General introduction and outline of the thesis
Cancer therapy

Cancer is one of the most concerning diseases of our time and it affects a large part of the population. Although the global prevalence of cancer remains high, the 5-year survival rate of patients continues to increase due to the improved efficacy of contemporary anticancer therapies. While these advances are certainly underpinned by the development of newer agents (e.g., immunotherapy), traditional cytotoxic treatments (e.g., chemotherapy/radiotherapy) continue to form the basis of the vast majority of treatment strategies, underscoring their continued relevance in effective cancer control. Cytotoxic agents act by interfering with DNA synthesis and chromosome division thereby causing cell death of rapidly dividing malignant cells. However, due to the non-selective nature of cytotoxic therapies, other rapidly dividing cell populations are at risk of being targeted resulting in a range of side effects. In the setting of radiotherapy, these off-target effects are controlled to a degree, through precise administration that avoids highly sensitive tissues. However, for systemically administered chemotherapeutic drugs, it remains a significant challenge to control side effects.

A number of cell populations throughout the body are at risk of cytotoxic injury by chemotherapeutic drugs, most of which are mucosal in their origins. The mucosal lining of the gastrointestinal (GI) tract is particularly susceptible due to its extremely high net turnover rate. This acute cytotoxic injury to the GI mucosa activates a number of inflammatory cascades that culminate in the disruption of the GI microenvironment and consequently in impaired intestinal barrier function, clinically referred to as GI mucositis (GI-M). Despite the high prevalence, GI-M remains without effective intervention, with current clinical guidelines recommending only symptomatic management using largely in-effective anti-diarrheal medications. This highlights the necessity to identify targetable mechanisms in its pathobiology to develop new strategies to control its development and clinical impact.

Chemotherapy-induced gastrointestinal mucositis

The 5-phase model of gastrointestinal mucositis

GI-M was initially thought to be a consequence of DNA damage caused by consecutive cycles of radiotherapy and chemotherapy. It is now accepted that GI-M is initiated by this damage, but ultimately dictated by subsequent inflammatory events resulting in indirect tissue injury and ulceration. The 5-phase model of GI-M was initially described by Sonis (2004) and it encompasses a complex sequence of biological events that occur in five interdependent stages. However, during the past years, the pathobiology of GI-M has been updated and new mechanisms of injury have been suggested.
Initiation phase. The penetration of chemotherapeutic agents from the submucosal blood supply induces direct DNA damage to the basal-epithelial cells, causing cellular stress and apoptosis. Consequently, the injured cells activate a variety of stress mechanisms which leads to the generation of reactive oxygen species (ROS) as well as the release of many pro-inflammatory cytokines.

Primary damage response. The ROS previously produced lead to an intense inflammatory response in the submucosa that eventually results in the death of basal epithelial cells. Several transduction pathways are activated including protein 53 (p53) and nuclear factor-kappa B (NF-κB). As result, pro-inflammatory cytokines such as tumour necrosis factor (TNF) and Interleukins (IL)-1β and IL-6 are produced. These pro-inflammatory cytokines eventually cause cell death of epithelial cells and injury compromising the mucosa.

Signal amplification. Upon NF-κB activation, a positive-feedback system is activated resulting in the production of more pro-inflammatory cytokines, such as TNF and IL-1β. TNF is also an activator of adhesion molecules and cyclooxygenase-2, leading to enhanced injury and apoptosis of the epithelial lining. This feedback system amplifies the primary damage initiated by chemotherapy.

Ulceration phase. This phase is the most severe phase of mucositis pathobiology where symptoms and secondary complications arise, including fever, rectal bleeding and malnutrition. The mucosal integrity is lost, causing painful ulcerations that are prone to bacterial and fungal colonization. Bacteria colonize the ulcers and penetrate into the submucosa, enhancing infiltration of macrophages to produce more pro-inflammatory cytokines. Ultimately, bacterial translocation leads to bacteraemia and sepsis specially in neutropenic patients.

Healing phase. This phase occurs through the renewal of proliferation and differentiation of the epithelium, as well as the re-population of local gut microbiota. This last phase is a self-healing condition characterized by signals from the submucosa that influence the rate of epithelial cell migration and proliferation.

