The role of the gut microbiota in human cancer

The role of the gut microbiota in human cancer

The power of an equilibrium

Janine Ziemons

The research presented in this dissertation was conducted at GROW-School for Oncology and Reproduction, Department of Surgery, Maastricht University.

Cover Design: © evelienjagtman.com
Layout: David Ziemons
Printed by: Ridderprint | www.ridderprint.nl
Printing of this thesis was financially supported by the Netherlands Society of Medical Microbiology (NVMM) and the Royal Netherlands Society of Microbiology (KNVM).

ISBN: 978-94-6483-681-3

The role of the gut microbiota in human cancer

The power of an equilibrium

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de
Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. dr. Pamela Habibović
volgens het besluit van het College van Decanen,
In het openbaar te verdedigen
op donderdag 8 februari 2024 om 16.00 uur

door

Janine Ziemons

Promotores
Prof. dr. Maria L. Smidt
Prof. dr. Koen Venema

Co-promotor
Dr. John Penders

Beoordelingscommissie
Prof. dr. Daniel Keszthelyi (voorzitter)
Em. Prof. dr. Frans C.S. Ramaekers
Prof. dr. Matthew R. Redinbo (University of North Carolina)
Dr. Dieuwertje E.G. Kok (Wageningen University & Research)

Het onderzoek beschreven in dit proefschrift werd financieel ondersteund door de Stichting Jules Coenegracht Sr., Danone Nutricia Research en TKI Topsector Agri & Food.

Table of Contents

Chapter 1

Introduction

1. The ecosystem within us

The human body is a habitat for trillions of microorganisms. Most of them reside in the gastrointestinal tract and are collectively called the **gut microbiota** or intestinal microbiota. Although the human gut is also colonized by other microorganisms, such as archaea, viruses, and yeasts, bacteria represent the most extensively studied part of the microbiota. The MetaHIT (Metagenomics of the Human Intestinal Tract) project revealed that 99.1% of the genes identified in human feces were of bacterial origin, while the remainder was mostly archaeal, with only 0.1% of eukaryotic and viral origins (1).

The gut microbiota is a diverse and complex ecosystem, which oscillates around a **dynamic equilibrium** under normal circumstances (2). Previous studies showed that longitudinal samples from the same adult individual are normally more similar to each other than samples obtained from distinct individuals, suggesting that the gut microbiota is a relatively stable and highly personalized community (3, 4). This equilibrium is the result of a fine-tuned set of interactions among gut bacteria themselves as well as with the human host and the environment.

The interaction between the gut microbiota and the **environment** already starts early in human life. Previous research indicated that birth mode and breastfeeding have a major role in shaping an individual's gut microbiota profile (5). Afterwards, the nutrient composition of the diet has been shown to have strong modulating effects on bacterial abundances as well as the production of relevant metabolites (6). Besides this, increasing evidence supports an influence of dietary and non-dietary xenobiotics on gut microbes. In a recent review, Lindell *et al*. elaborated on the effects of different groups of xenobiotics on gut microbiota composition and concluded that not only antibiotics but also several human-targeted drugs (e.g. metformin, proton-pump-inhibitors, laxatives, antipsychotics) can alter gut microbiota composition in a drug-dependent manner (7). Also, natural food components (e.g. phytochemicals, curcumin, tea polyphenols), food additives (e.g. artificial sweeteners and emulsifiers), pesticides (e.g. glyphosate), and other environmental pollutants showed interactions with gut bacteria (7). Other extrinsic factors which are known to influence the gut microbiota are the living environment, as well as travelling to foreign countries (8, 9).

In addition, **host** intrinsic factors such as genetics, age, and sex might influence gut microbiota composition, although their contribution is considered to be outweighed by environmental factors (6). In reverse, the gut microbiota has been shown to influence various metabolic and immunologic processes of the host, for instance, energy harvest, metabolism of food constituents, insulin sensitivity, gut barrier function, and inflammation (10-12). Hereby, gut microbiota-derived metabolites serve as linking molecules between the gut bacteria and the human host. In particular short-chain fatty acids (SCFA), main end-products of carbohydrate fermentation, have been shown to fulfill crucial physiological roles (11). Amongst others, the SCFA butyrate is important for intestinal homeostasis by acting as an energy source for colonocytes (13). Furthermore, SCFA directly interact with the immune system, fortify the intestinal barrier, and exert beneficial metabolic as well as anti-carcinogenic effects (10, 13).

As in each dynamic ecosystem, gut bacteria are not only residing next to each other but also show diverse **interactions among different species**. These interactions can be mutualistic or commensalistic, with a benefit for all species involved, but could also be competitive or promote colonization resistance against (opportunistic) pathogens (2). In general, we can assume that every modulation, intervention, or disturbance of the gut microbiota will affect several bacterial species, either directly or indirectly via microbe-microbe interactions.

If this fine-tuned microbial equilibrium is disturbed by perturbations, for instance, a change in dietary habits or the administration of antibiotics or other xenobiotics, the **resilience** of the gut microbiota determines the extent of consequences this might have. Resilience refers to the ability to restore the equilibrium after a perturbation and describes the amount of stress that a system can tolerate before it shifts towards a new equilibrium that might have different functional capacities (14).
If resilience is low or the disturbing trigger too strong or persistent, the gut microbiota can shift from its homeostatic state to a dysregulated state which is called **dysbiosis**. This dysbiotic state can be transient, after which the gut microbiota recovers to its original equilibrium. However, perturbations can also lead to the manifestation of an alternative (dysbiotic) stable state. Consequently, crucial microbial functions and interactions might be lost or altered, and the microbiota-host balance can be disturbed.
Microbial dysbiosis is often characterized by a loss of **microbial diversity** and increased inflammation and has been found to be associated with several diseases, for instance, inflammatory bowel disease (15), type 2 diabetes (16), or multiple sclerosis (17). On the other hand, high microbial diversity is generally considered to have a positive impact on the resilience of the gut microbiota, since it results in an increased level of **functional redundancy** (the ability of a number of different taxa within a community to perform the same function) (2). Consequently, if diversity is high, there are more species that can compensate for the disappearance of another species during perturbations, preventing the loss of crucial microbial functions.

2. The gut microbiota in human cancer

Human cancer is a condition that represents a major challenge for gut microbiota resilience and is considered to significantly impact the microbial equilibrium. Research has shown that the gut microbiota plays a role in different aspects of human cancer (Figure 1).

First of all, some gut bacteria have been shown to promote **carcinogenesis** either directly or via interaction with oncogenic factors and/or the immune system (18, 19). One of the best-described examples in this field is the association between *Helicobacter pylori* and gastric cancer (20). Similarly, *Fusobacterium nucleatum* is considered to be involved in tumorigenesis of intestinal cancers (21).
In addition, several research groups described cancer-associated gut microbiota profiles (e.g. (22, 23)), which might be useful to predict the risk to develop cancer but will first require further validation. A meta-analysis of eight studies with shotgun metagenomic sequencing of samples derived from patients with colorectal cancer (CRC) identified a core set of 29 taxa that were

significantly enriched in CRC. Amongst others, *Fusobacterium*, *Porphyromonas*, *Parvimonas*, *Peptostreptococcus, Gemella*, *Prevotella*, *Solobacterium,* and genera from the *Clostridiales* order seemed to be positively associated with CRC (24). Also in non-gastrointestinal cancers, the gut microbiota has been shown to play a role. For instance, an altered gut microbiota (compared to healthy controls) was described for postmenopausal breast cancer patients (25). These studies suggest that the homeostatic state of the gut microbiota might already be altered at the time of cancer diagnosis. However, what remains to be investigated is the causality: *Does microbial dysbiosis causes tumor growth? Or is microbial dysbiosis just a reflection of an unhealthy lifestyle which causes the cancer to develop? Or does microbial dysbiosis evolve as a consequence of the tumor? And what is the role of the immune system in this complex interplay?* Amongst others, these questions will be an important framework for the next years of research in this field.

Moreover, during the course of cancer treatment, the gut microbiota might affect **anti-cancer therapy** via different mechanisms. The most commonly applied treatments for human cancer include chemotherapy, hormonal therapy, immunotherapy, radiation therapy or surgery. Although the gut microbiota might interact with all of them, the current thesis focuses on the interactions between the gut microbiota and **chemotherapy**.
For decades, there has been a lack of attention for the potential role of the gut microbiota during chemotherapy, and standardized dosing regimens were applied to patients. Considering the complex interplay between the gut microbiota and the human host, as described above, it is not surprising that an increasing body of evidence describes gut microbiota-induced modulation of chemotherapy efficacy and toxicity (26, 27).
With the development of the new research field of **pharmacomicrobiomics**, our understanding of gut microbiota–drug interactions is expanding and the gut microbiota is considered to be an important player in personalized medicine (28). Important evidence in this field comes from two large-scale drug screening studies, mapping the effects of drugs on microbes and vice versa (29, 30). Maier *et al.* showed that several human-targeted drugs (including e.g. the chemotherapeutics 5-Fluorouracil (5-FU) and doxorubicin) affected the growth of different bacterial strains (29). Likewise, Zimmermann *et al*. demonstrated that a large set of drugs (including e.g. the chemotherapeutics capecitabine, cyclophosphamide, and paclitaxel) can be metabolized by gut bacteria (30).
In addition, some smaller pre-clinical studies provided evidence for a complex interplay between the gut microbiota and chemotherapeutic agents. For instance, Daillère *et al.* showed that bacterial species belonging to the genera *Enterococcus* and *Barnesiella* facilitated the anti-cancer efficiency of cyclophosphamide, through modulation of the immune system in mice (31). Similarly, it was suggested that *Lactiplantibacillus* (previously *Lactobacillus) plantarum*-derived metabolites might sensitize CRC cells to the anti-cancer effects of 5-FU and butyrate (32, 33). On the other hand, *F. nucleatum* has been shown to be not only involved in CRC carcinogenesis, but also in the development and manifestation of chemotherapy resistance towards 5-FU (21, 34, 35).
An interesting direct interaction between tumor-derived bacterial species in the context of chemotherapy has been recently described by LaCourse *et al.* (36). This study showed that 5-FU inhibited the growth of *F.nucleatum,* suggesting that the anti-cancer effect of 5-FU could also be partly attributed to the inhibition of this oncogenic species. However, different *Escherichia coli* isolates were able to decrease 5-FU concentrations, thereby reducing its toxicity towards *F.nucleatum* and colon cancer cells. These results indicate not only that *E.coli*

is capable of metabolizing 5-FU, but also that the co-occurrence of *F.nucleatum* and *E.coli* in tumor tissue might potentially protect the tumor against 5-FU, with a potential negative impact on its efficacy and the patient's prognosis.
In line with this, a recent study showed that mechanisms of 5-FU metabolism are also present in gut bacteria. In the human host, 5-FU is catabolized by the enzyme dihydropyrimidine dehydrogenase (DPD) to form the inactive metabolite DHFU (37). In *E.coli*, a bacterial DPD is encoded within the preTA operon (38). Spanogiannopoulos *et al.* evaluated the role of this operon in different experiments and found that the bacterial preTA operon was necessary and sufficient for *E.coli*-induced inactivation of 5-FU and that presence of this operon interferes with the efficiency of 5-FU treatment in mice (39). More specifically, the anti-cancer effect of 5-FU was stronger in the absence of preTA-associated inactivation of 5-FU. Next to *E.coli,* preTA was also found in strains from other bacterial genera, for instance, *Salmonella*, *Citrobacter*, *Anaerostipes,* and *Limosilactobacillus* (previously *Lactobacillus*) (39).

This study confirmed a theory that is not unexpected but has been neglected for a long time: pathways and enzymes involved in drug metabolism are conserved among bacteria and humans. Consequently, bacteria are able to metabolize human-targeted drugs. It might be speculated that the preTA operon is only the tip of the iceberg and many more metabolic (degradation) pathways remain to be investigated, but it highlights the necessity to look beyond the human genome in the context of drug metabolism. Furthermore, it remains to be investigated whether chemotherapy itself leads to the manifestation of a resilient dysbiotic state, favoring the overgrowth of potentially pathogenic bacteria, such as *F.nucleatum* and *E.coli*. Given the results described above, the consideration that this would induce a vicious cycle of microbial dysbiosis and resistance towards 5-FU treatment seems evident.

Besides this, chemotherapy-induced microbial dysbiosis could also lead to increased intestinal inflammation, which could enhance symptoms of gastrointestinal toxicity, such as mucositis, nausea or diarrhea. For instance, Sougiannis *et al.* showed that 5-FU administration affected gut microbiota composition as well as the colonic immune profile in mice (40). Particularly a bloom of bacteria belonging to the family of Enterobacteriaceae, also encompassing *E.coli*, has been repeatedly linked to increased inflammation (41).

Interestingly, Enterobacteriaceae have also been shown to play a role in another aspect of human cancer: **cancer cachexia**. According to the international consensus definition, cancer cachexia refers to a *'multifactorial syndrome characterized by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment'* (42). Next to an abnormal muscle and energy metabolism, systemic inflammation is a further hallmark of cancer cachexia (43). Previous studies repeatedly described an expansion of bacteria belonging to the Enterobacteriaceae family in cachectic mice (44-46). Pötgens *et al*. suggested that a reduction of SCFA-producing bacteria in cachectic cancer patients might contribute to the overgrowth of Enterobacteriaceae (47), as high concentrations of SCFAs might act inhibitory to members of the Enterobacteriaceae. Also in humans, differences in gut microbiota composition were observed between cachectic and non-cachectic patients (48).

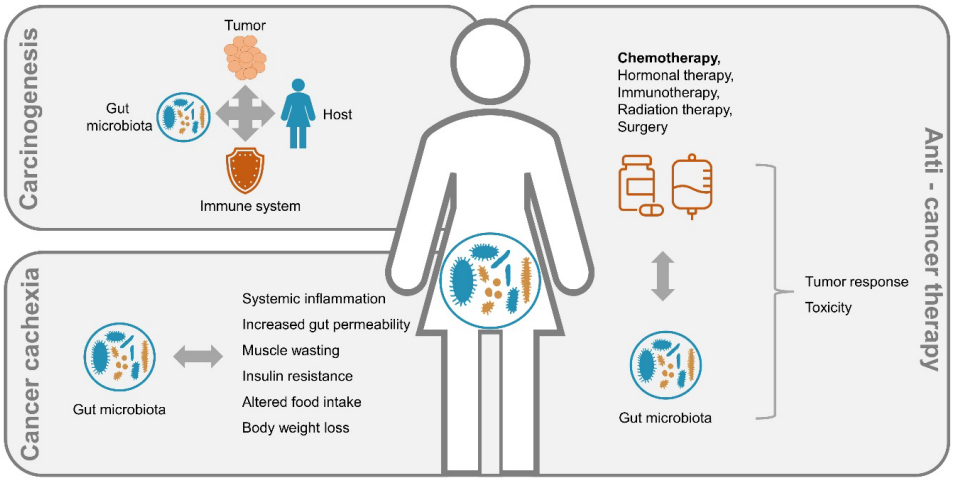


Figure 1: *Potential role of the gut microbiota in three different aspects of human cancer. The current thesis focusses on interactions between the gut microbiota and chemotherapy as well as cancer cachexia.*

3. Modulation of the gut microbiota

Altogether, the studies described above provide a rationale for the hypothesis that microbial activity plays a crucial role in human cancer. Based on this, targeted modulation of the gut microbiota could likely contribute to the optimization of chemotherapy efficacy, more specifically to the improvement of tumor response and reduction of toxicity. Assuming that the different aspects of human cancer (e.g. presence of a tumor, anti-cancer therapy, cancer cachexia) would be associated with perturbations of the microbial equilibrium and a switch to a dysbiotic state, the primary aim of microbiota modulation would be the **restoration or maintenance of the equilibrium.** Consequently, microbiota-modulating interventions should be designed to counteract cancer-associated alterations and to increase the resilience of a healthy state and/or to overcome the resilience of an altered, dysbiotic state. Currently, there are different strategies available for gut microbiota modulation, which all have advantages and disadvantages (Figure 2).

In the context of the current thesis, the potential of **prebiotics** as microbiota-modulating intervention in cancer patients was explored. Prebiotics are substrates that are selectively utilized by microorganisms, conferring a health benefit to the host (49). Prebiotics cannot be digested by the host, but are preferentially fermented by certain gut bacteria, thereby stimulating the growth of these bacteria, as well as associated taxa through microbe-microbe interactions (50). In other words, prebiotics are the favorite food of some gut bacteria. Although most prebiotics stimulate the growth of bacteria belonging to the genera *Bifidobacterium* and/or *Lactobacillus,* the working mechanisms and target species might differ between prebiotic compounds (50). Consequently, the selection of a prebiotic for an intervention should be well-thought through, and in accordance with the specific needs of the target patient population.

Preclinical as well as clinical studies have shown health benefits of prebiotic supplementation in the context of different diseases, although not all interventions were effective (as reviewed in (49, 51)). The observed beneficial effects could be (partly) attributed to the stimulation of potentially beneficial gut bacteria, and the prevention of overgrowth of potentially pathogenic and pro-inflammatory bacteria, thereby maintaining homeostasis. In addition, fermentation of prebiotic compounds leads to the formation of SCFA, which exert various health-promoting and anti-inflammatory effects as previously described (50).

Alternatively, **probiotics** could be used to introduce bacteria with beneficial properties into the gut microbiota. In contrast to prebiotics, probiotics are living microorganisms that (when administered in adequate amounts) confer a health benefit on the host (52). Due to their well-described beneficial effects, *Bifidobacterium* or *Lactobacillus* species are usually used as probiotics. However, also specific non-pathogenic *E.coli* strains (e.g. *E.coli* Nissle 1917) are applied as probiotics with the aim to replace pathogenic *E.coli* strains (e.g. (53)).

Synbiotics are mixtures of pre- and probiotics that combine their beneficial properties (54). Motoori *et al*. evaluated the effects of synbiotics in patients with esophageal cancer receiving neoadjuvant chemotherapy and concluded that the intervention successfully modulated the gut microbiota, increased SCFA levels, and reduced the occurrence of severe lymphopenia and diarrhea (55). However, in contrast to prebiotics, probiotics and synbiotics contain living bacteria, which could potentially cause infections in severely ill and immunocompromised cancer patients with inadequate gut barrier function (56).

A further, relatively new strategy is the use of **postbiotics**, which are preparations of inanimate microorganisms and/or their components (57). Postbiotics does not contain living bacteria, but inactivated microbial cells with or without associated metabolites or cell components that exert health benefits to the host. In this context, the use of pasteurized *Akkermansia muciniphila* has been proposed as promising approach (58, 59).

An alternative, more holistic, but also more complex approach, would be the use of **dietary interventions** to support chemotherapy (60). For instance, Ghosh *et al.* showed that a Mediterranean diet intervention was able to induce shifts in gut microbiota composition in elderly people (61). On the other hand, Kolodziejczyk *et al*. suggested the use of machine learning or artificial intelligence pipelines for the design of microbiota-based personalized nutrition, because response of the gut microbiota to dietary interventions would be expected to differ between individuals (6).

A more invasive strategy is the use of allogenic **fecal microbiota transplantation (FMT)**, which was first mentioned in 1958, but was then neglected for a long time (62). During FMT, a donor microbiome is transferred to a recipient in the form of a stool suspension, which can be done endoscopically, via an enema or capsule. De Clercq *et al.* recently applied FMT in patients with advanced gastroesophageal cancer treated with capecitabine and oxaliplatin (CAPOX). While this procedure did not affect parameters of cancer cachexia, disease control rate (percentage of patients who achieved complete response, partial response or stable disease) and survival were significantly increased due to FMT (63). In addition, FMT has been already successfully applied in melanoma patients during therapy with anti-programmed cell death protein 1 (PD-1) (64). In the Netherlands, there is currently a FMT trial on-going in

patients with immune checkpoint inhibitor (ICI)-refractory metastatic melanoma (Clinicaltrials.gov: NCT05251389). The aim is to assess whether transfer of the gut microbiota of patients who either responded well or did not respond to ICI could convert the response in the recipient.

Of course, also **antibiotics** can be used to modulate gut microbiota composition or to inhibit the growth of potentially pathogenic gut bacteria. However, antibiotics are considered to be inferior to the other strategies, due to their collateral damage on gut commensals, which might induce or promote microbial dysbiosis (65, 66).

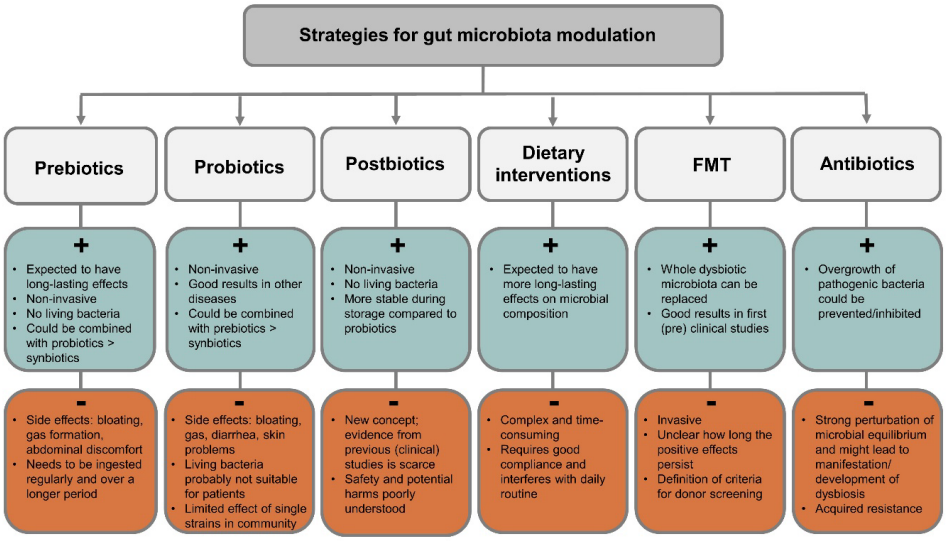


Figure 2: *Summary of different strategies for gut microbiota modulation as well as advantages (+) and disadvantages (-) of each strategy which are considered to be most crucial in the cancer setting. Please note that the figure probably might not encompass all possible advantages and disadvantages and contains own interpretation of results of the recent literature.*

4. Aims and outline of the thesis

The current thesis aims to contribute to unravelling the role of the gut microbiota in human cancer, with a primary focus on two aspects: chemotherapy and cancer cachexia. New knowledge in this field will not only enhance the attention given to the gut microbiota in cancer patients, but will also enable the design of evidence-based microbiota-modulating interventions to improve efficacy and reduce toxicity of anti-cancer treatment, as well as to counteract the development or manifestation of cancer cachexia.

In **Chapter 2**, the literature concerning the clinical link between the gut microbiota and systemic cancer therapies is reviewed. Included cancer therapies comprise chemotherapy, hormonal therapy, and immunotherapy. While most studies were performed in the pre-clinical

setting at that time (2019), our review provides an overview of especially clinical studies describing the association between the gut microbiota and systemic cancer therapy outcome as well as therapy-related changes in gut microbiota composition. In **Chapter 3**, we present an observational longitudinal cohort study in 33 patients with metastatic or unresectable CRC during treatment with capecitabine. Capecitabine is an oral prodrug of 5-FU. In this study, we analyze microbial diversity (α-diversity), community structure (β-diversity), and bacterial abundances on phylum and genus levels before, during, and after three cycles of capecitabine. In addition, we examine whether these microbial parameters differ between responders and non-responders. Furthermore, this pilot study provides a framework and crucial insights into potential challenges for future longitudinal studies in similar complex patient cohorts.

In **Chapter 4**, we look beyond bacterial abundances and focus on gut microbiota-derived SCFA and branched-chain fatty acids (BCFA) in 44 CRC patients, originating from the same cohort study as in chapter 3. For this study, we explore capecitabine-induced shifts in fecal SCFA and BCFA levels and relate them to various clinical parameters, for instance, tumor response, nutritional status, physical performance, chemotherapy-induced toxicity as well as blood inflammatory markers. In addition, fecal SCFA and BCFA levels are correlated with bacterial abundances.

Next to these two clinical studies in patients receiving 5-FU-based chemotherapy, we conducted an *in vitro* study to elucidate molecular interactions between 5-FU and gut bacteria as well as the potential of prebiotics to counteract 5-FU-induced microbiota changes. The results of these experiments are presented in **Chapter 5**. According to our experiences, the detection of consistent 5-FU-induced effects is challenging in the clinical setting due to complex medical histories and interference by various confounding factors. Therefore, we perform experiments in the TNO *in vitro* model of the colon (TIM-2), which closely mimics the physiological conditions in the colon and provides a more controlled setting to analyze interactions. In the TIM-2 model, gut bacteria derived from healthy volunteers were treated with 5-FU with and without the addition of prebiotic mixtures. The effects on gut microbiota composition and diversity, as well as on SCFA and BCFA levels are closely monitored.

In **Chapter 6** we examine shifts in gut microbiota composition and diversity in another type of chemotherapy. For this study, we included 44 breast cancer patients receiving (neo)adjuvant treatment with four cycles of Adriamycin and Cyclophosphamide (AC), followed by four cycles of Docetaxel (D). In these patients, we analyze gut microbiota composition and diversity at baseline, during AC, during D, and after the completion of chemotherapy. In addition, relationships between the gut microbiota and chemotherapy-induced toxicity, as well as pathologic response are explored.

Chapter 7 contains a narrative review highlighting the role of the gut microbiota in the context of cancer cachexia. Here, we describe cachexia-associated gut microbiota profiles and elaborate on the relationship between the gut microbiota and different metabolic aspects of cancer cachexia, including systemic inflammation, gut permeability, muscle wasting, insulin sensitivity, food intake, as well as body weight regulation. In addition, we summarize different strategies for gut microbiota modulation and ongoing trials in this field of research.

In **Chapter 8**, we present our results from a cross-sectional clinical study in cachectic and non-cachectic cancer patients, as well as healthy controls. Patients with pancreatic-, breast-, ovarian- or lung cancer are classified as being cachectic or non-cachectic, based on previous weight loss. In order to identify cachexia-associated microbiota profiles, we compare gut

microbiota composition and diversity, fecal SCFA levels as well as fecal calprotectin among cachectic and non-cachectic cancer patients and cancer-free controls.
Finally, **Chapter 9** integrates all results from the previous chapters and discusses the lessons learned from the studies presented in this thesis. Furthermore, recommendations and points of attention for future research are provided. **Chapter 10** provides a summary of the whole thesis, while **Chapter 11** highlights the implications the current thesis has on scientific as well as societal domains.

5. Some closing remarks concerning microbiota research

The gut microbiota is a complex and challenging research field which has attracted increasing interest in the last decades. To ensure good communication and valuable translation of research findings, it is crucial to use adequate terminology and to be aware of potential pitfalls. Therefore, I would like to conclude this introduction with some general remarks about the terminology used in this thesis and in microbiota research in general.
First of all it should be noted that the terms “microbiota” and “microbiome” describe slightly different concepts. While the gut microbiota refers to the microorganisms themselves, the gut microbiome defines their genetic material and biological activity.
Secondly, it should be noted that the current thesis focusses on gut bacteria only and does not investigate other microorganisms, which would require the use of other techniques.
Thirdly, our current understanding of the gut microbiota does not allow us to clearly distinguish between “beneficial” and “detrimental” bacteria since the effect of a microbe on the human host might also depend on the specific context. In addition, the exact effect might also be strain or species-specific. Since all bioinformatic analyses for this thesis were conducted on genus level only, we cannot draw valid conclusions concerning the role of specific strains or species. Therefore, the current thesis avoids distinguishing between “good” or “bad” bacteria, but uses the terms “potentially beneficial” or “potentially pathogenic” instead.
Fourthly, the nomenclature of bacterial taxa is changing regularly. If necessary for a better understanding, both old and new taxa names are provided in the text of this thesis.

