Microbiome-targeted interventions during gastrointestinal mucositis

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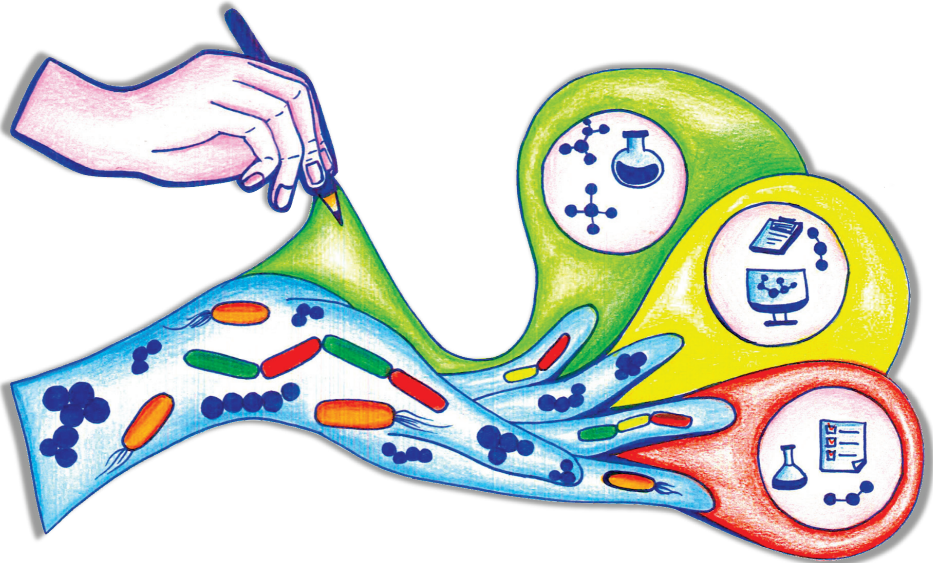
9

General discussion and future perspectives

General discussion and future perspectives

Although a plethora of studies have implicated the gut microbiome in the pathobiology of several gastrointestinal (GI) complaints, the translation of microbial interventions into clinical practice has been poor [1]. This is particularly pertinent in the context of gastrointestinal mucositis (GI-M) where, despite the distinct lack of effective therapeutic approaches and the well-characterised adverse shifts in gut microbial communities following chemotherapy, there has been relatively scarce research into the development of microbial interventions [2]. It is certainly true that efforts have been made to dissect the causative mechanisms of host-microbe interactions with the aim of developing novel interventions that can be translated into the clinical setting [3–5]. However, this work has been hindered by the highly heterogenous landscape of GI-M and the oversimplified experimental approaches. As such, studies in this thesis described key mechanisms involved in host-microbe interactions through in-depth longitudinal analyses in preclinical and clinical samples and investigated the efficacy of several microbial interventions, including vitamins and *Blautia luti*. Furthermore, we explored how alterations in the GI tract, including the composition of the gut microbiome, could affect the absorption of anti-infective drugs during severe stages of GI-M.

Exploring new microbial interventions during gastrointestinal mucositis

In order to explore novel interventions in the setting of GI-M, work performed in this thesis provided an overview of the current literature and dissected key mechanisms by which the gut microbiome is likely contributing to GI-M development (**Chapter 2**). We concluded that the gut microbiota may potentially drive mucosal injury through the modulation of drug metabolism, bile acid synthesis and intestinal barrier function. As such, a mechanistic understanding of such factors is necessary to understand the extent to which the gut microbiota can influence GI-M pathobiology.

As a mechanistic appreciation of the physiological factors contributing to GI-M was investigated, the impact of different microbial interventions on GI-M development was explored. This was achieved by the use of different *in vitro* and *in vivo* models. In **Chapter 3**, we investigated the impact of prophylactic treatment with vitamins C and B2 in a rat model of methotrexate (MTX)-induced mucositis. Although vitamin B2 did not show any beneficial effect during its administration, vitamin C significantly improved clinical outcomes such as body weight and food intake at severe stages of GI-M (day 4). Interestingly, neither of the vitamins were able to modulate the gut microbiota composition, although in vitro results indicated their ability to favourable stimulate the growth of commensal bacteria under oxidative stress conditions. This study is one of only a few studies which have documented the potential of vitamin C as prophylactic approach to attenuate GI-M [6]. Importantly, this vitamin was also shown to reduce the severity of oral mucositis in patients undergoing chemoradiotherapy, confirming the role of vitamin C not as modulator of the gut microbiota but rather as an emerging antioxidant approach for the management of mucositis

symptoms [7].

