an altered microbial biogeography and to have a dysbiotic bacterial profile more likely to cause colitis-like symptoms.[41] A recent paper has shown microbiome-modulated metabolites are able to regulate the NLRP6 inflammasome and subsequent IL-18 production.[42] These microbial metabolic products are also known to regulate the role of colonic regulatory T cell homeostasis among a raft of other effects.[43]

The Gut Microbiome in CIGT

The gut microbiome plays a variety of roles in the development of CIGT. One study showed that compared to germ-free

Mini Review

Table 1. Summary of studies investigating gut microbiome changes due to cancer treatment

Study Type/ Sample Size	Cancer treatment	Study population age (years)	Detection Method	Increases	Decreases	Outcome/commentary	Reference
Pre-clinical (tumour bearing rats) $n = 30$	Irinotecan		RT-PCR, PCR-denaturing gel electrophoresis	*Clostridium* cluster XI (0.5 log) and *Enterobacteriaceae* (1.5 log) ($p < 0.05$)	Total bacteria number (by day 3, 1 log), *Clostridium* cluster XIVa (1–3 log), *Lactobacillus* and *Bifidobacterium* ($p < 0.05$)	Pathogenic bacteria increased	Lin *et al.*[45]
Pre-clinical (mice) $n = 100$	Irinotecan		Differences in gastrointestinal toxicity response between germ-free and conventionalised mice. Conventionalised mice = increased inflammation, lesions of gastrointestinal epithelium and increased permeability. Conventionalisation of germ-free reversed phenotype to conventionalised.				Pedroso *et al.*[44]
Pre-clinical (mice) $n = 120$	Irinotecan		Differences in digestive toxicity response between germ-free and holoxenic mice. Holoxenic mice = higher intestinal damage score, more diarrhoea. Germ-free mice were more resistant to irinotecan than holoxenic (higher lethal dose).				Brandi *et al.*[45]
Pre-clinical (rats) $n = 81$	Irinotecan		Microbiological culture	*Escherichia* spp., *Clostridium*, *Enterococcus*, *Serratiam* *Staphylococcus* (qualitative results)	*Peptostreptococcus*, *Bifidobacterium* (qualitative results)	Microbiome changes corresponded with diarrhoea incidence	Stringer *et al.*[44]
Pre-clinical (rats) $n = 81$	Irinotecan		RT-PCR	*E. coli*, *Staphylococcus* spp. ($p < 0.05$)	*Lactobacillus* spp. ($p < 0.05$)	Species that decreased were beneficial, major components of gut microbiome	Stringer *et al.*[47]
Pre-clinical (rats) $n = 48$	Irinotecan, 5-FU or oxaliplatin		16S pyrosequencing	Irinotecan: *Fusobacteria* (relative abundance 13-fold) and *Proteobacteria* (relative abundance 17-fold)		Also noted changes in serum and urine metabolome	Forsgard *et al.*[48]
Pre-clinical (rats) $n = 27$	Irinotecan		Microbiological culture and RT-PCR	*E. coli* (qualitative results)	*Bifidobacterium* (qualitative results)	Changes also observed in mucous secretion and release	Stringer *et al.*[49]
Pre-clinical (rats) $n = 75$	5-FU		Microbiological culture	Large intestine: Gram-negative facultatives (1-fold change in proportion)	Large intestine: Gram-positive facultatives (9-fold change in proportion)	Small intestine: shift from domination by Gram-positive cocci to Gram-negative rods	Von Bultzingslowen *et al.*[69]
Clinical $n = 16$	Various chemotherapies	Cohort 1: mean = 71 (range 36–82), Cohort 2: mean = 63 (range 40–77)	Microbiological culture, qRT-PCR	*E. coli* and *Staphylococcus* spp. (no statistics due to low patient number)	*Lactobacillus* spp., *Bifidobacterium* spp., *Bacteroides* spp., and *Enterococcus* spp.		Stringer *et al.*[53]
Clinical $n = 9$	Various chemotherapies + antibiotics	Paediatric cohort, age not reported	PCR-denaturing gel electrophoresis fingerprinting and *in situ* hybridisation	*Enterococci* (100-fold)	Anaerobic bacteria (10,000-fold). Commensal species (*Bacteroides* spp., *Clostridium* cluster XIVa, *Faecalibacterium prausnitzii* and *Bifidobacterium* spp., 3,000-6,000-fold)	Prophylactic and therapeutic antibiotic use did not explain changes in microbiome composition	van Vliet *et al.*[54]

Clinical $n = 17$	Various chemotherapies +/− antibiotics	Treatment group: mean = 59 ± 13, healthy group: mean = 65 ± 18	TaqMan qPCR and gel electrophoresis fingerprinting	*Bacteroides* (2%), *Clostridium* cluster IV (2%)	*Bifidobacteria* (0.9%) and *Clostridium* cluster XIVa (22% to 19%)	Decrease in total number and abundance of bacteria	Zwielehner *et al.*[72]
Clinical $n = 8$	Camustine, etoposide, aracytine and melphalan	Mean = 50.5 ± 10.8	16S rRNA 454 high throughput—pyrosequencing	*Bacteroidetes* (32%), *Proteobacteria* (14%) ($p = 0.008$)	*Firmicutes* (56%) and *Actinobacteria* (3%) ($p = 0.008$)	Reduced diversity and microbiome metabolic capacity	Montassier *et al.*[70]
Clinical $n = 9$	Pelvic radiotherapy (concurrent chemotherapy in subset of patients)	Range = 35–63	16S rRNA 454 high throughput—pyrosequencing	*Fusobacteriaceae* (6-fold) and *Streptococcaceae* ($p < 0.05$)	*Firmicutes* (10%)	Number of species-level taxa significantly reduced after therapy	Nam *et al.*[71]

Increase and decrease columns refer to bacterial species that changed after cancer treatment
qRT-PCR, qualitative real time polymerase chain reaction; 5-FU, 5-fluorouracil.

mice, conventional mice treated with the same dose of irinotecan had more lesions within the jejunal intestinal epithelium and higher gastrointestinal permeability.[44] This was also reflected in another study, which showed that diarrhoea was more common in conventional mice compared to germ-free mice, which also had a lowered intestinal damage score.[45] Rigby *et al.*[46] also showed the role of gastrointestinal bacteria in mediating doxorubicin-induced gastrointestinal damage by showing that germ-free mice did not display the changes in crypt depth and proliferative cell numbers that conventional mice treated with doxorubicin showed. Closely linked are the inflammatory pathways that are markedly upregulated in all cases of CIGT. It is now known that microbiome-host interactions modulate inflammatory cytokine production capacity.[26] Dysbioses of gut microbes are often linked to aberrant immune responses, often complemented by abnormal production of inflammatory cytokines. Additionally, commensal bacteria have protective effects on the integrity of the gastrointestinal mucosal barrier, including interactions with tight junctions and regulation of mucous layer.[47]

Research in pre-clinical models conducted over the past decade has shown a variety of changes to microbiome composition and diversity in the gastrointestinal tract due to chemotherapy treatment. There are some limitations to using pre-clinical microbiome models with 85% of bacterial sequences seen in a mouse representing genera not detected in humans.[48] However, there is also significant similarity in the distal gut microbiome between human and mice at a divisional level, and both have the same two most abundant bacterial divisions (*Firmicutes* and *Bacteroidetes*). Therefore pre-clinical models are often routinely used in this field.

Pre-clinical studies show a decrease in commensal species after chemotherapy, which causes reduced protective effects and decreased resistance to pathogenic colonisation (Table 1). This increase in pathogenic species also corresponds to an increase in gram negative species, which release LPS that is known to initiate the inflammatory pathways involved in CIGT development.[27] These pre-clinical studies generally show decreases in *Lactobacillus* and *Bifidobacterium* and increases in *Escherichia coli* (*E. coli*) and *Staphylococcus*.[49] However, many of these studies used polymerase chain reaction (PCR) or microbial culture techniques to delineate species and changes. While these methods were standard at the time, with more sophisticated pyrosequencing techniques now the norm, these results may have limited reproducibility.

A small number of clinical studies have also been conducted with patients undergoing chemotherapy, often replicating what has been shown in pre-clinical studies (Table 1). However, due to a focus on clinical outcomes (e.g. diarrhoea severity), these studies have failed to conclusively link pre-treatment to post-treatment microbiome composition. Results overall have included a decrease in total bacteria numbers and diversity.[50,51] At a species specific level, findings have shown increases in *Bacteriodetes*, *Clostridium* cluster IV and

Table 2. Summary of studies investigating probiotics and antibiotics in significantly modulating cancer treatment-induced gastrointestinal toxicity.

Study Type/ Sample Size	Cancer treatment	Study population age (years)	Probiotic/ antibiotic	Administration	Endpoint	Outcome	Reference
Pre-clinical (rats) $n = 48$	Irinotecan		VSL#3	Oral gavage, different schedules: 7, 21, 28 days pre-, pre- and post- or post-treatment	Clinical and histological injury markers	Probiotic reduced weight loss compared to Irinotecan alone (5.3% *vs* 12.6%, $p < 0.05$), increased crypt proliferation ($p < 0.05$), inhibited apoptosis ($p < 0.05$)	Bowen *et al.*[55]
Pre-clinical (rats) $n = 8$	MTX		Cow or sheep milk yoghurt with probiotics	Oral gavages twice daily 7 days prior to MTX and for remainder of trial	Intestinal barrier function, lactulose/ mannitol ratio	Sheep yoghurt probiotic improved histological damage and small intestinal permeability ($p < 0.05$)	Southcott *et al.*[54]
Pre-clinical (rats) $n = 27$	MTX		*Streptococcus thermophilus*	7 oral gavages from 48 h prior to MTX to 72 h after	MPO levels, 13(C) sucrose breath test, histological injury markers	High-dose probiotic partially attenuated mucositis (↓ MPO, ↓ histological damage, $p < 0.05$)	Tooley *et al.*[56]
Pre-clinical (tumour bearing rats) $n = 36$	MTX		*Streptococcus thermophilus*	7 oral gavages from 48 h prior to MTX to 72 h after	MPO levels, 13(C) sucrose breath test, histological injury markers	No difference between groups	Tooley *et al.*[57]
Clinical $n = 15$	Irinotecan + 5-FU/ leucovorin	Not reported	Neomycin + bacitracin	Oral, 3 x daily, from second chemotherapy cycle, days 2–5 and 16–19 of each cycle	Diarrhoea reduction or resolution	Antibiotic = complete resolution of diarrhoea from 2nd to 4th chemotherapy cycle in all patients	Almonti *et al.*[60]
Clinical $n = 62$	Irinotecan	Range = 36–80	Neomycin	Oral, 3 x daily for 3 days, starting 2 days before Irinotecan	Grade 3 diarrhoea levels	No difference between groups	de Jong *et al.*[62]
Clinical $n = 7$	Irinotecan	Median = 57, Range = 49–71	Neomycin	Oral, 3 x daily, if developed diarrhoea in first chemo course, oral, days −2 to 5 of other cycles	Diarrhoea reduction	Diarrhoea ameliorated in 6 of 7 patients ($p = 0.033$)	Keefer *et al.*[61]
Clinical $n = 150$	Adjuvant 5-FU (colorectal cancer patients)	Median = 60, Range = 31–75	*Lactobacillus rhamnosus*	Oral, 2 x daily, 24 weeks of treatment	Reduction of severe diarrhoea	Less severe diarrhoea (22 *vs* 37%, $p = 0.027$), less abdominal discomfort (2 *vs* 12%, $p = 0.025$), less dose reductions (22% *vs* 47%, $p = 0.0008$)	Osterlund *et al.*[58]
Clinical $n = 42$	Various chemotherapies-paediatric population	Range = 14 months –13 years 4 months	*Bifidobacterium breve* strain Yakult	Oral, 3 x daily, 2 weeks before treatment, 6 weeks after (8 weeks total)	Effect on infectious complications	Reduced need for antibiotics (3.2 *vs* 6.9 days, $p = 0.04$) and frequency of fever (44 *vs* 68%)	Wada *et al.*[72]

Clinical $n = 63$	Pelvic radiotherapy with cisplatin	Placebo group: median = 52, treatment group: median = 47	*Lactobacillus acidophilus* + *Bifidobacterium bifidum*	Oral, 2 x daily, 7 days before treatment then every day of treatment	Diarrhoea reduction	Less grade 2 and 3 diarrhoea (9 vs 45% control, $p = 0.002$)	Chitapanarux *et al.*[73]
Clinical $n = 85$	Adjuvant radiotherapy: cervical or endometrial cancer + concomitant cisplatin	Placebo group: median = 59.34 ± 12.77, treatment group: median = 60.91 ± 11.8	*Lactobacillus casei* DN-114001	Oral	Diarrhoea reduction	No difference	Giralt *et al.*[74]

5-FU, 5-fluorouracil; MTX, methotrexate.