References

1. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59-65.
2. Fassarella M, Blaak EE, Penders J, Nauta A, Smidt H, Zoetendal EG. Gut microbiome stability and resilience: elucidating the response to perturbations in order to modulate gut health. Gut. 2021;70(3):595-605.
3. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature. 2012;489(7415):220-30.
4. Human Microbiome Project C. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486(7402):207-14.
5. Galazzo G, van Best N, Bervoets L, Dapaah IO, Savelkoul PH, Hornef MW, et al. Development of the Microbiota and Associations With Birth Mode, Diet, and Atopic Disorders in a Longitudinal Analysis of Stool Samples, Collected From Infancy Through Early Childhood. Gastroenterology. 2020;158(6):1584-96.
6. Kolodziejczyk AA, Zheng D, Elinav E. Diet-microbiota interactions and personalized nutrition. Nat Rev Microbiol. 2019;17(12):742-53.
7. Lindell AE, Zimmermann-Kogadeeva M, Patil KR. Multimodal interactions of drugs, natural compounds and pollutants with the gut microbiota. Nat Rev Microbiol. 2022;20(7):431-43.
8. Davies M, Galazzo G, van Hattem JM, Arcilla MS, Melles DC, de Jong MD, et al. Enterobacteriaceae and Bacteroidaceae provide resistance to travel-associated intestinal colonization by multi-drug resistant Escherichia coli. Gut Microbes. 2022;14(1):2060676.
9. Rothschild D, Weissbrod O, Barkan E, Kurilshikov A, Korem T, Zeevi D, et al. Environment dominates over host genetics in shaping human gut microbiota. Nature. 2018;555(7695):210-5.
10. Tremaroli V, Backhed F. Functional interactions between the gut microbiota and host metabolism. Nature. 2012;489(7415):242-9.
11. Fan Y, Pedersen O. Gut microbiota in human metabolic health and disease. Nat Rev Microbiol. 2021;19(1):55-71.
12. Blander JM, Longman RS, Iliev ID, Sonnenberg GF, Artis D. Regulation of inflammation by microbiota interactions with the host. Nat Immunol. 2017;18(8):851-60.
13. van der Beek CM, Dejong CHC, Troost FJ, Masclee AAM, Lenaerts K. Role of short-chain fatty acids in colonic inflammation, carcinogenesis, and mucosal protection and healing. Nutr Rev. 2017;75(4):286-305.
14. Sommer F, Anderson JM, Bharti R, Raes J, Rosenstiel P. The resilience of the intestinal microbiota influences health and disease. Nat Rev Microbiol. 2017;15(10):630-8.
15. Pascal V, Pozuelo M, Borruel N, Casellas F, Campos D, Santiago A, et al. A microbial signature for Crohn's disease. Gut. 2017;66(5):813-22.
16. Karlsson FH, Tremaroli V, Nookaew I, Bergström G, Behre CJ, Fagerberg B, et al. Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature. 2013;498(7452):99-103.
17. Miyake S, Kim S, Suda W, Oshima K, Nakamura M, Matsuoka T, et al. Dysbiosis in the Gut Microbiota of Patients with Multiple Sclerosis, with a Striking Depletion of Species Belonging to Clostridia XIVa and IV Clusters. PLoS One. 2015;10(9):e0137429.
18. Sepich-Poore GD, Zitvogel L, Straussman R, Hasty J, Wargo JA, Knight R. The microbiome and human cancer. Science. 2021;371(6536).
19. Sholl J, Sepich-Poore GD, Knight R, Pradeu T. Redrawing therapeutic boundaries: microbiota and cancer. Trends Cancer. 2022;8(2):87-97.
20. Amieva M, Peek RM, Jr. Pathobiology of Helicobacter pylori-Induced Gastric Cancer. Gastroenterology. 2016;150(1):64-78.
21. Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, et al. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. Cell Host Microbe. 2013;14(2):207-15.
22. Feng Q, Liang S, Jia H, Stadlmayr A, Tang L, Lan Z, et al. Gut microbiome development along the colorectal adenoma-carcinoma sequence. Nat Commun. 2015;6:6528.
23. Zeller G, Tap J, Voigt AY, Sunagawa S, Kultima JR, Costea PI, et al. Potential of fecal microbiota for early-stage detection of colorectal cancer. Mol Syst Biol. 2014;10(11):766.
24. Wirbel J, Pyl PT, Kartal E, Zych K, Kashani A, Milanese A, et al. Meta-analysis of fecal metagenomes reveals global microbial signatures that are specific for colorectal cancer. Nat Med. 2019;25(4):679-89.

25. Zhu J, Liao M, Yao Z, Liang W, Li Q, Liu J, et al. Breast cancer in postmenopausal women is associated with an altered gut metagenome. Microbiome. 2018;6(1):136.
26. Panebianco C, Andriulli A, Pazienza V. Pharmacomicrobiomics: exploiting the drug-microbiota interactions in anticancer therapies. Microbiome. 2018;6(1):92.
27. Alexander JL, Wilson ID, Teare J, Marchesi JR, Nicholson JK, Kinross JM. Gut microbiota modulation of chemotherapy efficacy and toxicity. Nat Rev Gastroenterol Hepatol. 2017;14(6):356-65.
28. Doestzada M, Vila AV, Zhernakova A, Koonen DPY, Weersma RK, Touw DJ, et al. Pharmacomicrobiomics: a novel route towards personalized medicine? Protein Cell. 2018;9(5):432-45.
29. Maier L, Pruteanu M, Kuhn M, Zeller G, Telzerow A, Anderson EE, et al. Extensive impact of non-antibiotic drugs on human gut bacteria. Nature. 2018;555(7698):623-8.
30. Zimmermann M, Zimmermann-Kogadeeva M, Wegmann R, Goodman AL. Mapping human microbiome drug metabolism by gut bacteria and their genes. Nature. 2019;570(7762):462-7.
31. Daillère R, Vétizou M, Waldschmitt N, Yamazaki T, Isnard C, Poirier-Colame V, et al. Enterococcus hirae and Barnesiella intestinihominis Facilitate Cyclophosphamide-Induced Therapeutic Immunomodulatory Effects. Immunity. 2016;45(4):931-43.
32. An J, Ha EM. Combination Therapy of Lactobacillus plantarum Supernatant and 5-Fluouracil Increases Chemosensitivity in Colorectal Cancer Cells. J Microbiol Biotechnol. 2016;26(8):1490-503.
33. Kim HJ, An J, Ha EM. Lactobacillus plantarum-derived metabolites sensitize the tumor-suppressive effects of butyrate by regulating the functional expression of SMCT1 in 5-FU-resistant colorectal cancer cells. J Microbiol. 2022;60(1):100-17.
34. Zhang S, Yang Y, Weng W, Guo B, Cai G, Ma Y, et al. Fusobacterium nucleatum promotes chemoresistance to 5-fluorouracil by upregulation of BIRC3 expression in colorectal cancer. J Exp Clin Cancer Res. 2019;38(1):14.
35. Yu T, Guo F, Yu Y, Sun T, Ma D, Han J, et al. Fusobacterium nucleatum Promotes Chemoresistance to Colorectal Cancer by Modulating Autophagy. Cell. 2017;170(3):548-63.e16.
36. LaCourse KD, Zepeda-Rivera M, Kempchinsky AG, Baryiames A, Minot SS, Johnston CD, et al. The cancer chemotherapeutic 5-fluorouracil is a potent Fusobacterium nucleatum inhibitor and its activity is modified by intratumoral microbiota. Cell Rep. 2022;41(7):111625.
37. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer. 2003;3(5):330-8.
38. Hidese R, Mihara H, Kurihara T, Esaki N. Escherichia coli dihydropyrimidine dehydrogenase is a novel NAD-dependent heterotetramer essential for the production of 5,6-dihydrouracil. J Bacteriol. 2011;193(4):989-93.
39. Spanogiannopoulos P, Kyaw TS, Guthrie BGH, Bradley PH, Lee JV, Melamed J, et al. Host and gut bacteria share metabolic pathways for anti-cancer drug metabolism. Nat Microbiol. 2022;7(10):1605-20.
40. Sougiannis AT, VanderVeen BN, Enos RT, Velazquez KT, Bader JE, Carson M, et al. Impact of 5 fluorouracil chemotherapy on gut inflammation, functional parameters, and gut microbiota. Brain Behav Immun. 2019;80:44-55.
41. Zeng MY, Inohara N, Nuñez G. Mechanisms of inflammation-driven bacterial dysbiosis in the gut. Mucosal Immunol. 2017;10(1):18-26.
42. Fearon K, Strasser F, Anker SD, Bosaeus I, Bruera E, Fainsinger RL, et al. Definition and classification of cancer cachexia: an international consensus. Lancet Oncol. 2011;12(5):489-95.
43. Argiles JM, Busquets S, Stemmler B, Lopez-Soriano FJ. Cancer cachexia: understanding the molecular basis. Nat Rev Cancer. 2014;14(11):754-62.
44. Bindels LB, Neyrinck AM, Claus SP, Le Roy CI, Grangette C, Pot B, et al. Synbiotic approach restores intestinal homeostasis and prolongs survival in leukaemic mice with cachexia. Isme j. 2016;10(6):1456-70.
45. Pötgens SA, Brossel H, Sboarina M, Catry E, Cani PD, Neyrinck AM, et al. Klebsiella oxytoca expands in cancer cachexia and acts as a gut pathobiont contributing to intestinal dysfunction. Sci Rep. 2018;8(1):12321.
46. Bindels LB, Neyrinck AM, Loumaye A, Catry E, Walgrave H, Cherbuy C, et al. Increased gut permeability in cancer cachexia: mechanisms and clinical relevance. Oncotarget. 2018;9(26):18224-38.
47. Pötgens SA, Thibaut MM, Joudiou N, Sboarina M, Neyrinck AM, Cani PD, et al. Multi-compartment metabolomics and metagenomics reveal major hepatic and intestinal disturbances in cancer cachectic mice. J Cachexia Sarcopenia Muscle. 2021.

48. Ni Y, Lohinai Z, Heshiki Y, Dome B, Moldvay J, Dulka E, et al. Distinct composition and metabolic functions of human gut microbiota are associated with cachexia in lung cancer patients. Isme j. 2021;15(11):3207-20.
49. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nat Rev Gastroenterol Hepatol. 2017;14(8):491-502.
50. Rastall RA, Diez-Municio M, Forssten SD, Hamaker B, Meynier A, Moreno FJ, et al. Structure and function of non-digestible carbohydrates in the gut microbiome. Benef Microbes. 2022;13(2):95-168.
51. Markowiak P, Śliżewska K. Effects of Probiotics, Prebiotics, and Synbiotics on Human Health. Nutrients. 2017;9(9).
52. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 2014;11(8):506-14.
53. Kruis W, Fric P, Pokrotnieks J, Lukás M, Fixa B, Kascák M, et al. Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine. Gut. 2004;53(11):1617-23.
54. Swanson KS, Gibson GR, Hutkins R, Reimer RA, Reid G, Verbeke K, et al. The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of synbiotics. Nat Rev Gastroenterol Hepatol. 2020;17(11):687-701.
55. Motoori M, Yano M, Miyata H, Sugimura K, Saito T, Omori T, et al. Randomized study of the effect of synbiotics during neoadjuvant chemotherapy on adverse events in esophageal cancer patients. Clin Nutr. 2017;36(1):93-9.
56. Redman MG, Ward EJ, Phillips RS. The efficacy and safety of probiotics in people with cancer: a systematic review. Ann Oncol. 2014;25(10):1919-29.
57. Salminen S, Collado MC, Endo A, Hill C, Lebeer S, Quigley EMM, et al. The International Scientific Association of Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics. Nat Rev Gastroenterol Hepatol. 2021;18(9):649-67.
58. Cani PD, de Vos WM. Next-Generation Beneficial Microbes: The Case of Akkermansia muciniphila. Front Microbiol. 2017;8:1765.
59. Plovier H, Everard A, Druart C, Depommier C, Van Hul M, Geurts L, et al. A purified membrane protein from Akkermansia muciniphila or the pasteurized bacterium improves metabolism in obese and diabetic mice. Nat Med. 2017;23(1):107-13.
60. Greathouse KL, Wyatt M, Johnson AJ, Toy EP, Khan JM, Dunn K, et al. Diet-microbiome interactions in cancer treatment: Opportunities and challenges for precision nutrition in cancer. Neoplasia. 2022;29:100800.
61. Ghosh TS, Rampelli S, Jeffery IB, Santoro A, Neto M, Capri M, et al. Mediterranean diet intervention alters the gut microbiome in older people reducing frailty and improving health status: the NU-AGE 1-year dietary intervention across five European countries. Gut. 2020;69(7):1218-28.
62. Eiseman B, Silen W, Bascom GS, Kauvar AJ. Fecal enema as an adjunct in the treatment of pseudomembranous enterocolitis. Surgery. 1958;44(5):854-9.
63. de Clercq NC, van den Ende T, Prodan A, Hemke R, Davids M, Pedersen HK, et al. Fecal Microbiota Transplantation from Overweight or Obese Donors in Cachectic Patients with Advanced Gastroesophageal Cancer: A Randomized, Double-blind, Placebo-Controlled, Phase II Study. Clin Cancer Res. 2021;27(13):3784-92.
64. Davar D, Dzutsev AK, McCulloch JA, Rodrigues RR, Chauvin JM, Morrison RM, et al. Fecal microbiota transplant overcomes resistance to anti-PD-1 therapy in melanoma patients. Science. 2021;371(6529):595-602.
65. Maier L, Goemans CV, Wirbel J, Kuhn M, Eberl C, Pruteanu M, et al. Unravelling the collateral damage of antibiotics on gut bacteria. Nature. 2021;599(7883):120-4.
66. McDonnell L, Gilkes A, Ashworth M, Rowland V, Harries TH, Armstrong D, et al. Association between antibiotics and gut microbiome dysbiosis in children: systematic review and meta-analysis. Gut Microbes. 2021;13(1):1-18.

Chapter 2

The clinical link between human intestinal microbiota and systemic cancer therapy

Romy Aarnoutse*, **Janine Ziemons***, John Penders, Sander S. Rensen, Judith de Vos-Geelen, Marjolein L. Smidt
** Shared first authorship*

International Journal of Molecular Sciences. 2019 Aug; 20(17):4145.
DOI: 10.3390/ijms20174145

Abstract

Clinical interest in the human intestinal microbiota increases considerably. However, an overview of clinical studies investigating the link between the human intestinal microbiota and systemic cancer therapy is lacking. This systematic review summarizes all clinical studies describing the association between baseline intestinal microbiota and systemic cancer therapy outcome as well as therapy-related changes in intestinal microbiota composition. A systematic literature search was performed and provided 23 articles. There were strong indications for a close association between the intestinal microbiota and outcome of immunotherapy. Furthermore, the development of chemotherapy-induced infectious complications seemed to be associated with the baseline microbiota profile. Both chemotherapy and immunotherapy induced drastic changes in gut microbiota composition with possible consequences for treatment efficacy. Evidence in the field of hormonal therapy was very limited. Large heterogeneity concerning study design, study population and methods used for analysis limited comparability and generalization of results. For the future, longitudinal studies investigating the predictive ability of baseline intestinal microbiota concerning treatment outcome and complications as well as the potential use of microbiota-modulating strategies in cancer patients are required. More knowledge in this field is likely to be of clinical benefit since modulation of the microbiota might support cancer therapy in the future.

1. Introduction

The human microbiota is the collection of bacteria, archaea, viruses and eukaryotic microorganisms that live in and on the human gastrointestinal tract, mucosae and skin. The microbiome is the collective genome of the microbiota and encodes approximately 100 fold more genes than the human genome itself (1). The majority of the microbiota resides in the gastrointestinal tract and belongs to the “intestinal microbiota” or “gut microbiota”.
It has been established that cross-talk between the gut microbiota and the human host is essential for maintaining homeostasis and human health (2). Therefore, it is not surprising that microbial dysbiosis has been shown to be associated with various metabolic and inflammatory diseases, such as ulcerative colitis, obesity, diabetes mellitus and hypertension (3-5).
Next to the taxonomic composition of the gut microbiota, the intra- and inter-individual diversity of the microbial community are considered to be of great importance (3, 4, 6). Microbial diversity can be quantified by means of two metrics: α-diversity and β-diversity. α-diversity describes the number (richness) and distribution (evenness) of taxa in a given sample (7). Common indices to describe α-diversity are the Shannon index, Simpson index and the Chao 1 index (7). β-diversity defines the number of taxa shared between different samples and can be seen as a (dis)similarity score (7). Generally, a healthy state is characterized by a species-rich, diverse and stable microbiota, which fulfills various and complex metabolic roles (8).

In recent years, increasing evidence shows that the gut microbiota has an important role in carcinogenesis and the pathophysiology of human cancer. For instance, infection with *Helicobacter pylori* is considered to stimulate the development of gastric carcinoma by producing virulence factors and enhancing chronic inflammation and subsequent carcinogenesis (9). Similarly, abundance of *Fusobacterium nucleatum* has been found to be increased in colorectal cancer and it is suggested that this bacterial species might be involved in intestinal tumorigenesis and modulation of the tumor microenvironment (10, 11).
Interestingly, the involvement of the gut microbiota is not limited to gastrointestinal cancers. It has been suggested that gut bacteria affect the development of breast cancer through modulation of estrogen metabolism (12, 13). In line with this, it has been demonstrated that gut microbiota composition as well as several functional features differ between postmenopausal breast cancer patients and healthy controls (14). Furthermore, Rajagopala et al. (2016) demonstrated that patients with leukemia already had reduced microbial diversity and dysbiosis at the time of diagnosis and could be distinguished from healthy controls based on their microbiota profiles (15).

While there are strong indications for the role of the gut microbiota in carcinogenesis, evidence concerning its role in the context of cancer treatment is scarce. Currently, most of the results concerning interactions between the gut microbiota and cancer therapy originate from *in vitro* studies using culturing methods (16-18). A comprehensive overview of clinical studies in this field of research is lacking.

This systematic review summarizes clinical studies investigating the influence of the intestinal microbiota on systemic cancer therapy as well as the influence of systemic cancer therapy on the intestinal microbiota (Figure 1). We focused on chemotherapy, immunotherapy and hormonal therapy. In addition, Table A2 in Appendix B provides an overview of important

terms used in microbiota research. By providing a comprehensive overview of clinical studies on the interaction between the gut microbiota and systemic cancer therapy, this review will provide pivotal information on current gaps of knowledge and will facilitate the evidence-based design of future studies in this field.

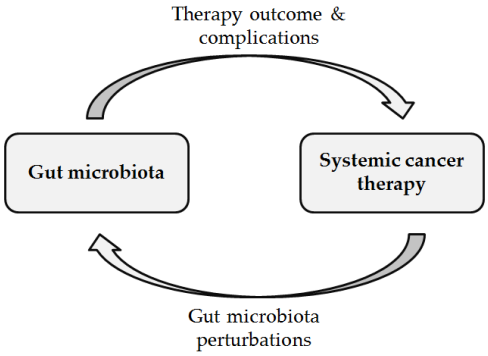


Figure 1: *Overview of the main questions addressed in this review*

2. Baseline human intestinal microbiota characteristics are associated with the development of complications and systemic cancer therapy outcome

2.1. Chemotherapy

Infectious complications are a common side effect of cancer therapy and have a considerable impact on patients' prognosis and quality of life (19). Research indicated that the development of chemotherapy-related infections might be associated with intestinal microbiota composition.

Galloway-Pena et al. (2016) demonstrated that baseline α-diversity was significantly lower in patients with acute myeloid leukemia (AML) suffering from infectious complications after induction chemotherapy compared to patients without infections (20). Consequently, a lower microbial diversity before the start of chemotherapy might increase the risk for the development of infections, potentially as a result of a reduced colonization resistance. Additionally, the same group also analyzed stool temporal variability as indicator of microbial instability and its association with induction chemotherapy outcome (21). Baseline samples were collected up to eight days before and 24h following chemotherapy initiation. It was concluded that AML patients who developed an infection within 90 days post-neutrophil recovery had significantly higher microbial instability (21). Moreover, patients developing an infection during induction chemotherapy had a significantly higher relative abundance of *Stenotrophomonas* (21). Intra-patient α-diversity variability was not associated with response to chemotherapy. Multivariate regression analysis indicated that age, antibiotic type and duration or chemotherapy regime were not significantly correlated with intra-patient temporal variability (21). In conclusion, baseline stool microbiota with low α-diversity, high temporal variability and increased potentially pathogenic *Stenotrophomonas* are linked to infectious complications during and after induction chemotherapy. Consequently, patients with a less diverse and less stable gut microbiota might be at higher risk to develop infections.

In a study among 28 patients suffering from non-Hodgkin lymphoma, eleven were reported to develop bloodstream infections (BSI) (22). Principal coordinate analysis (PCoA) of fecal samples collected before start of the treatment demonstrated differences between patients with or without subsequent BSI (22). This means that the overall microbial community structure (β-diversity) was already different at baseline and that this might be predictive for future development of BSI. Similar to the results of Galloway-Pena et al. (2016), it was also shown that α-diversity was significantly lower in fecal samples from patients who developed subsequent BSI (22). Furthermore, abundance of several bacteria was altered in these patients (Table 1). In addition, it was tested whether relative abundance of specific microbes could be used to discriminate between patients who did or did not develop subsequent BSI. In this context, Barnesiellaceae (AUC=0.94), Christensenellaceae (AUC=0.86) and *Faecalibacterium* (AUC=0.84), which were all reduced in patients with subsequent BSI, were found to be promising candidates (22). Based on these results, it was concluded that patients having a high risk to develop BSI could potentially be identified based on their microbial profile prior to therapy initiation.

In contrast with this study, the development of diarrhea in patients with metastatic renal cell carcinoma (RCC) was not related to differences in α-diversity of the gut microbiota (23). However, clustering of these patients based on relative abundance at genus level revealed a low-risk and a high-risk group. The high-risk group had a high abundance of *Bacteroides* (42%) and a low level of *Prevotella* (3%) (23). In the low-risk group, the opposite pattern was apparent with 47% *Prevotella* and 13% *Bacteroides* (23). This suggests that there might be an interaction between intestinal microbiota composition and VEGF-TKI-induced diarrhea.

2.2. Immunotherapy

Six articles were available describing the association between baseline human intestinal microbiota and immunotherapy outcome (24-29). Patients received anti-PD-1 or anti-CTLA-4 therapy for either metastatic or unresectable melanoma, renal cell carcinoma (RCC) or non-small cell lung cancer (NSCLC). Four out of these studies analyzed fecal microbiota composition with metagenomic shotgun sequencing (24-26, 28).

Matson et al. (2018) compared the baseline microbiota composition of 42 patients with metastatic melanoma that received anti-PD-1 (N=38) or anti-CTLA-4 (N=4) immunotherapy (24). Baseline stool samples were collected prior to immunotherapy initiation. Sixteen patients showed a response following immunotherapy, whereas 26 patients did not respond. Intestinal microbiota analysis indicated that one Operational Taxonomic Unit (OTU) belonging to the family of Bifidobacteriaceae was significantly more abundant in the responder group compared to the non-responder group. Another Bifidobacteriaceae OTU (559527) was borderline significantly (p=0.058) more abundant (24). Principal Component Analysis (PCA) showed a separation of responders and non-responders (24). Furthermore, eight species were more abundant in the responder group*: Enterococcus faecium, Collinsella aerofaciens, Bifidobacterium adolescentis, Klebsiella pneumoniae, Veillonella parvula, Parabacteroides merdae, Lactobacillus sp.,* and *Bifidobacterium longum* — whereas two were more abundant in the non-responder group: *Ruminococcus obeum* and *Roseburia intestinalis* (Table 1). As conclusions did not change when removing the 4 anti-CTLA-4 treated patients, these patients were retained in the analysis (24). This means that the baseline composition of the intestinal microbiota in patients with metastatic melanoma was associated with therapeutic efficacy of anti-PD-1 therapy. *Bifidobacteriam longum* and multiple other bacteria may contribute to improved anti-tumor immunity in patients. In addition, the ratio between potential “beneficial” and “non-beneficial” OTU’s might be a strong predictor of clinical response to anti-PD-1 therapy. The authors concluded that a higher ratio between beneficial and non-beneficial OTUs may predict the most favorable clinical outcome (24).

Gopalakrishnan *et al.* (2018) compared the microbiota composition of 43 metastatic melanoma patients treated with anti-PD-1 therapy (25). Baseline stool samples were collected prior to therapy initiation. Median time from initial fecal sampling and therapy initiation was nine days with a broad range between -481 and +14 days. There were 30 responders and 13 non-responders. Pre-treatment α-diversity was significantly higher ($p<0.01$) in responders compared to non-responders (25). In addition, patients with a higher α-diversity prior to anti-PD-1 therapy had a significantly prolonged progression-free survival (PFS) compared to patients with an intermediate ($p=0.02$) or low ($p=0.04$) α-diversity (25). The β-diversity at family level between responders and non-responders visualized with PCoA showed a clustering of samples ($p<0.05$). Linear Discriminant Analysis (LDA) demonstrated that Clostridiales and Ruminococcaceae were enriched in responders and Bacteroidales enriched in non-

responders (p<0.01). Pairwise comparison identified that the *Faecalibacterium* genus was significantly enriched in responders. Using Whole Metagenome Sequencing (WMGS), *Faecalibacterium sp., Clostridium sp., Clostridiales, Eubacterium sp., Oscillibacter sp*. and *Ruminococcaceae* were found to be enriched in responders (N=14). *Bacteroides thetaiotaomicron, Escherichia coli, Oxalobacter formigenes, Anaerotruncus colihominis* and *Klebsiella variicola* were significantly enriched in non-responders (N=11) (25). Nineteen out of 39 patients had a high abundance of *Faecalibacterium*, accompanied by a significantly prolonged PFS compared to patients with lower abundance (p=0.03). Twenty of 39 patients had a high abundance of Bacteroidales, accompanied by a shortened PFS compared to patients with a lower abundance (P=0.05). Cox proportional hazard analysis demonstrated that α-diversity and the abundance of *Faecalibacterium* and Bacteriodales were significant strong predictors of response to anti-PD-1 therapy in metastatic melanoma patients (25). Patients with a high α-diversity and abundance of Ruminococcaceae and *Faecalibacterium* were found to have an enhanced systemic and antitumor immune response mediated by increased antigen presentation and improved effector T-cell function. Conversely, patients with low α-diversity and high relative abundance of Bacteroidales had an impaired immune response (25).

Routy *et al.* (2018) analyzed 100 patients who received anti-PD-1 therapy for NSCLC (N=60) or RCC (N=40) (26). Baseline fecal samples were collected before anti-PD-1 infusion. Since there were no statistically significant differences in gene count and metagenomic species before and during anti-PD-1 therapy samples, T1 samples (collected after two anti-PD-1 infusions) were used if baseline samples were not available. A significantly higher α-diversity (richness at gene count (p=0.002) and metagenomic species level (p=0.003)) of fecal samples was correlated with clinical response at six months, but not at three months after therapy initiation (26). Response was defined as the absence of progression defined by the Response Evaluation Criteria in Solid Tumors (RECIST) (26). By means of the RECIST criteria, tumor response can be graded as complete response (CR), partial response (PR), progressive disease (PD) or stable disease (SD) (30). In addition, Routy *et al.* (2018) identified that for instance Firmicutes, *Akkermansia* and *Alistipes* were significantly associated with response (PR and SD) (26). *Akkermansia muciniphila* was most significantly (p=0.004) overrepresented at diagnosis in the feces of responders and patients with a PFS>3 months after anti-PD-1 therapy initiation (p=0.028). These results were independent of antibiotic use (26). Moreover, several additional bacterial species were significantly increased or decreased in patients with a PFS>3 months excluding those who took antibiotics (N=78), see Table 1. Similar results were seen when all patients were included (N=100). Comparable results were obtained in the cohort of NSCLC patients (N=58). In particular, it was notable that when high levels of *Akkermansia muciniphila* were present in the feces, patients would later benefit from anti-PD-1 therapy (26).

Chaput *et al.* (2017) analyzed the predictive value of baseline fecal microbiota samples of 26 patients with metastatic melanoma receiving ipilimumab (27). Baseline fecal samples were collected before the first ipilimumab infusion. PCA analysis at genera level (p=0.0090), species level (p=0.0050) or OTU level (p=0.0080) indicated that metastatic melanoma patients could be clustered into groups with long-term versus poor clinical benefit, based on gut microbiota composition at baseline (27). Main genera which contributed to this stratification were *Faecalibacterium, Gemmiger, Bacteroides* and *Clostridium XIVa (*27*)*. Before treatment, patients with poor clinical benefit had a high proportion of *Bacteroides* (p=0.034). The relative abundance of *Faecalibacterium, Clostridium XIVa,* and *Gemminger* was higher in patients

with long term benefit (27). Additionally, patients with higher levels of *Ruminococcus* and Lachnospiraceae (relatives of *Facealibacterium prausnitzii L2-L6, Gemmiger formicilis* and *butyrate-producing bacterium SS2-1*) at baseline had an overall survival (OS) longer than 18 months. These results were independent of previous antibiotic use and antibiotic use did not influence baseline dominant microbiota (27).

Three clusters could be identified based on baseline microbiota composition at the genus level. The first cluster (N=12) was enriched in *Faecalibacterium,* and other Firmicutes *(*unclassified Ruminococcaceae*, Clostridium XlVa* and *Blautia)*, had a longer PFS (p=0.0039), OS (p=0.051) and greater clinical benefit (p=0.0017) compared to patients in the second cluster with baseline samples enriched in *Bacteroides* (N=10). The third cluster of patients was enriched in *Prevotella* (N=4), but was not included in the analysis due to the low number (27). It was further shown that patients with baseline samples enriched in Firmicutes were more prone to develop colitis (p=0.009), while patients with enhanced baseline Bacteroidetes did not develop colitis (p=0.011) (27). These findings indicate that gut colonization with Firmicutes is associated with a better anti-cancer response and colitis in metastatic melanoma patients that will be treated with ipilimumab. On the other hand, gut colonization with Bacteroidetes appears to be associated with a poor response without colitis (27).