In line with previous observations pointing out the crucial role of bacteria in GI-M progression, in **Chapter 4** we explored the potential of autologous faecal microbial transplantation (FMT) in the modulation of mucosal barrier injury (MBI) during chemotherapy. FMT is a powerful tool that delivers a diverse and metabolically active microbial community to the host with greater durability compared with commercial probiotics [9,14]. However, due to the damage caused to the intestinal epithelium by chemotherapeutic agents, FMT has been met with caution as it can lead to bacterial translocation [15]. In our rat model, FMT promoted microbial stability during MTX toxicity but it did not impact clinical manifestations, including diarrhoea and anorexia. MTX treatment, in combination with a broad spectrum of antibiotics (ABX) led to impaired mucosal recovery and exacerbated diarrhoea. These symptoms were accompanied by microbial disruption, particularly by the expansion of Proteobacteria. However, this phenotype was partly reverted by adjuvant administration of FMT that mitigated the detrimental effects of ABX and MTX-induced diarrhoea by promoting colonisation of the rodent specific family *Muribaculaceae* (S24-7). Observations in this work are paralleled with recent work by Chang *et al.* (2020) that demonstrated the potential and safety of autologous FMT in attenuating intestinal injury caused by 5- Fluorouracil/Oxaliplatin 16. Hence, this work offers good prospects on FMT as a future microbial intervention to deliver functional microbes (e.g., *Faecalibacterium* and *Blautia*) before chemotherapy treatment to repopulate or shift composition of the host microbiome. Interesting to note, the beneficial impact of FMT on mucosal recovery after disruption of the host microbiome with ABX demonstrates the causal contribution of the gut microbiota to mucosal healing, which answers one of the first research questions of this thesis. Ultimately, FMT may be uniquely positioned to minimise the duration of mucosal injury, decreasing the intensity of symptoms and the opportunity for translocation events.

Although FMT provided microbial stability following ABX and MTX treatment, it was not overly effective in attenuating the clinical symptoms (**Chapter 4**). Additionally, microbial disruption observed after ABX treatment seemed to exacerbate MTX-induced GI-M by impairing mucosal recovery. These results therefore suggest that the baseline microbiome composition may have a higher impact on GI-M outcome than maintaining the gut microbiota composition throughout the disease. This hypothesis is in accordance with recent studies that show the ability of the gut microbiota to modulate the risk of toxic side-effects, including GI-M [9–12]. While an increased body of research has described this phenomenon, none have moved beyond superficial observations to provide a reliable approach to modify the risk of GI-M [9–11]. Hence, identifying microbial patterns that enable prediction of the risk of GI-M would guarantee an optimal delivery of anticancer therapy. Through collaboration with the University of Adelaide (Australia), work in **Chapter 5** provided mechanistic insight on data regarding the potential for the commensal microbe, *Blautia luti*, to regulate GI-M (specially diarrhoea) risk. We showed that *B. luti* was significantly more abundant

9

in patients that did not present clinical signs of GI-M. We demonstrated *in vitro* using a T84 cell model the ability of *B. luti* to attenuate prophylactically and therapeutically mucosal injury caused by chemotherapeutic agent 5-Fluouracil. These beneficial effects are thought to be the result of increased tight junction formation by *B. luti*, which might be explained by the production of acetate [13]. This bacterium also presented anti-inflammatory properties, demonstrating its wide-range of therapeutic applications. Additionally, *B. luti* also presented cross-feeding with other important commensal bacteria, including the beneficial butyrate-producer *Faecalibacterium prausnitzii*. This suggests the ability of this bacterium to modulate the gut microbiota composition, even under the inflammatory oxidative conditions frequently observed during GI-M. Ultimately, this work provides crucial evidence that supports *B. luti* as a risk predictor for GI-M during chemotherapy, but also as a potential microbial intervention to prevent GI-M progression.