E. coli[51,52] and decreases in *Lactobacillus*, *Bifidobacterium* and *Clostridium* cluster XIV.[52,53]

Attempts to ameliorate chemotherapy-induced changes to the microbiome profile have been variably successful in lowering damage severity. Administration of probiotics in pre-clinical models have attenuated gastrointestinal damage from chemotherapy by preventing apoptosis, reducing barrier disruption and promoting crypt survival (Table 2).[54,55] However there have been inconsistencies, with level of diarrhoea reduction varying due to probiotic strains, dosing and treatment plan. For example in pre-clinical studies using *Streptococcus thermophiles*, one study showed promising results, with non-tumour bearing rats treated with methotrexate having attenuation of gastrointestinal damage,[56] while another study utilising the same probiotic and chemotherapeutic in tumour bearing rats showed no benefit.[57] These studies additionally demonstrate a potential role of the tumour itself in regulating CIGT and gut microbiome changes, however further research is required to more fully understand this.

Multiple clinical trials have used probiotics in patients undertaking chemotherapy, with *Lactobacillus* species as a particular focus (Table 2). For example, Osterlund *et al.*[58] showed that *Lactobacillus* supplementation led to less severe diarrhoea, less abdominal discomfort and fewer dose reductions after 5-fluorouracil (5-FU) treatment for colorectal cancer. Subsequently, the Multinational Association for Supportive Care in Cancer (MASCC) released new clinical guidelines in 2014 suggesting the use of 'probiotic agents containing *Lactobacillus* species for the prevention of chemotherapy and radiation-induced diarrhoea in patients with a pelvic malignancy'.[1] Despite this, a recent meta-analysis found insufficient current evidence to support widespread implementation of probiotics after chemotherapy.[59]

Clinical trials have also investigated the impact of the antibiotic neomycin in combination with irinotecan, and results have shown less diarrhoea.[60,61] However, a larger study conducted by de Jong *et al.*[62] did not find a substantial role for neomycin in reducing diarrhoea severity. After inadequate/conflicting evidence, a 2013 systematic review was conducted and concluded that no clinical guideline for the use of neomycin was possible.[63] Another study administering antibiotics showed compromised anti-tumour efficacy of chemotherapy and an increase in potentially pathogenic bacteria.[64] These results may suggest that the removal of the entire microbiome with broad spectrum antibiotics does more harm than good, and that restoring microbial diversity is more important in maintaining damage-defence mechanisms.

Gastrointestinal Toxicity, the Microbiome and the Innate Immune System

Modifications to both immune function and bacterial profile may influence severity of gastrointestinal injury after chemotherapy.[75] This could possibly occur *via* an altered capacity to mount an immune response. For example, commensal

Table 3. Evidence of pre-treatment microbiome as a predictive factor in cancer treatment-induced gastrointestinal toxicity

Study Type/ Sample Size	Cancer treatment	Study population age (years)	Detection method	Predictive factor	Pre-therapy significant differences compared to controls		Outcome	Reference
					Increases	Decreases		
Clinical $n = 20$	Pelvic radiotherapy	Median = 51 years, range = 41–64	16S rRNA pyrosequencing	Development of diarrhoea	*Bacteroidetes*, *Dialister*, *Veillonella* ($p < 0.05$)	*Clostridium* cluster XI and XVIII, *Faecalibacterium*, *Oscillibacter*, *Parabacteroides*, *Prevotella* ($p < 0.05$)	Patients who developed diarrhoea: lower bacterial diversity (Shannon's index = 2.74 *vs* 3.78, $p < 0.01$), higher *Firmicutes*/ *Bacteroidetes* ratio ($p < 0.05$)	Wang *et al.*[83]
Clinical $n = 90$	Radiotherapy—head and neck cancers	Mean = 47.2, range = 22–75	16S rRNA pyrosequencing	Severe oral mucositis	*Actinobacillus* during erythmea	–	Healthy controls: greater diversity of oropharyngeal bacteria. Built predictive model (AUC = 0.89) for mucositis aggravation i.e. mild progressing to severe. Could not construct model to predict aggravation prior to irradiation.	Zhu *et al.*[11]
Clinical $n = 34$	Ipilimumab	Range = 28–85	16S rRNA pyrosequencing	Checkpoint blockade-induced colitis development	–	*Bacteroidetes*	Only study finding a protective phenotype. Model had sensitivity of 70% and specificity of 83%.	Dubin *et al.*[10]
Clinical $n = 23$	Pelvic radiotherapy—gynaecological malignancies	Mean = 71.5	Used electronic nose and Field Asymmetric Ion Mobility Spectrometry	Gastrointestinal toxicity (Irritable Bowel Syndrome scale)	–	–	Clear separation between high and low toxicity groups after 4 weeks of treatment (90% accuracy of reclassification).	Covington *et al.*[82]

Increase and decrease columns refer to differences in bacterial species from healthy controls or low toxicity groups that led to gastrointestinal toxicity symptoms
AUC, area under the curve.

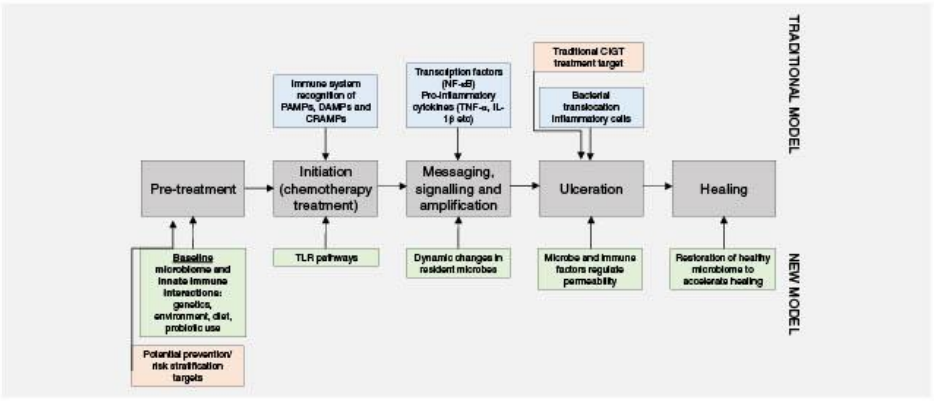

Figure 1. Pathobiology of CIGT. Chemotherapy treatment leads to direct DNA damage and release of Reactive Oxygen Species, causing transcription factor activation and release of pro-inflammatory cytokines. This leads to an amplification of apoptosis and epithelial damage, eventually causing intestinal ulceration and severe pain, in some cases caused by bacterial translocation through these ulcers. Removal of chemotherapy treatment will allow healing. Since the five-phase model was introduced in 2004,[12] significant research has been done to improve this model. We now suggest that bacterial and immune cells not only play a role in the ulceration phase, but also have an important baseline role in setting up risk of severe toxicity. [Color figure can be viewed at wileyonlinelibrary.com]

bacteria are able to induce CD4+ T cell differentiation. Specifically, *Bacteroides fragilis* can induce the development of a systemic Th1 response through polysaccharide A molecules.[76] It has been well established that *Bacteroides* genus levels are decreased by chemotherapy in both pre-clinical and clinical models.[49,64,65] This therefore leads to a potential decreased ability to mount a Th1 response after chemotherapy, which may affect the severity of gastrointestinal injury.

The TIMER (translocation, immunomodulation, metabolism, enzymatic degradation, reduced diversity) model was recently proposed by Alexander *et al.*[9] to show the various ways the gut microbiome can influence chemotherapy efficacy and toxicity. It is possible that each part of this model is reliant on the microbiome's interaction with the innate immune system, and thus the gut microbiome and innate immune system henceforth should be investigated together as much as practicable.

There are a variety of ways this interaction could be studied in future. Currently, much CIGT research is undertaken in animals or relatively rudimentary cell cultures. Additionally, there are limitations to using animal pre-clinical models for microbiome models, with many strains present in the mouse microbiome not present in humans as discussed previously.[48] Reproducible and scientifically robust *in vitro* and *ex vivo* models are therefore needed to effectively study the microbiome in intestinal models. This may include using human tissue samples or stem cells to grow organoids, or the development of gut-on-a-chip technology to incorporate microbiome changes.[77] Additionally, recent trials of ingestible electronic capsules have showed the ability to sense gases produced by the microbiome in the gastrointestinal tract.[78] This may represent a real-time method of monitoring gut microbiome changes that could be paired with other analyses.

Future Opportunities for Risk Prediction and Modification

A personalised approach could soon be taken to manage CIGT, where a patient's individual risk of toxicity could be managed early in their treatment plan. This approach is supported by observations relating genetic testing on DNA extracted from saliva to severe CIGT, in which key immunogenetic factors relating to the *TNF-a* and *TLR2* have been correlated with more severe toxicity.[79] Whether the patient's gut microbiome profile pre-cancer treatment could also predict toxicity severity is largely unknown, particularly in the setting of chemotherapy-induced damage. However, this idea was first postulated by Touchefeu *et al.*,[50] and more recently by Wardill and Tissing.[75] Use of the microbiome as a predictive marker is gaining support in a variety of fields, including recent prediction of chemotherapy-related bloodstream infection.[80] Studies may employ methods such as machine learning or metabolomics techniques to create an algorithm or predictive model in which a patient's microbiome profile can be input to test risk of disease.[80]

There is some initial evidence that such an idea could also prove true in relation to other cancer treatment-induced toxicities (Table 3), although as yet no study has investigated this after chemotherapy. One study of patients undergoing pelvic radiotherapy[81] found that patients who went on to develop diarrhoea had lower bacterial diversity and a higher

Firmicutes/Bacteroidetes ratio. A study of patients suffering from oral mucositis after radiotherapy for head and neck cancers was also able to build a predictive model for the aggravation of oral mucositis (similar to CIGT in the oral cavity).[11] Only one study has identified a protective phenotype of the gut microbiome for gastrointestinal toxicity.[10] This study measured development of checkpoint blockade-induced colitis in melanoma patients receiving the immunomodulatory therapy ipilimumab. Using 16S rRNA pyrosequencing techniques, it was shown that an increased representation of bacteria in the *Bacteroidetes* phylum before treatment was associated with resistance to development of colitis.

Finally, one study[82] did not use a bacterial sequencing approach, but rather used the relatively novel method of an electronic nose and the Field Asymmetric Ion Mobility Spectrometry method, to analyse stool samples. Gases and other metabolic by-products of microbiome fermentation emitted from the samples were analysed. The patient pre-radiotherapy samples were successfully separated, using principal component analysis and linear discriminant analysis, into those who suffered from gastrointestinal toxicity and those who did not. This method represents a translatable clinical test, where pre-treatment samples could be analysed, and future cancer treatment and supportive care measures adapted to suit.

This emergent data on the role of the microbiome and the immune system in determining severity of cancer treatment-induced toxicities represents an additional opportunity to personalise cancer treatment, and allow the identification of patients at risk of severe symptoms. Alexander *et al.*[9] summarised evidence of being able to adapt the microbiome profile of the gastrointestinal tract using an enzyme inhibitor such as a β-glucuronidase inhibitor,[83] changes in diet[84] or probiotics.[55] Of these, diet modification has been shown to be one of the most consistent and predictable ways of remodelling the microbiome, able to induce rapid shifts in composition and subsequent function.[85] An enzyme inhibitor may be particularly useful for patients undergoing irinotecan treatment, as the enzyme β-glucuronidase is crucial in irinotecan toxicity. SN-38, the active form of irinotecan, is conjugated in the liver to a less toxic metabolite, SN-38G. When excreted to the gastrointestinal tract *via* bile, it is hydrolysed back to the toxic SN-38 form by microbe-derived β-glucuronidase.[61,67] As discussed above, probiotics have previously been used in CIGT studies, with mixed results (Table 2). There is currently a renewed call for carefully planned studies with new types of probiotics to better understand the potential benefits they could play in modulating CIGT risk.[86] Also required is better characterisation of the microbial profiles associated with toxicities caused by different agents to properly identify and develop the ideal microbial protectant. Finally, the developing area of faecal microbiota transplants and even faecal capsules represent methods of directly modifying the gut microbiome yet to be investigated in this context.

Conclusion

Effective treatments or predictive strategies for gastrointestinal toxicity caused by chemotherapy are urgently required. This under-reported but common adverse event has a substantial effect on quality of life and economic burden. Understanding the effect the gut microbiome and the innate immune system has on CIGT is key to developing treatment and prevention strategies. This review has summarised the key interactions between the gut microbiome and the innate immune system, and how these interactions may adversely affect gastrointestinal toxicity after chemotherapy. We have also drawn emphasis to new data suggesting that the bidirectional interaction between microbiome and immune system is distinct to each patient and how we may be able to predict high-risk patients, thereby adjusting supportive care measures to each person. It is hoped that through pragmatic and rigorous scientific investigation, effective CIGT treatment may be within reach.