Frankel *et al.* (2017) collected baseline fecal samples of 39 unresectable or metastatic melanoma patients before treatment with anti-PD-1 or anti-CTLA-4 (ipilimumab (I), nivolumab (N), ipilimumab + nivolumab (IN) or pembrolizumab (P)) (28). Response was quantified by means of the RECIST criteria and was defined as stable or responsive disease. Metagenomic shotgun sequencing indicated that responders (N=24) were significantly enriched with *Bacteroides caccae* (p=0.032) and *Streptococcus parasanguinis* (p=0.048) (28). In the IN+N group there were 16 responders and eight non-responders (28). Within this group, responders treated with IN (N=16) and N (N=1) were significantly enriched with *Faecalibacterium prausnitzii* (p=0.032), *Holdemania filiformis* (p=0.043) and *Bacteroides thetaiotamicron* (p=0.046). Responders treated with P had significantly higher levels of *Dorea formicigenerans* (p=0.045). The P group contained six responders and seven non-responders (28).

Interestingly, overall microbial diversity was not significantly different between responders and patients with progressive disease (28). Overall, this study identified specific gut microbiota species associated with response to anti-PD-1 and anti-CTLA-4 therapy.

Dubin *et al.* (2016) correlated fecal microbiota composition with subsequent colitis development in 34 patients with metastatic melanoma to be treated with ipilimumab (29). In general, fecal samples were obtained from patients before the first dose of ipilimumab (30/34). Ten patients with metastatic melanoma developed colitis between 13 and 57 days after ipilimumab initiation. Colitis free patients (N=24) had an increased relative abundance of Bacteroidaceae, Bacteroidaceae, *Bacteroides*, Barnesiellaceae, unclassified Barnesiellaceae, Rikenellaceae, unclassified Rikenellaceae, Bacteroidetes, Bacteroidia and Bacteroidales. Patients that developed colitis (N=10) had a decreased relative abundance of Bacteroidetes in fecal samples collected before ipilimumab infusion (29). Based on this, the authors concluded that increased fecal abundance of Bacteroidetes, Bacteroidaceae, Rikenellaceae and Barnesiellaceae correlated with a reduced risk to develop ipilimumab-induced colitis (29).

Table 1: *Clinical studies investigating the association between baseline intestinal microbiota composition and systemic cancer therapy outcome and complications*

STUDY DESIGN					MAIN FINDINGS	
Study	**Cancer type**	**N**	**Type of therapy**	**Analysis method**	**Therapy outcome**	**Microbial outcomes found to be different**
Chemotherapy						
Galloway-Peña et al. (2017)	AML	N=35	Induction chemotherapy	16S rRNA gene sequencing	Increased risk for infections	↑ intra-patient temporal variability of α-diversity (CV of Shannon index) ↑ *Stenotrophomonas*
Galloway-Peña et al. (2016)	AML	N=34	Induction chemotherapy	16S rRNA gene sequencing	Increased risk for infections	↓ baseline α-diversity (Shannon index)
Pal et al. (2015)	Metastatic RCC	N=20	VEGF-TKI	16S rRNA gene sequencing	Increased risk to develop diarrhea	↑ *Bacteroides* ↓ *Prevotella*
Immunotherapy						
Matson et al. (2018)	Metastatic melanoma	N=42	Anti-PD-1 (N=38) Anti-CTLA-4 (N=4)	- 16S rRNA gene sequencing - Metagenomic shotgun sequencing - qPCR	Response (N=16)	↑ Bifidobacteriaceae ↑ *Enterococcus faecium* ↑ *Collinsella aerofaciens* ↑ *Bifidobacterium adolescentis* ↑ *Klebsiella pneumoniae* ↑ *Veillonella parvula* ↑ *Parabacteroides merdae* ↑ *Lactobacillus sp.* ↑ *Bifidobacterium longum* ↓ *Ruminococcus obeum* ↓ *Roseburia intestinalis*
Gopalakrishnan et al. (2018)	Metastatic melanoma	N=43	Anti-PD-1	16S rRNA gene sequencing	Response (N=30)	↑ α-diversity (inverse Simpson score) ↑ between-group β-diversity ↑ Clostridiales ↑ Ruminococcaceae ↑ *Faecalibacterium* ↓ Bacteroidales
		N=25	Anti-PD-1	Metagenomic whole-genome shotgun sequencing	Response (N=14)	↑ *Faecalibacterium sp.* ↑ *Clostridium sp.* ↑ *Clostridiales* ↑ *Eubacterium sp.*

					↑ *Oscillibacter sp.* ↑ *Ruminococcaceae* ↓ *Bacteroides thetaiotaomicron* ↓ *Escherichia coli* ↓ *Oxalobacter formigenes* ↓ *Anaerotruncus colihominis* ↓ *Klebsiella variicola*
		N=39 Anti-PD-1	Metagenomic whole-genome shotgun sequencing	Prolonged PFS (N=19)	↑ *Faecalibacterium* ↓ Bacteroidales
Routy et al (2018)	NSCLC (N=60) RCC (N=40)	N=100 Anti-PD-1	Metagenomic shotgun sequencing	Response	↑ α-diversity (richness) ↑ *Akkermansia muciniphila* ↑ *Firmicutes* and *4x unclassified* ↑ *Eubacterium sp.* ↑ *Lachnospiraceae* ↑ *Erysipelotrichaceae bacterium* ↑ *Cloacibacillus porcorum* ↑ *Enterococcus faecium* ↑ *Intestinimonas* ↑ *2x unclassified Clostridialis* ↑ *Alistipes* ↑ *Bacteroides sp.* ↑ *Alistipes indistinctus* ↑ *Firmicutes bacterium* ↑ *Prevotella* ↓ *Prevotella* ↓ *Clostridium sp.* ↓ *unclassified Firmicutes* ↓ *Prevotella sp.* ↓ *Clostridiales* ↓ *Clostridium bolteae* ↓ *Firmicutes bacterium* ↓*Closteridiales bacterium* ↓ *Blautia* ↓ *Bacteroides clanus* ↓ *Proteobacteria* ↓ *Bacteroides nordii* ↓ *Parabacteroides distasonis*

	NSCLC + RCC	N=78	Anti-PD-1	Metagenomic shotgun sequencing	PFS > 3 months	↑ *unclassified Firmicutes 6x* ↑ *Eubacterium sp.* ↑ *Alistipes 2x* ↑ *Akkermansia muciniphila* ↑ *Intestinimonas* ↑ *Bacteroides nordii* ↑ *Bacteroides xylanisolvens* ↑ *Blautia* ↑ *Lachnospiraceae* ↑ *Firmicutes bacterium* ↑ *Firmicutes* ↑ *unclassified Clostridiales 2x* ↑ *Clostridialis* ↑ *Ruminococcaceae* ↑ *Clostridium sp.* ↑ *Flavonifractor* ↑ *Bacteroides caccae* ↑ *unclassified Ruminococcaceae* ↑ *Ruminococcus sp.* ↓ *unclassified Clostridialis* ↓ *Parabacteroides distasonis* ↓ *Firmicutes bacterium* ↓ *Clostridiales* ↓ *Clostridialis VE202-14* ↓ *Anaerotruncus colihominis* ↓ *Lachnospiraceae* ↓ *Erysipelotrichaceae*
Chaput et al. (2017)	Metastatic melanoma	N=26	Ipilimumab	16S rRNA gene sequencing	Colitis and good response	↑ Firmicutes ↓ Bacteroidetes
					↑ PFS ↑ OS ↑ % clinical benefit	↑ *Faecalibacterium* ↑ Firmicutes ↑ unclassified *Ruminococcaceae* ↑ *Clostridium XIVa* ↑ *Blautia*
Frankel et al. (2017)	Metastatic/ unresectable melanoma	N=39	Ipilimumab, Nivolumab Ipilimumab + Nivolumab Pembrolizumab	Metagenomic shotgun sequencing	Response (N=24)	↑ *Bacteroides caccae* ↑ *Streptococcus parasanguinis*

		N=24	Ipilimumab + Nivolumab	Metagenomic shotgun sequencing	Response (N=16)	↑ *Faecalibacterium prausnitzii* ↑ *Holdemania filiformis* ↑ *Bacteroides thetaiotamicron*
		N=13	Pembrolizumab	Metagenomic shotgun sequencing	Response (N=6)	↑ *Dorea formicigenerans*
Dubin et al. (2016)	Metastatic Melanoma	N=34	Ipilimumab	16S rRNA gene sequencing	Colitis free	↑ Bacteroidaceae ↑ Bacteroides ↑ Barnesiellaceae ↑ Barnesiellaceae unclassified ↑ Rikenellaceae ↑ Rikenellaceae unclassified ↑ Bacteroidetes ↑ Bacteroidia ↑ Bacteroidales ↑ *Bacteroidetes*
Other						
Montassier et al. (2016)	Non-Hodgkin lymphoma	N=28	HSCT	16S rRNA high-throughput DNA sequencing	Increased risk to develop bloodstream infections	↑ Erysipelotrichaceae ↑ *Veillonella* ↓ α-diversity (phylogenetic diversity, observed species, Chao1 & Shannon indices) ↓ Barnesiellaceae ↓ *Butyricimonas* ↓ Christensenellaceae ↓ *Faecalibacterium* ↓ *Oscillospira* ↓ *Christensenella* ↓ *Dehalobacterium* ↓ *Desulfovibrio* ↓ *Sutterella* ↓ *Oxalobacter* ↓ Coriobacteriaceae

↑: Increase, ↓: Decrease, **AML:** Acute myeloid leukemia, **CV:** coefficient of variation, **RCC:** renal cell carcinoma, **PFS:** progression-free survival, **OS:** overall survival, **NSCLC:** non-small cell lung cancer, **HSCT:** hematopoietic stem cell transplantation

2.3. Hormonal therapy

To the best of our knowledge, clinical studies investigating the association between baseline human intestinal microbiota and the outcome of hormonal therapy have not been reported so far.

3. Human intestinal microbiota changes during systemic cancer therapy

3.1. Chemotherapy

Several studies investigated the effect of systemic cancer therapy on gut microbiota composition in different types of cancer. These studies included gastrointestinal and non-gastrointestinal cancers as well as different chemotherapeutic agents and treatment settings. In patients with neuroendocrine tumors (NET), it was observed that systemic chemotherapy increased the concentration of *Faecalibacterium prausnitzii* in patients with midgut NET (31). While this study used fluorescent in situ hybridization (FISH) targeting selected species only, more recent articles use sequencing-based approaches in order to extensively profile the bacterial species composition.

Using sequencing of the 16S rRNA gene, Montassier *et al.* (2014) observed a remarkable shift of the intestinal microbiota composition during five day high-dose chemotherapy as conditioning regimen for bone marrow transplantation (6). More precisely, there was a significant reduction in the observed microbial richness (number of bacterial taxa), estimated microbial richness (Chao1 index) as well as microbial diversity (Shannon index), indicating a significant reduction in α-diversity due to chemotherapy ($p<0.001$) (6). Furthermore, PCoA showed a clear separation of pre-chemotherapy and post-chemotherapy samples ($p<0.001$) (6). Thus, it can be concluded that high-dose chemotherapy induced a marked decrease in overall microbial diversity and shifted the microbial community structure. On the phylum level, abundance of Bacteroidetes and Proteobacteria was increased, while Firmicutes and Actinobacteria were decreased (6). On the genus level, *Bacteroides* ($p=0.0008$) and *Escherichia* ($p=0.008$) were significantly higher in the post-chemotherapy samples compared to pre-chemotherapy samples. On the other hand, *Blautia* ($p=0.008$), *Faecalibacterium* ($p=0.04$), *Roseburia* ($p=0.008$) and *Bifidobacterium (p=0.04)*, which are considered health promoting and anti-inflammatory bacteria, were decreased after chemotherapy (6). Furthermore, there was a statistically significant shift from Gram-positive bacteria to Gram-negative bacteria during chemotherapy ($p<0.001$) (6). Interestingly, this study also described that several less abundant bacterial genera appeared after chemotherapy treatment (6). A similar observation was described by Zwielehner *et al.* (2011) (32).

In a subsequent study, Montassier *et al.* (2015) verified the previously described results concerning microbial diversity and differences at the phylum level (33). Additionally, abundance of *Ruminococcus, Oscillospira, Blautia, Lachnospira, Roseburia, Dorea, Coprococcus, Anaerostipes, Clostridium, Collinsella, Adlercreutzia* and *Bifidobacterium* were decreased after chemotherapy ($p<0.05$) while the abundance of *Citrobacter*, *Klebsiella*, *Enterococcus*, *Megasphaera* and *Parabacteroides* was increased ($p<0.05$) (33). Besides these profound changes in intestinal microbiota composition, shifts in microbial functions were observed by means of a computational approach. Amino acid metabolism ($p=0.0004$),

nucleotide metabolism (p=0.0001), energy metabolism (p=0.001) as well as metabolism of cofactors and vitamins (p=0.006) were depleted in samples collected after chemotherapy compared to samples collected before chemotherapy (33). Concurrently, signal transduction (p=0.0002), xenobiotics biodegradation (p=0.002) and glycan metabolism (p=0.0002) were enhanced (33). Furthermore, several other metabolic pathways, amongst others pathways involved in bacterial motility, virulence and epithelial repair, were altered after chemotherapy (33).

Galloway-Peña *et al.* (2016) observed similar dramatic changes in the intestinal microbiota composition in AML patients during induction chemotherapy (20). Using 16S rRNA gene sequencing, they identified a statistically significant progressive decrease in overall microbial diversity as well as decreased abundance of the anaerobic genus *Blautia* (20). On the other hand, chemotherapy caused increased abundance of *Lactobacillus* (20). Interestingly, chemotherapy also increased the occurrence of a phenomenon called intestinal domination, which means that more than 30% of the intestinal bacteria belong to a single taxon. After completion of chemotherapy, 50% of the domination events was caused by opportunistic pathogenic bacteria, known to induce bacteremia (e.g. *Staphylococcus*, *Enterobacter*, *Escherichia*). Before chemotherapy, this was only 20% (20). In addition, induction chemotherapy resulted in a high variation in temporal stability, as assessed by calculating the coefficient of variation (CV) of the Shannon index (21). Furthermore, high intra-patient temporal instability was associated with increased abundance of opportunistic pathogenic genera (21). High CV values were positively correlated with pathogenic genera such as *Staphylococcus* and *Streptococcus* and negatively associated with the non-pathogenic *Akkermansia* (21). Thus, a high relative abundance of *Akkermansia*, *Subdilogranulum* and *Pseudobutvrivibrio* was associated with a more stable microbiome during induction chemotherapy. Potentially pathogenic bacteria such as *Streptococcus* and *Staphylococcus* were more abundant in patients with a more variable microbiome (21).

Different studies focused on the effect of chemotherapy on gut microbiota composition in gastrointestinal cancers. For instance, Sze *et al.* (2017) collected pre- and post-treatment fecal samples of 26 colorectal cancer (CRC) patients treated with different types of chemotherapy (34). A significant change in community structure (β-diversity) between pre- and post-treatment samples was observed (p=0.005). Using random forest models, collections of OTUs were identified that differentiated between pre- and post-treatment samples (AUC 0.82-0.98) (34). However, no significant change in α-diversity between pre- and post-treatment samples was identified (34). The authors concluded that the community structure was affected by the treatment, but the effect of treatment was not consistent across patients (34). No subgroup analysis was performed for these very heterogeneous small groups receiving chemotherapy or chemoradiation. Next, Sze *et al.* constructed a random forest model using CRC patients (N=94) and healthy controls (N=172) in order to define a normal gut microbiota profile. Afterwards, it was indicated that gut microbiota composition of 19 out of 26 treated CRC patients (73%) shifted towards this normal profile (p=0.001) (34). Hence, it was concluded that the treatment induced a shift towards a microbial profile that has great similarity to the gut microbiota of healthy participants (34). These results are contradictory to the studies described before, which indicated deterioration of the gut microbiota instead of improvement.

Youssef *et al.* (2018) collected fecal samples of 20 treated patients with gastrointestinal neoplasms and 13 healthy controls (35). Gastrointestinal neoplasms included neoplasms of the stomach (N=6), small intestine (N=1) or rectum (N=13). Treatment included chemotherapy

and/or radiotherapy (35). 16S rRNA gene sequencing indicated that at the genus level, the α-diversity, genus richness and β-diversity did not significantly differ between controls (N=13) and non-treated patients (N=43) compared to treated patients (N=20). Patients treated with chemotherapy and/or radiotherapy had a significantly higher relative abundance of Lactobacillaceae and *Lactobacillus* compared to untreated patients with gastrointestinal neoplasms. In comparison to healthy controls, treated patients had a significantly lower relative abundance of Bifidobacteriaceae *Ruminiclostridium*, *Lachnoclosteridium* and *Oscillibacter* (35).

Similarly, Deng *et al.* (2018) compared fecal microbiota composition of 14 CRC patients treated with chemotherapy with 33 healthy controls (36). Chemotherapy consisted of the 5-fluoruouracil (5-FU) precursor tegafur and oxaliplatin. Compared to healthy controls, *Veillonella* at the genus level and *Veillonella dispar* at the species level were only present in CRC patients. *Prevotella copri and Bacteroides plebeius* were enriched in patients treated with chemotherapy compared to controls (36).

In a cohort of patients with different cancer types, Zwielehner *et al.* (2011) indicated that species richness within the *Clostridium cluster IV* was remarkably reduced immediately after chemotherapy, but recovered within 5-9 days after chemotherapy (32). Likewise, total bacterial abundance declined after chemotherapeutic treatment ($p=0.037$) and was also restored within a few days (32). Next to *Clostridium cluster IV*, the bacteria found to be affected most by chemotherapy were *Bacteroides*, *Bifidobacteria*, as well as *Clostridium cluster XIVa* (32).

In a similar study, it was demonstrated that cancer patients receiving chemotherapy for different cancer types were characterized by a decreased relative abundance of *Lactobacillus spp*., *Bacteroides spp*., *Bifidobacterium spp*. and *Enterococcus spp*. when compared to healthy controls (37). Increased relative abundance was found for *Escherichia coli* and *Staphylococcus spp*. (37). These findings were complemented with the observation that the abundance of *Escherichia coli* gradually increased during chemotherapy, while the initial increase of *Lactobacillus spp*. was followed by a decreased abundance after ten days (37).

Besides, some studies investigated the effect of chemotherapy on the gut microbiota in pediatric patients. In this context, Wada *et al.* (2010) reported that the start of chemotherapy induced an increase of the facultative anaerobic Enterobacteriaceae in children with malignancies (38).

In addition, another study with pediatric AML patients revealed that there was a considerable decrease in bacterial diversity during chemotherapy treatment, which restored quickly after chemotherapy (39). Furthermore, the total number of bacteria was found to be significantly reduced in patients during treatment but resembled the bacterial count in healthy samples six weeks after the last chemotherapy cycle (39). This reduced number of bacteria was caused by a 3000-6000 fold decrease of the anaerobic *Bacteroides*, *Clostridium cluster XIVa*, *Faecalibacterium prausnitzii* and *Bifidobacterium*. Interestingly, only *Clostridium XIVa* and *Faecalibacterium prausnitzii* levels were restored six weeks after treatment (39). The number of aerobic enterococci was significantly higher in patients compared to healthy controls, while the number of streptococci was 100-1000 fold decreased in patient samples (39). Of note, the disturbed balance marked by a dramatic reduction of anaerobic bacteria and increased enterococci levels might have negative consequences for the risk of infection and colonization with potentially pathogenic bacteria (39).

On the contrary, Rajagopala *et al.* (2016) indicated that there was no difference in microbial diversity before and during induction chemotherapy in patients with pediatric and adolescent

ALL (15). It was also shown that microbial diversity was significantly higher during maintenance chemotherapy compared to baseline, which is not in line with the results described above (15, 39). Of note, this study also demonstrated that microbial dysbiosis was already present at the time of diagnosis. By comparing ALL patients and their healthy siblings, it was found that ALL patients were characterized by decreased diversity and decreased relative abundance of Lachnospiraceae (including Clostridium XIVa, IV) *Roseburia, Anaerostipes, Coprococcus and Ruminococcus 2*. (15). *Bacteroides* occurrence was increased in these patients (15). In view of the fact that ALL patients suffer from an impaired immune system at the time of diagnosis (40), it might be suggested that the increasing microbial diversity during therapy might be interpreted as an indication for the anti-cancer effect of the therapy.

3.2. Immunotherapy

Six articles of five human clinical studies were identified that described human intestinal microbiota changes during immunotherapy assed by longitudinal sampling (25-28, 31, 41). Patients received anti-PD-1, anti-CTLA-4 or interferon alpha-2b therapy for either metastatic or unresectable melanoma, renal cell carcinoma (RCC), non-small cell lung cancer (NSCLC) or neuroendocrine tumors (NET).

Routy *et al.* (2018) collected longitudinal fecal samples of 32 patients that received two months anti-PD-1 therapy for NSCLC (N=15) or RCC (N=17) (26). Feces were collected before start of the treatment, as well as after the 2nd (one month), 4th (two months) and 12th (six months) anti-PD-1 infusion. The stool α-diversity (richness at metagenomic species (MGS) level) increased. Stool richness at MGS level increased more in RCC patients (p=0.033) compared to NSCLC and RCC patients together (p=0.046). None of the 32 patients received antibiotics. After two months anti-PD-1 therapy, the following bacteria were enriched: *Candidatus Alistipes marseilloanorexicus*, *Clostridium scindens*, *Eubacterium sp*., *Clostridium sp*., *Streptococcus salivarius*, *Clostridiales*, *Eubacterium eligens* (26).

Chaput *et al.* (2017) collected longitudinal fecal samples of 26 patients with metastatic melanoma (27). Patients received four cycles of ipilimumab every three weeks. Fecal samples were collected before the first ipilimumab infusion (N=26), before each following infusion (V2: N=14, V3: N=15, V4: N=13) and 3 weeks after the last infusion (N=4). It was observed that the phyla Firmicutes and Bacteroidetes remained stable during treatment with ipilimumab. Additionally, Shannon and Simpson α-diversity indices did not change during ipilimumab treatment, thereby suggesting that ipilimumab treatment did not modify the gut microbiota (27). However, it should be noted that the number of fecal samples analyzed decreased to four over time (27). While there was no direct effect of ipilimumab on the gut microbiota in this study, the authors reported changes in gut microbiota composition at the time of colitis occurrence during ipilimumab treatment. Therefore, fecal samples of seven patients with colitis were collected and compared with baseline samples. At family level (p=0.0049) as well as at genus level (p=0.0059), significant differences in microbiota composition were observed. Relative abundance of seven dominant genera (*Ruminococcus, Lachnospiracea incertae sedis, Blautia, Clostridium IV, Eubacterium, unclassified Lachnospiraceae* and *Pseudoflavonifracto*) was significantly reduced in metastatic melanoma patients with ipilimumab-induced colitis (27). They all belong to the Firmicutes phylum. Furthermore, 18 other bacteria, mostly Firmicutes, were significantly reduced (Table 2). Ipilimumab-induced

colitis was also associated with lower α-diversity (27). However, these microbial perturbations were most likely caused by the colitis instead of the therapy itself.
Prior to this study, Vetizou *et al.* (2015) already published results concerning gut microbiota composition in patients with metastatic melanoma before (N=19) and after (N=18) treatment with ipilimumab (41). These patients were later also described in the article of Chaput *et al.* (2017) in relation to ipilimumab-induced colitis (27). Patients were divided into three clusters based on genus composition. Cluster A was enriched in *Alloprevotella* and *Prevotella;* cluster B was enriched with relatives of *Prevotella copri, Bacteroides sp. CCUG 39913, Barnesiella intestinohominis YIT 11860 and Parabacteroides distasonis M86695* and cluster C was enriched in *Bacteroides salyersiae WAL 10018, Bacteroides acidifaciens AB021157* and *Bacteroides uniformis JCM 5828T (*41*)*. During ipilimumab treatment, the proportion of patients in cluster C increased (p=0.05) whereas it decreased in cluster B (p=0.007) (41). Interestingly, it has been shown that tumors in mice treated with ipilimumab respond better to fecal microbiota transplantation (FMT) of cluster C patients compared to FMT with cluster B enterotypes. This suggests that ipilimumab might modify the enterotype to the more favorable cluster C (41).
Additional to the studies of Routy, Chaput and Vetizou, three studies performed longitudinal fecal sampling of a limited number of melanoma patients treated with immunotherapy. Gopalakrishnan *et al.* (2018) tested the stability of the gut microbiome during anti-PD-1 therapy in only three patients. Median time to repeat collection was 49 days (31 – 78 days) after initial sampling. They concluded that the α-diversity and microbiome composition at the order level was relatively stable during longitudinal sampling (25). On the contrary, Frankel *et al.* (2017) performed longitudinal sampling, within one month after therapy initiation, of five patients (four responders and one patient with progressive disease) who received ipilimumab with nivolumab (N=4) or pembrolizumab (N=1) (28). They concluded that specific gut microbiota abundances changed, but that these numbers were too small to draw conclusions (28). In 2012, Dörffel *et al.* collected fecal samples in eleven patients with NET before and during interferon alpha-2b therapy. After four weeks of therapy, they observed by using FISH that interferon alpha-2b therapy was able to increase the concentration of *Faecalibacterium prausnitzii* to almost normal levels (31).

3.3. Hormonal therapy

Currently, only two studies are available that investigated human intestinal microbiota changes during hormonal therapy (31, 42).
Dörffel *et al.* (2012) collected fecal samples of 27 patients receiving somatostatin analogs for NET. It was observed that somatostatin analogs had no influence on the abundance of specific bacterial groups in these patients (31).
Sfanos *et al.* (2018) compared intestinal microbiota of patients with prostate cancer treated with androgen axis-targeted therapies compared to no hormonal medication use (42). Androgen axis-targeted therapies included treatment with gonadotropin-releasing hormone (GNRH) (N=5) or androgen receptor axis-targeted therapies (ATT) (N=9). The group without hormonal medication included healthy controls (prostatic hyperplasia, N=6), benign tumors (negative biopsy for prostate cancer, N=3) and prostate cancer patients without therapy (N=7). This study indicated no significant difference in α-diversity between prostate cancer patients treated with or without hormonal medication. The β-diversity was smallest within the ATT group compared to GNRH and the group without hormonal medication. The greatest β-

diversity was seen between the ATT and the no medication group (42). Together, these results indicate that the gut microbiota was most similar within the group of patients receiving ATT, while their microbiota was most dissimilar to that of the no medication group. Furthermore, ATT seemed to induce a low β-diversity. In the fecal samples of men taking oral ATT (N=9) compared to no medication use (N=16), several bacteria were significantly altered at species and/or family level (Table 3) (42). In addition, it was confirmed that *Akkermansia muciniphila* was significantly more prevalent in men taking oral ATT, using quantitative Polymerase Chain Reaction (qPCR), (42). As indicated in table 3, abundance of several bacteria at the species and family level was altered in men taking oral GNRH (N=5) when compared to the group without use of hormonal medication (N=16) (42).