Breakdown of the intestinal barrier (intestinal barrier dysfunction) is a critical event in the pathobiology of GI-M, reflecting the important homeostatic functions of the intestinal epithelium. The intestinal barrier forms a crucial barrier between the lumen and the underlying tissue, facilitating the exchange of nutrients whilst preventing the entrance of toxins and antigens trafficking into the body. Additionally, the intestinal epithelium also serves as a physical barrier to microbes onto which a mucosal-associated microbial community is formed and will prevent translocation of bacteria to other sites of the body. The intestinal barrier is in part maintained through the action of tight junctions, multi-protein complexes that maintain intravascular volume and regulate the flux of fluids and solutes between vessels and organ parenchyma. During GI-M, morphological
changes, including flattening of the villi and apoptosis are associated with the internalization and downregulation of tight junctions (e.g., Claudin-1), resulting in increased intestinal permeability 17–19. Such alterations were elegantly described in both pre-clinical and clinical studies by Keefe et al. (2000) and Wardill et al. (2014) that reported a significant increase in the amount of open tight junctions in intestinal crypts of the small intestine 18,19. As tight junctions seal the paracellular space between epithelial cells, their damage results in an increased permeability, which may compromise transport over the epithelium and thus the absorption of nutrients and drugs 20.

**Clinical presentation and impact of gastrointestinal mucositis**

Clinically, GI-M results in ulcerative lesions and significant atrophy throughout the GI mucosa. These changes in GI architecture lead to a reduced absorptive capacity of the gut, resulting in diarrhea and malnutrition 5,21. GI-M has been linked to symptoms such as nausea, vomiting, bleeding and pain, and its development depends on the type of cancer drug administered (e.g., 5-Fluouracil and methotrexate present a higher risk of inducing mucositis), the type of therapy (radiation, chemotherapy, or combined chemo-radiotherapy), dosage, and delivery schedule 7,11,22. Moreover, patient-specific risk factors such as age, ethnicity and gender have also been shown to influence the disease’s progression 9. GI-M is also associated with psychological problems, particularly a depressive disposition, which results in social isolation, and a reduced well-being and quality of life 4,23. Although the incidence depends on the type of therapy and its dose, it has been estimated that 40% of patients receiving standard dose chemotherapy and up to 100% of patients undergoing high dose chemotherapy or haemopoietic stem cell transplantation (HSCT) might experience GI-M 7,24,25.

Patients with GI-M also suffer from an increased risk of secondary complications such as bacteremia, fungaemia, and sepsis, resulting from translocation of enteric pathogens across an impaired barrier into systemic circulation 5,26. Furthermore, patients also require additional supportive care measures such as parenteral nutrition, oral opioids, antibiotics/fungal and hospitalizations 7,10. Ultimately, these impediments commonly instigate the need for chemotherapeutic dose reductions, consequently reducing the overall efficacy of the anticancer treatment, and prolonging the duration of costly hospital stays 10. These additional supportive care measures, particularly the higher prevalence of hospitalization, have large economical costs associated, with Nonzee et al. (2008) reporting incremental costs of more than 20,000 USD for severe forms of GI-M 27. In addition to the economic burden, GI-M can also become life threatening, as in some cases the severity of GI-M symptoms require the complete treatment cessation 4,9. Despite GI-M being a major oncological problem, chemotherapy/radiotherapy remain the gold standard in many oncology cases and no universal preventative treatments have been accepted in the onset of GI-M 28,29.
General introduction and outline of the thesis