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Diarrhea Induced by Small Molecule Tyrosine Kinase Inhibitors Compared With Chemotherapy: Potential Role of the Microbiome

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Abstract

Small molecule receptor tyrosine kinase inhibitors (SM-TKIs) are among a group of targeted cancer therapies, intended to be more specific to cancer cells compared with treatments, such as chemotherapy, hence reducing adverse events. Unfortunately, many patients report high levels of diarrhea, the pathogenesis of which remains under investigation. In this article, we compare the current state of knowledge of the pathogenesis of chemotherapy-induced diarrhea (CID) in comparison to SM-TKI–induced diarrhea, and investigate how a similar research approach in both areas may be beneficial. To this end, we review evidence that both treatment modalities may interact with the gut microbiome, and as such the microbiome should be investigated for its ability to reduce the risk of diarrhea.

Keywords

microbiome, diarrhea, supportive care, tyrosine kinase inhibitors, chemotherapy

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Introduction

Gastrointestinal toxicity, commonly manifesting as diarrhea, is a common side effect of a range of cancer treatments including chemotherapy and tyrosine kinase inhibitor targeted therapy. This toxicity is currently without a specific prevention or treatment strategy, and can affect between 50% and 80% of patients, depending on their treatment protocol.[1] Diarrhea, and associated intestinal ulceration, can lead to a host of severe issues including dehydration, malnutrition, fatigue, renal insufficiency, and increased risk of systemic infection.[2] In addition to the severe compromise in quality of life, for people experiencing one or more of these side effects, treatment breaks or dose reductions are likely, leading to less effective cancer care and compromised remission rate.[3] Available economic data suggest that each episode of severe diarrhea requiring hospitalization may cost up to US$6616; however, this may be accompanied by broader costs related to emergency medical procedures or loss of productivity from time out of work.[4-6] As such, effective preventative and treatment strategies for cancer treatment–induced diarrhea are critically needed. By understanding the pathogenesis of this diarrhea, identification of appropriate treatment targets may be expedited.

Small molecule tyrosine kinase inhibitors (SM-TKIs) are used to treat a variety of solid tumor types including lung, breast, and head and neck cancers.[7] They are used in

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combination or as a monotherapy, increasingly as first-line therapy.[8] Most act by binding to the intracellular adenosine triphosphate (ATP) domain of the tyrosine kinase, preventing downstream signaling and subsequent cell division and growth.[9] While there are a large variety of targets, the most common are the epidermal growth factor receptors (EGFRs) and vascular endothelial growth factor receptors (VEGFRs).[8] SM-TKI diarrhea usually occurs in the first week of the treatment course and is typically managed with antidiarrheal agents such as loperamide.[10,11] However, for many patients, loperamide does not sufficiently reduce diarrhea, and/or causes side effects including fatigue, constipation, and abdominal pain.[11,12] Thus, there is merit in investigating new targets for this diarrhea.

Comparatively, the mechanism of diarrhea stemming from chemotherapy has had far more research than mechanisms of diarrhea from SM-TKI treatments.[13] This is, in part, likely due to the length of time these treatments have been available. Chemotherapy-related diarrhea occurs due to direct damage to the intestine, initiating a host of inflammatory pathways eventually leading to ulceration and potential bacterial translocation.[14] It was previously assumed that SM-TKI and chemotherapy toxicity had the same pathogeneses; however, recent research has posited that they have different initiating events and mechanisms, and are therefore specific, separate toxicities.[13,15] While it is now clear that there are treatment-specific mechanisms that differ between SM-TKI–induced diarrhea and chemotherapy-induced diarrhea (CID; similarly to CID and radiation-induced diarrhea), there is a strong evidence base, explored here, to suggest that they share core mechanisms relating to direct mucosal damage, changes to chloride secretion, and upregulation of inflammatory processes.[16,17] Given the variance in the literature surrounding the mechanisms of cancer treatment–induced diarrhea, we propose that taking a similar research approach to elucidate the causes of both chemotherapy- and SM-TKI–induced diarrhea may be beneficial in further building our understanding.

The gut microbiome is gaining momentum as a key research and therapeutic target in cancer therapy–induced toxicities.[14] Changes in the microbiome following chemotherapy have been clearly shown; however, we do not yet fully understand how to manipulate the microbiome, or determine the microbial-mucosal interactions that accelerate gut toxicity and diarrhea.[14] In SM-TKI treatments, initial indications suggest altered microbial composition in people experiencing more severe diarrhea.[18] While there appears to be differences in the mechanisms of SM-TKI–induced diarrhea and CID, these results suggest that both are underpinned by changes in the microbiome. Additionally, when the microbiome is compromised via antibiotics in both treatment modalities, diarrhea and treatment efficacy outcomes are altered.[19-21] Given the suggestion that the microbiome may be a common and targetable mechanism of both SM-TKI–induced diarrhea and CID, we propose that similar to the research into the microbiome in CID, a similar research approach is taken with SM-TKI–induced diarrhea.

This review will focus on comparing the pathogenesis of diarrhea stemming from highly mucotoxic chemotherapies (5-fluorouracil [5-FU] and irinotecan) with diarrhea induced by EGFR and VEGFR inhibitors used for the treatment of solid tumors. The benefits and drawbacks of taking a similar research approach to determining the mechanisms of both of these types of diarrhea will be explored. Additionally, we will examine the potential of the gut microbiome to play a key role in both of these treatment modalities.

Diarrhea Incidence in Chemotherapy and SM-TKI Cancer Treatments

Chemotherapy

Cytotoxic chemotherapies irinotecan and 5-FU are 2 agents known to cause high levels of gastrointestinal toxicity, with up to 80% of patients developing at least some level of diarrhea.[2] The National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) classifies a diarrhea grading over 3 (increase of ≥7 stools per day over baseline; hospitalization indicated; severe increase in ostomy output compared with baseline; limiting self-care activities of daily life) as severe.[22] As single agents, 5-FU (bolus) has rates of up to 32% severe diarrhea, and weekly irinotecan has rates of up to 22% severe diarrhea.[2] Combination therapy, often in the form of FOLFIRI (5-FU, folinic acid, and irinotecan), is commonly used in a variety of cancer types (Table 1) and is also associated with high risk of gastrointestinal damage (severe diarrhea = 12% to 20%).[23,24] Adverse effects of these highly mucotoxic chemotherapies are often managed with loperamide, octreotide, or nonpharmacological interventions, such as oral rehydration, but still commonly require treatment breaks and dose reductions.[23,25]

SM-TKIs

SM-TKIs are growing in use as cost-effective, orally administered agents known to inhibit extremely important oncological targets in a range of cancers (Table 1).[48,49] After dermatologic toxicity, diarrhea is the most common side effect of SM-TKI treatment, and it is often severe enough to require a break in treatment or a dose reduction.[7] Unlike chemotherapy, it is important to consider that SM-TKI treatments are often taken daily for long periods (months-years), meaning that even low-grade diarrhea can have significant impacts on patient quality of life. Therefore, proactive and clinically effective management of side effects is required to enable treatment to continue successfully and reduce the risk of chronic side effects that are less common in the relatively acute chemotherapy setting.[50]

Table 1. Comparison of Diarrhea Incidence in Highly Mucotoxic Chemotherapies and VEGFR and EGFR TKIs[a].

Treatment type	Agent	Target	Indication	Diarrhea incidence		Reference
				All grades (%)	Severe, grade 3+, (%)	
Chemotherapy	Irinotecan	Topoisomerase I	Primarily colorectal cancer	60% to 90%	20% to 40% (single agent)	Mego et al,[26] Stein et al,[2] and Michael et al[27]
	5-Fluorouracil	Thymidylate synthase	Primarily colorectal cancer	Up to 50%	32% (bolus), 6% to 13% (CI)	Stein et al[2]
VEGFR TKI	Sorafenib	VEGFRs, PDGFRs, BRAF, KIT, FLT3, RET	Renal, hepatocellular, and prostate cancers	30% to 50%	>10%	Stein et al[2] and Que et al[28]
	Sunitinib	VEGFRS, PDGFRs, FLT3, TIE-2, RET, KIT, CSF1-R	Gastrointestinal stromal tumor and renal cancers	30% to 50%	>10%	Stein et al[2] and Que et al[28]
	Regorafenib	VEGFRs, PDGFRs, RAF, KIT, TIE-2, FGFR, PIGF, RET, and BRAF	Colorectal cancers and gastrointestinal stromal tumors	33%	4% to 8.5%	Carrato et al[29] and Yin et al[30]
	Pazopanib	VEFGRs, PDGFRs, KIT, FGFR, TIE-2	Renal cancers and soft tissue sarcomas	33% to 52%	2% to 4%	Santoni et al,[31] Que et al,[28] and Sternberg et al[32]
	Axitinib	VEGFR, eNOS, AKT, ERK1/2, PDGFR, KIT	Renal cell carcinoma	55%	11%	Rini et al[33]
	Vandetanib	VEGFR, MET, RET, EGFR	Advanced carcinomas (eg, medullary thyroid cancers and non–small cell lung cancer [NSCLC], small cell lung cancer [SCLC], breast cancer, malignant glioma, hepatocellular cancer)	52.1%	5.6%	Miller et al,[34] Huo et al,[35] Pozo et al,[36] and Yin et al[37]
	Lenvatinib	VEGFR, EGFR, RET	Thyroid cancer, renal cell carcinoma	45% to 67%	4% to 9%	Berdelou et al[38] and Haddad et al[39]
	Cabozantinib	VEGFRs, EGFR MET, KIT, RET, AXL, TIE2, TRKB, and FLT3	Medullary thyroid cancers	72% to 75%	10% to 13%	Schmidinger et al[40] and Fazio et al[41]
EGFR TKI	Gefitinib	HER1/EGFR	NSCLC	27% to 69%	1% to 25%	Rugo et al[7]
	Erlotinib	HER1/EGFR	NSCLC and pancreatic cancer	18% to 68%	1% to 12%	Becker et al,[42] Rugo et al,[7] and Hirsh et al[43]
	Lapatinib	HER1/2	Breast cancer	65%	14%	Rugo et al[7] and Azim et al[44]
	Afatinib	HER 1, 2, 3, and 4	NSCLC	87% to 95%	5% to 17%	Tagliamento et al[45]
	Neratinib	HER 1, 2, and 4	HER2+ breast cancer	95%	40%	Rugo et al[7] and Chan et al[46]
	Osimertinib	T790M EGFR mutation	NSCLC	41%	1%	Rugo et al[7]
	Dacomitinib	HER 1, 2, and 4	NSCLC	72% to 78%	12% to 13%	Van Sebille et al[47]

Abbreviations: VEGFR, vascular endothelial growth factor receptor; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; FDA, Food and Drug Administration.
[a]VEGFR and EGFR TKIs listed are FDA approved for cancer treatment with diarrhea incidence results from phase III trials or clinical use studies.