Table 2. *Clinical studies assessing intestinal microbiota changes during systemic cancer therapy by longitudinal sampling*

STUDY DESIGN						MAIN FINDINGS
Study	**Type of cancer**	**N**	**Type of therapy**	**Sampling time points**	**Method used for microbiota analysis**	**Effects of therapy on microbiota**
Chemotherapy						
Galloway-Peña et al. (2017)	AML	N=35	Induction chemotherapy	Baseline: before or within first 24h of chemotherapy; Follow-up: every 96h until neutrophil recovery	16S rRNA gene sequencing	↑ intra-patient temporal variability of α-diversity (CV of Shannon) ↑ *Staphylococcus* ↑ *Streptococcus* ↑ *Akkermansia* ↑ *Subdilogranulum* ↑ *Pseudobutyrivibrio*
Sze et al. (2017)	CRC	N=26	12 surgery 9 surgery + chemotherapy 5 surgery + chemotherapy + radiation	Before and after treatment	16S rRNA gene sequencing	Change in community structure Shift towards healthy microbiota
Galloway-Peña et al. (2016)	AML	N=34	Induction chemotherapy	Baseline: before therapy; Follow-up: every 96h until neutrophil recovery	16S rRNA gene sequencing	↑ *Lactobacillus* ↓ α-diversity (Shannon index) ↓ *Blautia*
Rajagopala et al. (2016)	ALL	N=28	Chemotherapy	(1) Before therapy, (2) during induction chemotherapy (3) during consolidation chemotherapy (4) during maintenance chemotherapy	16S rRNA gene sequencing	↑ α-diversity (Shannon index)
Montassier et al. (2015)	Non-Hodgkin's lymphoma	N=28	Chemotherapy	Baseline: before chemotherapy; Follow-up: 7 days later	16S rRNA gene sequencing	↑ Proteobacteria ↑ *Citrobacter* ↑ *Klebsiella* ↑ *Enterococcus* ↑ *Megasphaera* ↑ *Parabacreroides* ↓ α-diversity (Faith's phylogenetic diversity, observed species) ↓ Firmicutes ↓ Actinobacteria

						↓ *Ruminococcus* ↓ *Oscillospira* ↓ *Blautia* ↓ *Lachnospira* ↓ *Roseburia* ↓ *Dorea* ↓ *Coprococcus* ↓ *Anaerostipes* ↓ *Clostridium* ↓ *Collinsella* ↓ *Adlercreutzia* ↓ *Bifidobacterium*
Montassier et al. (2014)	Non-Hodgkin's lymphoma	N=8	Chemotherapy	Baseline: before chemotherapy; Follow-up: 1 week after chemotherapy	16S rRNA gene pyrosequencing / dHPLC	↑ Bacteroidetes ↑ Proteobacteria ↑ *Bacteroides* ↑ *Escherichia* ↓ α-diversity (OTUs, Chao index, Shannon index) ↓ Firmicutes ↓ Actinobacteria ↓ *Blautia* ↓ *Faecalibacterium* ↓ *Roseburia* ↓ *Bifidobacterium*
Stringer et al. (2013)	Breast cancer, gastrointestinal cancer	N=10	Chemotherapy (FOLFOX4, FOLFOX6, FOLFIRI, capecitabine)	(1) Before chemotherapy (2) Day 2 of chemotherapy (3) Day 5 (4) Day 10	Bacterial growth tests with selective media, real-time PCR	↑ *E.coli* ↑ *Lactobacillus spp.* (until day 5, then decrease)
Dörffel et al. (2012)	NET	N=13	Chemotherapy	Before and during therapy	FISH	↑ *Faecalibacterium prausnitzii* (midgut NET only)
Zwielehner et al. (2011)	Different types of cancer	N=17	Chemotherapy	(1) Before chemotherapy (2) Day 1-4 after chemotherapy (5) Day 5-9 after chemotherapy	qPCR/PCR-DGGE	↓ *Bacteroides* ↓ *Bifidobacteria* ↓ *Clostridium cluster IV* ↓ *Clostridium cluster XIVa*
		N=2	Chemotherapy	(1) Before chemotherapy (2) Day 1-4 after chemotherapy	High throughput sequencing	↑ *Enterococcus faecium* ↑ *Clostridium difficile* ↑ Peptostreptococcaceae ↓ *Faecalibacterium prausnitzii*

						↓ Lactobacilli ↓ *Veillonella spp.* ↓ Bifidobacteria ↓ *E.coli/Shigella*
Wada et al. (2010)	Different types of cancer	N=23	Chemotherapy	(1) Before chemotherapy (2) Within 24h after initiation (3) Once weekly	Bacterial cultures (N=3)	↑ Enterobacteriaceae
Van Vliet et al. (2009)	Pediatric AML	N=9	Chemotherapy	(1) Day 2 of chemotherapy (2) Day 11 of chemotherapy (3) ≥6 weeks after treatment	PCR-DGGE	↓ α-diversity
Immunotherapy						
Routy et al. (2018)	NSCLC (N=15) RCC (N=17)	N=32	anti-PD-1	(1) Before treatment (2) After 2nd injection (1 month) (3) After 4th injection (2 months) (4) After 12th injection (6 months	Metagenomic shotgun sequencing	↑ α-diversity (Richness) ↑ *Candidatus Alistipes marseilloanorexicus* ↑ *Clostridium scindens* ↑ *Eubacterium sp.* ↑ *Clostridium sp.* ↑ *Streptococcus salivarius* ↑ *Clostridiales* ↑ *Eubacterium eligens*
Chaput et al. (2017)	Metastatic melanoma with colitis	N=7	Ipilimumab	At baseline and at the time of colitis occurrence	16S rRNA gene sequencing	↓ α-diversity (Shannon index) ↓ *Ruminococcus* ↓ *Lachnospiracea incertae sedis* ↓ *Blautia* ↓ *Clostridium IV* ↓ *Eubacterium* ↓ *unclassified Lachnospiraceae* ↓ *Pseudoflavonifracto* ↓ Butyrate producing bacterium L2-21

						↓ *Ruminococcus bromii* ↓ *Blautia obeum 1-33* ↓ *Eubacterium coprostanoligenes HL* ↓ *Clostridium clostridioforme LCR24* ↓ *Alistipes spe 627* ↓ *Blautia obeum* ↓ Butyrate producing bacterium PH08AY04 ↓ *Clostridium leptum DSM 753T* ↓ *Bacterium ASF500* ↓ *Clostridium sp JC3* ↓ *Rumen bacterium 2-293-25* ↓ *Bacterium ic* ↓ Butyrate producing bacterium M21-2 ↓ Unidentified bacterium CCCM23 ↓ Unidentified bacterium CCCM41 ↓ *Ruminococcus bromii L2-63* ↓ *Clostridiales bacterium JN18-V41*
Vetizou et al. (2015)	Metastatic melanoma	N=18	Ipilimumab	See Chaput et al. (2017)	16S rRNA gene sequencing	↑ *Bacteroides salyersiae* ↑ *Bacteroides acidifaciens* ↑ *Bacteroides uniformis* ↓ *Prevotella copri* ↓ *Bacteroides sp.* ↓ *Barnesiella intestinohominis* ↓ *Parabacteroides distasonis*
Dörffel et al. (2012)	Midgut NET	N=11	Interferon alpha-2b	Before and during therapy	FISH	↑ *Faecalibacterium prausnitzii*

↑: Increase, ↓:Decrease, **AML:** Acute myeloid leukemia, **CRC:** Colorectal cancer, **ALL:** Acute lymphoblastic leukemia, **dHPLC:** Denaturing High-Performance Liquid Chromatography, **NET:** neuroendocrine tumor, **PCR-DGGE:** Polymerase chain reaction denaturing gradient gel electrophoresis**, RCC:** renal cell carcinoma, **NSCLC:** non-small cell lung cancer, **FISH:** Fluorescent in situ hybridization

Table 3: *Clinical studies assessing intestinal microbiota changes during systemic cancer therapy by cross-sectional sampling*

STUDY DESIGN						MAIN FINDINGS
Study	**Type of cancer**	**Type of therapy**	**N Cases**	**N Controls**	**Method used for microbiota analysis**	**Effects of therapy on microbiota**
Chemotherapy						
Youssef et al. (2018)	Gastrointestinal cancer	Chemotherapy and / or radiotherapy	N=20 (treated patients)	Non-treated patients: N=43	16S rRNA gene sequencing	↑ Lactobacillaceae ↑ *Lactobacillus*
				Healthy controls: N=13	16S rRNA gene sequencing	↓ Bifidobacteriaceae ↓ *Ruminiclostridium* ↓ *Lachnoclosteridium* ↓ *Oscillibacter*
Deng et al. (2018)	CRC	Oxaliplatin + tegafur	N=14	N=33	16S rRNA gene sequencing	↑ *Veillonella* ↑ *Veillonella dispar* ↑ *Prevotella copri* ↑ *Bacteroides plebeius*
Stringer et al. (2013)	CRC, breast cancer, laryngeal cancer, esophageal cancer, melanoma	Chemotherapy	N=16	N=2	Bacterial growth tests with selective media, real-time PCR	↑ *Escherichia coli* ↑ *Staphylococcus spp.* ↓ *Lactobacillus spp.* ↓ *Bacteroides spp.* ↓ *Bifidobacterium spp.* ↓ *Enterococcus spp.*
Van Vliet et al. (2009)	Pediatric AML	Chemotherapy	N=9	N=11	FISH	↑ Enterococci ↓ total number of bacteria ↓ *Bacteroides* ↓ *Clostridium cluster XIVa* ↓ *Faecalibacterium prausnitzii* ↓ *Bifidobacterium* ↓ Streptococci
Hormonal therapy						
Sfanos et al. (2018)	Prostate cancer	ATT / GNRH	ATT: N=9 GNRH: N=5	N=16 (no medication)	16S rDNA sequencing	Smallest β-diversity within ATT compared to GNRH and controls Greatest β-diversity between ATT and no medication

	ATT	N=9	N=16 (no medication)	16S rDNA sequencing	↑ *Akkermansia muciniphila* ↑ Ruminococcaceae ↑ *Blautia wexlerae* ↑ *Clostridium oroticum* ↑ *Lachnospiraceae_Clostridium_XlVa* ↑ *Robinsoniella peoriensis* ↑ *Anaerococcus tetradius* ↑ *Bacteroides stercoris* ↑ Verrucomicrobiaceae ↑ Lachnospiraceae ↑ *Clostridiales insertae sedis XIII* ↑ Staphylococcaceae ↑ Bacillales ↑ Aerococcaceae ↑ Selenomonadales ↓ Clostridiales ↓ Brevibacteriaceae ↓ Erysipelotrichaceae ↓ Streptococcaceae ↓ Clostridiales_unassigned ↓ Prevotellaceae
	ATT	N=9	N=16 (no medication) N=5 (GNRH)	qPCR	↑ *Akkermansia muciniphila*
	GNRH	N=5	N=16 (no medication)	16S rDNA sequencing	↑ *Blautia wexlerae* ↑ *Clostridium oroticum* ↑ *Anaerococcus tetradius* ↑ Lachnospiraceae ↑ Staphylococcaceae ↑ Aerococcaceae ↑ Selenomonadales

↑: Increase, ↓: Decrease, **AML:** Acute myeloid leukemia, **CRC:** Colorectal cancer, **FISH:** Fluorescent in situ hybridization, **ATT:** androgen receptor axis-targeted therapy, **GNRH:** gonadotropin-releasing hormone

4. Discussion

Awareness of the interaction between the human intestinal microbiota and systemic cancer therapy is increasing and results gained in this field of research potentially have considerable clinical implications. This review provided a detailed overview about all clinical studies describing the association between baseline intestinal microbiota and systemic cancer therapy as well as the influence of systemic cancer therapy on gut microbiota composition. We focused on systemic cancer therapy with chemotherapy, immunotherapy and hormonal therapy.

4.1. Baseline human intestinal microbiota is associated with the development of complications and systemic cancer therapy outcome

It became evident that baseline microbiota composition is associated with the development of (infectious) complications as well as with the outcome of systemic cancer therapy.
In the context of chemotherapy, research mainly focused on the association between baseline human intestinal microbiota composition and the development of chemotherapy associated complications, such as infections or diarrhea. It seems that patients with a particular intestinal microbiota are more prone to develop infections, likely as a result of a reduced colonization resistance, and that a beneficial intestinal microbiota might be protective. This is particularly interesting, since early identification of patients at risk for the development of complications would enable targeted interventions and the prevention of infectious complications in the future. Generally, gut microbiota composition of patients with infectious complications was characterized by reduced microbial diversity and increased microbial instability. Furthermore, *Stenotrophomonas* and *Bacteroides* were found to be increased, while *Barnesiellaceae*, *Christensenellaceae*, Faecalibacterium and *Prevotella* were reduced. This suggests that these characteristics might be useful to identify patients at risk to develop subsequent infections.

To our knowledge, there is currently no clinical study published investigating the effect of the intestinal microbiota on chemotherapy efficacy. However, results from *in vitro* studies strongly suggest an interaction between the gut microbiota and chemotherapy. For instance, it has been shown that the addition of *Lactobacillus plantarum* supernatant potentiates the therapeutic effect of 5-fluorouracil (5-FU) in chemoresistant cells (16). In line with this, administration of an antibiotic cocktail markedly diminished the antitumor efficacy of 5-FU in mice (43). Considering the complexity of the interaction, various metabolic pathways might be involved in microbial metabolism of chemotherapeutic drugs. The field of pharmacomicrobiomics focusses on unravelling these interactions between drugs and the human microbiome (44).
In this context, Alexander *et al.* (2017) suggested the TIMER mechanistic framework (**T**ranslocation, **I**mmunomodulation, **M**etabolism, **E**nzymatic degradation, **R**educed diversity) to describe the mechanisms through which the gut microbiota might modulate chemotherapy treatment (45). According to this concept, bacterial **T**ranslocation might be facilitated by chemotherapeutic drugs which damage the intestinal barrier (45). Subsequently, intestinal bacteria or their products can shape the chemotherapy-induced immune response by **I**mmunomodulation (45). In support of this, Viaud *et al.* (2013) described that the intestinal

microbiota influences the anticancer immune effects of cyclophosphamide by modulation of T-helper cells (17).
The most direct effect of the gut microbiota on drug metabolism is through **M**etabolism and **E**nzymatic degradation. Several gut bacteria-derived enzymes metabolize chemotherapeutic drugs and their metabolites, thereby modulating efficacy as well as toxicity (44, 45). Zimmermann *et al.* (2019) indicated that the orally administered capecitabine, cyclophosphamide, melphalan and paclitaxel can be metabolized by specific bacterial strains (46). A further example for direct microbial metabolism of chemotherapeutic drugs is a thymidine phosphorylase encoded by *Mycoplasma hyorhinis*. Activity of this enzyme has been shown to reduce the cytotoxic activity of several pyrimidine nucleoside analogues (47) In contrast, the same thymidine phosphorylase enhanced cytotoxicity of capecitabine, probably by converting the pro-drug into the cytotoxic 5-FU (47).
Another factor with a considerable impact on cancer therapy outcome is chemotoxicity. High chemotoxicity often results in dose reduction or premature termination of the therapy, thereby severely limiting effectivity. In the case of the chemotherapeutic drug irinotecan, it is well described that microbial metabolism enhances chemotoxicity. It has been indicated that the bacterial enzyme β-glucuronidase reactivates previously detoxified SN-38G into the active metabolite SN-38, leading to severe toxicity in the gut (48). In support of this, targeted inhibition of bacterial β-glucuronidase has been shown to alleviate gastrointestinal toxicity in mice (49).
Lastly, **R**educed microbial diversity and ecological variation might also affect the chemotherapy response of the host (45). As shown in the present review, chemotherapy induces changes in gut microbiota composition and diversity. As a result, dysbiosis and overgrowth of potentially pathogenic bacteria might also have negative consequences for the treatment response.
To conclude, it can be stated that we are currently only beginning to understand the whole biological complexity of microbiota-chemotherapy interactions.

Limited (N=6), but recently published articles describe the association of baseline human intestinal microbiota with immunotherapy outcome. The results of these studies suggest that a diverse and specific human intestinal microbiota (enriched in *B. longum* (24), *Faecalibacterium (*25*,* 27*,* 28*)* and *A. muciniphila (*26*)* and a reduced number of Bacteroidales (25) and Bacteroides (27)) stimulates and trains the immune system. This might result in increased antigen presentation, an improved effector T-cell function (increased CD4+, CD8+ T-cells) and lower levels of regulatory T-cells (25). An active and well developed immune system stimulates beneficial T-cell activation and consequently a diverse repertoire of T-cells (50). Subsequently, this diverse pool of beneficial T-cells will be able to combat cancer cells by expressing multiple PD-1 receptors. PD-1 promotes apoptosis, reduces suppressive T-cells and stimulates inflammation, resulting in an increased tumor response as well as inflammatory side effects like colitis. Consequently, it might be speculated that tumors had to be better developed and probably need multiple keys (PD-L1's) in order to lock all the PD-1 receptors and to escape this efficient immune system. Based on this, it can be concluded that a more diverse T-cell repertoire, stimulated by a diverse intestinal microbiota, might inhibit tumor growth besides tumor suppressive effects of anti-PD-1 therapy. This theoretical basis evokes the question whether the immune modulatory effect of immunotherapy can be potentiated by the action of specific gut bacteria.

Results from mouse experiments provided further evidence for the immune modulatory effects of the intestinal microbiota. By transplanting baseline fecal microbiota of responder and non-responder patients to germ-free and tumor bearing mice, it was revealed that the clinical response was repeated in the majority of mice (24-26). Moreover, the immune stimulatory effect of anti-CTLA-4 blockade reactivated T-cells, which resulted in anti-cancer response (27), but also immune-mediated colitis (27, 29). Bacteroidetes seemed to be associated with this clinical presentation (27, 29). Bacteroidetes could stimulate differentiation of regulatory T-cell (51) and consequently suppress the immune systems anti-cancer potency on the one hand and reduce colitis on the other hand.
The above presented mechanisms and interpretations indicate that the intestinal microbiota stimulates the immune system via multiple pathways. This might suggest that the addition of immunomodulation by microbiota modulation could be much more efficient compared to immunotherapy alone, since simultaneous activation of multiple tumor-suppressing pathways will inhibit tumor growth in a more efficient way.

Currently, there are no studies available that describe the role of the human intestinal microbiota in hormonal therapy, which is administered in hormone related malignancies like breast cancer, prostate cancer and ovarian cancer. Since these are common malignancies with high morbidity, research is urgently required to investigate if baseline human intestinal microbiota is associated with hormonal therapy outcome.

4.2. Human intestinal microbiota changes during systemic cancer therapy

By definition, systemic cancer therapies affect the whole body. Therefore, it is not surprising that several studies investigated the effect of systemic cancer therapy on intestinal microbiota composition and that dramatic changes were reported.

Different studies investigated chemotherapy-induced changes of human gut microbiota composition by collecting fecal samples before and during chemotherapy. While only one study reported increased α-diversity during chemotherapy compared to baseline (15), the majority of the studies reported a chemotherapy-induced decrease of microbial diversity (6, 20, 33, 39). Furthermore, we identified key species which were shown to be affected in several studies. Abundance of Proteobacteria and Staphylococcus was found to be elevated due to chemotherapy. On the other hand, the phyla Firmicutes and Actinobacteria seemed to be negatively influenced by chemotherapy, leading to decreased levels of these bacteria. More specifically, *Blautia*, *Roseburia*, *Bifidobacterium*, as well as *Clostridium cluster IV* and *XIVa* were consistently found to be decreased during chemotherapy. For *Lactobacillus*, *Bacteroides*, *E.coli* and *Faecalibacterium prausnitzii* the results were divergent, meaning that some studies reported increased abundance while others showed the opposite.
Interestingly, the bacteria found to be reduced during chemotherapy are prominent short-chain fatty acid (SCFA) producing bacteria. SCFA are produced by microbial fermentation of non-digestible carbohydrates and are considered to fulfill a crucial role in colonic health. In particular butyrate is essential for gut barrier integrity, since it serves as energy source for colonocytes (52). Moreover, *in vitro* studies showed that SCFA regulate the expression of tight-junction proteins and mucins (52). In addition, SCFA have been shown to have potent anti-inflammatory as well as direct anti-carcinogenic effects (52). Therefore, it might be

suggested that the observed decrease in SCFA producing bacteria during chemotherapy might also have consequences for colonic SCFA concentrations and subsequently for the development of colonic inflammation and anti-cancer efficacy of the therapy.
Next to SCFA, there are also other metabolites of the gut microbiota that fulfill crucial physiological roles. In this context, secondary bile acids, branched chain fatty acids as well as amino acids are repeatedly suggested as key metabolites (53). The gut microbiota produces secondary bile acids by converting primary bile acids which were produced in the liver (54). Mikó *et al.* (2018) showed that the secondary bile acid lithocholic acid inhibited cancer cell proliferation, tumor infiltration as well as metastasis formation and improved the anti-tumor immune response (55). Very recently, Colosimo *et al.* (2019) applied large-scale functional screening of molecules produced by gut bacteria in order to identify bacterial metabolites agonizing G-protein-coupled receptors (GPCRs) (56). Amongst others, they identified phenylpropanoic acid, the amino acid cadaverine as well as the branched-chain fatty acid 12-methyltetradecanoic acid as promising molecules capable of modulating human signaling pathways through GPCR agonism (56). Furthermore, microbial metabolites might also play a role in the indirect modulation of drug response (44).
Another interesting result of the current overview about changes in gut microbiota composition during chemotherapy is that specific bacteria might be more vulnerable to chemotherapy, compared to others. This might facilitate colonization with potentially pathogenic bacteria, such as Staphylococcus and many species belonging to the Proteobacteria phylum. As described by Zwielehner *et al.* (2011) (32) and Montassier *et al.* (2014) (6), elimination of specific bacteria might also lead to the appearance of less abundant bacterial genera. This phenomenon can be characterized by the term ‘functional response diversity’ which describes the different sensitivity of species to changes in the ecosystem (8). This, in combination with the reduction of potentially health-promoting bacteria, might lead to severe dysbiosis in patients during chemotherapy, with possible negative consequences for chemotoxicity and treatment outcome.

In the field of immunotherapy, six studies collected longitudinal samples to determine human intestinal microbiota changes during immunotherapy with anti-PD-1, anti-CTLA-4 or interferon alpha-2b. Longitudinal microbiota sampling during four cycles of immunotherapy showed increases of microbiota richness and specific genera in one study (26), but did not affect microbiota diversity and the abundance of Firmicutes and Bacteroides in another (27). In this study, only patients who developed colitis showed a reduced diversity and a significant difference in bacteria belonging to the Firmicutes phylum (27). Vetizou *et al.* (2015) (41), who used the same patient population as described by Chaput *et al.* (2017) (27), observed microbiota changes during ipilimumab treatment.
Mechanisms by which immunotherapy influences intestinal microbiota composition are sparsely studied and are mainly based on mouse studies (57). There are indications that anti-PD-1 therapy stimulates T-cell responses against intestinal bacteria and consequently improves cancer cell surveillance and detection (26, 58). In mice, anti-CTLA-4 therapy promoted pro-inflammatory pathways and induced intestinal epithelial cell death and proliferation. In patients, anti-CTLA-4 therapy led to microbial dysbiosis at the genus level by a not yet fully explored mechanism. Dysbiosis promoted T-helper 1 and dendritic cell maturation in humans. This consequently affected anti-cancer therapy efficacy (41).
Based on the available studies and proposed mechanisms, no strong conclusions could be drawn. Additional clinical research should reveal if immunotherapy influences the human

intestinal microbiota composition and its relation with anti-cancer therapy efficacy. If immune modulatory effects could be attributed to the intestinal microbiota composition changes, future systemic cancer therapies could probably be independent of one specific targeted immunotherapy and should instead, focus on microbiota composition changes.

Unfortunately, microbiota changes during hormonal therapy remain poorly described. We identified only two studies published in this field. One study collected longitudinal microbiota samples and the other performed cross-sectional microbiota sampling. Hormonal therapies consisted of somatostatin analogs or androgen axis-targeted therapies with ATT or GNRH (31, 42). No explanation is available why somatostatin analogs had no influence on human intestinal microbiota. Treatment with ATT resulted in a microbiota with low β-diversity. Both ATT and GNHR therapy were related to significant microbiota composition changes in patients with prostate cancer. However, this cross-sectional study compared patients treated with hormonal therapy with healthy controls but also patients without treatment, resulting in small and heterogeneous groups.
The observations could be explained by the potential influence of androgen axis therapy on bacterial steroid biosynthesis (59). Assuming that hormonal therapy interacts with the intestinal microbiota involved in steroid/hormone synthesis, this might modulate steroid biosynthesis, thereby affecting systemic hormone levels and therapy efficacy (12, 60, 61).

4.3 Strengths and Limitations

Gut microbiota research is a field with great biological complexity, imposing considerable challenges on the researchers. A strength of particularly the more recent studies is the use of 16S rRNA gene sequencing or even WMGS. These techniques are superior to other microbiota profiling techniques, like qPCR, PCR-DGGE or FISH and provide a detailed overview of microbiota composition, with high taxonomic resolution. WMGS even offers the possibility to quantify functional capacity of the gut microbiota.
However, we also identified several limitations, reducing generalization of the results. First of all, it is difficult to compare the different studies under investigation, since sampling time points, study design and methods used for microbiota profiling were highly heterogeneous. For example, in the study of Gopalakrishnan *et al.* (2018), baseline sampling took place over a broad range of days. Furthermore, different approaches were used in order to distinguish between responders and non-responders or to quantify treatment response and complications.
Next to the heterogeneity between studies, some studies also suffer from high heterogeneity within the study, due to the inclusion of patients with different cancer types and/or different drugs. This leads to a study population with a high level of heterogeneity and inadequate comparability. Another limitation is the relatively small population size studied in the majority of the studies. Since gut microbiota composition is known to be highly different between individuals (62), greater sample sizes are needed. The problem of small sample sizes gets even worse due to substantial loss to follow up, resulting in small groups to draw conclusions on.
Since gut microbiota composition is influenced by several external factors, the risk of bias is generally high in microbiota research. Strong confounding factors in this field are antibiotic use, age,BMI and diet (62). Attention and correction for these confounders was very different

between the studies (Appendix A). In most of the studies, the measurement of potential confounders was considered to be insufficient. Particularly the regularly observed insufficient assessment of and correction for previous antibiotic use might be problematic, since antibiotics have also been shown to be associated with (breast) cancer risk (63). Besides, it has been recently demonstrated that antibiotics modulate gut microbiota composition and metabolite production as well as key metabolic processes and tumor growth (64). These findings support the necessity that antibiotic use should be adequately reported in clinical microbiota studies and that patients with previous antibiotic use should be distinguished from patients without use of antibiotics.
Despite the fact that systemic cancer therapy most likely also affects stool consistency and bacterial biomass, changes in these parameters were hardly assessed in the studies under investigation. Vandeputte *et al.* (2016) showed that stool consistency was strongly associated with microbiota richness as well as with community composition and abundance of specific enterotypes (65). Consequently, studies neglecting these parameters might imply the risk that reported changes in gut microbiota composition and diversity can be attributed to changes in microbial biomass. Therefore, it is considered to be of great benefit for microbiota research to correct for this strong confounding effect.

5. Materials and Methods

5.1. Review questions

Main questions for this review were if:

- Baseline human intestinal microbiota was associated with systemic cancer therapy outcome
- Human intestinal microbiota changed during systemic cancer therapy

5.2. Review search

A thorough systematic literature search was performed using the following databases: Annual review, BioMed Central, Cochrane Library, EBMR, EMBASE, Informa Healthcare, Medline and PubMed.
By using the Boolean Search Operator, the following query was created: “(((((Microbiota OR microbiome OR “gut microbiota” OR “gut microbiome” OR “intestinal microbiota” OR “intestinal microbiome” OR “gastrointestinal microbiota” OR “gastrointestinal microbiome”)) AND (“cancer treatment” OR “cancer treatments” OR “cancer therapy” OR “cancer therapies” OR “anticancer therapy” OR “anticancer therapies” OR “systemic therapy” OR “systemic therapies” OR chemotherapy OR chemotherapies OR chemotherapeutics OR “hormone treatment” OR “hormone treatments” OR “hormone therapy” OR “hormone therapies” OR immunotherapy OR immunotherapies OR “antineoplastic”)) AND (Cancer OR neoplasm OR neoplasms)) AND (Human OR humans)) NOT (Murine OR mice OR mouse OR rat OR rats)”. No limits were set in any database. Predefined inclusion and exclusion criteria were used for article selection. The last search was performed April 22nd 2019.

5.3. Eligibility criteria

The systematic search was structured by means of the PICOS acronym (Participants, Interventions, Comparators, Outcome measures, Study design).

The PICOS criteria were identified as follows:

- Types of participants: human participants with any type of cancer
- Types of interventions: systemic cancer therapy with chemotherapy, immunotherapy or hormone therapy.
- Types of Comparators: studies comparing baseline and or follow up intestinal microbiota composition in patients starting and/or receiving systemic cancer therapy with either healthy controls, no intervention, follow up samples and/or therapy outcomes.
- Types of outcome measures: intestinal microbiota associated therapy outcomes and intestinal microbiota composition changes analyzed with any type of detection method.
- Types of study design: observational studies or intervention studies with a control and/or placebo group.

All studies that did not fulfill the PICOS characteristics were excluded. In addition, animal studies, conference papers, abstracts as well as articles that were not available in full text in Dutch or English were excluded.