Altogether, results from this thesis supports the beneficial role of microbe-targeted interventions during GI-M. In fact, while vitamin C holds promise to reduce GI-M symptomology, *in vitro* and *in vivo* studies performed in **Chapters 4** and **5** indicated that *B. luti* and FMT may support the gut microbiome with greater efficacy. Therefore, targeting the gut microbiota with specific microbes could certainly reduce mucosal barrier injury, ultimately resulting in intestinal homeostasis. Additionally, the identification of *Blautia* as a risk predictor in cancer patients undergoing chemotherapy supports the evidence suggesting that an individual's baseline gut microbiota is a predictor for toxicity outcomes, underscoring the multifunctional role of the gut microbiota in GI-M.

"Mini-gut" organoids: a new era for the study of host-microbe interactions in GI-M
Given the inherent challenges to access the gut microbiota in humans, the study of host-microbe interactions remains difficult, which has led to a reduced number of translatable interventions [1,17]. Animal models were used to study and develop new microbial interventions in this thesis. While informative, they cannot be used in a high-throughput manner, making screening of numerous microbial interventions logistically challenging, time-consuming and resource intensive. Recognising the challenges of this approach, work in this thesis also sought to develop new *in vitro* models that could facilitate the study of such interactions. In **Chapter 6** we developed a model of chemotherapy-induced GI-M using intestinal organoids cultured in a 3D fashion. The robustness of this model was demonstrated by characterizing the metabolic activity, citrulline levels and cytokine/chemokine production at different stages of MTX treatment. An important finding of this study was the ability to reverse MTX cytotoxicity by incubation of intestinal organoids with folinic acid, thus demonstrating its clinical relevance. To further investigate its utility to study host-microbe interactions, we investigated the protective effects of the short-chain fatty acids butyrate, propionate and acetate, which have been previous demonstrated to exert beneficial anti-inflammatory and proliferative properties in numerous studies. We observed prevention of mucosal injury by butyrate and propionate, hypothesised to be mediated by the modulation of the ABC transporter which

promotes MTX efflux [18,19]. This provides the first mechanistic evidence of how microbial metabolites minimise MTX-induced mucosal injury. Despite exhibiting some limitations (e.g., lack of an immune system), to our best knowledge, this is the first MTX-induced mucositis model of intestinal organoids to be developed. Notably, our work displays significant similarities to other organoid models of mucositis, including the oral model of mucositis developed by Driehuis *et al.* (2020), thus showing the reliability of this model to screen microbial interventions [20]. Additionally, the presence of specialized cell types in this intestinal organoid model could also overcome the current limitation faced with the use of single cell-type cultures such as intestinal epithelial cells (e.g., Caco-2 and T84), as they fail to address certain host-microbe interactions that drive mucosal injury.

Drug absorption and gastrointestinal mucositis: a complex relationship
The efficacy of anti-infectives, including antibiotics and antifungal agents, is greatly dependent on optimal intestinal function, in particular its capacity to absorb luminal contents and to metabolise key compounds [21]. However, as hypothesized in **Chapter 7**, alterations in the GI microenvironment caused by chemotherapy may lead to mucosal barrier injury (MBI), resulting in a compromised intestinal barrier functions, thus impairing drug absorption. This is particularly problematic in supportive oncology as many patients rely on prophylactic treatment with anti-infectives to prevent secondary complications such as septicaemia [22,23]. Surprisingly, few studies have focused on the impact of MBI on drug absorption during chemotherapy, with those that are limited by small sample size and contradictory results [24–26]. Given the lack of more in-depth investigations, in **Chapter 8**, a prospective and observational pilot study was performed in 21 hematopoietic stem cell transplantation (HSCT) patients, routinely receiving oral anti-infectives. We opted to use a study design that was the least harmful for this vulnerable patient group, collecting left-over blood samples and faecal swabs from routine clinical care and conducted analysis on these samples. Due to their frequent use, we measured the exposure of fluconazole, ciprofloxacin and valacyclovir as reference anti-infectives. We observed severe GI-M in all patients, confirmed by a reduction of plasma citrulline. However, no statistical correlation was found between plasma citrulline and the anti-infective agents. Furthermore, no significant associations between the gut microbiota and drug concentration were found.