EGFR TKIs, a common group of SM-TKIs, are associated with diarrhea that typically occurs early in a treatment course (often within the first week), similar to chemotherapy.[7] Severe diarrhea is common, occurring in up to 25% of cases (Table 1), requiring individualized management (lowered dosing, diet modification, and intravenous fluids and electrolytes) to reduce the risk of hospitalization and treatment delays.[7] Clinical data have shown patients receiving second-generation multi-EGFR SM-TKIs, such as afatinib and dacomitinib, have a higher incidence of severe, grade 3 diarrhea compared with first-generation agents (eg, gefitinib), which are more target specific and less likely to inhibit other tyrosine kinase groups.[10] Diarrhea associated with VEGFR-TKIs in monotherapy is predominantly mild to moderate (Table 1) but needs to be managed as early as possible (with antidiarrheal agents including loperamide) in order to avoid symptoms progressing to more severe, higher grade diarrhea.[51] VEGFR-TKIs are also often given in combination with chemotherapy, which can cause a compound effect of more severe diarrhea.[51] For example, in the ICON6 trial of combination platinum-based chemotherapy and VEGF inhibitor cediranib, 39% of people in the intervention arm had to stop the trial early with highly toxic diarrhea and fatigue.[52] Anecdotal evidence has also suggested that diarrhea following cediranib treatment leads to a sustained reduction in health-related quality of life.[51]

Chemotherapy Toxicity in Comparison to SM-TKI Toxicity

Chemotherapy

Currently, diarrhea from chemotherapy treatment is largely understood to follow the 5-phase model proposed in 2004, and updated in 2010 and 2019.[14,53,54] Briefly, initiating events such as reactive oxygen species generation and DNA damage lead directly to transcription factor activation (eg, nuclear factor κB [NF-κB]) and pro-inflammatory cytokine release, leading to apoptosis and mucosal ulceration.[14,55] This eventual thinning of the mucosal layer from activation of these inflammatory pathways can lead to bacterial translocation and an inability to properly absorb fluids from the intestine, eventuating in diarrhea. In this vein, indirect biological signaling is the basis of much of the damage, as opposed to direct tissue damage by chemotherapy itself. Current research is attempting to understand the connection of the microbiome to the host immune response underlying this signaling, specifically targeting the role of inflammation and the enteric nervous system.[14]

SM-TKI: Direct Target Inhibition

SM-TKI–induced diarrhea is likely to be multifactorial, and there are multiple hypotheses currently under investigation. One such hypothesis surrounds the inhibition of the specific kinase targets in the intestinal epithelium. EGFR and VEGFR are both highly expressed in the gut, and inhibition in the intestine leads to lowered cell proliferation and reduced capillary networks in the intestinal villi, respectively.[56-59] EGFR pathways also have stimulatory effects on enterocyte proliferation and nutrient and electrolyte transport, causing structural and functional changes when inhibited.[16] These changes in the intestinal architecture may lead to mucosal atrophy and thereby a reduction in the absorptive capacity of the gut. However, many SM-TKI agents have multi-targeted activity. For example, sorafenib is able to block tyrosine kinases in the VEGFR, PDGFR, BRAF, KIT, FLT3, and RET families. It is, therefore, challenging to determine how inhibition of which particular kinase targets affects diarrhea levels and how this could be used to reduce diarrhea without affecting treatment efficacy.[7,60] In EGFR SM-TKIs, second-generation agents, which have a broader inhibitory profile, have more diarrhea of any level, and also more severe diarrhea compared with the first-generation agents.[7] Diarrhea is also the most frequent adverse event for the Food and Drug Administration–approved third-generation agent osimertinib, which targets the T790M mutation of EGFR, but has a low affinity for wild-type EGFR.[61] In a phase I escalation trial, diarrhea increased with escalating doses of osimertinib, suggesting that direct target inhibition by osimertinib may be involved in the development of diarrhea.[61] In a larger phase III clinical trial of 253 patients with non–small cell lung cancer, 47% of patients developed diarrhea.[62,63] Similar to multi-kinase EGFR SM-TKIS, in VEGFR SM-TKIs, diarrhea is observed at higher levels in the more common multi-kinase inhibitors such as sorafenib compared with pure VEGFR inhibitors.[51,64]

SM-TKI: Chloride Secretion

Despite evidence that direct inhibition of EGFR and VEGFR may drive diarrhea following treatment, preclinical SM-TKIs have shown differing results around intestinal histopathological damage. Multiple SM-TKI studies have demonstrated a lack of this histopathological damage.[17,65,66] As this damage is a hallmark of chemotherapy-induced gastrointestinal toxicity, this vast histopathological difference has been a driving factor for the hypothesis that SM-TKI–induced diarrhea has a distinctly different mechanism than CID. However, in a recent preclinical study, the EGFR-targeting SM-TKI dacomitinib caused significant blunting and fusion of the villi in the ileum,[66] challenging the notion that chemotherapy and SM-TKI–induced toxicities are unrelated. One recent hypothesis has theorized that diarrhea from SM-TKIs (in particular EGFR TKIs) had a secretory phenotype.[13,16] In secretory diarrhea, activation of chloride channels including the cystic fibrosis transmembrane conductance regulator (CFTR) and calcium-activated channels increases fluid secretion into the lumen, and inhibition of intestinal sodium transport lowers fluid absorption.[67] In the

intestine, EGFR has an inhibitory effect on chloride secretion, and it has, therefore, been hypothesized that SM-TKI inhibition of EGFR allows excessive chloride secretion into the gut lumen.[16] Studies have additionally shown that in ex vivo models, potassium channels as well as CFTR chloride channels are directly activated by EGFR TKI treatments including afatinib, gefitinib, and lapatinib.[68] Additionally, preclinical rat studies have suggested that EGFR inhibitor dacomitinib-induced diarrhea was of a secretory form, and clinical studies of neratinib show a fecal osmotic gap consistent with secretory diarrhea.[66,69] Crofelemer is an anti-chloride secretory medication currently being trialed clinically to reduce diarrhea in HER2+ breast cancer being treated with EGFR targeting monoclonal antibodies (NCT02910219).[70,71] However, targeting secretory changes in a dacomitinib rat model using crofelemer actually worsened diarrhea levels.[16,66] In addition, preclinical studies of neratinib and lapatinib have both shown no changes in serum chloride levels.[17,50] Collectively, this leaves the role of chloride secretion unclear and requiring further investigation.

SM-TKI: Inflammation

Inflammation is known to have a key role in the development of CID[72]; however, it has only more recently been considered as a factor in SM-TKI–induced diarrhea. In chemotherapy, upregulation of inflammatory pathways is a key part of the 5-phase model. Inflammasome activation and pro-inflammatory cytokine release has been shown to mediate irinotecan-induced gastrointestinal damage and diarrhea.[73,74] In addition, 5-FU treatment causes upregulation of a host of pro-inflammatory cytokines including tumor necrosis factor-α, interleukin-1β, interleukin-6, interleukin-17A, and interleukin-22.[75] Activation of the Toll-like receptor (TLR) signaling pathways leads to upregulation of many of these inflammatory mediators, and it has recently been implicated in irinotecan-induced diarrhea.[76] A mouse model demonstrated that TLR4 knockout mice had reduced diarrhea and other clinical indicators of gastrointestinal toxicity.[76] Similarly, a preclinical study of diarrhea from neratinib also showed potential for inflammation to be involved by demonstrating that budesonide, a locally acting corticosteroid, reduced levels of diarrhea and colonic injury, as well as increasing levels of anti-inflammatory interleukin-4 levels.[17] This result has been reflected in data reported by Liu and Kurzrock showing a marked alleviation in EGFR-TKI–induced diarrhea following budesonide administration.[77] Furthermore, the STEPP trial of panitumumab in colorectal cancer showed that patients receiving prophylactic doxycycline for skin toxicity developed less diarrhea than patients on a reactive skin treatment regimen (15% vs 32%, n = 95).[78] While panitumumab is an EGFR-targeting monoclonal antibody, its side effects display many similar features of SM-TKI treatment.[79] It has since been suggested that as doxycycline may have acted as an anti-inflammatory, the diarrhea may have had an inflammatory component.[43] Alternatively, it could be speculated that the antibiotic nature of doxycycline altered the gut microbiota to influence diarrhea development through other pathways. Together, these findings have suggested that while SM-TKIs and chemotherapies induce diarrhea via different preliminary mechanisms, initiation of inflammatory processes may be a key contributing factor to its development following both treatment types.

Potential Role of the Microbiome in Chemotherapy and SM-TKI Treatment Diarrhea

While it has been widely posited that chemotherapy and SM-TKI treatments induce diarrhea via different mechanisms, interventions targeting these hypotheses have so far been unable to definitively reduce diarrhea. Therefore, using similar research methods to that used to elucidate CID may be useful to rule out or more fully understand mechanisms underlying SM-TKI–induced diarrhea. The gut microbiome could potentially play a key role in both treatments due to its relation to inflammatory responses and chloride secretion (Figure 1).

Intestinal Inflammation

The gut microbiome has been shown to play an integral role in mediating intestinal inflammation. This has been demonstrated in inflammatory bowel disease, where there is a marked decrease in microbial diversity and richness compared with healthy controls.[84] Similarly, in Crohn's disease, the treatment-naïve microbiome is strongly correlated with disease status, with increased abundance of *Enterobacteriaceae, Pasteurellaceae, Veillonellaceae*, and *Fusobacteriaceae* occurring along with decreases in Erysipelotrichales, Bacteroidales, and Clostridiales in people with the disease.[85]

The gut microbiome is proposed to mediate these inflammatory responses via the innate immune system and has a particularly important role in the development of this system. For example, preclinical studies have demonstrated that early life exposure to commensal bacteria is required to develop appropriate invariant natural killer T-cell tolerance.[86] Additionally, dysbiosis of the microbiome can alter levels of metabolites from the microbiome such as butyrate. Butyrate is a short-chain fatty acid produced by colonic bacteria by fermenting elements from our dietary intake. It can induce regulatory T (Treg) cell development to maintain immune tolerance and maintain the balance between Th17 and Treg cells.[87] This balance is highly important in modulating intestinal inflammation. Finally, the gut microbiome and innate immune system are intrinsically linked via many types of pattern recognition receptors (PRRs). TLRs are important in sensing molecular patterns originating from the

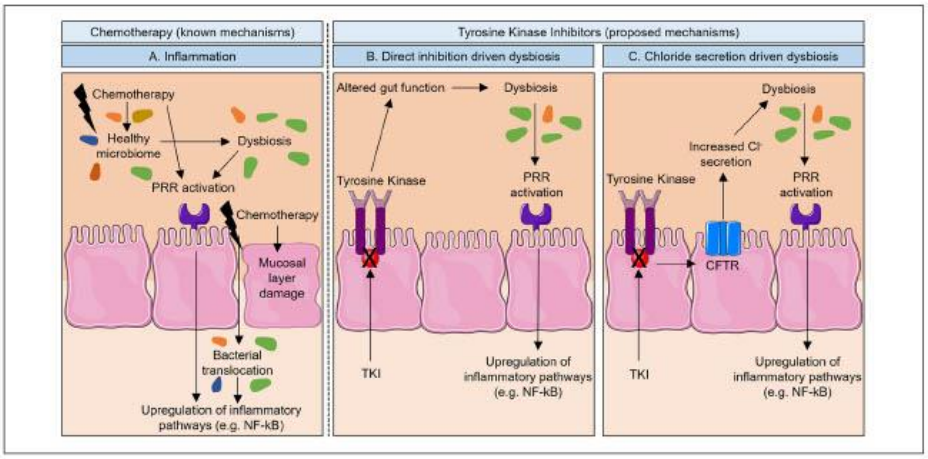

Figure 1. Potential interactions of the gut microbiome with tyrosine kinase inhibitor (TKI) treatments leading to diarrhea. (A) Chemotherapy causes vast changes to the gut microbiome,[80,81] as well as activation of inflammatory pathways via pattern recognition receptors (PRRs), such as Toll-like receptor 4,[53,76] that lead to ulceration and eventual diarrhea. TKI treatment also leads to diarrhea, but the mechanism is not well understood. We propose that the gut microbiome may play a key role. (B) Long-term TKI treatment may lead to a dysbiotic microbiome.[18] Additionally, direct inhibition of EGFRs or VEGFRs in the gut can lead to altered gut function (eg, changes in cell proliferation and capillary networks) that can alter microbial composition.[56-59] This could lead to similar inflammatory outcomes as in chemotherapy.[17] (C) Alternatively, increased chloride secretion in the gut (causing diarrhea itself)[16] could lead to a significant shift in the microbiome that may lead to additive effect on the diarrhea.[18,82,83]

gut microbiome, such as lipopolysaccharide (LPS), that cause activation of downstream signaling pathways of transcription factor (eg, NF-κB) upregulation and pro-inflammatory cytokine release.

Chloride Secretion

In addition, there is an emerging link between gut microbiome composition and intestinal chloride secretion, particularly via CFTR, which allows exit of chloride ions across the apical membrane. Two studies have investigated this link with lubiprostone, used clinically to treat constipation and known to stimulate electrogenic chloride secretion.[88,89] Upregulation of chloride secretion with this agent caused large shifts in the stool microbiome, with an increased abundance of *Lactobacillus* spp in the stool of lubiprostone-treated mice. It was concluded that epithelial chloride secretion may have a key role in influencing bacterial-epithelial interactions. In addition, changes to the CFTR have also shown to cause significant gut microbial changes. In a mouse model, CFTR gene mutations were sufficient to alter the gut microbiome,[82] and in a clinical study of 31 patients aged 1 to 6 years with cystic fibrosis (who have mutations in the CFTR), it was suggested that gut microbiota enterophenotypes were direct expressions of altered intestinal function.[83] These studies show the close links between chloride secretion and the gut microbiome. As excess chloride secretion into the intestinal lumen may cause diarrhea in some SM-TKI treatments, this provides further evidence for SM-TKI–induced diarrhea to be influenced by gut microbial changes. However, while there is some evidence that probiotic bacteria or pathogenic bacteria can alter chloride secretion,[90,91] there are low levels of evidence to suggest that the native gut microbiome changes are able to drive chloride channel dysfunction. Future work needs to be done to understand whether microbial dysbiosis is a direct driver of diarrhea, or whether the diarrhea itself causes dysbiosis as an outcome.