5.4. Study selection

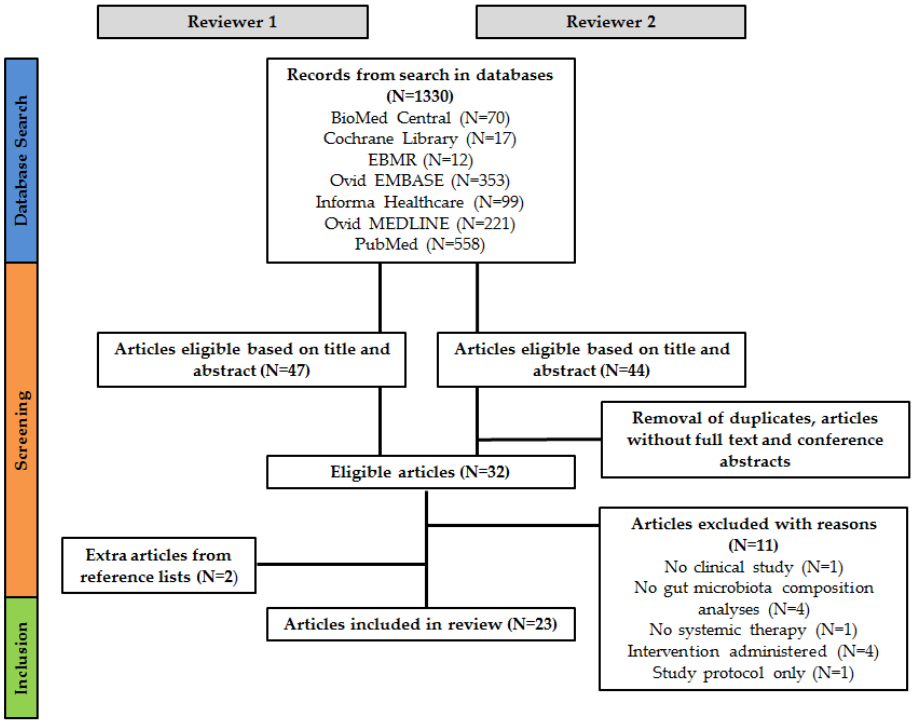


Figure 2. *Schematic overview of the article selection procedure*

Two researchers (R.A. and J.Z.) independently examined the databases for eligible articles based on title and abstract. Duplicates, articles without full text available and conference abstracts were removed. With regard to the remaining articles, discrepancies between the two reviewers were discussed until agreement for in- or exclusion was reached. This generated a list of 32 articles. Subsequently, both researchers read the full text of the articles which led to the exclusion of another eleven articles. Additionally, the reference lists of included articles were screened for additional articles and these were included after approval of the second reviewer. Finally, 23 articles were included in the current review. Figure 2 provides an overview of the article selection process.

5.5. Data Collection Process

Data extraction was conducted following a data extraction sheet conform Tables 1-3. Data extraction was performed in an unblinded and independent manner by the two reviewers (R.A. and J.Z.). Disagreements were discussed and resolved until consensus was reached.

5.6. Risk Of Bias Assessment

The risk of bias in the individual studies was evaluated with the Quality In Prognosis Studies (QUIPS) tool, which is recommended by the Cochrane Prognosis Methods Group to assess the risk of bias in prognostic studies (67). The QUIPS tool consisted out of the following domains: (1) Study Participation, (2) Study Attrition, (3) Prognostic Factor Measurement, (4) Outcome measurement, (5) Study Confounding, (6) Statistical analysis and reporting. Based on whether specific criteria were fulfilled or not, the risk of bias per domain was defined as low, moderate or high (Appendix A). Two reviewers (R.A. and J.Z.) independently assessed the risk of bias and consensus was reached afterwards.

6. Future directions

The rapidly growing number of publications concerning microbiota – cancer therapy interactions emphasizes the great relevance of the topic. However, evidence obtained in a clinical setting is still limited. Therefore, there is an urgent need for well controlled human studies to further elucidate the role of the gut microbiota in human cancers and to evaluate its potential as therapeutic target. From our perspective, future research should focus on two main aspects:

The predictive ability of pre-treatment intestinal microbiota concerning development of complications and response to cancer treatment.

The potential use of microbiota-modulating strategies in order to improve cancer therapy outcome.

It would be a breakthrough in cancer therapy if patients at risk of developing complications or having a lower chance of success could be identified in advance, based on their microbiota profile. This would not only facilitate precision medicine but would also give the opportunity to intervene at an early stage by means of microbiota-targeted interventions. Therefore, future research should evaluate the potential of the gut microbiota as a biomarker for therapy success.

Concerning the sampling of feces, we recommend that future research should perform longitudinal sampling, since this provides important information concerning changes over time and is considered superior to cross-sectional comparisons. In addition, it is strongly advised to take the different confounders into consideration for the study design of upcoming studies. In particular, the quality of research will greatly benefit by assessing antibiotic use, BMI and dietary intake.

Furthermore, there is currently a shortage of studies investigating the functional capacity of the gut microbiome, since most of the studies focus on gut microbiota composition. This lack of knowledge might be filled by future studies measuring microbial metabolites (meta-metabolomics) or gene expression (metatranscriptomics).

In the present review, it also became clear that scientific evidence is particularly scarce in the field of hormonal therapy. Two years ago, our research group started an observational cohort study with longitudinal fecal sample collection to study the microbiota composition before and during hormonal therapy in postmenopausal breast cancer patients receiving aromatase inhibitors or tamoxifen. Specifically in the aromatase group (N=60), we will study circulating hormonal levels related to the human intestinal microbiota composition and therapy efficacy. In the tamoxifen group (N=60), endoxifen levels and tamoxifen related human intestinal microbiota changes will be studied. The upcoming results will bridge the knowledge gap and will provide novel insights into hormonal therapy efficacy. In addition, similar longitudinal studies are on-going in CRC and breast cancer patients receiving chemotherapy (66).

Finally, proven interaction of human intestinal microbiota with systemic cancer therapy should lead to the evidence-based design of clinical trials targeting the gut microbiota. Possible strategies would be prebiotics, probiotics as well as FMT. Currently, several clinical trials using FMT (e.g. NCT03341143) or probiotics (e.g. NCT00197873, NCT03642548 or NCT03705442) are on-going. Results of these studies may reveal the potential of microbiota-targeted interventions in cancer patients, although more fundamental knowledge is likely needed to guide the selection of specific intervention strategies.

Author Contributions:

Romy Aarnoutse and Janine Ziemons equally contributed to the conceptualization, data curation, investigation, methodology, project administration, visualization and writing of the manuscript. John Penders, Sander S. Rensen, Judith de Vos-Geelen and Marjolein L. Smidt all provided supervision and contributed to the visualization and critical review and editing process of the manuscript. All authors provided final approval of the manuscript and are accountable for its accuracy and integrity.

Funding

This research received no external funding

Conflicts of Interest

Judith de Vos-Geelen has received non-financial support from BTG, and Servier, and has served as a consultant for Shire. Marjolein L. Smidt and Judith de Vos-Geelen received institutional research funding from Servier.

Abbreviations

5-FU	5-fluorouracil
AML	Acute myeloid leukemia
ALL	Acute lymphoblastic leukemia
ATT	Androgen receptor axis-targeted therapies
BSI	Bloodstream infections
CPIs	Checkpoint inhibitors
CRC	Colorectal cancer
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CV	Coefficient of variation
dHPLC	Denaturing High-Performance Liquid Chromatography
FISH	Fluorescent in situ hybridization
FMT	Fecal microbiota transplantation
GNRH	Gonadotropin-releasing hormone
GPCR	G protein-coupled receptor
I	Ipilimumab
IN	Ipilimumab + nivolumab
LDA	Linear Discriminant Analaysis
N	Nivolumab
NET	Neuroendocrine tumors
NSCLC	Non-small cell lung cancer
OTU	Operational Taxonomic Unit
OS	Overall survival
P	Pembrolizumab
PCA	Principal Component Analysis
PCoA	Principal coordinate analysis
PCR-DGGE	Polymerase chain reaction denaturing gradient gel electrophoresis
PD-1	Programmed cell death protein 1
PFS	Progression free survival
PICOS	Participants, Interventions, Comparators, Outcome measures, Study design
qPCR	quantitative Polymerase Chain Reaction
QUIPS	Quality In Prognosis Studies
RECIST	The Response Evaluation Criteria in Solid Tumors
RCC	Renal cell carcinoma
SCFA	Short-chain fatty acids
WMGS	Whole Metagenome Sequencing

References

1. Savage DC. Microbial ecology of the gastrointestinal tract. Annu Rev Microbiol. 1977;31:107-33.
2. Morrison DJ, Preston T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes. 2016;7(3):189-200.
3. Lepage P, Hasler R, Spehlmann ME, Rehman A, Zvirbliene A, Begun A, et al. Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis. Gastroenterology. 2011;141(1):227-36.
4. Verdam FJ, Fuentes S, de Jonge C, Zoetendal EG, Erbil R, Greve JW, et al. Human intestinal microbiota composition is associated with local and systemic inflammation in obesity. Obesity (Silver Spring). 2013;21(12):E607-15.
5. Noce A, Marrone G, Di Daniele F, Ottaviani E, Wilson Jones G, Bernini R, et al. Impact of Gut Microbiota Composition on Onset and Progression of Chronic Non-Communicable Diseases. Nutrients. 2019;11(5).
6. Montassier E, Batard E, Massart S, Gastinne T, Carton T, Caillon J, et al. 16S rRNA gene pyrosequencing reveals shift in patient faecal microbiota during high-dose chemotherapy as conditioning regimen for bone marrow transplantation. Microb Ecol. 2014;67(3):690-9.
7. Morgan XC, Huttenhower C. Chapter 12: Human microbiome analysis. PLoS computational biology. 2012;8(12):e1002808.
8. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature. 2012;489(7415):220-30.
9. Wang F, Meng W, Wang B, Qiao L. Helicobacter pylori-induced gastric inflammation and gastric cancer. Cancer Lett. 2014;345(2):196-202.
10. Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, et al. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. Cell Host Microbe. 2013;14(2):207-15.
11. Kostic AD, Gevers D, Pedamallu CS, Michaud M, Duke F, Earl AM, et al. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. Genome Res. 2012;22(2):292-8.
12. Kwa M, Plottel CS, Blaser MJ, Adams S. The Intestinal Microbiome and Estrogen Receptor-Positive Female Breast Cancer. J Natl Cancer Inst. 2016;108(8).
13. Flores R, Shi J, Fuhrman B, Xu X, Veenstra TD, Gail MH, et al. Fecal microbial determinants of fecal and systemic estrogens and estrogen metabolites: a cross-sectional study. J Transl Med. 2012;10:253.
14. Zhu J, Liao M, Yao Z, Liang W, Li Q, Liu J, et al. Breast cancer in postmenopausal women is associated with an altered gut metagenome. Microbiome. 2018;6(1):136.
15. Rajagopala SV, Yooseph S, Harkins DM, Moncera KJ, Zabokrtsky KB, Torralba MG, et al. Gastrointestinal microbial populations can distinguish pediatric and adolescent Acute Lymphoblastic Leukemia (ALL) at the time of disease diagnosis. BMC Genomics. 2016;17(1):635.
16. An J, Ha EM. Combination Therapy of Lactobacillus plantarum Supernatant and 5-Fluouracil Increases Chemosensitivity in Colorectal Cancer Cells. J Microbiol Biotechnol. 2016;26(8):1490-503.
17. Viaud S, Saccheri F, Mignot G, Yamazaki T, Daillere R, Hannani D, et al. The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. Science. 2013;342(6161):971-6.
18. Florez AB, Sierra M, Ruas-Madiedo P, Mayo B. Susceptibility of lactic acid bacteria, bifidobacteria and other bacteria of intestinal origin to chemotherapeutic agents. International journal of antimicrobial agents. 2016;48(5):547-50.
19. Attman E, Aittoniemi J, Sinisalo M, Vuento R, Lyytikainen O, Karki T, et al. Etiology, clinical course and outcome of healthcare-associated bloodstream infections in patients with hematological malignancies: a retrospective study of 350 patients in a Finnish tertiary care hospital. Leuk Lymphoma. 2015;56(12):3370-7.
20. Galloway-Pena JR, Smith DP, Sahasrabhojane P, Ajami NJ, Wadsworth WD, Daver NG, et al. The role of the gastrointestinal microbiome in infectious complications during induction chemotherapy for acute myeloid leukemia. Cancer. 2016;122(14):2186-96.
21. Galloway-Pena JR, Smith DP, Sahasrabhojane P, Wadsworth WD, Fellman BM, Ajami NJ, et al. Characterization of oral and gut microbiome temporal variability in hospitalized cancer patients. Genome Med. 2017;9(1):21.
22. Montassier E, Al-Ghalith GA, Ward T, Corvec S, Gastinne T, Potel G, et al. Pretreatment gut microbiome predicts chemotherapy-related bloodstream infection. Genome Med. 2016;8(1):49.

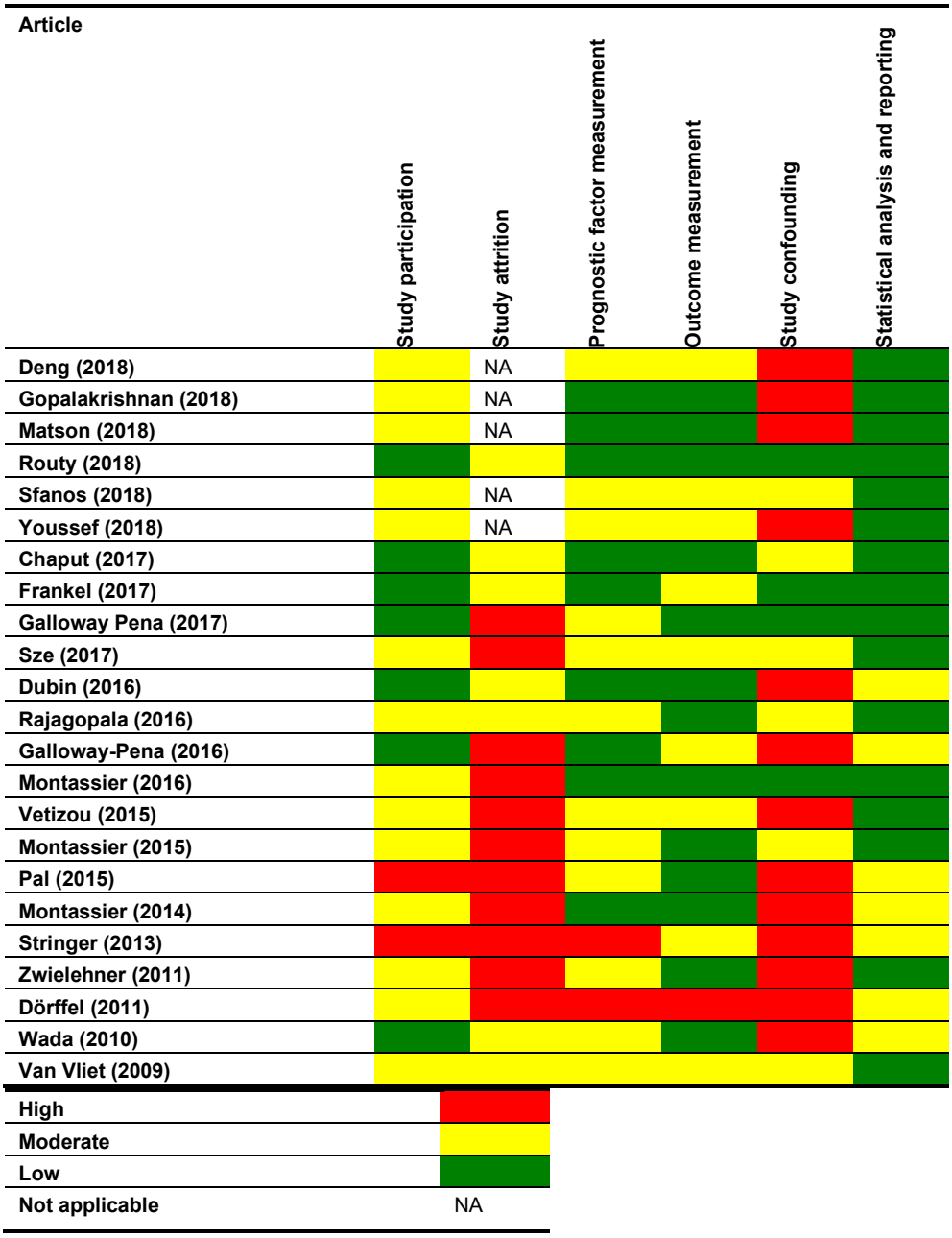
23. Pal SK, Li SM, Wu X, Qin H, Kortylewski M, Hsu J, et al. Stool Bacteriomic Profiling in Patients with Metastatic Renal Cell Carcinoma Receiving Vascular Endothelial Growth Factor-Tyrosine Kinase Inhibitors. Clin Cancer Res. 2015;21(23):5286-93.
24. Matson V, Fessler J, Bao R, Chongsuwat T, Zha Y, Alegre ML, et al. The commensal microbiome is associated with anti-PD-1 efficacy in metastatic melanoma patients. Science. 2018;359(6371):104-8.
25. Gopalakrishnan V, Spencer CN, Nezi L, Reuben A, Andrews MC, Karpinets TV, et al. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. Science. 2018;359(6371):97-103.
26. Routy B, Le Chatelier E, Derosa L, Duong CPM, Alou MT, Daillere R, et al. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. Science. 2018;359(6371):91-7.
27. Chaput N, Lepage P, Coutzac C, Soularue E, Le Roux K, Monot C, et al. Baseline gut microbiota predicts clinical response and colitis in metastatic melanoma patients treated with ipilimumab. Ann Oncol. 2017;28(6):1368-79.
28. Frankel AE, Coughlin LA, Kim J, Froehlich TW, Xie Y, Frenkel EP, et al. Metagenomic Shotgun Sequencing and Unbiased Metabolomic Profiling Identify Specific Human Gut Microbiota and Metabolites Associated with Immune Checkpoint Therapy Efficacy in Melanoma Patients. Neoplasia. 2017;19(10):848-55.
29. Dubin K, Callahan MK, Ren B, Khanin R, Viale A, Ling L, et al. Intestinal microbiome analyses identify melanoma patients at risk for checkpoint-blockade-induced colitis. Nat Commun. 2016;7:10391.
30. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer. 2009;45(2):228-47.
31. Dorffel Y, Swidsinski A, Loening-Baucke V, Wiedenmann B, Pavel M. Common biostructure of the colonic microbiota in neuroendocrine tumors and Crohn's disease and the effect of therapy. Inflamm Bowel Dis. 2012;18(9):1663-71.
32. Zwielehner J, Lassl C, Hippe B, Pointner A, Switzeny OJ, Remely M, et al. Changes in human fecal microbiota due to chemotherapy analyzed by TaqMan-PCR, 454 sequencing and PCR-DGGE fingerprinting. PLoS One. 2011;6(12):e28654.
33. Montassier E, Gastinne T, Vangay P, Al-Ghalith GA, Bruley des Varannes S, Massart S, et al. Chemotherapy-driven dysbiosis in the intestinal microbiome. Aliment Pharmacol Ther. 2015;42(5):515-28.
34. Sze MA, Baxter NT, Ruffin MTt, Rogers MAM, Schloss PD. Normalization of the microbiota in patients after treatment for colonic lesions. Microbiome. 2017;5(1):150.
35. Youssef O, Lahti L, Kokkola A, Karla T, Tikkanen M, Ehsan H, et al. Stool Microbiota Composition Differs in Patients with Stomach, Colon, and Rectal Neoplasms. Dig Dis Sci. 2018;63(11):2950-8.
36. Deng X, Li Z, Li G, Li B, Jin X, Lyu G. Comparison of Microbiota in Patients Treated by Surgery or Chemotherapy by 16S rRNA Sequencing Reveals Potential Biomarkers for Colorectal Cancer Therapy. Front Microbiol. 2018;9:1607.
37. Stringer AM, Al-Dasooqi N, Bowen JM, Tan TH, Radzuan M, Logan RM, et al. Biomarkers of chemotherapy-induced diarrhoea: a clinical study of intestinal microbiome alterations, inflammation and circulating matrix metalloproteinases. Support Care Cancer. 2013;21(7):1843-52.
38. Wada M, Nagata S, Saito M, Shimizu T, Yamashiro Y, Matsuki T, et al. Effects of the enteral administration of Bifidobacterium breve on patients undergoing chemotherapy for pediatric malignancies. Support Care Cancer. 2010;18(6):751-9.
39. van Vliet MJ, Tissing WJ, Dun CA, Meessen NE, Kamps WA, de Bont ES, et al. Chemotherapy treatment in pediatric patients with acute myeloid leukemia receiving antimicrobial prophylaxis leads to a relative increase of colonization with potentially pathogenic bacteria in the gut. Clin Infect Dis. 2009;49(2):262-70.
40. Zhang XL, Komada Y, Chipeta J, Li QS, Inaba H, Azuma E, et al. Intracellular cytokine profile of T cells from children with acute lymphoblastic leukemia. Cancer immunology, immunotherapy : CII. 2000;49(3):165-72.
41. Vetizou M, Pitt JM, Daillere R, Lepage P, Waldschmitt N, Flament C, et al. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. Science. 2015;350(6264):1079-84.

42. Sfanos KS, Markowski MC, Peiffer LB, Ernst SE, White JR, Pienta KJ, et al. Compositional differences in gastrointestinal microbiota in prostate cancer patients treated with androgen axis-targeted therapies. Prostate Cancer Prostatic Dis. 2018;21(4):539-48.
43. Yuan L, Zhang S, Li H, Yang F, Mushtaq N, Ullah S, et al. The influence of gut microbiota dysbiosis to the efficacy of 5-Fluorouracil treatment on colorectal cancer. Biomed Pharmacother. 2018;108:184-93.
44. Sharma A, Buschmann MM, Gilbert JA. Pharmacomicrobiomics: The Holy Grail to Variability in Drug Response? Clinical pharmacology and therapeutics. 2019;106(2):317-28.
45. Alexander JL, Wilson ID, Teare J, Marchesi JR, Nicholson JK, Kinross JM. Gut microbiota modulation of chemotherapy efficacy and toxicity. Nat Rev Gastroenterol Hepatol. 2017;14(6):356-65.
46. Zimmermann M, Zimmermann-Kogadeeva M, Wegmann R, Goodman AL. Mapping human microbiome drug metabolism by gut bacteria and their genes. Nature. 2019;570(7762):462-7.
47. Bronckaers A, Balzarini J, Liekens S. The cytostatic activity of pyrimidine nucleosides is strongly modulated by Mycoplasma hyorhinis infection: Implications for cancer therapy. Biochem Pharmacol. 2008;76(2):188-97.
48. Wallace BD, Wang H, Lane KT, Scott JE, Orans J, Koo JS, et al. Alleviating cancer drug toxicity by inhibiting a bacterial enzyme. Science. 2010;330(6005):831-5.
49. Roberts AB, Wallace BD, Venkatesh MK, Mani S, Redinbo MR. Molecular insights into microbial beta-glucuronidase inhibition to abrogate CPT-11 toxicity. Mol Pharmacol. 2013;84(2):208-17.
50. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. Cell. 2014;157(1):121-41.
51. Round JL, Mazmanian SK. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. Proc Natl Acad Sci U S A. 2010;107(27):12204-9.
52. van der Beek CM, Dejong CHC, Troost FJ, Masclee AAM, Lenaerts K. Role of short-chain fatty acids in colonic inflammation, carcinogenesis, and mucosal protection and healing. Nutr Rev. 2017;75(4):286-305.
53. Miko E, Kovacs T, Sebo E, Toth J, Csonka T, Ujlaki G, et al. Microbiome-Microbial Metabolome-Cancer Cell Interactions in Breast Cancer-Familiar, but Unexplored. Cells. 2019;8(4).
54. Ramírez-Pérez O, Cruz-Ramón V, Chinchilla-López P, Méndez-Sánchez N. The role of the gut microbiota in bile acid metabolism. Annals of hepatology. 2018;16(1):21-6.
55. Miko E, Vida A, Kovacs T, Ujlaki G, Trencsenyi G, Marton J, et al. Lithocholic acid, a bacterial metabolite reduces breast cancer cell proliferation and aggressiveness. Biochim Biophys Acta Bioenerg. 2018;1859(9):958-74.
56. Colosimo DA, Kohn JA, Luo PM, Piscotta FJ, Han SM, Pickard AJ, et al. Mapping Interactions of Microbial Metabolites with Human G-Protein-Coupled Receptors. Cell Host Microbe. 2019;26(2):273-82.e7.
57. Yi M, Yu S, Qin S, Liu Q, Xu H, Zhao W, et al. Gut microbiome modulates efficacy of immune checkpoint inhibitors. J Hematol Oncol. 2018;11(1):47.
58. Asmar N, Ibrahim T, Rey JF. Checkpoint Inhibitors: Conquering Cancer with a Little (T)-Help from Our Microbial Friends. Dig Dis Sci. 2018;63(9):2177-9.
59. Wei JH, Yin X, Welander PV. Sterol Synthesis in Diverse Bacteria. Front Microbiol. 2016;7:990.
60. Ridlon JM, Ikegawa S, Alves JM, Zhou B, Kobayashi A, Iida T, et al. Clostridium scindens: a human gut microbe with a high potential to convert glucocorticoids into androgens. J Lipid Res. 2013;54(9):2437-49.
61. Fuhrman BJ, Feigelson HS, Flores R, Gail MH, Xu X, Ravel J, et al. Associations of the fecal microbiome with urinary estrogens and estrogen metabolites in postmenopausal women. J Clin Endocrinol Metab. 2014;99(12):4632-40.
62. Rinninella E, Raoul P, Cintoni M, Franceschi F, Miggiano GAD, Gasbarrini A, et al. What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. Microorganisms. 2019;7(1).
63. Velicer CM, Heckbert SR, Lampe JW, Potter JD, Robertson CA, Taplin SH. Antibiotic use in relation to the risk of breast cancer. JAMA. 2004;291(7):827-35.
64. Kirkup B, McKee A, Makin K, Paveley J, Caim S, Alcon-Giner C, et al. Perturbation of the gut microbiota by antibiotics results in accelerated breast tumour growth and metabolic dysregulation. BioRxiv. 2019:553602.
65. Vandeputte D, Falony G, Vieira-Silva S, Tito RY, Joossens M, Raes J. Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates. Gut. 2016;65(1):57-62.

66. Aarnoutse R, de Vos-Geelen J, Penders J, Boerma EG, Warmerdam F, Goorts B, et al. Study protocol on the role of intestinal microbiota in colorectal cancer treatment: a pathway to personalized medicine 2.0. Int J Colorectal Dis. 2017;32(7):1077-84.
67. Higgins JPT GS. Cochrane Handbook for Systematic Reviews of Interventions Version 5.1.0: The Cochrane Collaboration; 2019 [updated March 2011. Available from: www.handbook.cochrane.org.

Appendix A

Table A1: *Risk of bias of included studies assessed by Quality in Prognosis Studies tool (QUIPS).*



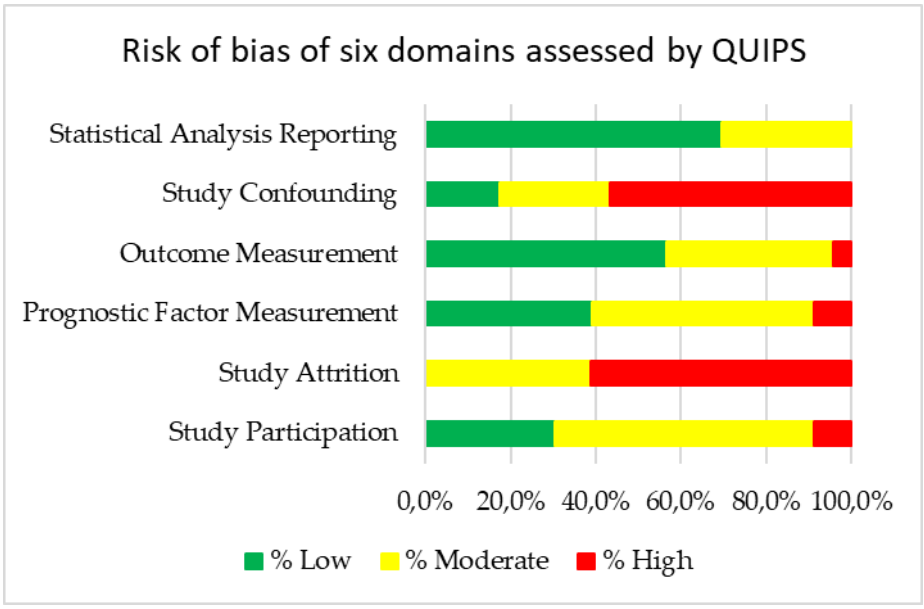


Figure A1. *Risk of bias of six domains assessed by QUIPS*

Appendix B

Table A2. *Definition of terms used in microbiota research*

α-diversity	Number and evenness of distribution of taxa within a given sample
β-diversity	The difference in diversity of taxa from one sample to another, i.e. the number of taxa that are not the same (or not similarly distributed) in two different samples.
16S rRNA gene	Marker gene for bacterial identification, containing evolutionary conserved universal as well as variable regions
Operational taxonomic unit (OTU)	Cluster of nearly identical sequences (e.g. 97% similarity), often used in microbiota research instead of 'species'
16S rRNA gene sequencing	Sequencing of the 16S rRNA marker gene
Metagenomic sequencing	Sequencing of the entire metagenome (all the genetic material in a sample) , also allowing analysis of the functional capacity of the microbiome

Chapter 3

The role of intestinal microbiota in metastatic colorectal cancer patients treated with capecitabine

Romy Aarnoutse[*], **Janine Ziemons**[*], Judith de Vos-Geelen, Liselot Valkenburg-van Iersel, Aurelia C.L. Wildeboer, Anne Vievermans, Geert-Jan M. Creemers, Arnold Baars, Hanneke J.H.M.J. Vestjens, Giang N. Le, David J.M. Barnett, Sander S. Rensen, John Penders[#], Marjolein L. Smidt[#]

** Shared first authorship*
 # Shared last authorship

Clinical Colorectal Cancer, 2022 Jun;21(2): e87-e97.
DOI: 10.1016/j.clcc.2021.10.004

Abstract

Background

Previous pre-clinical research has indicated that the intestinal microbiota can potentiate anti-tumor efficacy of capecitabine and that capecitabine treatment impacts intestinal microbiota composition and diversity. Using a longitudinal design, this study explores the associations between the intestinal microbiota and treatment response in patients with metastatic colorectal cancer (mCRC) during capecitabine treatment.