Contribution of the gut microbiome to health

One of the factors involved in GI-M is the gut microbiota. The gut microbiota is defined as the collection of bacteria and other microorganisms present in and around tissues from the mouth through the anus and is made up of almost one hundred trillion ($10^{14}$) microorganisms. The gut microbiome not only comprises the microorganisms but also includes genes, gene functions and the entire ecosystem (“biome”) in which the microbes reside. The highest number of bacteria can be found in the colon, where more than 400 bacterial species reside. In the adult gut, four numerically important bacterial phyla have been described: Bacteriodetes, Firmicutes, Actinobacteria and Proteobacteria. While Gram-negative Bacteriodetes are represented by the anaerobic genera Bacteroides, Alistipes, Porphyromonas and Prevotella, the Gram-positive Firmicutes are represented by the anaerobic genera Clostridium, Blautia, Faecalibacterium, Eubacterium, Roseburia, Ruminococcus, and the aero-tolerant genera Streptococcus and Lactobacillus. Gram-positive Actinobacteria are represented by the anaerobic genera Bifidobacteria, Atopobium and Collinsella. In turn, the Gram-negative Proteobacterial genera are represented by Enterobacteriaceae, which includes the facultative anaerobic genera Escherichia, Enterobacter and Klebsiella. Although each individual’s gut microbiome is unique, Bacteroides and Firmicutes phyla comprise over 90% of the microbiome and have pivotal functions, such as metabolism, vitamin synthesis, colonization resistance and induction of immune tolerance. In the colon, the anaerobic fermentation of metabolites by bacteria results mainly in the production of short chain fatty acids (SCFAs), including butyrate, acetate and propionate. These compounds act by maintaining intestinal homeostasis via inducing an anti-inflammatory response and by affecting host cell processes such as proliferation, differentiation and gene expression.

The immune system is strongly influenced by the GI microenvironment and vice versa. This bidirectional relationship has been demonstrated in germ-free (GF) models that show abnormal number of several immune cell types and immune cell products, as well as deficits in local and systemic lymphoid structures. The mucosal immune system has the complex role of maintaining tolerance towards harmless bacteria while controlling the gut microbiota to prevent its overgrowth of pathobionts and their translocation to systemic sites. It is therefore not surprising that perturbations between the gut microbiota and the immune system may lead to the development of gastrointestinal diseases, including inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and GI-M.

Microbial dysbiosis as a hallmark of gastrointestinal mucositis

In the most recent update on the pathobiological framework of mucositis, the contribution of the gut microbiota was highlighted as a potentially overlooked aspect in the pathobiology of GI-M. This is reflected in a growing body of work demonstrating that the microbiota composition is
severely disrupted after chemotherapy, and appears to align with key events in its pathobiology. In fact, multiple clinical studies have demonstrated that chemotherapy induces a profound adverse impact on the diversity and composition of the gut microbiota. Specifically, decreases in faecal *Lactobacillus* spp., *Bifidobacterium* spp. and *Bacteroides* spp. were observed following treatment with 5-fluouracil and irinotecan. Paralleled increases in *Escherichia* spp., *Clostridium* spp., *Enterococcus* spp. *Serratia* spp. and *Staphylococcus* spp. were also identified. Furthermore, since the GI tract harbours the largest number of immune cells within the human body, gut dysbiosis and the resultant inflammation leads to the activation of the mucosal immune system. Consequently, a positive feedback loop is generated whereby gut dysbiosis and the ensuing inflammation intensifies the expansion of pathobionts and induces a worsened disease state.

Evidence for the contribution of the gut microbiota to GI-M development was previously demonstrated in GF mice. In fact, Brandi et al. (2006) and Pedroso et al. (2015) showed less intestinal damage caused by irinotecan administration in GF mice compared to conventional mice. Similar results were observed in humans, with clinical studies reporting a reduction in the number of anaerobic bacteria (e.g., *Bacteroides*, *Clostridium cluster XIVa* (Lachnospiraceae), *Faecalibacterium prausnitzii* and *Bifidobacterium*) and streptococci, and an increase of enterococci.

Whilst an exact causal relationship between gut dysbiosis and GI-M pathobiology has not been yet shown, evidence points to gut dysbiosis as a pivotal driver of GI-M. Anecdotal evidence has shown that commensal bacteria and their secreted products play a crucial role in gut homeostasis. As such, modulation of the gut microbiota may present a promising approach to improve GI-M symptomology. Occurring at the colonic host-microbiome interface, these beneficial effects were shown to include the modulation of oxidative stress, regulation of intestinal permeability, and the development of immune tolerance.