Microbiome Changes Due to Cancer Treatment

Preclinical studies have shown marked changes to overall microbiome composition in the gut following chemotherapy treatment, toward a dysbiotic state. The key finding has been a decrease in commensal bacterial species, along with a corresponding increase in pathogenic species.[80,81,92-94] These pathogenic species were usually gram-negative species, which can release LPS known to initiate the inflammatory pathways that are key mediators in development of diarrhea.[95,96] Clinical studies have shown similar findings,

with a decrease in total bacterial abundance and diversity, as well as decreases in commensals such as *Lactobacillus* and *Bifidobacteria*, with increases in *Bacteroidetes* and *Escherichia coli*. In addition to these dynamic changes during chemotherapy, the TIMER (translocation, immunomodulation, metabolism, enzymatic degradation, reduced diversity) model proposed by Alexander and colleagues has outlined how the functions of the microbiome may itself have a central role in determining the extent and intensity of diarrhea.[97] Key to this model is the facilitation of inflammatory responses to chemotherapy by the microbiome.[72]

Evidence of Microbial Changes in SM-TKI Treatment

More recently, there has been some direct evidence suggesting links between gut microbial changes and diarrhea following SM-TKI treatments. A preclinical study of the EGFR inhibitor lapatinib showed that lapatinib-treated rats had significantly lowered microbial diversity.[98] In addition, decreases in *Betaproteobacteria* were seen following lapatinib treatment. In contrast, chemotherapy studies have shown changes in *Gammaproteobacteria*, and this difference was suggested to be a key difference between chemotherapy- and SM-TKI–induced diarrhea outcomes.[99] A study of 20 patients receiving VEGF-TKI treatment for metastatic renal cell carcinoma (mRCC) assessed the microbiome via 16S sequencing of stool samples. Patients with diarrhea had higher levels of *Bacteroides* spp and lower levels of *Prevotella* spp.[18] However, it was inconclusive whether these microbial changes were simply due to the occurrence of diarrhea, or the drivers of this diarrhea.

Probiotics and Fecal Microbiota Transplant

Probiotics and dietary modification have also been suggested as a treatment or preventative measure for cancer treatment–induced diarrhea. In chemotherapy, probiotics have had varying levels of success in reducing diarrhea.[72] While some studies have shown lowered gastrointestinal damage levels and less diarrhea, others have shown no benefit. A meta-analysis recently found insufficient current evidence to support widespread implementation of probiotics after chemotherapy.[100] The authors noted the wide variety in probiotic types and dosing schedules, and stressed the need for rationally designed probiotic mixtures and trials. Probiotics are commonly used alongside some forms of SM-TKI treatment.[101] However, to date, there is no robust evidence for probiotic use during SM-TKI treatment.[64] One study of the EGFR inhibitor dacomitinib in 173 non–small cell lung cancer patients demonstrated that VSL#3 probiotics were unsuccessful in reducing diarrhea or intestinal damage.[102] Subsequent commentary on this article suggested some issues with the study, further highlighting the need for consistently designed probiotic studies.[103,104] Currently, a clinical trial is underway to assess the use of probiotic yoghurt in reducing diarrhea following VEGF inhibitor treatment for kidney cancer (NCT02944617).[105] Another form of microbiome modulation under investigation for use in cancer treatment is fecal microbiota transplant (FMT).[106] Preclinically, FMT was able to reduce gut dysbiosis caused by 5-FU.[107] Clinically, a recent study of 21 patients having treatment with VEGFR inhibitors pazopanib and sunitinib for mRCC was completed.[101] Patients with diarrhea received FMT via colonoscopy or *Lactobacillus casei* DG probiotics as control. After 7 days, all patients in the FMT group had resolution of diarrhea compared with 54.5% of patients in the probiotic group. At a longer term 15- and 30-day follow-up, 90% of FMT patients had no diarrhea compared with 0% of patients in the probiotic group, demonstrating the potential of the microbiome to be a key part of SM-TKI–induced diarrhea.

While there is only a small amount of direct evidence suggesting that gut microbiome changes occurs in SM-TKI treatment, the close relationship of a dysbiotic microbiome with both inflammatory activation and chloride secretion processes is an incentive to further elucidate the use of the microbiome in treating diarrhea from SM-TKI treatments.

Predicting Toxicity and Treatment Response: Role of the Microbiome

In the future, individual microbial composition could be used as a predictor of risk of a range of gastrointestinal conditions. Recent reviews have summarized the initial work that has been done to characterize microbial profiles putting people at severe risk of diarrhea following radiotherapy[72] and how this work is now being translated into the chemotherapy setting.[108] In the past, toxicities have previously been used as an indicator of SM-TKI treatment response,[109] and moving forward, the microbiome may represent a unique opportunity to be able to predict both toxicity and response to treatment.

Aside from diarrhea, the other main side effect from SM-TKI treatment is skin rash. Patients receiving EGFR TKI treatment are at particular risk and may develop secondary skin infections. Multiple studies have suggested a correlation between incidence of rash and subsequent response to treatment, where occurrence of rash was associated with better response.[77,110] Commonly, broad-spectrum antibiotics are used to treat this rash. These antibiotics have a deleterious effect on the gut microbiome, and importantly, their use has been shown to be a negative predictor of efficacy and toxicity of EGFR-TKI treatment in non–small cell lung cancer populations.[19] This study retrospectively grouped 102 patients into antibiotic and nonantibiotic-treated groups and found that people who took antibiotics had worse progression-free survival and more instances of

severe diarrhea.[19] However, this retrospective study may not take into account the reasons for antibiotic use and whether the results may actually reflect that patients receiving antibiotics were a more vulnerable group overall. In immunotherapy settings, there is a growing link between antibiotic use and treatment response.[111] Similarly, antibiotic use during chemotherapy may also have detrimental effects on treatment efficacy.[20] In addition, chemotherapies including oxaliplatin and cyclophosphamide are reliant on an immune response to induce tumor cell death. A study showed that the antitumor efficacy of cyclophosphamide was reduced in germ-free mice or mice treated with antibiotics.[21] These results suggest that the gut microbiome is integral in shaping an antitumor immune response during both chemotherapy and SM-TKI treatment.

Finally, it has also been shown that occurrence of diarrhea relates to treatment success during SM-TKI treatment.[112] An analysis of 4 phase I trials (total 179 patients) showed that patients who had diarrhea with VEGFR inhibitor sorafenib treatment had a significantly increased time to progression compared with patients who did not develop diarrhea.[113] Another study of 223 patients showed that diarrhea from gefitinib (EGFR inhibitor) treatment was predictive of lowered risk of progression in multivariate analysis,[114] while increased progression-free survival was seen when patients receiving first line VEGF-TKI treatment for mRCC were given antibiotics that gave protection from *Bacteroides* spp.[115] This is contradictory to the results seen in EGFR-targeted treatment discussed above; and hence, these findings may be target, or antibiotic-specific. Also in mRCC, a small US study of 6 patients showed significant differences in the gut microbiome compositions of responders and nonresponders to sunitinib.[116]

Taken together, these findings suggest that levels of diarrhea and antibiotic use could be predictive for a person's positive and negative responses, respectively, to chemotherapy and SM-TKI treatment. Due to the widespread effects of antibiotics on the gut microbiome, and the proposed role of the microbiome in causing diarrhea in these treatments, future research should focus on connecting gut microbial composition with overall survival and treatment response. It should be noted that a potential drawback of taking a similar research approach is that current research provides evidence for overlapping mechanisms in both toxicity and treatment efficacy. This also suggests that exploiting the gut microbiome to enhance efficacy may lead to increased levels of toxicity. Recent research into microbial changes relating to diarrhea from Crohn's disease has suggested that microbial dysbiosis drives clinical symptomology, despite a lack of mucosal injury.[117] This suggests that the microbiome is likely to govern the duration of symptoms via mechanisms independent of mucosal injury. As some SM-TKIs do not cause overt tissue injury, this is of potential importance.

Future studies should be careful to determine methods to manipulate the microbiome in a way to minimize toxicity while simultaneously enhancing efficacy. This may be via specialized pre- or probiotics that ensure the production of specific bacterial metabolites, or defined microbial modulation techniques including FMT that could be used prophylactically.

Conclusion

Diarrhea from any cancer treatment can have negative effects on a person's ability to complete their treatment course, as well as affecting financial welfare and quality of life. Precision treatment and ideally preventative strategies are required to reduce the burden of diarrhea. In this article, we have compared gastrointestinal toxicity stemming from SM-TKI treatment with the more well-understood toxicity seen with traditional, highly mucotoxic chemotherapy treatment, in order to understand key commonalities. Although past research has shown differences in the mechanism of pathogenesis, we hypothesize that the gut microbiome may play a key role in the gastrointestinal response to both treatments. A common mechanism between the 2 would allow for a more rapid development of targeted treatments and prophylactic medications. In addition, as SM-TKIs and chemotherapy are often given in combination, a common way to target this diarrhea would be highly beneficial. Current research in SM-TKI toxicity has focused on the use of interventional treatments to reduce diarrhea severity; however, these interventions may be unable to reduce gut microbial changes and subsequent inflammatory responses. It may, therefore, be advantageous to alter this research direction to focus on how to predict toxicity and treatment efficacy using pretreatment microbiome profiling techniques.

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ORIGINAL ARTICLE

Pathophysiology of neratinib-induced diarrhea in male and female rats: microbial alterations a potential determinant

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Abstract

Background Neratinib is a potent irreversible pan-ErbB tyrosine kinase inhibitor, approved by the FDA for extended adjuvant treatment of HER2-positive breast cancer. Diarrhea is the most frequently observed adverse event with tyrosine kinase inhibitor therapy. In this study, we developed a reproducible model for neratinib-induced diarrhea in male and female rats.

Methods At first, male rats were treated with neratinib at 15, 30 or 50 mg/kg or vehicle control via oral gavage for 28 days (total $n=12$). Secondly, we compared outcomes of male ($n=7$) and female ($n=8$) rats, treated with 50 mg/kg neratinib.

Results Rats treated with a 50 mg/kg daily dose of neratinib had a reproducible and clinically relevant level of diarrhea and therefore was confirmed as an appropriate dose. Male rats treated with neratinib had significant changes to their gut microbiome. This included neratinib-induced increases in *Ruminococcaceae* ($P=0.0023$) and *Oscillospira* ($P=0.026$), and decreases in *Blautia* ($P=0.0002$). On average, female rats experienced more significant neratinib-induced diarrhea (mean grade 1.526) compared with male rats (mean grade 1.182) ($P<0.0001$). Neratinib caused a reduction in percentage weight gain after 28 days of treatment in females ($P=0.0018$) compared with vehicle controls. Females and males both showed instances of villus atrophy and fusion, most severely in the distal ileum. Serum neratinib concentration was higher in female rats compared to male rats ($P=0.043$).

Conclusions A reproducible diarrhea model was developed in both female and male rats, which indicated that diarrhea pathogenesis is multifactorial, including anatomical disruption particularly evident in the distal ileum, and alterations in microbial composition.

Keywords Tyrosine kinase inhibitors · Diarrhea · Breast cancer · Rat model · Neratinib

Introduction

Small molecule tyrosine kinase inhibitors (TKIs) are increasingly used for the treatment of cancers in blood and solid tissues. These drugs compete with the intracellular ATP binding site of oncogenic tyrosine kinases including the ErbB group [1]. Over-expression of one ErbB member, HER2, is known to be an oncogenic driver in approximately 20% of breast cancers [2]. Neratinib (HKI-272) is an orally available, high affinity, irreversible small molecule pan-ErbB TKI. It has been FDA approved for extended adjuvant treatment of HER2-positive breast cancer and is now being assessed for other HER2 related cancers (NCT03289039, NCT02932280). It has been shown to be beneficial in metastatic and early settings, and also for patients who previously received trastuzumab [3].

Diarrhea has been a common adverse effect in multiple clinical trials for neratinib. In the ExteNET trial of 2840 patients randomized to 12 months of treatment with oral neratinib or placebo [4], 40% of patients developed severe, grade 3–4 diarrhea. Fifty-five percent of patients developed grade 1 and 2 diarrhea. Most grade 3 diarrhea occurrences happened in the first month of treatment, with a reduction in frequency thereafter [2]. Diarrhea management guidelines advise prophylactic loperamide, and in the I-SPY 2 trial of 115 patients receiving neoadjuvant neratinib plus

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chemotherapy, prophylactic loperamide reduced severe diarrhea from 47 to 36% [5].