Patients and methods

Patients with mCRC treated with capecitabine were prospectively enrolled in a multicenter cohort study. Patients collected a fecal sample and completed a questionnaire before, during, and after three cycles of capecitabine. Several clinical characteristics, including tumor response, toxicity and antibiotic use were recorded. Intestinal microbiota were analyzed by amplicon sequencing of the 16S rRNA V4 gene-region.

Results

33 patients were included. After three cycles of capecitabine, six patients (18%) achieved a partial response, 25 (76%) showed stable disease, and one (3%) experienced progressive disease. 90 fecal samples were collected. Microbial diversity (α-diversity), community structure (β-diversity), and bacterial abundance on phylum and genus level were not significantly different between responders and non-responders and were not significantly affected by three cycles of capecitabine.

Conclusions

This is the first clinical study with longitudinal intestinal microbiota sampling in mCRC patients that explores the role of the intestinal microbiota during treatment with capecitabine. Intestinal microbiota composition and diversity before, during, and after three cycles of capecitabine were not associated with response in this study population. Capecitabine did not induce significant changes in the microbiota composition and diversity during the treatment period. Individual effects of antibiotics during capecitabine treatment were observed.

Introduction

Colorectal cancer (CRC) is the third most common cancer in the world (1). Despite recent developments in systemic therapy, classical chemotherapeutic agents such as fluoropyrimidines, e.g. capecitabine, an oral prodrug of 5-fluorouracil (5-FU), remain the backbone of most systemic therapies. Capecitabine, with or without the vascular endothelial growth factor inhibitor bevacizumab (2), is often applied in mCRC patients who are not eligible for intensive chemotherapy combinations because of comorbidity or impaired performance score, resulting in an objective response rate of only 21-25% (3, 4). Besides controlling tumor growth, capecitabine potentially induces toxicity, severely impacting quality of life. The most common CTCAE grade 3 toxic events are diarrhea (24%), hand-foot syndrome (18%), and stomatitis (3%) (3).

In order to optimize treatment outcome, factors that impact individual response and safety profile to capecitabine need to be identified. During the last decade, evidence of the interaction between systemic cancer therapies and the human intestinal microbiota has rapidly expanded. The human intestinal microbiota consists of bacteria, archaea, viruses, and fungi (5). It has been shown that trillions of intestinal bacteria stimulate the immune system, might be involved in carcinogenesis and influence human metabolism of dietary components and medication, including chemotherapeutic agents (6).

Pre-clinical microbiota studies indicate significant interactions between the intestinal microbiota and 5-FU or capecitabine. Sougiannis *et al.* demonstrated that 5-FU treatment affects intestinal microbiota composition, the colonic morphology and immune profile, as well as functional outcomes of fatigue in a mouse model of colon cancer (7). Furthermore, *Lactobacillus plantarum* supernatant sensitized CRC cell lines to 5-FU and stimulated apoptosis in chemo-resistant cells (8). Administration of an antibiotic cocktail (vancomycin, ampicillin, neomycin, and metronidazol) reduced anti-tumor efficacy of 5-FU in mice (9). Very recently, Zimmermann *et al.* provided the first in vitro evidence that capecitabine can be metabolized by several bacterial species (10).

Clinical evidence for a potential influence of intestinal microbiota on chemotherapy efficacy or toxicity is limited. This is mainly due to a lack of studies with longitudinal microbiota sampling during chemotherapy (11, 12). With respect to CRC and capecitabine, no clinical studies are available. Only one study, in which 31 patients with rectal cancer were treated with a combination of 5-FU and oxaliplatin (FOLFOX), partly supports the pre-clinical data (12).

We hypothesized that pre-treatment intestinal microbiota composition and diversity and its changes during capecitabine therapy are associated with response and/or therapy-related toxicity in mCRC patients. We conducted a prospective study to evaluate changes in intestinal microbiota composition and diversity during chemotherapy, assessing chemotherapy toxicity and response to capecitabine in mCRC patients.

Patients and methods

Patients

Between March 2017 and September 2019, patients were prospectively enrolled in four Dutch Hospitals (13). Patients with histologically proven mCRC to be treated with capecitabine with or without bevacizumab, aged 18 years or older were eligible. Exclusion criteria included microsatellite instability (MSI), impaired renal function as defined by creatinine clearance (Cockroft-Gault) < 30 ml/min, abdominal radiotherapy within two weeks prior to starting capecitabine, systemic cancer therapy within four weeks prior to starting capecitabine, and therapeutic antibiotics use within three months prior to starting capecitabine.
The study was registered in the Dutch Trial Register (NTR6957) and approved by the Medical Ethics Committee azM/UM (METC 16-4-234.1) and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. Each patient provided written informed consent.

Treatment

During the study period, patients received three cycles of capecitabine (1000-1250 mg/m2 orally, twice daily on days 1-14 in a 3 week cycle) with or without bevacizumab (7.5 mg/kg intravenously on day 1 every 3 weeks).

Materials and methods

According to the previous published study protocol (14), patients collected pure fecal samples in preservation free feces tubes (Sarstedt) and completed questionnaires at three time points: before the start of the first capecitabine cycle (T1, one or two days before the start of the cycle), between days 7 and 14 of the third cycle (T2), and at day 20 or 21 of the third cycle (T3) (Figure S1). After collection, samples were immediately stored in the freezer at home and transported to the hospital in a cooled container (Sarstedt), where samples were stored at -20°C first and at -80°C for long-term storage. Patient characteristics including history of gastrointestinal surgery, Karnofsky performance score (KPS), nutritional status assessed with the Malnutrition Universal Screening Tool (MUST), chemotherapy compliance, dose reductions, antibiotic/prebiotic/probiotic use, and the use of nutritional supportive drinks were registered.

Response measurement

Tumor response was assessed using CT or MRI scans before and at the end of three cycles of capecitabine by means of RECIST (Response Evaluation Criteria in Solid Tumors) version 1.1 (15). Response was defined as complete response (CR): disappearance of all target lesions and partial response (PR): ≥30% decrease in the sum of the target lesions. Non-response was defined as progressive disease (PD): ≥20% increase in the sum of target lesions and stable disease (SD): small changes that do not meet above criteria (15).

Toxicity was scored with CTCAE version 4.0 (16). The following aspects were scored: diarrhea with or without colostomy, peripheral sensory neuropathy, hand-foot syndrome, fatigue, nausea, oral mucositis, vomiting, and constipation.

Fecal microbiota analyses

Metagenomic DNA was isolated using the Ambion MagMaxTM Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific) and consisted of mechanical disruption with bead-beating, as well as chemical and thermal disruption. The manual pre-processing was followed by automated nucleic acid purification with the KingFisher FLEX (Thermo Fisher Scientific). Upon PCR-amplification of the 16S ribosomal RNA (rRNA) hypervariable V4 gene-region according to current international accepted standards (17), amplicons were sequenced on a MiSeq platform, as described previously (18).

Pre-processing of the sequencing data was performed using R. A standardized in-house pipeline using the software package DADA2 (R version 4.0.3) was applied (19). After pre-processing, 908 taxa remained for downstream analysis. For further details on DNA isolation, sequencing, and data pre-processing see the supplementary methods.

Statistical analysis of clinical data

Baseline characteristics were analyzed in IBM SPSS version 26. For continuous data, normality was tested using the Shapiro-Wilk test. Depending on whether the variable was normally distributed or not, an unpaired t-test or the non-parametric Mann-Whitney U test was applied. Levene's test was used to test for equal variances. For categorical variables, the non-parametric Chi-square test or a Fisher's exact test, in case of low frequencies for binary variables, was performed. For longitudinal analysis with two time points of quantitative variables, a paired sample t-test or the non-parametric Wilcoxon signed-rank sum test was used. For longitudinal analysis with three time points, repeated-measures ANOVA (sphericity assumed) or Friedman's ANOVA were used for normally and non-normally distributed data, respectively. Significant results were subjected to a post hoc Wilcoxon signed-rank sum tests with Bonferroni correction. Two-tailed tests were use and p-values below 0.05 were considered statistically significant.

Statistical analysis of intestinal microbiota data

Bioinformatic analysis of the sequencing data was performed using R version 4.0.3 (20). For the calculation of α-diversity indices on Amplicon Sequencing Variant (ASV) level (Shannon effective and observed richness) and prior data normalization, the standard script and settings of the Rhea pipeline were used (21). Testing the assumptions of normality, homogeneity of variance and subsequent statistical testing was performed as described for clinical data.

In order to quantify microbial community structure (β-diversity), generalized UniFrac and Bray-Curtis distances were calculated on ASV level, using Rhea (21) and the R packages GUniFrac (22) and phyloseq (23) respectively. Temporal (in)stability of microbial community structure was expressed as generalized UniFrac/Bray-Curtis distances between T1/T2, T2/T3 and T1/T3 within the same patient. Mann Whitney U test was used to compare differences between responders and non-responders at all time points. The R packages, phyloseq (23), vegan (24), microbiome (25), dplyr (26), ggplot2 (27), and microViz (28) were used for ordination and visualization of taxonomic composition. Taxa present in less than 5

samples were filtered out for ordination and all subsequent analyses. Permutational multivariate analysis of variance (PERMANOVA) was applied to examine associations between variation in overall microbial community structure and treatment response and study time point variables. Aitchison distance on phylum and genus level was used for ordination as well as for PERMANOVA. Differential abundance analysis of individual microbial taxa was conducted using the workflow of ANCOM v.2.1 which accounts for the underlying structure of microbiota data and the presence of zeros (29). We tested for differential abundance between responders and non-responders at T1 and T2, and for differential abundance over time within individuals. We set $p < 0.05$ at 70% of comparisons as a threshold for significance.

Results

In total, 33 patients with mCRC treated with capecitabine (+/- bevacizumab) were included. Baseline characteristics were stratified by response evaluation (Table 1 and S1). After three cycles of capecitabine, six patients (18%) achieved a partial response, 25 (76%) showed stable disease, and one (3%) had progressive disease. In one patient (3%), response could not be evaluated due to withdrawal of study participation. Consequently, 6 patients were classified as responders and 26 patients as non-responders. In total, 90 fecal samples were collected. Figure S2 provides an overview of all samples available for 16S rRNA gene sequencing.

Baseline characteristics

Median age was 75 years. Mean BMI was 27 kg/m2. Men (76%) were predominant in the total group. Most patients presented with synchronous metastatic disease, of which eight patients had metastasis at one site and 25 had multiple organs involved. Twenty-one (66%) patients had a left-sided tumor. In total 88% underwent resection of the primary tumor (Table 1). A low anterior resection was performed in twelve patients, a sigmoid resection in five patients, a left-sided hemicolectomy in two patients, an extended left-sided hemicolectomy in one patient, and a right-sided hemicolectomy in eight patients. Of the patients who underwent resection of the primary tumor, 30% still had a colostomy at the time of inclusion in the current study. Nearly half of the patients (48%) received previous systemic therapy in any setting with any type of chemotherapy. In the year prior to inclusion, 24% of the patients used therapeutic antibiotics (none within three months before T1). The mean time in days between the last intake of antibiotics and the baseline fecal sample collection was 197 days. In total, 30% used prophylactic antibiotics in the last year, with a mean of 96 days between the last intake of prophylactic antibiotics and fecal sample collection. None of the patients used prednisone (one month), prebiotics, or probiotics (1 year) prior to T1. Men were predominant in the non-responders group (85%, p=0.023). All other baseline characteristics were not significantly different between responders and non-responders (Table 1 and S1).

Clinical characteristics before, during, and after three cycles of capecitabine

During capecitabine treatment, there were no significant differences in capecitabine dose intensity, compliance, and antibiotic use between responders and non-responders (Table S2). In total, 83% of the responders and 81% of the non-responders received co-treatment with bevacizumab.

After three cycles of capecitabine, non-responders indicated significantly higher grades of fatigue compared to responders (p=0.026). All other toxicity measures were not significantly different between responders and non-responders before, during or after three cycles of capecitabine (Table S3-S5). Toxicity grades of peripheral sensory neuropathy, hand foot syndrome, oral mucositis, and bone marrow toxicity increased significantly over the study period (Figure 1, Table S6 and S7). All other toxicity measures, including diarrhea, did not change during three cycles of capecitabine (Table S6).

Compared to baseline, KPS was significantly lower after three cycles of capecitabine (p=0.002) (Table S8). The MUST score was not significantly different before, during or after three cycles of capecitabine (Table S8).

Table 1: *Clinical characteristics*

Baseline characteristics	Total *N*=33	Responders *n*=6	Non-responders *n*=26	*p*-value
Age – Years				
Median (IQR)	75 (14)	74 (19)	75 (13)	0.981
BMI – kg/m^2				
Mean (SD)	27 (±5)	26 (±7)	27 (± 4)	0.439
CTCAE Unintentional weight loss – No. (%)*				
Gr. 0	28 (88)	6 (100)	21 (84)	
Gr. 1	4 (13)	0 (0)	4 (16)	0.561
Male – No. (%)	25 (76)	2 (33)	22 (85)	0.023
Sidedness tumor – No. (%)				
Left sided	21 (66)	5 (83)	16 (64)	
Right sided	11 (34)	1 (17)	9 (36)	0.634
Time to metastasis – No. (%)				
Synchronous	20 (61)	3 (50)	16 (62)	
Metachronous	13 (39)	3 (50)	10 (38)	0.666
Colorectal surgery in the past – No. (%)	29 (88)	6 (100)	22 (85)	0.566
Colostoma in situ at T1– No. (%)	10 (30)	2 (33)	8 (31)	1.000
Previous systemic treatment – No. (%)	16 (48)	4 (66)	12 (46)	0.654
MUST score – No (%)				
Low risk	26 (79)	5 (83)	21 (81)	
Medium risk	4 (12)	0 (0)	3 (11)	
High risk	3 (9)	1 (17)	2 (8)	0.823
Karnofsky Performance Score – No (%)*				
50-60	3 (10)	1 (17)	2 (8)	
70-80	9 (29)	1 (17)	8 (34)	
90-100	19 (62)	4 (67)	14 (58)	0.372

Response could not be evaluated in one patient.
**Percentages do not add up to 100% due to rounding.*

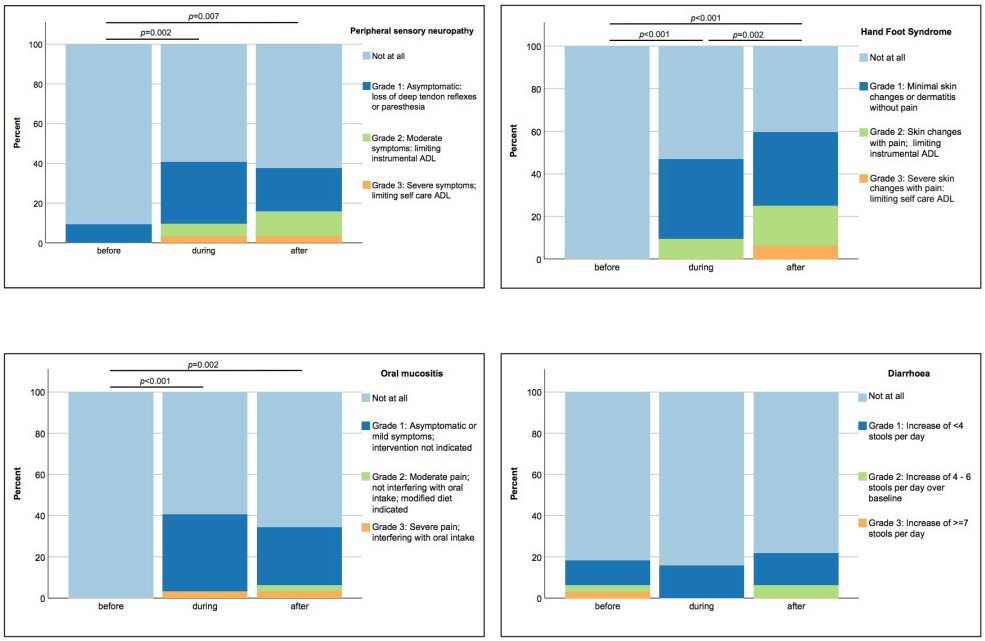


Figure 1: *Stacked bar charts presenting percentage toxicity grades before, during, and after three cycles of capecitabine. For peripheral sensory neuropathy and oral mucositis significant differences were observed between T1-T2 and T1-T3. For hand foot syndrome significant differences were observed between all time points.*

Intestinal microbiota composition and diversity

Similar α-diversity in responders and non-responders

Before (Figure S3 and Table S9) and during (Figure 2A and Table S10) three cycles of capecitabine, Shannon effective as well as observed richness were similar between responders and non-responders. In addition, both α-diversity indices did not significantly change over the course of three cycles of capecitabine (Figure 2B and Table S11).

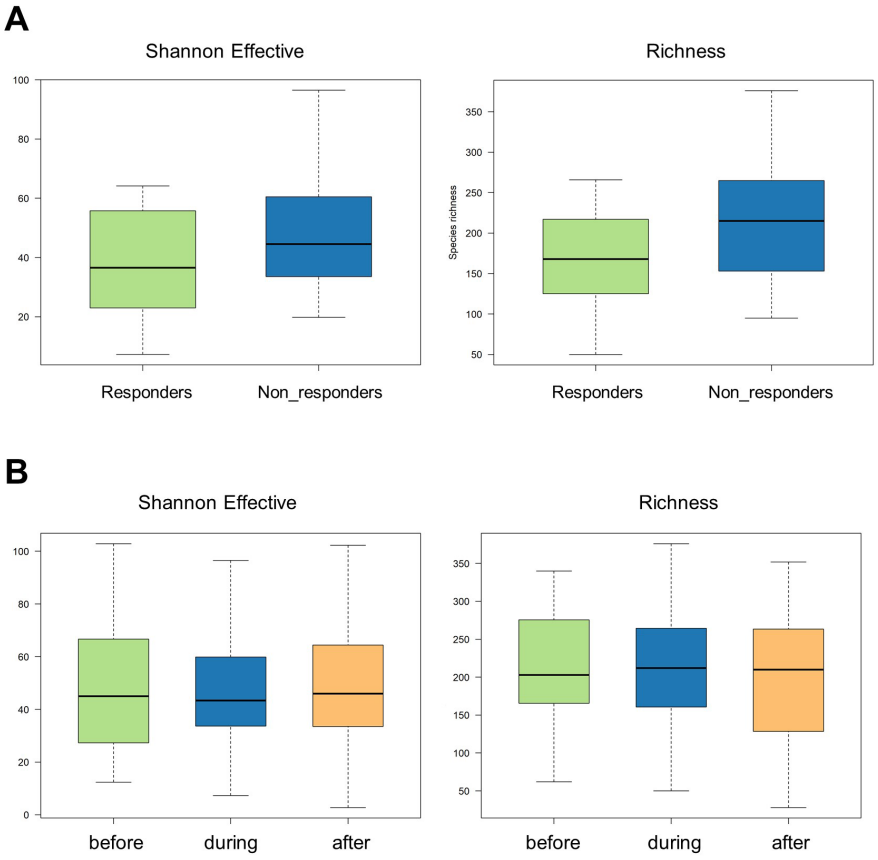


Figure 2: *α-diversity measures* ***A:*** *Microbial diversity and richness of responders and non-responders at T2, measured in terms of Shannon effective (p=0.301) and observed richness (p=0.145) (Table S10).* ***B:*** *α-diversity before, during, and after three cycles of capecitabine, measured in terms of Shannon effective (p=0.640) and observed richness (p=0.240) (Table S11).*
Numbers presented in median (IQR).

Microbial community structure (β-diversity) and abundance of specific bacteria

No differences between responders and non-responders

Principal Component Analysis (PCA) showed large heterogeneity in individual microbial community structures. PERMANOVA revealed that there was no statistically significant association between treatment response and the overall microbial community structure at T2 on phylum (p=0.07) and genus (p=0.41) level (Figure 3). However, on phylum level, responders tended to cluster in the direction of Proteobacteria and Actinobacteria (Figure 3A). In addition, in the entire population the abundance of Euryarcheota and Verrucomicrobia had a major contribution to the first and second PCA axis, respectively (Figure 3A). On genus level, *Lachnospiraceae ND3007 group*, *Dialister*, *Veillonella*, *Anerostipes*, and *Flavonifractor* contributed the most to the variation in the overall microbiota community structure (Figure 3B). At T1, there was also a large heterogeneity and no association between treatment response and overall microbial community structure on phylum (p=0.38) and genus (p=0.73) level (Figure S4). Furthermore, there were no differences found between responders and non-responders concerning within-subject temporal (in)stability of β-diversity between the various time points, using generalized UniFrac as well as Bray-Curtis distances. P08 showed considerably large instability between T2-T3 and T1-T3 (Figure S5 and Table S12).
Differential abundance analysis on phylum and genus level identified no taxa which were differentially abundant between responders and non-responders at T1 and T2.

Large intra-individual microbiota alterations during capecitabine treatment

In the present research population, Firmicutes were the most abundant phylum, followed by Bacteroidetes and Actinobacteria. At phylum level, no major shifts were observed during the course of three cycles of capecitabine (Figure 4A). This was confirmed by PERMANOVA, which revealed no association between sampling time point and microbial community structure on phylum (p=0.96) level.
Figure 4B shows the most abundant genera before, during, and after three cycles of capecitabine. We observed large inter-individual heterogeneity but no prominent universal capecitabine-induced pattern. On group level, PERMANOVA revealed no association between sampling time point and microbial community structure on genus (p=1.0) level. In line with this, ANCOM analysis with treatment response as covariate identified no phyla or genera that significantly differed in abundance before, during, and after three cycles of capecitabine.
During the study period, large intra-individual shifts of the intestinal microbiota composition were observed (Figure 4B), which could partly be explained by clinical data. P20 displayed a high relative abundance of bifidobacteria. This patient showed partial response (40% decrease in the sum of target lesions) after three cycles of capecitabine. P08 received oral amoxicillin/ciprofloxacin before collection of the last fecal sample and showed relatively high levels of *Bacteroides* and *Streptococcus* in this sample. P01 received oral ciprofloxacin 24 days before the second fecal sample collection, resulting in relatively high levels of *Streptococcus*.

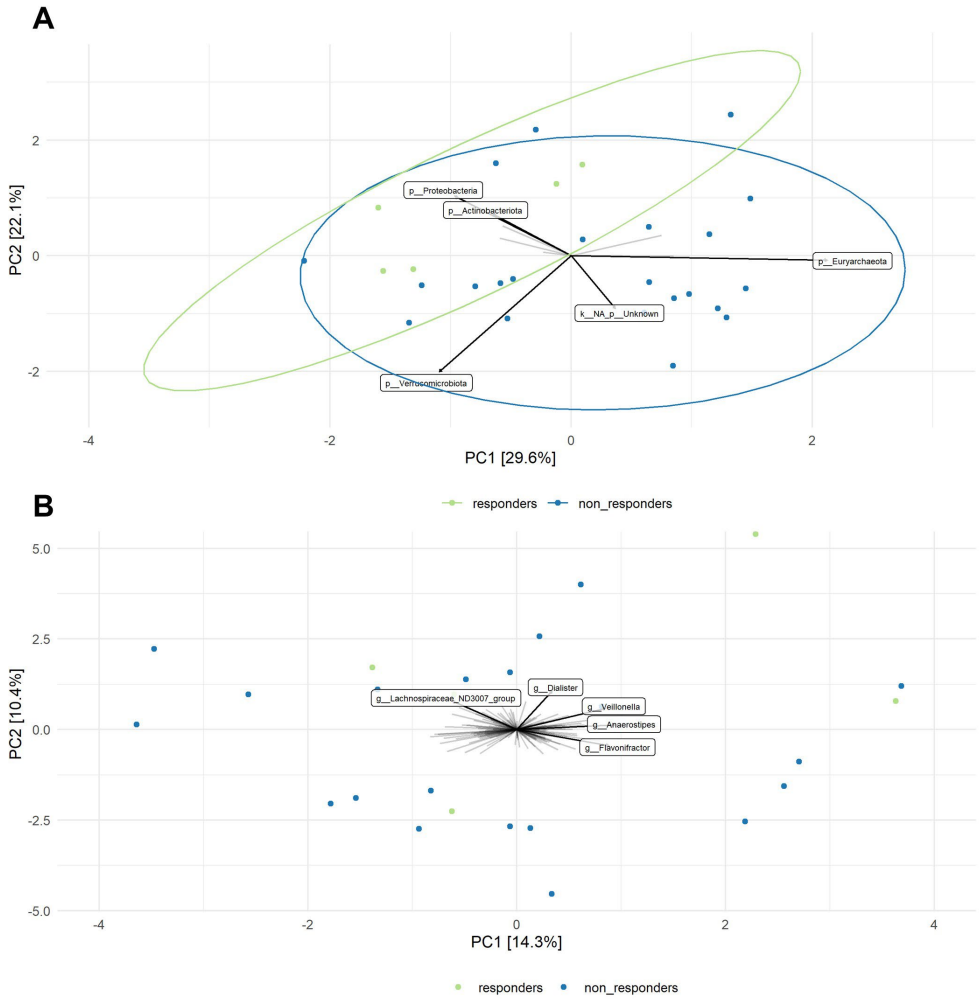


Figure 3: *Ordination plots derived from unconstrained Principal Components Analysis (PCA), showing overall composition of the microbial community on phylum (A) and genus level (B) at T2. Aitchison distance was used. 10 phyla and 150 genera were included for this analysis. Data were transformed using centre-log-ratio transformation. Names are given for genera which contributed most to overall microbial variation.*

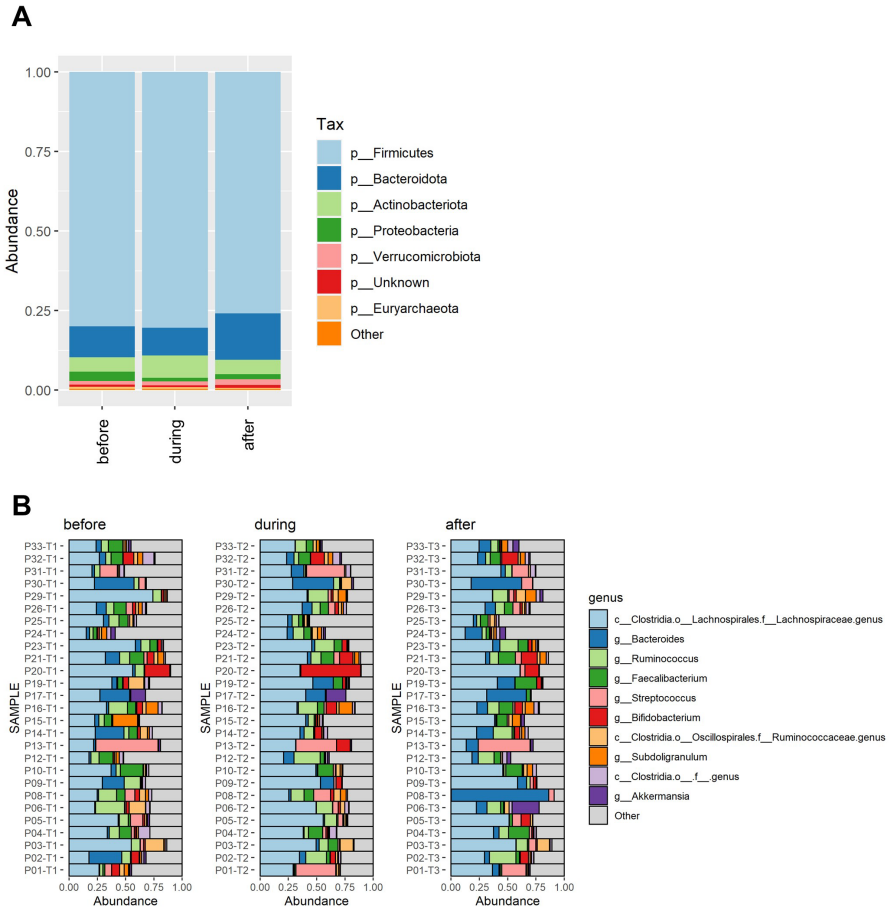


Figure 4: A: *Composition plot at phylum level, before, during, and after three cycles of capecitabine, indicating relative abundance of the most common phyla* ***B:*** *Changes in relative abundance of the most common genera before, during, and after three cycles of capecitabine indicate a large inter-individual heterogeneity and no prominent universal capecitabine-induced effect.*

Discussion

This is the first clinical study with longitudinal intestinal microbiota sampling in mCRC patients that explored the role of the intestinal microbiota during treatment with capecitabine (without surgery, radiation or chemotherapy or combinations thereof). Intestinal microbiota composition and diversity before, during or after three cycles of capecitabine were not associated with treatment response in the current small study population. Furthermore, capecitabine treatment did not alter the microbiota composition and diversity during the course of three cycles of capecitabine. In contrast to the minor effect of capecitabine on the intestinal microbiota, individual effects of antibiotic treatment during capecitabine treatment were observed in two patients.