Although it provided insights into the drug exposure of fluconazole and ciprofloxacin, it is important to acknowledge the limitations of this work which may have masked any potential findings. Firstly, the small number of patients included in this pilot study almost certainly resulted in an underpowered study. Another limiting factor is the challenge to investigate the exposure of fluconazole within this timeframe, as it takes up to 10 days to reach steady state. As the sampling was performed mainly before steady state was reached, our results may not completely reflect fluconazole's exposure in this cohort of patients. Regardless the limitations, this pilot study complements existing research on the impact of mucositis on the intestinal absorption of anti-infective agents and, given the potential

9

implications for inaccurate administration of anti-infective drugs, highlights a need to investigate this question in a more robust setting. Importantly, the limitations faced during this study are certainly a lesson that will contribute to the improvement of new clinical studies to explore the role of GI-M on drug absorption.

Clinical implications

Results from this thesis provide unique mechanistic understanding on host-microbe interactions and offer new potential microbial intervention that may influence GI-M pathobiology. We demonstrated the potential of high dose vitamin C as a prophylactic approach to minimise GI-M symptomology and reported the *in vitro* abilities of *B. luti* to attenuate mucosal injury and secondary disruption of the GI microenvironment. Moreover, the potential of vitamin C as a therapeutic approach suggests that other vitamins may have similar effects. Such beneficial effects provide an exciting opportunity to develop symbiotic formulations of probiotics and vitamins that can be used in patients undergoing conditioning regimen with different chemotherapeutic agents. This could be achieved by determining the timeframe in which this formulation could be administrated in patients in order to minimize the duration of mucosal injury and to contribute to stabilization of the gut microbiota. Alternatively, *B. luti* could be combined with other commensal bacteria such as F. prausnitzii as a probiotic formulation. *F. prausnitzii* is a gram-positive and obligate anaerobe and it has been shown to prevent barrier disruption and to attenuate intestinal inflammation [27,28]. In this scenario, FMT could present as a promising approach to deliver these bacteria in the gut, among other commensal microbes that exhibit similar properties. As such, methods could be designed to rapidly select and isolate a wide range of bacteria from faecal samples collected at baseline (e.g., at the time of chemotherapy) in order to deliver a more personalized FMT to patients. Nonetheless, it is necessary to acknowledge that colonization of these bacteria could be a great challenge since the gut is not a favourable environment to the growth of commensal bacteria during GI-M. To overcome such limitation, the use of bacterial secreted products could present as a promising alternative to the use of the bacteria itself.

In this thesis, we also provided evidence supporting an individual's gut microbiota baseline as a risk predictor for chemotherapy-induced diarrhoea. Whilst this idea has been previously recognized in the setting of other intestinal inflammatory disorders, only superficial observations had been done in the setting of GI-M [29,30]. This work therefore supports the clinical applicability of the gut microbiota as a risk predictor for GI-M in patients undergoing chemotherapy. Similar to the results obtained with *B. luti*, the identification of novel microbial patterns associated with predisposition to GI-M would contribute to more personalized anticancer treatments.

Finally, we identified numerous factors (e.g., nature the drug and GI microenvironment) that may

contribute to altered drug absorption. Importantly, work performed in the clinical prospective study demonstrates the challenges in assessing absorption of anti-infectives during GI-M. As such, this work may draw attention to the need to investigate the exposure of other anti-infectives routinely used during chemotherapy regimens. This would ultimately allow the development of new guidelines for the prophylactic treatment with anti-infectives, thus optimizing supportive care in cancer patients.