While in some cases loperamide is able to somewhat decrease diarrhea incidence, there may be other, more effective diarrhea management strategies that are more specific to the mechanism of neratinib-induced diarrhea. Models of diarrhea have previously been developed with other TKIs such as the dual-HER inhibitor lapatinib [6], however pan-HER inhibitors such as neratinib are becoming more commonly used to overcome resistance to these other treatments. Therefore we attempted to develop a reproducible animal model of neratinib-induced diarrhea in both male and female rats that could be used in future to target and test possible interventions.

Materials and methods

Chemicals

Neratinib was kindly provided by Puma Biotechnology (USA). Neratinib was diluted in 0.5% (w/w) hydroxypropyl methylcellulose (HPMC) buffer (Sigma-Aldrich, USA).

Animals and ethics

All experiments were conducted on Albino Wistar (AW) rats obtained from the University of Adelaide Laboratory Animal Service, Waite Campus. Rats were housed in groups of up to 4 in ventilated cages. The environmental controls were set to maintain temperature within the range of 19–23 °C and relative humidity within the range of 45–65%; with a 12 h light/dark cycle. Food and water were consumed ad libitum. If rats were experiencing moderate to severe treatment-related toxicity (e.g. diarrhea, weight loss, stress marks) they were allowed soaked chow (normal feed softened in water to ease mastication) in addition. Rats were acclimatized to local housing conditions for a minimum of 7 days prior to the first day of dosing. On day 1 of treatment, the rats were between 7–9 weeks old. This study was approved by the Animal Ethics Committee of the University of Adelaide (study number M-2015-006), and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training (2013).

Experimental design

All rats were randomly allocated to experimental groups and were identified by a unique animal number written with an indelible marker on their tail. During the 28 day treatment period, rats received daily oral gavages using a soft plastic feeding tube. Neratinib or vehicle controls were given at a constant dose volume of approximately 5 mL/kg. Individual dose volumes were adjusted daily according to most recent body weight records. The first day of dosing was designated day 1. The final dose was given on the day before scheduled necropsy. The 28-day treatment schedule aimed to mimic long term clinical administration. All rats were deeply anaesthetized via isoflurane inhalation (Isothesia, Henry Schein, USA), and culled by cardiac exsanguination with death confirmed by cervical dislocation.

Dose finding study

Male rats were treated for 28 days with either vehicle control (0.5% (w/w) HPMC buffer) ($n=3$) or neratinib at 15 mg/kg ($n=3$), 30 mg/kg ($n=3$) or 50 mg/kg ($n=3$).

Outcome comparison in males and females

Comparisons were made between female rats, treated for 28 days with either vehicle control (0.5% (w/w) HPMC buffer) ($n=4$) or 50 mg/kg neratinib ($n=8$) and male rats treated for 28 days with vehicle control ($n=7$) or 50 mg/kg neratinib ($n=7$).

Clinical gut toxicity assessment

Rats were weighed daily, and there was a twice daily comprehensive clinical symptom recording. Diarrhea was graded by two assessors according to a validated grading system [6] with four grades: 0, no diarrhea,1, mild (soft unformed stools); 2, moderate (perianal staining and loose stools); 3, severe (watery stools and staining over legs and abdomen). Rats were euthanized if displaying 15% or over weight loss from baseline or significant distress and clinical deterioration.

Tissue collection and preparation

At necropsy, the gastrointestinal tract was removed from the pyloric sphincter to the rectum. The small and large intestine were flushed with chilled, sterile phosphate buffered saline and weighed. Samples of duodenum, jejunum, ileum and colon were collected and fixed in 10% formalin (ChemSupply, Australia) for embedding in paraffin (Sigma-Aldrich, USA). As ileal damage is common in other models of TKI-induced diarrhea, samples were taken of both distal and proximal ileum. Other organs (stomach, spleen, liver, brain, kidney, lung and heart) were also collected, weighed, fixed in formalin and embedded in paraffin.

Histological examination

All histology was conducted on paraffin embedded intestinal samples, which were cut using a rotary microtome

(RM2235, Leica, Germany) and 4 µm sections mounted onto Superfrost glass slides (Menzel-Glaser, Germany). All histology had images taken using a Nanozoomer digital slide scanner (Hamamatsu Photonics, Japan) and viewed using the Nanozoomer Digital Pathology Software (NDP View v1.2) (Histalim, France). All analysis was done in a blinded fashion.

Serum analysis

Blood samples were collected by cardiac puncture using a 23 gauge needle (Becton–Dickinson, USA) in K3EDTA Vacuette tubes (Greiner Bio-One, Austria). Serum was separated by centrifugation at 300*g* for 5 min before being analyzed via a multiple blood analysis (MBA-20). This analysis was completed by the Department of Clinical Pathology, SA Pathology, Adelaide, South Australia.

Serum neratinib concentration was measured by liquid chromatography tandem mass spectrometry/time of flight (LC–MS/TOF) (validated range 10–10,000 ng/ml). This work was conducted in the Good Laboratory Practice (GLP)-certified Pharmaceutical Science Sector Laboratory, School of Pharmacy and Medical Sciences, University of South Australia. Calibrator and quality control working control solutions were prepared by dissolving neratinib powder in methanol. Sample supernatant was aspirated and eluted through a Kinetex 1.7 µm C18 2.1 × 100 mm column (Phenomenex, USA) using a 30 series UHPLC system (Shimadzu, Japan). All rats were assessed for neratinib concentration 24 h after the final dose (C_{min}).

Mucosal damage analysis

Routine hematoxylin and eosin (H&E) staining was completed and an injury score assigned using a well-established system of histological criteria [7, 8]. Criteria were villus fusion, villus atrophy, disruption of brush border and surface enterocytes, crypt losses/architectural disruption, disruption of crypt cells, infiltration of polymorphonuclear cells and lymphocytes, dilation of lymphatics and capillaries and edema. The latter six criteria were examined in the colon. Each criterion was scored as present = 1 or absent = 0.

Microbial analysis

In a subset of male rats treated with 50 mg/kg neratinib ($n = 4$) or vehicle control ($n = 4$) for 28 days, cecal contents were aseptically collected during dissection into a sterile tube at each time point, and stored at − 80 °C. Samples were sent to the Australian Genome Research Facility (AGRF) for DNA extraction and 16S ribosomal RNA (rRNA) gene region analysis. DNA was extracted from 250 mg of cecal sample using the Qiagen DNeasy PowerLyzer PowerSoil Kits with the PowerLyzer 24 Homogenizer. 16S analysis sequencing details are as follows:

Target: 16S: 341F (V3–V4) (V1–V3), read length = 300 bp.
Forward sequence: 5′ CCTAYGGGRBGCASCAG 3′
Reverse sequence: 5′ GGACTACNNGGGTATCTAAT 3′

Image analysis was performed in real time by the MiSeq Control Software (MCS) v2.6.2.1 and Real Time Analysis (RTA) v1.18.54, running on the instrument computer. The Illumina bcl2fastq 2.20.0.422 pipeline was used to generate the sequence data. CLC Genomics Workbench 12.0 (https://www.qiagenbioinformatics.com/) was used to complete bacterial diversity profiling. Paired-ends reads were assembled by aligning forward and reverse reads. Primers were identified and trimmed. Trimmed sequences were quality filtered, duplicate sequences removed and sorted by abundance. Reads were assigned to taxonomic identities using the Greengenes 97% similarity database version 13.8. Alpha-diversity was calculated using the Shannon diversity index. Beta diversity was calculated using Principal Coordinate Analysis (PCoA) based on generalized UniFrac distances [9]. The program BURRITO [10] was used to visualise the links between taxonomic composition and function in the dataset. Statistical Analysis of Metagenomic Profiles (STAMP) [11] was used with the predicted metagenome using Welch's *t* test with the Benjamini–Hochberg correction for the false-discovery rate (FDR).

Statistical analysis

Data were compared using Prism version 7.0 (GraphPad Software, USA). The vehicle control and neratinib treated groups were compared separately in males and females. If data was normally distributed, bars on graphs are mean ± SEM. If not, median is displayed in graphs. The assumptions of equality of variance for each group and normally distributed data were tested using Bartlett's test and D'Agostino and Pearson omnibus normality test, respectively. If these assumptions were violated, non-parametric equivalent tests were performed, including Kruskal–Wallis for independent data and Freidman's test for repeated measures. When assumptions held, ANOVA's were performed using the two-way analysis of variance (ANOVA). Diarrhea proportions were analyzed by Chi Squared test. *P* values less than 0.05 were considered statistically significant.

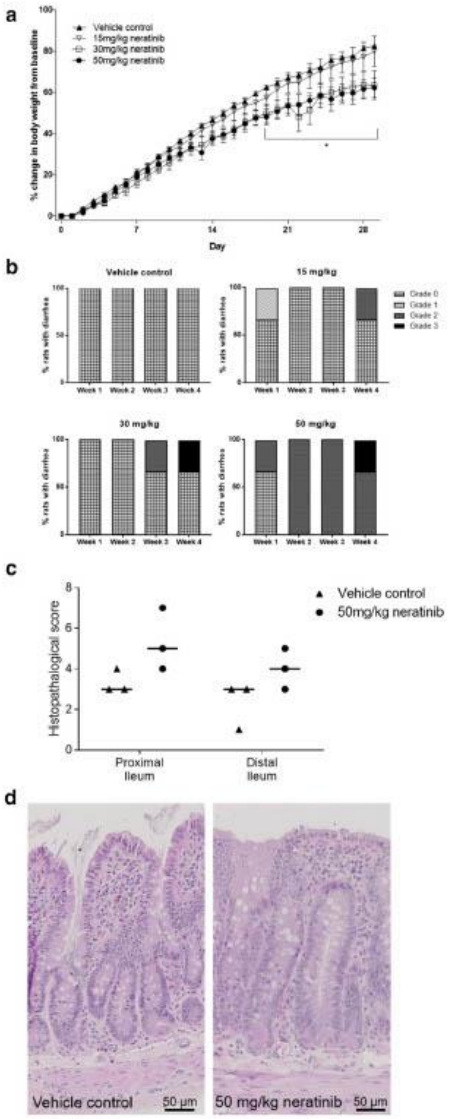

Fig. 1 **a** Graph showing the weight change from baseline in each treatment group. Rats in the 30 mg/kg and 50 mg/kg group had significantly less weight gain from day 18 onwards, compared to the vehicle control group (30 mg/kg: $P=0.043$, 50 mg/kg: $P=0.0183$, two-way ANOVA). Data presented as mean ± SEM. **b** Graph shows percentage of rats at their highest grade of diarrhea after each week of treatment. 100% of rats receiving a 50 mg/kg dose of neratinib developed moderate diarrhea throughout the treatment course. Rats treated with 50 mg/kg neratinib had significantly more severe diarrhea than other dosages ($P<0.0001$, Chi-squared test). **c** There was no significant difference in histopathological scoring in the ileum between the vehicle control and 50 mg/kg neratinib group ($P>0.05$, Kruskal–Wallis test). Non-parametric data, line represents median. **d** Photomicrographs of distal ileum at ×200 original magnification (stained with H&E) showing evidence of blunting and fusion of villi, flattening of surface enterocytes and crypt elongation. Scale bars shown in diagram

Results

Model and dose development in male rats

50 mg/kg neratinib causes reproducible diarrhea and weight loss in male rats

From day 19 of treatment until the end of the experiment, rats treated with 30 mg/kg and 50 mg/kg neratinib had significantly less weight gain than vehicle controls (30 mg/kg: $P=0.043$, 50 mg/kg: $P=0.0183$) (Fig. 1). No rats treated with vehicle control developed diarrhea at any time during the experiment. 15 and 30 mg/kg doses caused diarrhea in one-third of animals, whereas 50 mg/kg neratinib caused diarrhea in all animals, including one who developed grade 3, severe diarrhea ($P<0.0001$ compared to vehicle control) (Fig. 1).

Histopathological changes were most pronounced in the ileum

The groups treated with 15 mg/kg and 30 mg/kg neratinib had minimal histopathological changes, with minor evidence of lymphocyte infiltration in the ileum (data not shown). There were no statistically significant differences in histopathological scoring between the vehicle control group and the 50 mg/kg neratinib groups in any intestinal region, however there was evidence of polymorphonuclear cell and lymphocyte infiltration in the ileum and colon, as well as fusion of villi in the ileum of the 50 mg/kg neratinib group (Fig. 1). Due to low sample numbers in this pilot study, it may be possible that it was not sufficiently powered for statistical significance.