In this study, we showed that longitudinal fecal sample collection is feasible in mCRC patients. Our baseline characteristics indicate that we included a representative mCRC population; the disease control rate after three cycles of capecitabine is comparable to the study of Cutsem *et al.* (4).

In the current study population, microbial α-diversity was not significantly different between responders and non-responders and did not diminish during the course of three cycles of capecitabine. This is partially in line with data described by Li *et al.* (12). Li at al. studied rectal cancer patients without metastasis who received a combination of 5-FU and oxaliplatin (FOLFOX). They also did not observe a difference in α-diversity between responders and non-responders before FOLFOX treatment. However, a decrease in α-diversity after FOLFOX treatment in the responder group was found in that study. These different study outcomes could be due to an already altered intestinal microbiota at baseline in our study population. Nearly half of the patients (48%) received previous chemotherapy (more than one month before inclusion), which is associated with extensive hospitalization and lifestyle changes (desirable and undesirable) (30). As a consequence, microbial dysbiosis might have been already present at baseline, leading to only minor capecitabine related effects. It is possible that the potential capecitabine-induced effects on the microbiota diversity would be higher if the patients were included and collected fecal samples at primary diagnosis.

In line with the extensive medical history of these patients, we observed considerable heterogeneity in individual microbial community structure (β-diversity) before and during three cycles of capecitabine. This might have contributed to the lack of association between treatment response and microbial community structure.

Abundance of taxa at phylum and genus level did not significantly differ before, during or after chemotherapy in the whole group. This is in contrast with results from Sze *et al.* (31) who performed longitudinal microbiota analysis in patients with primary diagnosed CRC (n=26). After treatment, they observed a change in community structure and a shift towards a microbiota comparable to the profile of healthy controls. These findings were based on a heterogeneously treated group including surgery, with or without eight different types of chemotherapy, with or without radiation (31). Another study in patients with rectal cancer without metastasis treated with FOLFOX showed therapy-induced changes in genus

abundances, which were more pronounced in the patients achieving a partial or complete response (12). Additionally, they identified specific species (*Coprobacter fastidiosus*, *Alistipes finegoldii*, *Gemella* unclassified, *Granulicatella adiacens*, *Parvimonas micra*, and *Clostridium ramosum*) associated with the outcome of FOLFOX treatment, which might potentially be useful as a biomarker to predict therapy outcome (12). After different types of chemotherapy (n=23), Zwielehner *et al.* showed decreased levels of *Clostridium cluster IV*, *Bacteroides*, bifidobacteria, as well as *Clostridium cluster XIVa* in patients (n=17) with different types of cancer (n=13) (32). Recently, Zimmermann *et al.* provided *in vitro* evidence that capecitabine can be metabolized by several bacterial species including *Bifidobacterium ruminatum*, *Bacteroides xylanisolvens DSM18836*, and *Salmonella Typhimurium LT2* (10). Our results are not in line with these previous studies, which may be related to the complex medical history of our patients in combination with the relatively mild form of systemic therapy with capecitabine in contrast to FOLFOX treatment (2-4, 12).

Although there were no differences in microbiota composition between responders and non-responders, specific patients showed remarkable microbiota shifts during therapy, which could be explained based on clinical data. Two patients (P08/P01) received ciprofloxacin during chemotherapy. As a consequence of this broad-spectrum antibiotic, relatively high levels of possibly ciprofloxacin-resistant *Streptococcus* were observed (33). These individual changes indicate that the impact of antibiotics was substantial compared to the impact of the relatively mild chemotherapeutic capecitabine (2-4). Since antibiotics are commonly applied in mCRC patients receiving palliative chemotherapy due to several comorbidities, this should be taken into account for future studies in this field. The fecal sample of P20 contained a relatively high relative abundance of bifidobacteria. Surprisingly, this patient also showed the highest tumor response (40% decrease in the sum of target lesions). Bifidobacteria are known to have immune-modulating effects and contribute to the production of the short-chain fatty acid (SCFA) e.g. acetate (34). These observations in individual patients are interesting but surely need further investigation in larger groups in order to have clinical relevance. Furthermore, the potential role of SCFA-producing microbiota underlines the importance of performing functional microbiota analysis by performing metagenomic sequencing or measuring microbial metabolites in the future. Furthermore, it would be worthwhile to use full length 16S sequencing or metagenomic sequencing to acquire even higher taxonomic resolutions when studies evolve from explorative pilot studies to more causal designs (35).

In general, our study was limited by the small group size and an unequal distribution between responders (n=6) and non-responders (n=26). Large heterogeneity concerning inter and intra-individual microbiota composition and diversity further complicated the detection of differences on group level. This heterogeneity is most likely caused by a diverse medical history and other strong microbiota-modulating factors, such as the living environment, diet, and antibiotics (30, 36).
Furthermore, the relatively mild cytotoxic effects of capecitabine might have contributed to the lack of association between capecitabine treatment and microbiota modulation (37). It is known that capecitabine is converted in tumor tissue to its cytotoxic moiety 5-FU and that approximately 3% of the dose is excreted via the feces (37), thereby passing the colon. Compared to other chemotherapeutics, gastrointestinal toxicity was relatively low in the present study population. A possible explanation might be that the study period ended after

the third cycle of capecitabine. It is probable that manifestation of gastrointestinal toxicity takes more time and that it would be beneficial to extend the study period in future studies (38, 39). Due to the low prevalence of gastrointestinal toxicity in the current cohort, the association between gastrointestinal toxicity and the intestinal microbiota was not analyzed in the present study, but is considered to be highly relevant for future research.

Before proceeding to clinical interventions studies with pre- and/or probiotics or even fecal microbiota transplantation (FMT) in mCRC patients with a complex medical history, changes in intestinal microbiota composition and diversity should be evaluated in studies with larger and more equal group sizes between responders and non-responders supported with functional microbiota analysis. In view of the fact that the maintenance of quality of life is essential for mCRC patients, the design of targeted interventions improving treatment response and decreasing gastrointestinal toxicity for these patients is considered to be pivotal. Our study provides insights into potential challenges and points of attention for the design of upcoming microbiota studies in this complex patient population.

In conclusion, intestinal microbiota composition and diversity before, during, and after three cycles of capecitabine were not associated with response in the current small study population. High inter- and intra-individual microbiota variations were observed during capecitabine treatment. This is most likely due to an extensive medical history in this complex patient group. This highly variable microbiota composition and diversity is a great challenge for the application of personalized medicine and microbiota-based therapies. It should be noted that the results of the current study are limited by the small group sizes and large heterogeneity. However, we provide a framework and insights into potential challenges and points of attention for future studies in mCRC patients. Additional longitudinal studies using larger and equal cohorts will be highly relevant to further explore microbiota-therapy interactions in mCRC patients. Upcoming research should also focus on functional microbiota analysis by performing metagenomic sequencing or measuring microbial metabolites. This knowledge could support future interventions with pre- or probiotics and/or fecal microbiota transplantations.

Clinical practice points

With the current study, we provide a framework and insights into potential challenges and points of attention for future studies in mCRC patients. Additional longitudinal studies using larger and equal cohorts will be highly relevant to further explore microbiota-therapy interactions in mCRC patients. Upcoming research should also focus on functional microbiota analysis by performing metagenomic sequencing or measuring microbial metabolites. This knowledge could support future interventions with pre- or probiotics and/or fecal microbiota transplantations.

Author contributions

Romy Aarnoutse: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration, Funding acquisition. **Janine Ziemons:** Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration. **Judith de Vos-Geelen:** Conceptualization, Methodology, Resources, Data Curation, Writing - Review & Editing, Supervision. **Liselot Valkenburg-van Iersel:** Resources, Data Curation, Writing - Review & Editing. **Aurelia Wildeboer:** Data Curation, Writing - Review & Editing. **Anne Vievermans:** Data Curation, Writing - Review & Editing. **Geert-Jan Creemers:** Resources, Data Curation, Writing - Review & Editing. **Arnold Baars:** Resources, Data Curation, Writing - Review & Editing. **Hanneke Vestjens:** Resources, Data Curation, Writing - Review & Editing. **Giang Le:** Software, Formal analysis, Writing - Review & Editing. **David Barnett:** Software, Formal analysis, Writing - Review & Editing. **Sander Rensen:** Conceptualization, Methodology, Validation, Writing - Review & Editing. **John Penders:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - Review & Editing, Supervision. **Marjolein Smidt:** Conceptualization, Methodology, Validation, Writing - Review & Editing, Supervision, Funding acquisition

Disclosure

JdVG has served as a consultant for Amgen, AstraZeneca, MSD, Pierre Fabre, and Servier. All outside the submitted work. LV has served as a consultant for MSD, Pierre Fabre, and Servier. All outside the submitted work. JdVG, MLS, and RA has received institutional research funding from Servier. All outside the submitted work.
The other authors declare no potential conflicts of interest.

Acknowledgement

We thank all patients who participated in this study. Medical oncologists and research nurses from Catharina Hospital, VieCuri Medical Centre, Hospital Gelderse Vallei, and MUMC+ assisted with patient inclusion and sample collection. Especially, we are grateful to Marjan Laven, Ramon Bax, Sanne Achten, Eva de Jong, Alina van de Vendel, Janneke van den Brink, Ilona van Rooij-Tieleman, Wendy Heuts, Nicol Pepels, and Kim Puts-van der Burgt. We wish to acknowledge Christel Driessen and Wesley Nix for supporting the laboratory analyses. We also thank Renée Granzier for providing support for data analysis.

Funding

This work was supported by the Stichting Jules Coenegracht Sr.

References

1. Rawla P, Sunkara T, Barsouk A. Epidemiology of colorectal cancer: incidence, mortality, survival, and risk factors. Prz Gastroenterol. 2019;14(2):89-103.
2. Cunningham D, Lang I, Marcuello E, Lorusso V, Ocvirk J, Shin DB, et al. Bevacizumab plus capecitabine versus capecitabine alone in elderly patients with previously untreated metastatic colorectal cancer (AVEX): an open-label, randomised phase 3 trial. Lancet Oncol. 2013;14(11):1077-85.
3. Hoff PM, Ansari R, Batist G, Cox J, Kocha W, Kuperminc M, et al. Comparison of oral capecitabine versus intravenous fluorouracil plus leucovorin as first-line treatment in 605 patients with metastatic colorectal cancer: results of a randomized phase III study. J Clin Oncol. 2001;19(8):2282-92.
4. Van Cutsem E, Findlay M, Osterwalder B, Kocha W, Dalley D, Pazdur R, et al. Capecitabine, an oral fluoropyrimidine carbamate with substantial activity in advanced colorectal cancer: results of a randomized phase II study. J Clin Oncol. 2000;18(6):1337-45.
5. Team NIHHMPA. A review of 10 years of human microbiome research activities at the US National Institutes of Health, Fiscal Years 2007-2016. Microbiome. 2019;7(1):31.
6. Helmink BA, Khan MAW, Hermann A, Gopalakrishnan V, Wargo JA. The microbiome, cancer, and cancer therapy. Nat Med. 2019;25(3):377-88.
7. Sougiannis AT, VanderVeen BN, Enos RT, Velazquez KT, Bader JE, Carson M, et al. Impact of 5 fluorouracil chemotherapy on gut inflammation, functional parameters, and gut microbiota. Brain Behav Immun. 2019;80:44-55.
8. An J, Ha EM. Combination Therapy of Lactobacillus plantarum Supernatant and 5-Fluouracil Increases Chemosensitivity in Colorectal Cancer Cells. J Microbiol Biotechnol. 2016;26(8):1490-503.
9. Yuan L, Zhang S, Li H, Yang F, Mushtaq N, Ullah S, et al. The influence of gut microbiota dysbiosis to the efficacy of 5-Fluorouracil treatment on colorectal cancer. Biomed Pharmacother. 2018;108:184-93.
10. Zimmermann M, Zimmermann-Kogadeeva M, Wegmann R, Goodman AL. Mapping human microbiome drug metabolism by gut bacteria and their genes. Nature. 2019;570(7762):462-7.
11. Aarnoutse R, Ziemons J, Penders J, Rensen SS, de Vos-Geelen J, Smidt ML. The Clinical Link between Human Intestinal Microbiota and Systemic Cancer Therapy. Int J Mol Sci. 2019;20(17):4145.
12. Li J, Li J, Lyu N, Ma Y, Liu F, Feng Y, et al. Composition of fecal microbiota in low-set rectal cancer patients treated with FOLFOX. Ther Adv Chronic Dis. 2020;11:2040622320904293.
13. Aarnoutse R, de Vos-Geelen JMPGM, Penders J, Boerma EG, Warmerdam FARM, Goorts B, et al. Study protocol on the role of intestinal microbiota in colorectal cancer treatment: a pathway to personalized medicine 2.0. International Journal of Colorectal Disease. 2017:1-8.
14. Aarnoutse R, de Vos-Geelen J, Penders J, Boerma EG, Warmerdam F, Goorts B, et al. Study protocol on the role of intestinal microbiota in colorectal cancer treatment: a pathway to personalized medicine 2.0. Int J Colorectal Dis. 2017;32(7):1077-84.
15. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer. 2009;45(2):228-47.
16. Institute NIoHNC. Common Terminology Criteria for Adverse Events (CTCEA). U.S.: Department of Health and Human Services; 2010.
17. Liu PY, Wu WK, Chen CC, Panyod S, Sheen LY, Wu MS. Evaluation of Compatibility of 16S rRNA V3V4 and V4 Amplicon Libraries for Clinical Microbiome Profiling. bioRxiv. 2020.
18. Galazzo G, van Best N, Benedikter BJ, Janssen K, Bervoets L, Driessen C, et al. How to Count Our Microbes? The Effect of Different Quantitative Microbiome Profiling Approaches. Front Cell Infect Microbiol. 2020;10:403.
19. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13(7):581-3.
20. Team RC. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2020 [Available from: https://www.R-project.org/.
21. Lagkouvardos I, Fischer S, Kumar N, Clavel T. Rhea: a transparent and modular R pipeline for microbial profiling based on 16S rRNA gene amplicons. PeerJ. 2017;5:e2836.
22. Chen J. GUniFrac: Generalized UniFrac Distances. R package version 1.1. 2018 [Available from: https://CRAN.R-project.org/package=GUniFrac.

23. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One. 2013;8(4):e61217.
24. Jari Oksanen FGB, Michael Friendly, Roeland Kindt, Pierre Legendre, Dan McGlinn,, Peter R. Minchin RBOH, Gavin L. Simpson, Peter Solymos, M. Henry H. Stevens, Eduard Szoecs, Wagner aH. vegan: Community Ecology Package. R package version 2.5-6. 2019 [Available from: https://CRAN.R-project.org/package=vegan.
25. Lahti L, Ernst FGM, Huang R, Shetty S, Borman T, Braccia DC, et al. microbiome R package 2020 [Available from: http://microbiome.github.io.
26. Wickham H, François R, Henry L, Müller K. dplyr: A Grammar of Data Manipulation. R package version 1.0. 2 (2020). 2020.
27. Wickham H. ggplot2: elegant graphics for data analysis: springer; 2016.
28. Barnett DJM, Arts ICW, Penders J. microViz: an R package for microbiome data visualization and statistics. The Journal of Open Source Software. 2021;6(63):3201.
29. Mandal S, Van Treuren W, White RA, Eggesbø M, Knight R, Peddada SD. Analysis of composition of microbiomes: a novel method for studying microbial composition. Microbial ecology in health and disease. 2015;26(1):27663.
30. Balhareth A, Aldossary MY, McNamara D. Impact of physical activity and diet on colorectal cancer survivors' quality of life: a systematic review. World J Surg Oncol. 2019;17(1):153.
31. Sze MA, Baxter NT, Ruffin MTt, Rogers MAM, Schloss PD. Normalization of the microbiota in patients after treatment for colonic lesions. Microbiome. 2017;5(1):150.
32. Zwielehner J, Lassl C, Hippe B, Pointner A, Switzeny OJ, Remely M, et al. Changes in human fecal microbiota due to chemotherapy analyzed by TaqMan-PCR, 454 sequencing and PCR-DGGE fingerprinting. PLoS One. 2011;6(12):e28654.
33. Dubinsky V, Reshef L, Bar N, Keizer D, Golan N, Rabinowitz K, et al. Predominantly Antibiotic-resistant Intestinal Microbiome Persists in Patients With Pouchitis Who Respond to Antibiotic Therapy. Gastroenterology. 2020;158(3):610-24 e13.
34. Riviere A, Selak M, Lantin D, Leroy F, De Vuyst L. Bifidobacteria and Butyrate-Producing Colon Bacteria: Importance and Strategies for Their Stimulation in the Human Gut. Front Microbiol. 2016;7:979.
35. Durazzi F, Sala C, Castellani G, Manfreda G, Remondini D, De Cesare A. Comparison between 16S rRNA and shotgun sequencing data for the taxonomic characterization of the gut microbiota. Sci Rep. 2021;11(1):3030.
36. Manor O, Dai CL, Kornilov SA, Smith B, Price ND, Lovejoy JC, et al. Health and disease markers correlate with gut microbiome composition across thousands of people. Nat Commun. 2020;11(1):5206.
37. Reigner B, Blesch K, Weidekamm E. Clinical pharmacokinetics of capecitabine. Clin Pharmacokinet. 2001;40(2):85-104.
38. van Hellemond IEG, Thijs AM, Creemers GJ. Capecitabine-Associated Terminal Ileitis. Case Rep Oncol. 2018;11(3):654-9.
39. FDA. Description Xeloda (capecitabine) tablets for oral use. 2015. Report No.: 3718670

Supplementary methods

Fecal microbiota analyses

In order to extract metagenomic DNA, 250 mg of the frozen fecal samples were homogenized in phosphate buffered saline (PBS) and were centrifuged for 1 minute at 900 rpm. For cell lysis, a combination of chemical, mechanical and thermal disruption was used. A lysis buffer containing 1M Tris-HCl, 0.5M EDTA, 5M sterile NaCl and SDS (final concentration 4%) was filled into bead tubes of the Ambion MagMaxTM Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific) and mixed with 175 µl supernatant of feces in PBS. Mechanical disruption consisted of a bead-beating procedure using the Fastprep™ Homogenizer (5,5 ms for 3x1 min; resting 1 min in between, MP Biomedicals). Samples were subsequently incubated for 15 minutes at 95°C with gentle shaking. After centrifugation for five minutes at 11000 rpm, supernatant was filled in an Eppendorf tube. Afterwards, a second round of bead beating and incubation was performed and supernatants were pooled and stored at -20°C until further analysis. 200 µl of the supernatants were introduced into a KingFisher 96-wells deep well plate (Thermo Fisher Scientific), together with bead mix of the Ambion MagMaxTM Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific), isopropanol, and lysis buffer. Other plates were filled with wash buffers, elution buffer (+RNAse), and 96-tips for DW magnets (Thermo Fisher Scientific). Afterwards, the prepared plates were introduced into the KingFisher system and the DNA isolation was performed according to the manufacturer's standard protocol (Thermo Fisher Scientific). After removal of the plates from the system, the plate containing purified nucleic acids was incubated for 15 minutes at 37°C for degradation of RNA.

Subsequently, according to current international accepted standards (1) the V4 hypervariable region of the 16S rRNA gene was amplified in triplicate using the 515F/806R barcoded primer pair as described previously (2). Pooled amplicons from the triplicate reactions were purified using AMPure XP purification (Agencourt) according to the manufacturer's instructions and eluted in 25 µl 1 × low TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Quantification of amplicons was subsequently performed by the Quant-iT PicoGreen dsDNA reagent kit (Invitrogen) using a Victor3 Multilabel Counter (Perkin Elmer, Waltham, USA). Amplicons were mixed in equimolar concentrations to ensure equal representation of each sample and sequenced on an Illumina MiSeq instrument (MiSeq Reagent Kit v3, 2 × 250 cycles, 10% PhiX) to generate paired-end reads of 250 bases (~25.000 reads/sample) (3).

The pre-processing of sequencing data, using an in-house pipeline based upon DADA2 (R version 4.0.3) (4), consisted of the following steps: reads filtering, identification of sequencing errors, dereplication, and removal of chimeric sequences. In order to assign taxonomy, DECIPHER (5) was used to annotate to the genus level. Data were expressed as amplicon sequence variants (ASVs). Decontam was used with the either setting, which combines the two statistical methods prevalence and frequency for the identification of contamination in marker-gene and metagenomics data (6). Contaminated ASVs identified by decontam were filtered out together with ASVs presented in less than 5% of all samples and a total abundance of less than 0.001%. A total of 908 ASVs were maintained for downstream analysis. The final file was saved in the phyloseq format (7).

References

1. Liu PY, Wu WK, Chen CC, Panyod S, Sheen LY, Wu MS. Evaluation of Compatibility of 16S rRNA V3V4 and V4 Amplicon Libraries for Clinical Microbiome Profiling. bioRxiv. 2020.
2. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. Isme j. 2012;6(8):1621-4.
3. Galazzo G, Tedjo DI, Wintjens DSJ, Savelkoul PHM, Masclee AAM, Bodelier AGL, et al. Fecal Microbiota Dynamics and their Relation to Disease Course in Crohn's Disease. J Crohns Colitis. 2019;13(10):1273-82.
4. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13(7):581-3.
5. Wright ES. Using DECIPHER v2.0 to Analyze Big Biological Sequence Data in R. The R Journal. 2016;8(1):352-9.
6. Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome. 2018;6(1):226.
7. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One. 2013;8(4):e61217.

Supplementary tables

Table S1: *Clinical characteristics*

Baseline characteristics	Total	Responders	Non-responders	p-value
Total no. of weeks previous systemic treatment				
Median (IQR)	0 (24)	13 (31)	0 (24)	
25-75%	0-24	0-31	0-24	0.436
Time between collection fecal sample and last systemic treatment – days				
Median (IQR)	686 (1000)	425 (1452)	705 (869)	
25-75%	261-1261	140-1592	392-1261	0.446
Therapeutic antibiotic use last year – No. (%)	8 (24)	0 (0)	8 (31)	0.296
Time between collection fecal sample and last therapeutic antibiotic treatment – days				
Mean (SD)	197 (±101)	NA	197 (±101)	
Range	93-394		93-394	NA
Prophylactic antibiotics use last year – No. (%)	10 (30)	2 (33)	7 (27)	1.000
Time between collection fecal sample and last prophylactic antibiotic treatment – days				
Mean (SD)	96 (±92)	23 (±24)	91 (±74)	
Range	0-276	6-40	0-171	0.083
Previous chemoradiation – No. (%)	6 (18)	0 (0)	6 (23)	0.564
Use of PPI at T1– No. (%)	14 (42)	1 (17)	12 (46)	0.361
Co-treatment with bevacizumab – No. (%)	27 (82)	5 (83)	21 (81)	1.000
No. of years smoking until T1				
Mean (SD)	32 (±17)	26 (±22)	32 (±17)	
Range	2-68	7-50	2-68	0.559
Type of colorectal surgery – No. (%)*				
Rectal resection	12 (41)	2 (33)	10 (46)	
Sigmoid resection	5 (17)	0 (0)	5 (23)	
Hemicolectomy left	2 (7)	1 (17)	1 (5)	
Extended hemicolectomy left	1 (3)	1 (17)	0 (0)	
Transverse resection	0 (0)	0 (0)	0 (0)	
Hemicolectomy right	8 (28)	2 (33)	5 (23)	
Extended hemicolectomy right	0 (0)	0 (0)	0 (0)	
Subtotal colectomy	0 (0)	0 (0)	0 (0)	
Total colectomy	0 (0)	0 (0)	0 (0)	
Unknown	1 (3)	0 (0)	1 (5)	0.316
Tumor mutation status – No. (%)				
KRAS/NRAS/BRAF WT	9 (27)	1 (17)	8 (31)	
KRAS mutation	13 (40)	4 (66)	9 (34)	
NRAS mutation	1 (3)	0 (0)	1 (4)	
BRAF mutation	2 (6)	0 (0)	1 (4)	
Unknown	8 (24)	1 (17)	7 (27)	0.865

Response could not be evaluated in one patient.

** Percentages do not add up to 100% due to rounding.*

Table S2: *Clinical characteristics during chemotherapy*

Baseline characteristics	Total	Responders	Non-responders	p-value
Antibiotic use between T1 and T2 – No. (%)**	5 (16)	0 (0)	5 (19)	0.555
Days antibiotics use between T1 and T2				
Median (IQR)	7 (27)	NA	7 (27)	NA
% Capecitabine administered – *Median (IQR)*				0.356
Cycle 1	94 (19)	86 (23)	95 (17)	0.131
Cycle 2	95 (16)	84 (22)	96 (14)	0.119
Cycle 3	95 (16)	84 (21)	96 (15)	
Compliant at T2	24 (89)	4 (100)	20 (87)	1.000
% Tumor change				
Mean (SD)	-13 (17)	-34 (14)	-8 (13)	<0.001
Range	-53-22	-53 - -10	-29-22	
Continuation cycle 4 – No. (%)*	28 (88)	6 (100)	22 (85)	0.566

Response could not be evaluated in one patient.

** Percentages do not add up to 100% due to rounding.*

***Between T2-T3 only one non-responder received oral amoxicillin and ciprofloxacin to treat pneumonia.*

Table S3: *CTCAE at T1*

Toxicity grade	Total	Responders	Non-responders	*p*-value
Diarrhea without colostomy – No. (%)				
0	19 (86)	4 (100)	14 (82)	1.000
1	3 (14)	0 (0)	3 (18)	
Diarrhea with colostomy – No (%)*				
0	7 (70)	1 (50)	6 (75)	
1	1 (10)	0 (0)	1 (13)	0.186
2	1 (10)	0 (0)	1 (13)	
3	1 (10)	1 (50)	0 (0)	
Diarrhea with or without colostomy – No (%)				
0	27 (82)	5 (83)	21 (81)	0.384
1	4 (12)	0 (0)	4 (15)	
2	1 (3)	0 (0)	1 (4)	
3	1 (3)	1 (17)	0 (0)	
Peripheral Sensory Neuropathy – No (%)				
0	30 (91)	5 (83)	24 (92)	0.476
1	3 (9)	1 (17)	2 (8)	
Hand Foot Syndrome – No. (%)				NA
0	32 (100)	6 (100)	25 (100)	
Fatigue – No (%)*				
0	14 (42)	3 (50)	10 (39)	0.753
1	14 (42)	2 (33)	12 (46)	
2	5 (15)	1 (17)	4 (15)	
Nausea – No (%)				
0	27 (82)	6 (100)	20 (77)	0.226
1	5 (15)	0 (0)	5 (19)	
2	1 (3)	0 (0)	1 (4)	
Oral mucositis – No (%)				NA
0	33 (100)	6 (100)	26 (100)	
Vomiting – No. (%)				NA
0	33 (100)	6 (100)	26 (100)	
Constipation – No (%)				
0	27 (82)	6 (100)	21 (81)	0.773
1	6 (18)	0 (0)	5 (19)	

Response could not be evaluated in one patient.