**Modelling host-microbe interactions to dissect mechanisms of gastrointestinal mucositis**

Given the logistical and ethical obstacles in collecting biospecimens from people with cancer where colonoscopy is often contraindicated, *in vitro* and *in vivo* models remain heavily important for continued research in supportive care for oncology. Studying host-microbe interactions initially relied on cell models, including the intestinal epithelial cell lines Caco-2, HT29 and T84. Although these models could provide useful information on the role of bacteria in processes such as cell proliferation and metabolism, they lacked the complexity usually observed *in vivo*. In order to overcome this limitation, animal models became a reliable alternative as they represented more homology with the clinical setting. As such, systems such as GF and antibiotic-depleted mice became routinely used to dissect causative roles due to the facilitated manipulation of the gut microbiota. However, next to ethical concerns, certain limitations are associated with these
models, including their cost, maintenance, immunological deficiencies and the inability to control the exact composition and number of organisms in the gut 48. Alternatively, *ex vivo* models have been successfully implemented in this setting as they present as a reliable alternative to animal experimentation 52. For example, intestinal organoid systems present as a powerful tool to study disease processes due to the stem cell’s ability of self-renewal and to differentiate into specialized intestinal cell types 54,55. However, despite these clear advantages, few intestinal organoid models have been developed to study chemotherapy-induced mucosal injury. Despite the challenge associated with their development and maintenance, these innovative systems can provide crucial information on host–microbe interactions. Unravelling such interactions will help dissecting causality and underlying mechanisms, allowing the development of novel approaches to prevent GI-M.

**Modulating the composition of the gut microbiota**

With the increased evidence on the ability of the gut microbiota to modulate not only GI-M development but also the host response to chemotherapeutic drugs, new approaches that enable the manipulation of the composition of the gut microbiota have been investigated 56. The ability to easily modify the microbiome together with the new tools to study host-microbe interactions provide an exciting opportunity to intervene in GI-M development and progression.

**Prebiotics**

Prebiotic compounds stimulate growth, activate metabolism and promote protection of commensal bacteria in the host 57,58. They are defined as: a substrate that is selectively utilized by host microorganisms conferring a health benefit 59. Moreover, prebiotics can stimulate the production of SCFAs and lactic acid by beneficial gut bacteria such as bifidobacteria, lactobacilli and faecalibacteria. Ultimately, these bacteria contribute to a panoply of beneficial events including reinforcement of the intestinal barrier, growth of intestinal epithelium and restriction of expansion of enteric pathobionts (pathogens present in the gut that are non-pathogenic when kept to a low abundance) 60. Although a wide range of compounds have the potential to meet the requirements for definition as a prebiotic, not all have been studied sufficiently to be classified as such. To date, the most extensively studied prebiotics in the setting of intestinal inflammatory disorders include the fructose polysaccharide inulin, fructo-oligosaccharide (FOS), mannan-oligosaccharides (MOS) and galacto-oligosaccharide (GOS), as they are able to increase the amount of bifidobacteria, *Roseburia*, *Oscillospiraceae* and *Eubacterium* 61,62. In the recent update of the Multinational Association of Supportive Care in Cancer practice guidelines (MASCC/ISOO), the use of prebiotics in studies for prevention of GI-M shows conflicting results regarding effectiveness 10. In fact, while Yazbeck et al. (2019) reported no beneficial effects of FOS, MOS and GOS in rats receiving 5-FU, Garcia-Perez
et al. (2012) showed higher numbers of lactobacilli and bifidobacteria in patients undergoing radiotherapy after inulin and fructo-oligosaccharide treatment. Hence, a better understanding of the potential of prebiotics in the setting of GI-M is required.