Neratinib induced changes in serum biochemistry

All biochemical serum values were within normal ranges for Wistar rats [12]. There were no significant differences between doses for any parameter other than lactate dehydrogenase (LD). LD levels were significantly higher in the 30 mg/kg ($P=0.014$) and 50 mg/kg ($P=0.0006$) group compared to the 15 mg/kg group (Table 1). As expected,

Table 1 Blood biochemistry in male rats ($n=12$) (mean ± SEM)

	Vehicle		15 mg/kg		30 mg/kg		50 mg/kg	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Sodium	141.67	0.33	141.33	0.67	141.67	0.33	143.33	1.20
Potassium	5.00	0.10	5.13	0.09	5.27	0.19	5.20	0.06
Chloride	100.33	0.88	100.67	0.33	100.33	0.33	100.33	0.33
Bicarb	1.60	0.77	1.66	0.81	1.73	0.82	1.73	0.84
Anion gap	16.00	1.15	15.00	0.00	17.00	1.73	19.67	2.33
Glucose	8.47	0.03	8.90	0.36	8.37	0.28	8.23	0.71
Urea	6.67	0.07	6.50	0.25	6.40	0.21	6.63	0.39
Creatinine	21.00	1.15	20.00	0.58	20.00	0.58	22.67	1.86
Cholesterol	1.73	0.03	1.57	0.12	1.63	0.09	1.70	0.12
Urate	0.05	0.01	0.06	0.01	0.08	0.01	0.09	0.02
Phosphate	2.50	0.15	2.43	0.04	2.55	0.03	2.58	0.03
Tot. Ca	2.57	0.06	2.52	0.03	2.50	0.02	2.50	0.06
Albumin	16.67	0.33	15.67	0.33	15.00	0.00	15.33	0.33
Glob	41.67	0.88	38.67	0.88	38.67	0.88	39.67	1.76
Protein	58.33	1.20	54.33	1.20	53.67	0.88	55.00	2.08
Tot. Bili	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
GGT	3.00	0.00	3.00	0.00	3.00	0.00	3.00	0.00
ALP	217.67	22.30	258.33	40.34	248.33	40.71	256.67	39.96
ALT	58.33	3.28	74.33	4.91	69.33	5.24	76.33	6.36
AST	96.67	7.31	107.00	4.16	107.00	3.21	113.67	7.75
LD	786.00	149.95	718.00	45.40	830.00*	81.21	865.00*	141.48

One-way ANOVA used to determine statistical significance. No significance noted except for lactate dehydrogenase levels in the 30 and 50 mg/kg dosage

*Denotes significance. LD: 15 mg/kg vs 30 mg/kg: $P=0.014$, 15 mg/kg vs 50 mg/kg: $P=0.0006$

there was a dose-dependent increase in neratinib serum levels (15 mg/kg: mean = 111 ± 13.1 ng/mL, 30 mg/kg: mean = 286 ± 59.0 ng/mL, $P=0.045$ compared to 15 mg/kg, 50 mg/kg: mean = 407.3 ± 30.9 ng/mL, $P=004$ compared to 15 mg/kg) measured in serum 24 h after final dose (Fig. 2).

Overall, 15 mg/kg and 30 mg/kg doses of neratinib had little effect on clinically relevant markers of neratinib-induced injury. A 50 mg/kg dose of neratinib caused diarrhea in all animals and 23% less weight gained. 50 mg/kg was therefore confirmed as a robust and reproducible dose for use in a model for neratinib-induced diarrhea.

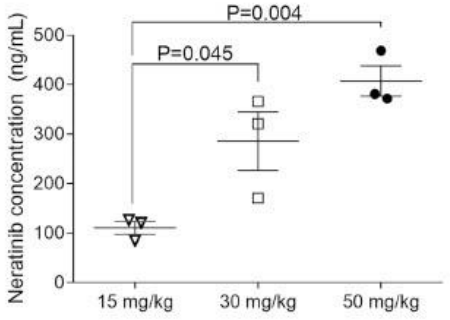

Fig. 2 Rats treated with 30 mg/kg ($P=0.004$) and 50 mg/kg ($P=0.045$) neratinib had significantly higher levels of serum neratinib after 28 days compared to rats treated with 15 mg/kg neratinib (total $n=9$). Parametric data, mean ± SEM shown, one-way ANOVA used

Neratinib induced vast changes in microbiome composition

Microbial changes were assessed in a subset of male rats treated with vehicle control or 50 mg/kg neratinib (Fig. 3). Cecal microbial diversity, measured using Shannon's diversity index at the genus level, was increased in the neratinib treated group compared to vehicle control ($P=0.0066$). However, there were no significant differences between the groups when the Simpson or Chao1 indexes were used. Principal coordinate analysis showed that neratinib treated rats and vehicle control treated rats clustered separately. This was confirmed with pairwise PERMANOVA testing showed significant differences between the groups ($P=0.0288$).

Rats treated with neratinib had significantly higher relative abundance of the family *Ruminococcaceae* ($P=0.0023$)

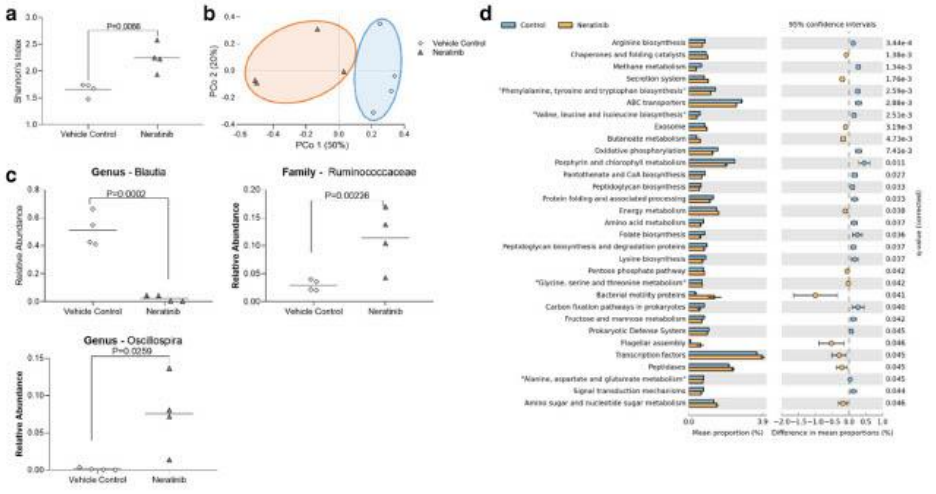

Fig. 3 Cecal microbial analysis of male rats. **a** Shannon's diversity index showed a significant increase in microbial alpha diversity in neratinib treated animals. Genus level shown in this graph. **b** Principal coordinate analysis showed separate clustering between vehicle control and neratinib treated animals. **c** Relative abundance of bacterial species. The genus *Blautia* was significantly higher in vehicle control animals, whereas *Ruminococcaceae* and *Oscillospira* were significantly higher in neratinib treated animals. **d** Significantly different predicted changes in the cecal metabolome based on changes in the microbiome. Mean proportions of pathways and Welch's *t* test with Benjamini–Hochberg false discover rate corrected significance are shown here

and the genus *Oscillospira* ($P=0.026$). The genus *Blautia* was highly significantly increased in vehicle control animals compared to neratinib treated ($P=0.0002$).

Metabolic pathways predicted to alter significantly due to changes in microbial composition are shown in Fig. 3. The most significantly increased pathway in the control group compared to neratinib treated was arginine biosynthesis. In the neratinib treated group, the most significantly increased pathway compared to the control group was chaperones and folding catalysts.

Outcome comparison of male and female rats

Females suffered more severe diarrhea and weight loss than males

Following the determination of 50 mg/kg as the optimal neratinib dose, the model was expanded to female rats. At 50 mg/kg, females suffered more severe diarrhea and weight loss than male rats receiving the same dose. Female rats had a mean diarrhea grade of 1.53 ± 0.37 compared to 1.18 ± 0.42 for males ($P<0.0001$) (Fig. 4). In females, all rats had diarrhea by day 3, and there were a total of 8 days where rats had severe diarrhea. In males, only 30% of rats had diarrhea by day 3, and none developed severe diarrhea.

Female rats treated with neratinib had significantly less weight gain than females treated with vehicle control at days 25 ($P=0.0498$) and 29 ($P=0.0490$). Neratinib did not cause any male rats to lose weight at any time throughout the experiment, however did in females in the first week of treatment. 88% of female rats were below their starting weight at day 7, compared to no male rats.

Moderate increase in systemic neratinib concentrations in females

LC–MS/TOF was used to assess serum neratinib level (female $n=8$, male $n=7$). There was an approximately two-fold increase in female (mean 827.5 ng/mL) serum neratinib values compared to male rats (mean 409.4 ng/mL) ($P=0.0012$) (Fig. 4). However, these samples were collected and assayed on different days, so inter-day/inter-assay variability cannot be ruled out.

Organ weights

Males had a significant increase, not seen in females, in small intestine ($P=0.013$) weight (normalised to brain weight) in neratinib treated animals compared to vehicle control (Fig. 4). Neratinib treated males also had a

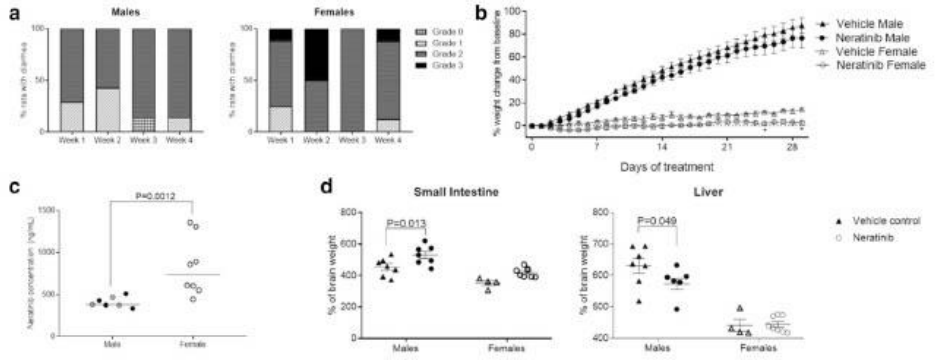

Fig. 4 Comparison of diarrhea levels in female and male rats after receiving 50 mg/kg neratinib. Figure represents highest grade of diarrhea for each animal after each week of treatment. Female rats had an overall significantly higher average grade of diarrhea ($P<0.0001$, Chi-squared test). **b** Percentage weight change from baseline after neratinib treatment in male and female rats. 88% of females lost weight in the first week of treatment, no male rats lost weight. Female neratinib treated rats had significantly less weight gain at day 25 (*$P=0.0498$) and 29 (*$P=0.0490$). Parametric data, data presented as mean ± SEM, two-way ANOVA used. **c** Serum neratinib concentration was significantly higher in female rats compared to male rats ($P=0.0012$). Data non-parametric, line shows median, Mann–Whitney test used. Grey data points in male column indicate data from initial dose finding study, these are included as 3 serum samples were missing from the second study dataset. **d** Organ weights in male and female mice. Males had a significant change in small intestine ($P=0.013$) and liver ($P=0.049$) weight not seen in females. Parametric data, two-way ANOVA used. Data shown as mean ± SEM

significantly lowered liver weight compared to vehicle control ($P=0.049$). Neither males nor females had a significant change in large intestine, spleen, kidney, stomach, heart or lung weight (data not shown).

Neratinib caused multiple blood biochemistry changes in female rats

Female neratinib-treated rats had a number of changes in serum biochemistry between vehicle control and the neratinib treated group that were not seen in male rats. These include liver toxicity markers alanine transaminase (ALT) and urate, and metabolic markers such as bicarbonate and glucose (Table 2). Males had significantly higher levels of alkaline phosphatase (ALP) in the neratinib treated group than vehicle control ($P=0.039$). Both males ($P=0.0023$) and females ($P=0.0020$) treated with neratinib had higher levels of albumin than vehicle control animals. Although a significant difference in LD was seen in the dose-finding pilot study, this finding was not replicated here.

Female rats treated with neratinib had significant histological damage in the ileum and colon

After 28 days of neratinib treatment, female rats had significantly higher levels of gastrointestinal damage in the proximal ileum, distal ileum and colon than vehicle controls (Fig. 5). In males, there was only a significant difference between groups in the distal ileum. Similar to the dose finding study, observations included blunting and fusion of villi and infiltration of polymorphonuclear cells and lymphocytes.

Discussion

This study aimed to develop a rat model of neratinib-induced diarrhea that can in future be used to test potential interventions. Neratinib is used primarily for breast cancer, so we sought to carry out this model in a pilot study of female rats. However, as previously developed similar models are in male rats, we began a dose finding study in male rats. It was concluded that a 50 mg/kg daily dose of neratinib most successfully models effects in humans. As seen in other small molecule TKI models of diarrhea, this is higher than the human equivalent dose [6, 13].