Conclusions and future directions

The studies carried out in this thesis aimed to better characterize the role that the gut microbiota plays in the development of GI-M pathobiology, and to identify novel microbial interventions to attenuate GI-M symptomology. Through a number of investigations, this thesis dissected the causal relationship between gut microbiota modulation and mucosal healing, therefore supporting the hypothesis that the gut microbiota contributes positively to GI-M pathobiology. Importantly, this thesis provided mechanistic evidence supporting the role of vitamin C, *B. luti* and FMT as potential interventions to modulate the microbiome during chemotherapy-induced GI-M. Final chapters of this thesis also draw attention to the impact that GI-M may have on drug absorption, particularly on anti-infectives, and stresses how alterations in the GI microenvironment can interfere with intestinal barrier function (**Figure 1**).
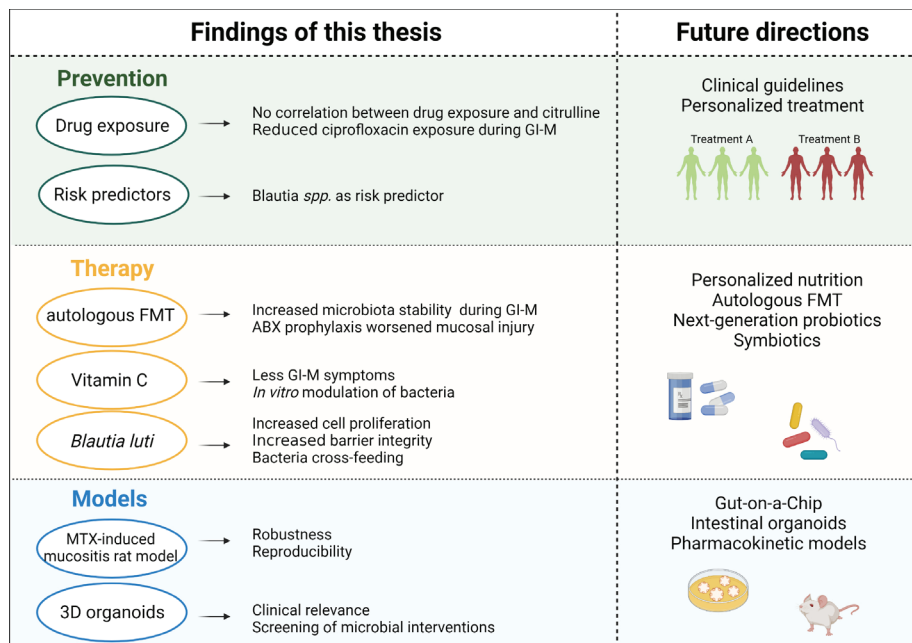
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Figure 1. Finding on GI-M of this thesis and future perspectives. Contribution of the studies included in this thesis to prevention and therapy approaches during GI-M. Pre-established *in vivo* models were used and new *ex vivo* models were used in the different studies. Created with BioRender.com

Although this work suggests a substantial number of clinical applications in the setting of supportive oncology, we believe some findings still warrant further investigation. First, as discussed in **Chapter 2**, we suggest focus on longitudinal studies across heterogeneous oncology cohorts to better characterize the contribution of the gut microbiota to GI-M pathology. Importantly, we need to understand the extent to which multiple factors such as inflammation and intestinal barrier may be compromised by the (lack of) gut microbiota.

Second, although we observed a partial reduction of GI-M symptomology after vitamin C prophylaxis, to ensure translation of our finding and integration into clinical practices, a longer prophylactic regimen with multiple vitamins and/or supplements (e.g., selenium) could be studied (**Chapter 3**). This would provide sufficient time to allow microbial modulation. Additionally, as inflammation plays a pivotal role in GI-M progression, these therapeutic approaches could reduce the inflammation observed during severe stages of GI-M and therefore prevent epithelial damage and consequently bacterial translocation. Similarly, further investigations are required to demonstrate B. luti abilities

displayed in cellular models, which should also include possible adverse effects. (**Chapter 5**). As such, we suggest the use of in vivo models (e.g., MTX-induced mucositis rat model) but also the intestinal organoid model developed in **Chapter 6**. This would determine the exact extent to which this bacterium may provide protection against mucosal injury and to find the optimal timeline of treatment.

Work performed in this thesis also suggested that the timing of microbial intervention is essential to prevent GI-M development. In fact, results obtained in **Chapters 3** and **4** showed that it is challenging to support the microbiome during active GI-M. This clearly demonstrates that the baseline microbiota composition is important and intervening before or after GI-M would be the best approach to minimize the depth and duration of GI-M.

Lastly, following results obtained in **Chapter 8**, we suggest as next step a clinical study for drug absorption and efficacy with a sufficient sample number that would allow more concise results. The inclusion of a more heterogeneous cohort would also allow to determine associations between drug exposure and other patient related factors. We propose the investigation of shorter half-life drugs, and in order to assess absorption of anti-infectives, we recommend that blood samples should be taken 1-2 hours after administration. Also, optimal sampling strategies could be used to calculate area under the concentration-time curve (AUC) that describes the exposure of the drug over time.

These recommendations will certainly contribute to more in-depth investigations into the role of the microbial-targeted interventions discussed in this thesis, thus facilitating their clinical applicability. We believe that the findings of the present thesis could rapidly contribute to the development of new clinical trials (e.g., prophylactic potential of vitamins, autologous FMT). It is therefore hoped that these findings can guide not only researchers but also physicians to develop strategies and guidelines with an ultimate mission: offering the best supportive care to cancer patients.

9

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9