**Probiotics**

Probiotics are generally preparations that contain sufficient numbers of viable bacteria that are able to exert beneficial effects. Commercialized probiotic formulations containing *Lactobacillus casei* such as VSL#3 and Dixentil have shown prophylactic efficacy in the reduction of diarrhoea following chemotherapy and radiotherapy treatments. Currently, only the use of *Lactobacillus*-containing probiotics for the prevention of chemotherapy and radiotherapy-induced diarrhoea in patients with pelvic malignancies has been recommended by MASCC/ISOO. Nevertheless, a considerable effort has been made to investigate potential bacterial targets to reduce GI-M. Wada et al. (2010) showed in a clinical study that administration of *Bifidobacterium breve* strain Yakult in cancer patients undergoing chemotherapy results in a decrease of mucositis incidence and severity of different adverse effects such as fever and diarrhoea. Sharma et al. (2016) reported that administration of *Lactobacillus brevis* in a cohort of patients undergoing chemotherapy for head and neck cancer reduced the incidence of grade III and IV anticancer therapy-induced oral mucositis. However, a more recent study by De Sanctis et al. (2019) showed that the same probiotic had no effect on a cohort of patients undergoing radiotherapy. Whilst promising in the oncological setting, there remains no consensus on recommendation for probiotics in the prevention of GI-M. These negative recommendations are supported by a comprehensive systematic review and meta-analysis by Wardill et al. (2018) that reported no beneficial effects of probiotics for the prevention of cancer-therapy induced diarrhoea. The heterogeneity in the impact of probiotics highlights the differences in approaches used by various studies, with no consistency in the strains used or microbial load administered. Further to this, there has been an overwhelming reliance on commercial probiotic strains which lack a biological rationale for their use in GI-M (e.g., no data to show that these strains are functionally-important in GI-M). As such, future attempts to develop probiotic interventions for GI-M must base their approaches on i) robust clinical data/phenomena, ii) prediction of a functional role for the probiotic (e.g., metabolite production or anti-inflammatory) and (iii) strong *in vitro* characterisation that supports investigation of key microbial strains.

**Vitamins and trace elements**

New research has focussed the attention to the role of vitamins and trace elements, including zinc and selenium in the management of oral and GI mucositis. Increasing evidence has demonstrated the antioxidant and immunomodulatory properties of vitamins C and B2. More importantly, both vitamins were shown to impact the gut microbiota, particularly under oxidative stress conditions. Vitamin C has been shown to attenuate 5-FU-induced GI-M by inhibiting the activation of the NF-κB pathway and by reducing lipid peroxidation and myeloperoxidase. Vitamin
B2, or riboflavin, has been mostly characterized for its impact on the microbiota. While not directly assessed in the setting of mucositis, *Faecalibacterium prausnitzii* – a key butyrate producer – is particularly dependent on riboflavin as a redox mediator for extracellular electron transfer. This therefore demonstrates the potential of riboflavin in protecting microbiota and thus mucositis prevention. The trace-element selenium has been shown to prevent 5-FU-induced mucositis in vivo with Lee et al. (2011) reporting reduced expression of IL-1β and TNF upon prophylactic treatment with selenium. This effect was accompanied by partial rescue of morphological structure in the small intestine indicating a mucosal protective effect. Although studies suggest the protective role of these compounds in the setting of GI-M, no current guidelines have been proposed due to conflicting results in both preclinical and clinical studies. Hence, an investigation on the exact mechanisms underlying such potential beneficial properties is required.

**Faecal microbiota transplantation**

Faecal microbiota transplantation (FMT) involves the administration of a faecal suspension into the digestive tract, with the aim of treating or preventing disease via modulation of the gut microbiota. This approach has been successfully used for recurrent or refractory *Clostridium difficile* infections and its use is included in several practical guidelines. FMT represents a new alternative to probiotics as it allows the collection and storage of autologous faecal samples of patients and/or the delivery of a more diverse microbiome. Currently, two studies have investigated the impact of FMT on GI-M toxicity. In a first study, Le Bastard et al. (2018) reported that autologous FMT delivery for 3 days to mice exposed to both 5-FU and antibiotics restored microbial diversity. Whilst promising, crucial clinical parameters including diarrhoea and body weight were not reported in this study, limiting its relevance. Later, a study by Chang et al. (2020) demonstrated the potential and safety of autologous FMT for suppressing GI-M in colorectal cancer mouse model. In fact, daily FMT administration following FOLFOX (5-fluorouracil, leucovorin, and oxaliplatin) treatment resulted in decreased diarrhoea, bacterial translocation and intestinal mucosal injury. Moreover, the authors also reported reduced intestinal mucosal inflammation and barrier integrity disruption, indicating that FMT may have clinical potential for the management of chemotherapy-induced intestinal dysbiosis and toxicity.