The present study showed that the changes observed in the gastrointestinal tract were mainly surrounding anatomical disruption of the ileum, and there was evidence of inflammatory infiltrate in the intestines. In male rats, significant microbial perturbations were observed in rats treated with neratinib.

This model was based on a previously developed model for targeted therapy-induced diarrhea, where the drug lapatinib was used [6]. In that model, moderate diarrhea was induced in 60% of rats, whereas we induced moderate

Table 2 Mean and SEM of blood serum biochemistry in male and female rats

	Male: vehicle		Male: neratinib		Female: vehicle		Female: neratinib	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Sodium	141.57	0.69	141.43	0.57	140.75	1.03	139.63	0.60
Potassium	4.86	0.087	5.21	0.14	4.53	0.25	5.2	0.10
Chloride	100.57	0.48	102.29	0.57	102.75	0.48	103.88	0.85
Bicarb	29.57	0.84	28.71	0.57	28.00	1.23	24.89*	0.44
Anion gap	16.29	0.81	15.43	0.61	14.50	0.65	15.88	0.67
Glucose	8.49	0.41	8.59	0.28	8.33	0.46	6.18‡	0.27
Urea	6.60	0.20	6.21	0.26	5.15	0.30	6.49	0.61
Creatinine	21.57	1.41	19.43	2.02	28.75	1.03	30.50	1.93
Cholesterol	1.84	0.072	1.70	0.04	1.93	0.11	1.89	0.11
Urate	0.039	0.003	0.053	0.006	0.050	0.004	0.098$^{\wedge}$	0.007
Phosphate	2.60	0.052	2.44	0.10	2.03	0.057	2.10	0.045
Tot. Ca	2.51	0.035	2.44	0.049	2.51	0.023	2.42	0.028
Albumin	15.86	0.14	14.43**	0.20	19.000	0.000	15.0$^{\#\#}$	0.27
Glob	38.57	0.65	36.71	0.36	40.25	0.63	42.88	1.32
Protein	54.43	0.75	51.14	0.34	59.25	0.63	57.88	1.46
Tot. Bili	1.00	0	1.00	0	1.00	0	1.00	0
GGT	3.00	0	3.00	0	3.00	0	3.13	0.13
ALP	229.43	12.65	337.14$^{\wedge\wedge}$	34.02	139.25	11.14	156.88	5.65
ALT	50.00	2.43	61.86	2.98	47.00	1.87	115.75***	14.67
AST	97.71	6.68	133.86	28.35	94.25	4.33	177.00	21.09
LD	685.57	180.37	730.71	193.22	546.50	126.43	944.63	113.91

Two-way ANOVA used for normally distributed data

Symbol denotes significance between vehicle control and neratinib group in either male or females. *$P=0.046$, $^{\ddagger}P=0.029$, $^{\wedge}P=0.0002$, **$P=0.0023$, $^{\#\#}P=0.002$, $^{\wedge\wedge}P=0.039$, ***$P=0.0006$

diarrhea in 100% of male rats (in the dose finding study). Interestingly however, there was no significant tissue pathology seen in that model. This is in contrast to another TKI animal model, of dacomitinib-induced diarrhea, which caused severe tissue pathology localized in the ileum. The main factors of this pathology were severe villus atrophy with increased inflammatory infiltrate [14], mirroring that seen in the development of this neratinib-induced diarrhea model. Results from the initial lapatinib model, where blood biochemistry showed a significant decrease in serum chloride, partly led to the hypothesis that TKIs may lead to a secretory type diarrhea [15]. In addition, a study of neratinib in healthy volunteers showed a fecal osmotic gap that was consistent with that of secretory diarrhea [16]. No evidence of this was seen in the current study when evaluating serum biochemistry.

Our results showed that neratinib caused more severe diarrhea, weight change and biochemical parameters in female compared to male rats. This may be due to a variety of reasons. In this study we saw a statistically significant difference in systemic drug concentration between males and females. However, as previous work has shown no relationship to diarrhea and serum concentration, it is unlikely to be responsible for the increase in diarrhea in female rats. For example, a rat model of lapatinib showed no relationship between serum lapatinib levels and diarrhea [6]. Additionally, our study of budesonide and neratinib in rats showed a decrease in diarrhea in rats treated with budesonide and neratinib compared to neratinib alone, however these rats did not have a corresponding change in serum neratinib [17]. Finally, a study of healthy volunteers having neratinib treatment failed to show a relationship between neratinib serum levels and diarrhea occurrence [16]. We also saw that females had a higher level of variability than males in serum levels, and therefore cannot rule out inter-day variability in this study. Differential levels of ErbB expression in males and females may also cause variances in response. While there is little current research surrounding this, it is known that there is a significant difference between males and females in liver expression levels of ErbB1 in mice [18]. A final possible mechanism is related to the female hormonal cycle. The hormonal cycles are well known to cause response changes in animal models of addiction and cardiovascular disease [19, 20]. Progesterone and estrogen can also affect intestinal contractility and transit, gastric emptying time and higher gastrointestinal pH [21]. In this study, female rats had higher levels of histopathological damage in the

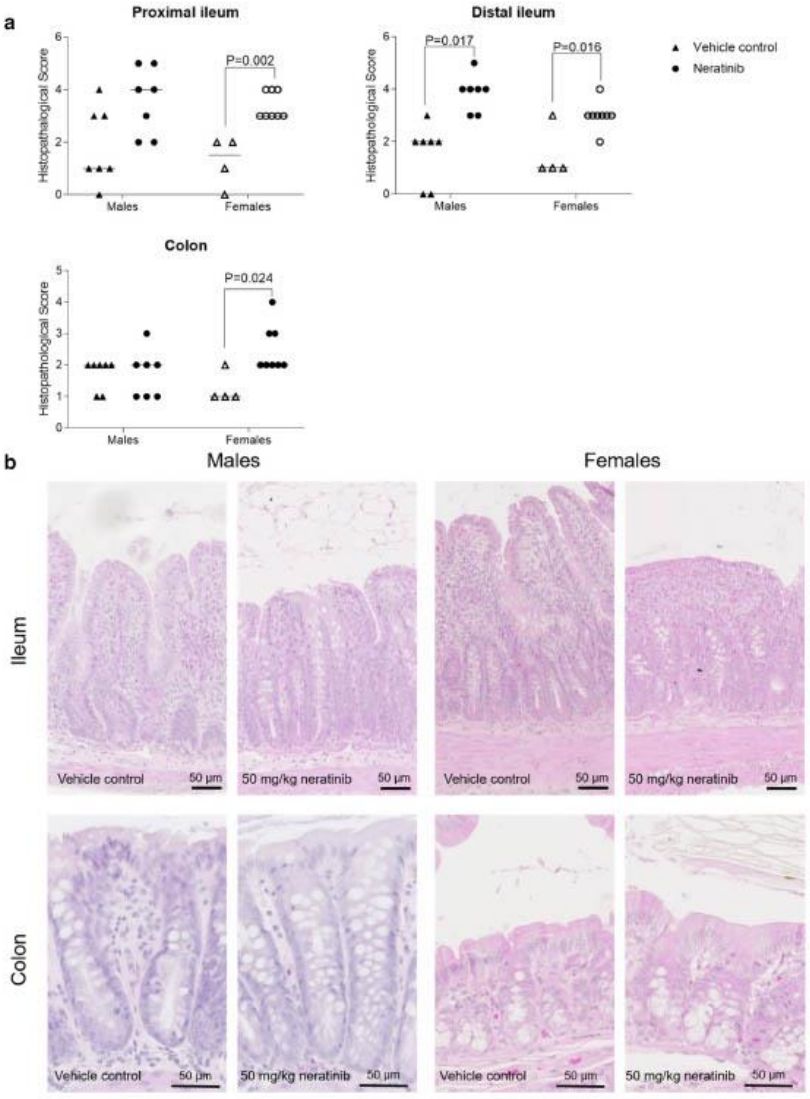

Fig. 5 **a** Female neratinib-treated rats had higher histopathological scores than vehicle controls in the ileum (proximal: $P=0.002$, distal: $P=0.0016$) and colon ($P=0.024$). Male neratinib treated rats had higher scores than vehicle controls in the distal ileum ($P=0.017$). Data non-parametric, line shows median. Mann–Whitney test used on male and female data, non-parametric data. **b** Photomicrographs of distal ileum at ×200 original magnification and colon at ×400 original magnification (stained with H&E) showing evidence of blunting and fusion of villi and crypt elongation. Scale bars shown in diagram

colon compared to males, and this may cause less fluid absorption and therefore diarrhea in the female rats.

One aim of developing this animal model was to be able to better understand the mechanisms of neratinib-induced diarrhea in order to identify potential interventions. Many past studies have hypothesized how TKIs cause diarrhea, and have included gut microbiome alterations, impaired water absorption or fluid transport and altered gut motility [22]. The results of this dose-finding study showed blunting and fusion of the villi in the ileum. The ileum is the region of most pronounced histopathological changes, which impacts on surface area and loss of transporters. Additionally, it has shown that ErbB1 is expressed at a relatively higher level in the ileum compared to the rest of the gastrointestinal tract [14]. Moreover, histopathological analysis showed an increase in inflammatory infiltrate in the ileum. This may suggest that the neratinib-induced injury has an inflammatory basis, and could be targeted with anti-inflammatory agents. In this study, we did not see any statistically significant increase in histopathological damage in the colon of male rats, only females. However, in another study where we showed the effects of neratinib in a male rat model [17], there were significant levels of damage in the colon. This may have been due to the use of a carboxymethylcellulose (CMC) buffer rather than a HPMC buffer. CMC has been shown to have negative effects on the colon in human studies [23].

In this study, we examined differences in the cecal microbiome of male rats treated with either vehicle control or neratinib. Examination of alpha diversity using Shannon's index showed a significant increase in diversity in rats treated with neratinib. This was an unexpected result, as a previous study in lapatinib showed decreases in alpha diversity as a result of treatment [24]. In addition, decreases in diversity have also been seen in chemotherapy studies [25]. The changes seen in chemotherapy may be attributed to the severe histological injury seen in these models, which was not seen in this neratinib model. In addition, the use of the cecal microbiome rather than the fecal microbiome may reflect these changes. Another potential hypothesis is that the large decrease in the *Blautia* genus in neratinib treated animals allowed a niche for a variety of other bacteria to multiply in. Despite the change in the Shannon's index, no significant differences between the groups were seen when using the Simpson and Chao1 index. This reflects a clinical study of the VEGF TKI sunitinib, where there was no significant difference in any diversity measure between patients with diarrhea and patients who did not develop diarrhea [26]. The genus *Blautia* has previously been implicated in another side-effect of cancer treatment, graft versus host disease (GvHD). In that study, higher levels of intestinal *Blautia* led to reduced levels of GvHD [27]. In addition, pilot work has shown that in patients having chemotherapy, higher levels of *Blautia* are related to less severe diarrhea [28]. These results may form a hypothesis that changes in *Blautia* abundance during cancer treatment are a key component of diarrhea development. Further research would be required to ascertain if the changes in *Ruminococcaceae* and *Oscillaspira* are due to the diarrhea or are direct drivers of the diarrhea. In addition, this study did not examine the female microbiome, and therefore it is unknown if differences in the microbiome between male and female rats may explain the more severe toxicity seen in females. Additionally, as this study was not a tumor-bearing model, we have been unable to determine if the microbiome, or the diarrheal effect of neratinib, would be altered by the presence of a tumor. Future work could employ shotgun metagenomic sequencing or functional tests of the microbial metabolome to gain a further understanding of neratinib-induced changes to microbiome function. In any case, these results suggest that gut microbial changes may be a beneficial avenue of research in determining how neratinib-induced diarrhea is exacerbated.

Conclusion

Severe neratinib-induced diarrhea occurs in around 40% of patients and therefore an effective solution is greatly needed. This study has successfully developed a rat model for neratinib-induced diarrhea, which can be used to test potential interventions for this diarrhea. The model was reproducible and manageable in both female and male rats, with females suffering from more severe diarrhea and colonic histopathological damage. The diarrhea seen was multi-factorial and focused on the distal small intestine, with elements of anatomical disruption and inflammation in the ileum. The vast microbial changes seen in rats treated with neratinib suggests that future research should interrogate manipulating the microbiome as a strategy to reduce diarrhea levels.

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Compliance with ethical standards

Conflict of interest Kate Secombe, Imogen Ball, Joseph Shirren and Anthony Wignall declare that they have no conflicts of interest. Joanne Bowen has received research funding from Puma Biotechnology, AstraZeneca, Helsinn and Pfizer. Dorothy Keefe is a consultant for and owns stock in Entrinsic Health Solutions, has received consulting fees from Zealand Pharma and is on the speakers' bureau of Mundipharma.

Ethics approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.

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