**Aims and outline of the thesis**

Despite anecdotal evidence that supports the role of the microbiome in GI-M development, it remains a challenge to modulate its composition in order to prevent or minimise GI-M. The lack of extensive studies is this field is likely due to the heterogeneity in oncology cohorts, and the clinical variables associated. Furthermore, and contrarily to oral mucositis, it is still difficult to
access the GI tract, with colonoscopy contraindicated and patients often reluctant to discuss GI symptoms. This has led to few well-defined and clinically accepted strategies for the modulation of the gut microbiota following chemotherapy. As such, this thesis aimed at (i) exploring novel microbial interventions to prevent GI-M development and progression, (ii) develop new models of chemotherapy-induced GI-M that are able to rapidly screen new interventions and to dissect host-microbe interactions relevant to its pathobiology and (iii) investigate how disruption of the GI microenvironment, including the microbiome, impacts the absorption of commonly administered anti-microbial drugs.

In Chapter 2, we provided an overview of the key mechanisms by which the microbiome is likely to causally contribute to GI-M development, including the microbial impact on drug metabolism, bile acid synthesis and barrier function. Moreover, we also suggested emerging approaches to model host–microbe interactions with clinical relevance and translational potential in the provision of supportive care. Among several approaches, we discussed how gut-on-a-chip and 3D-organoid systems may provide crucial information to dissect the causation mechanisms and to prevent GI-M.

As vitamins C and B2 have been increasingly recognized by their ability to reduce inflammation and enhance the growth of anaerobic bacteria in the gut, in Chapter 3 we investigated whether these vitamins are able to ameliorate GI-M caused by the commonly administered chemotherapy drug, methotrexate. This was performed using a previously validated animal model of GI-M to evaluate the impact of prophylactic treatment with vitamins C or B2. GI-M severity was assessed by measuring plasma citrulline levels (a clinically relevant biomarker of GI-M) and changes in the composition of the gut microbiota composition investigated through 16S rRNA gene sequencing.

In Chapter 4, we analysed, in collaboration with the University of Adelaide (Australia), the intestinal microbiota of a cohort of people undergoing standard dose chemotherapy and identified the genus Blautia as a predictor of GI-M, with the species *Blautia luti* strongly correlating with toxicity outcomes. We dissected the mechanisms whereby *B. luti* exerts such beneficial effects by: i) assessing its ability to induce differential growth of commensal bacteria under oxidative conditions, and ii) evaluating its mitogenic properties in intestinal epithelial cells treated with 5-FU. Additionally, we investigated the immunomodulatory properties of this bacterium upon concurrent and therapeutic inflammatory stimuli.

To further explore novel microbial interventions in the setting of GI-M, in Chapter 5 we investigated the potential of autologous faecal microbiota transplantation (FMT) in restoring the gut microbiota after administration of the chemotherapeutic agent MTX in rats. FMT was evaluated first as an adjunct to MTX and later as a restorative approach after antibiotic prophylaxis. To explore whether FMT led to augmentation of the gut microbiota, the gut microbiota composition was assessed.
by 16S rRNA gene sequencing and clinical manifestation of mucosal barrier injury evaluated by assessment of plasma citrulline levels, body weight, diarrhoea and food/water intake.

In Chapter 6, we developed and characterized a chemotherapeutic-induced model of mucositis using 3D intestinal organoids. Here, organoids derived from mouse ileum were grown and incubated with different concentrations of MTX. Metabolic activity, citrulline levels and cytokine/chemokine production were measured to determine the optimal dosage and incubation time. After optimization, we further investigated the impact that microbial-derived short-chain fatty acids could have in vivo, by supplementation with butyrate in the organoid model.

Whilst few studies have focused on the impact of GI-M on drug absorption, there are still many aspects that need to be addressed in order to optimize drug delivery in cancer patients. In Chapter 7, we discussed how chemotherapy leads to intestinal damage and consequently to impaired intestinal function. Given the cumulative evidence showing that both GI-M itself and GI-M-related factors such as intestinal luminal pH, intestinal motility and gut microbiota may alter intestinal function, we discussed how these factors contribute to altered drug absorption.

After discussing the possible causative mechanisms responsible for the altered intestinal absorption during GI-M, in Chapter 8 we conducted a prospective study to explore the exposure of three different anti-microbial agents, ciprofloxacin, fluconazole and (val)acyclovir, and the effect of mucositis on the absorption of these drugs in HSCT recipients. Moreover, we investigated potential associations between drug exposure and the gut microbiota.

In Chapter 9, we summarize and discuss the most relevant findings in this thesis, draw conclusions and describe perspectives for future mucositis related research.
Chapter 1

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Chapter 1