** Percentages do not add up to 100% due to rounding.*

Table S4: *CTCAE at T2*

Toxicity grade	Total	Responders	Non-responders	*p*-value
Diarrhea without colostomy – No. (%)				
0	17 (94)	3 (100)	14 (93)	1.000
1	1 (6)	0 (0)	1 (7)	
Diarrhea with colostomy – No (%)				
0	6 (60)	0 (0)	6 (75)	0.133
1	4 (40)	2 (100)	2 (25)	
Diarrhea with or without colostomy – No (%)*				0.228
0	27 (84)	4 (67)	23 (89)	
1	5 (16)	2 (33)	3 (12)	
Peripheral Sensory Neuropathy – No (%)*				
0	19 (59)	5 (83)	14 (54)	
1	10 (31)	1 (17)	9 (35)	0.193
2	2 (6)	0 (0)	2 (8)	
3	1 (3)	0 (0)	1 (4)	
Hand Foot Syndrome – No. (%)*				
0	17 (53)	3 (50)	14 (54)	0.800
1	12 (38)	3 (50)	9 (35)	
2	3 (9)	0 (0)	3 (12)	
Fatigue – No (%)*				
0	7 (22)	4 (67)	3 (12)	
1	20 (63)	1 (17)	19 (73)	0.067
2	4 (13)	1 (17)	3 (12)	
3	1 (3)	0 (0)	1 (4)	
Nausea – No (%)				
0	22 (69)	5 (83)	17 (65)	0.637
1	10 (31)	1 (17)	9 (35)	
Oral mucositis – No (%)				
0	19 (59)	5 (83)	14 (54)	
1	12 (38)	1 (17)	11 (42)	0.221
2	0 (0)	0 (0)	0 (0)	
3	1 (3)	0 (0)	1 (4)	
Vomiting – No. (%)				NA
0	32 (100)	6 (100)	26 (100)	
Constipation – No (%)				
0	26 (81)	5 (83)	21(81)	0.773
1	5 (16)	1 (17)	4 (15)	
2	1 (3)	0 (0)	1 (4)	

Response could not be evaluated in one patient.

** Percentages do not add up to 100% due to rounding.*

Table S5: *CTCAE at T3*

Toxicity grade	Total	Responders	Non-responders	*p*-value
Diarrhea without colostomy – No. (%)				
0	19 (86)	4 (100)	15 (83)	0.442
1	2 (9)	0 (0)	2 (11)	
2	1 (5)	0 (0)	1 (6)	
Diarrhea with colostomy – No (%)*				
0	6 (60)	1 (50)	5 (63)	1.000
1	3 (30)	1 (50)	2 (25)	
2	1 (10)	0 (0)	1 (13)	
Diarrhea with or without colostomy – No (%)				
0	25 (78)	5 (83)	20 (77)	0.592
1	5 (16)	1 (17)	4 (15)	
2	2 (6)	0 (0)	2 (8)	
Peripheral Sensory Neuropathy – No (%)*				
0	20 (63)	4 (67)	16 (62)	0.458
1	7 (22)	2 (33)	5 (19)	
2	4 (13)	0 (0)	4 (15)	
3	1 (3)	0 (0)	1 (4)	
Hand Foot Syndrome – No. (%)*				
0	13 (41)	2 (33)	11 (42)	
1	11 (34)	2 (33)	9 (35)	0.784
2	6 (19)	2 (33)	4 (15)	
3	2 (6)	0 (0)	2 (8)	
Fatigue – No (%)*				
0	5 (16)	3 (50)	2 (8)	
1	22 (69)	3 (50)	19 (73)	0.026
2	4 (13)	0 (0)	4 (15)	
3	1 (3)	0 (0)	1 (4)	
Nausea – No (%)*				
0	21 (66)	5 (83)	16 (62)	0.271
1	8 (25)	1 (17)	7 (27)	
2	3 (9)	0 (0)	3 (12)	
Oral mucositis – No (%)				
0	21 (66)	4 (67)	17 (65)	
1	9 (28)	2 (33)	7 (27)	0.692
2	1 (3)	0 (0)	1 (4)	
3	1 (3)	0 (0)	1 (4)	
Vomiting – No. (%)				NA
0	32 (100)	6 (100)	26 (100)	
Constipation – No (%)				
0	26 (81)	5 (83)	21 (81)	
1	5 (16)	1 (17)	4 (15)	0.716
2	0 (0)	0 (0)	0 (0)	
3	1 (3)	0 (0)	1 (4)	

Response could not be evaluated in one patient.

** Percentages do not add up to 100% due to rounding.*

Table S6: *Longitudinal CTCAE*

Toxicity grade	T1	T2	T3	*p*-value
Diarrhea without colostomy – No. (%)*				
0	19 (86)	17 (94)	19 (86)	0.449
1	4 (13)	1 (6)	2 (9)	
2	0 (0)	0 (0)	1 (5)	
Diarrhea with colostomy – No (%)				
0	7 (70)	6 (60)	6 (60)	
1	1 (10)	4 (40)	3 (30)	0.819
2	1 (10)	0 (0)	1 (10)	
3	1 (10)	0 (0)	0 (0)	
Diarrhea with or without colostomy – No (%)				
0	27 (82)	27 (84)	25 (78)	
1	4 (12)	5 (16)	5 (16)	0.407
2	1 (3)	0 (0)	2 (6)	
3	1 (3)	0 (0)	0 (0)	
Peripheral Sensory Neuropathy – No (%)*				
0	30 (91)	19 (59)	20 (63)	
1	3 (9)	10 (31)	7 (22)	0.002[1]
2	0 (0)	2 (6)	4 (13)	
3	0 (0)	1 (3)	1 (3)	
Hand Foot Syndrome – No. (%)				
0	32 (100)	17 (53)	13 (41)	
1	0 (0)	12 (38)	11 (34)	<0.001[2]
2	0 (0)	3 (9)	6 (19)	
3	0 (0)	0 (0)	2 (6)	
Fatigue – No (%)*				
0	14 (42)	7 (22)	5 (16)	
1	14 (42)	20 (63)	22 (69)	0.154
2	5 (15)	4 (13)	4 (13)	
3	0 (0)	1 (3)	1 (3)	
Nausea – No (%)				
0	27 (82)	22 (69)	21 (66)	0.132
1	5 (15)	10 (31)	8 (25)	
2	1 (3)	0 (0)	3 (9)	
Oral mucositis – No (%)				
0	33 (100)	19 (59)	21 (66)	
1	0 (0)	12 (38)	9 (28)	<0.001[3]
2	0 (0)	0 (0)	1 (3)	
3	0 (0)	1 (3)	1 (3)	
Vomiting – No. (%)				NA
0	33 (100)	32 (100)	32 (100)	
Constipation – No (%)				
0	27 (82)	26 (81)	26 (81)	
1	6 (18)	5 (16)	5 (16)	0.761
2	0 (0)	1 (3)	0 (0)	
3	0 (0)	0 (0)	1 (3)	

** Percentages do not add up to 100% due to rounding.*

[1] Post hoc Wilcoxon test with Bonferroni correction indicated a significant difference between T1-T2 (p=0.002) and T1-T3 (p=0.007).

[2] Post hoc Wilcoxon test with Bonferroni correction indicated a significant difference between T1-T2 (p=<0.001), T1-T3 (p=<0.001), and T2-T3 (p=0.002).
[3] Post hoc Wilcoxon test with Bonferroni correction indicated a significant difference between T1-T2 (p=<0.001) and T1-T3 (p=0.002).

***Table S7**: Bone marrow toxicity*

Variable	Pre	Post	*p*-value
Hemoglobin – in µ/L			
Mean (SD)	8.3 (1.1)	8.0 (1.0)	0.166
Leucocytes – in 10^9/l			
Median (IQR)	7.4 (1.8)	6.0 (2.9)	0.032
Neutrophils – 10^9/l			
Median (IQR)	5.1 (1.7)	3.7 (2.4)	0.006
Thrombocytes – in 10^9/l			
Median (IQR)	248 (111)	186 (110)	<0.001

***Table S8:** Longitudinal data*

	T1	T2	T3	
MUST score – No (%)*				
Low risk	26 (79)	25 (83)	29 (94)	0.554
Medium risk	4 (12)	4 (13)	0 (0)	
High risk	3 (9)	1 (3)	2 (7)	
Karnofsky Performance Score – No (%)*				
Median (IQR)	90 (20)	80 (20)	80 (23)	
50	1 (3)	1 (3)	1 (3)	0.013**
60	2 (7)	4 (14)	6 (20)	
70	3 (10)	6 (21)	5 (17)	
80	6 (19)	5 (17)	8 (27)	
90	11 (36)	7 (24)	8 (27)	
100	8 (26)	6 (21)	2 (7)	
Carcino Embryonic Antigen – in µ/L				0.234
Median (IQR)	28 (100)	23 (79)	23 (64)	

** Percentages do not add up to 100% due to rounding.*
*** Post hoc Wilcoxon test with Bonferroni correction indicated a significant difference between T1-T3 (p=0.002).*

Table S9: *α-diversity at T1*

α-diversity	Responders *n*=6	Non-responders *n*=26	*p*-value
Shannon effective*			0.786
Mean (SD)	47.0 (29.8)	50.1 (24.3)	
Median (IQR)	47.3 (46.7)	46.3 (36.6)	
Richness*			0.528
Mean (SD)	200.5 (86.8)	222.4 (73.3)	
Median (IQR)	198 (94.8)	209 (104.8)	

Response could not be evaluated in one patient.
** An independent t-test was performed.*

Table S10: *α-diversity at T2*

α-diversity	Responders n=5	Non-responders n=22	p-value
Shannon effective*			0.301
Mean (SD)	37.3 (23.3)	48.0 (19.6)	
Median (IQR)	36.6 (32.8)	44.5 (26.6)	
Richness*			0.145
Mean (SD)	165.2 (83.3)	220.4 (72.3)	
Median (IQR)	168 (92)	215 (102.2)	

Response could not be evaluated in one patient.
** An independent t-test was performed.*

Table S11: *α-diversity changes over time*

α-diversity	T1	T2	T3	*p*-value
Shannon effective*				0.640
Mean (SD)	48.9 (26.2)	46.6 (19.9)	48.5 (23.8)	
Median (IQR)	45.0 (39.3)	43.3 (26.2)	46.0 (31.0)	
Richness**				0.240
Mean (SD)	220.3 (79.5)	211.1 (75.4)	202.9 (85.6)	
Median (IQR)	203 (110)	212 (104)	210 (135)	

** The Friedman test was performed.*
*** Repeated measures ANOVA was performed.*

Table S12: *Within-subject temporal (in)stability of β-diversity between responders and non-responders*

β-diversity	T1 vs. T2	T2 vs. T3	T1 vs. T3
Generalized UniFrac*	*p*=0.8	*p*=0.9	*p*=0.3
Bray-Curtis*	*p*=0.6	*p*=0.4	*p*=0.07

**A Mann Whitney U test was performed.*

Supplementary figures

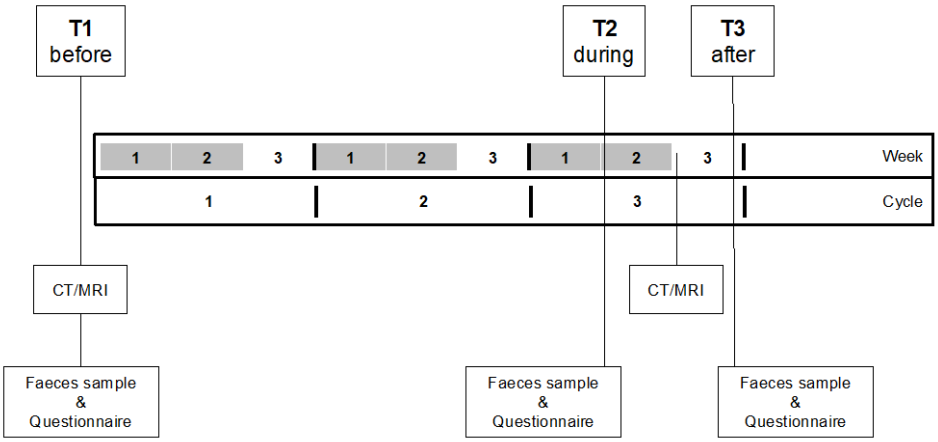


Figure S1: Study design. *Patients collected fecal samples and completed questionnaires at three time points. T1 is collected before the start of the first capecitabine cycle, T2 is collected between day 7-14 of the third cycle, and T3 is collected at day 20 or 21 of the third cycle. Tumor response was assessed using CT or MRI scans before and at the end of three cycles capecitabine by means of RECIST. During the observation period, patients received three cycles capecitabine (1000-1250 mg/m^2 orally, twice daily on days 1-14 in a three week cycle), this is illustrated in gray. Depending on the decision of the medical oncologist, patients continue with capecitabine treatment after the third treatment cycle.*

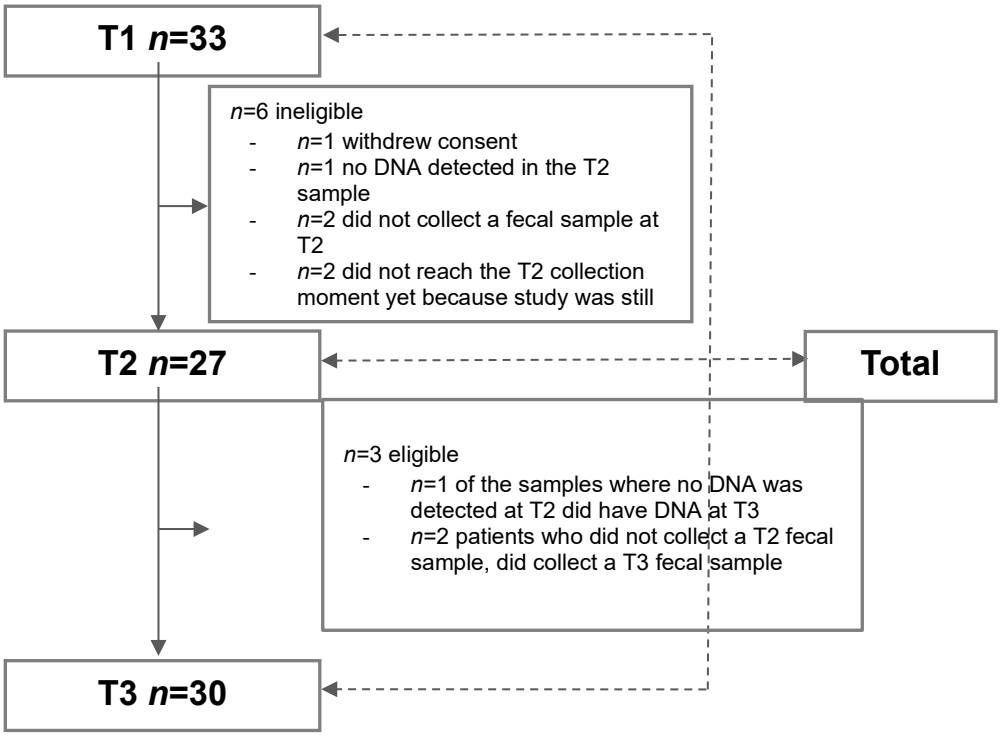


Figure S2: *Overview of fecal samples used for 16S rRNA gene sequencing of the V4 hypervariable region.*

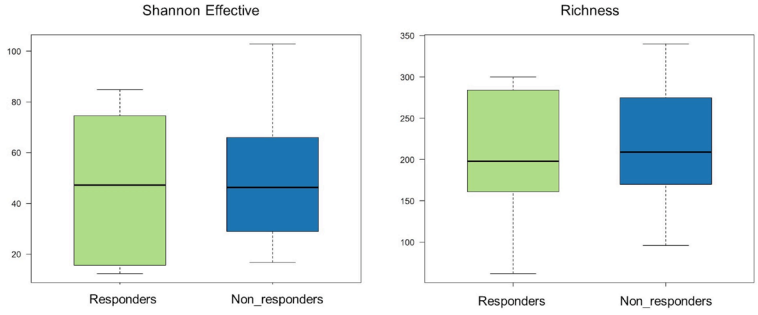


Figure S3: α-diversity measures. *Microbial diversity and richness of responders and non-responders at T1, measured in terms of Shannon effective (p=0.786) and observed richness (p=0.528) (Table S9). Numbers presented in median (IQR).*

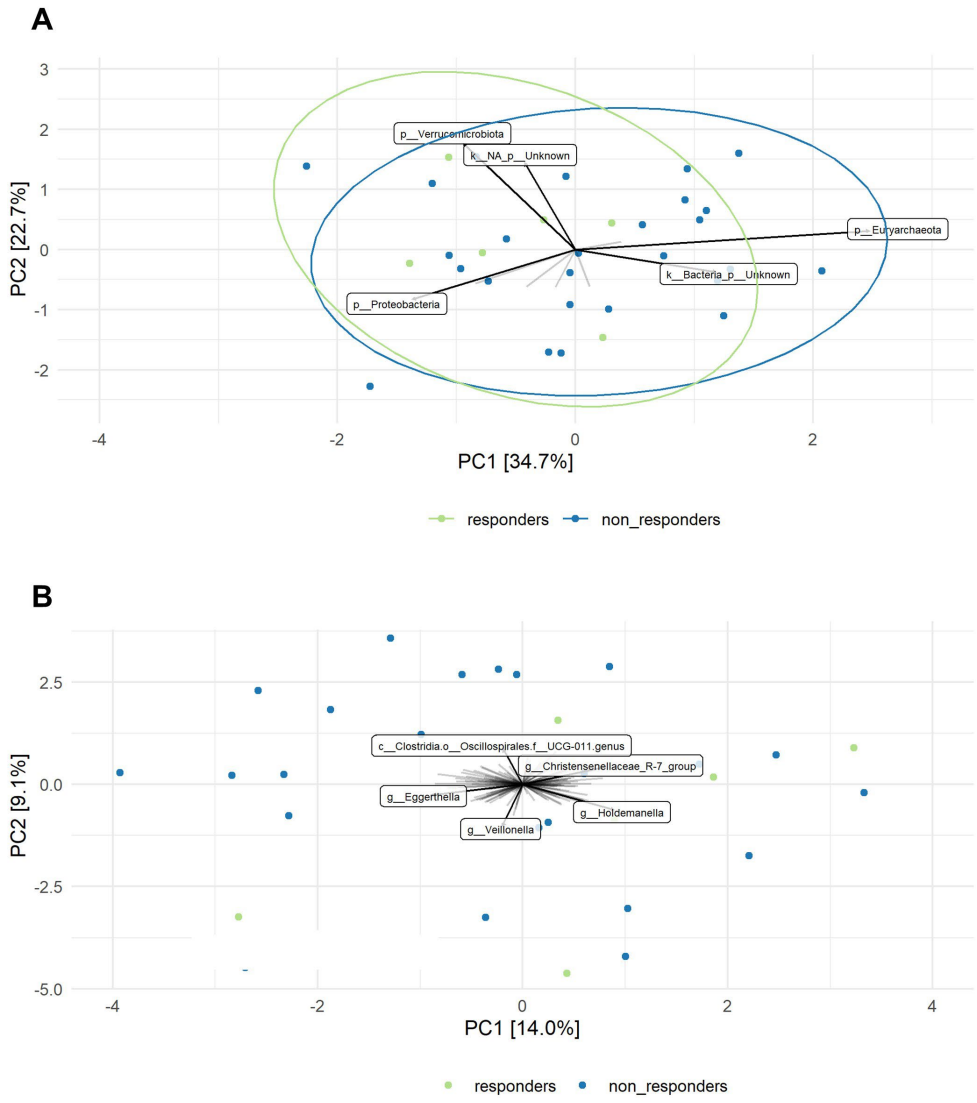


Figure S4: *Ordination plots derived from unconstrained Principal Components Analysis (PCA), showing overall composition of the microbial community on phylum (**A**) and genus level (**B**) at T1. Aitchison distance was used. 10 phyla and 156 genera were included for this analysis. Data were transformed using center-log-ratio transformation. Names are given for genera which contributed most to overall microbial variation.*

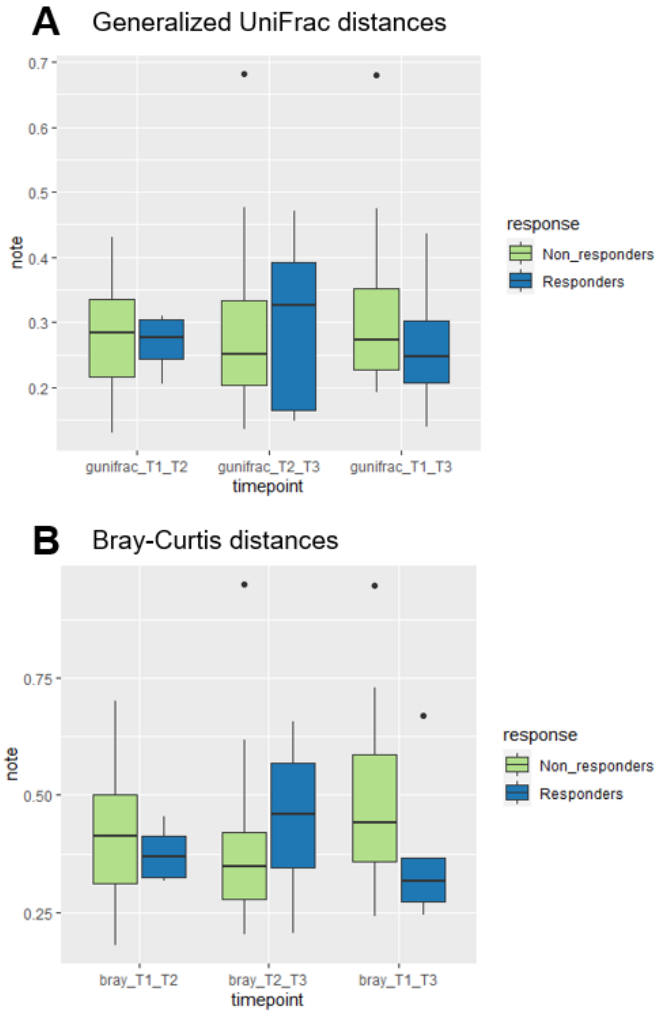


Figure S5: *Temporal (in)stability in microbial community structure (β-diversity). Changes of generalized UniFrac distances* ***(A)*** *and Bray-Curtis distances* ***(B)*** *between T1 versus T2, T2 versus T3 and T1 versus T3. At all time points, distances were not significantly different between responders and non-responders.*

Chapter 4

Fecal levels of SCFA and BCFA during capecitabine in patients with metastatic or unresectable colorectal cancer

Janine Ziemons, Romy Aarnoutse, Anne Heuft, Lars Hillege, Janneke Waelen, Judith de Vos-Geelen, Liselot Valkenburg-van Iersel, Irene E.G. van Hellemond, Geert-Jan M. Creemers, Arnold Baars, J.H.M.J. Vestjens, John Penders, Koen Venema & Marjolein L. Smidt

Clinical and Experimental Medicine. 2023 April;07.
DOI: 10.1007/s10238-023-01048-7

Abstract

Background

Gut bacteria-derived short-chain fatty acids (SCFA) and branched-chain fatty acids (BCFA) are considered to have beneficial metabolic, anti-inflammatory as well as anti-carcinogenic effects. Previous pre-clinical studies indicated bi-directional interactions between gut bacteria and the chemotherapeutic capecitabine or its metabolite 5-FU. This study investigated the effect of three cycles of capecitabine on fecal SCFA and BCFA levels and their associations with tumor response, nutritional status, physical performance, chemotherapy-induced toxicity, systemic inflammation, and bacterial abundances in patients with colorectal cancer (CRC).

Methods

Forty-four patients with metastatic or unresectable CRC, scheduled for treatment with capecitabine (±bevacizumab), were prospectively enrolled. Patients collected a fecal sample and completed a questionnaire before (T1), during (T2), and after (T3) three cycles of capecitabine. Tumor response (CT/MRI scans), nutritional status (MUST score), physical performance (Karnofsky Performance Score), and chemotherapy-induced toxicity (CTCAE) were recorded. Additional data on clinical characteristics, treatment regimen, medical history, and blood inflammatory parameters were collected. Fecal SCFA and BCFA concentrations were determined by gas chromatography-mass spectrometry (GC-MS). Gut microbiota composition was assessed using 16S rRNA amplicon sequencing.

Results

Fecal levels of the SCFA valerate and caproate decreased significantly during three cycles of capecitabine. Furthermore, baseline levels of the BCFA iso-butyrate were associated with tumor response. Nutritional status, physical performance, and chemotherapy-induced toxicity were not significantly associated with SCFA or BCFA. Baseline SCFA correlated positively with blood neutrophil counts. At all timepoints, we identified associations between SCFA and BCFA and the relative abundance of bacterial taxa on family level.

Conclusions

The present study provided first indications for a potential role of SCFA and BCFA during capecitabine treatment as well as implications for further research.

Trial registration

The current study was registered in the Dutch Trial Register (NTR6957) on 17/01/2018 and can be consulted via the International Clinical Trial Registry Platform (ICTRP).

Background

In recent years, it has become increasingly evident that the gut microbiota plays a crucial role in the development, manifestation, and treatment of different types of cancer. For instance, there is accumulating evidence that the gut microbiota interacts with chemotherapeutic drugs via various mechanisms (1-3). In this context, the role of gut microbiota-derived metabolites such as short-chain fatty acids (SCFA) and branched-chain fatty acids (BCFA) is of particular interest. A proportion of these metabolites is absorbed into the bloodstream, where they can exert not only local but also systemic effects, in that way functioning as a linking factor between the gut microbiota and human metabolism as well as carcinogenesis (4, 5).

The SCFA acetate, propionate, butyrate, valerate, and caproate are produced by gut bacteria through different metabolic pathways. In particular, dietary non-digestible carbohydrates are an important substrate for microbial fermentation and subsequent SCFA production (6). SCFA have been shown to have pivotal effects on human metabolism and the immune system. For instance, several studies showed that SCFA have potent anti-inflammatory effects by among others inhibition of histone deacetylases (HDACs) and NF-κB as well as by interaction with several G-protein coupled receptors and modulation of cytokine production (4, 7, 8). Particularly butyrate is essential for gut barrier function by serving as primary energy source for colonocytes and by modulating the expression of tight junction proteins and mucins (4, 8). In addition, SCFA have been shown to have various effects on human macronutrient metabolism and metabolic health (9).

Preclinical studies also indicated direct anti-carcinogenic effects of SCFA, as well as the potential that SCFA could sensitize cancer cells to chemotherapeutic agents. For instance, Encarnação *et al.* observed anti-proliferative effects of butyrate and a synergistic effect of irinotecan and butyrate in different colon cancer cell lines (10). Very recently, Kim *et al.* described anti-carcinogenic effects of butyrate in colon cancer cell lines, which could be potentiated by the addition of a growth medium from *Lactiplantibacillus plantarum* in butyrate-resistant cells (11). Furthermore, SCFA might reduce (gastrointestinal) side effects of the chemotherapy, which are commonly caused by intestinal barrier disruption and inflammation (12). In support of this, previous research indicated that the administration of prebiotics, alone or in combination with probiotics, might reduce the occurrence of serious side effects of chemotherapy (13, 14). Prebiotics are substrates that can be metabolized by several gut bacteria to produce SCFA, thereby stimulating the growth of SCFA-producing bacteria (15). However, it should be noted that the literature concerning the physiological roles of SCFA is divergent since some studies also described pro-inflammatory or oncogenic properties under certain circumstances (3, 7).

In contrast to SCFA, there is currently only limited knowledge concerning the exact physiological roles of BCFA. The BCFA iso-butyrate and iso-valerate are produced by the gut microbiota from branched-chain amino acids or are directly ingested via the diet (e.g. through beef and milk products) (16, 17). While BCFA are mostly studied in the context of the neonatal gut, evidence concerning physiological effects in the adult gut is scarce, but it has been suggested that BCFA also play a role in human energy metabolism and might have anti-inflammatory as well as anti-carcinogenic properties (16, 18). On the other hand, branched-

chain amino acids, the precursors of BCFA, are considered to play a role in insulin resistance, which could indicate a potentially negative effect on metabolic health (19).

In view of these physiological effects of SCFA and BCFA that are also relevant in the setting of cancer treatment, it might be expected that these microbial metabolites also play a role during treatment with chemotherapy, for instance capecitabine. Capecitabine is an orally administered prodrug that is converted intratumorally to the cytotoxic compound 5-fluorouracil (5-FU) and which is commonly administered in patients with metastatic colorectal cancer (mCRC) (20). In the last years, there is increasing evidence for bi-directional interactions between 5-FU-based therapies and the gut microbiota. For instance, large-scale *in vitro* screening studies showed that capecitabine/5-FU did not only impact the growth of several bacterial species (21), but could also be metabolized by specific gut bacteria (22). In addition, 5-FU induced shifts in gut microbiota composition in mice (23). Furthermore, it has been indicated that *Fusobacterium nucleatum* might be able to induce chemoresistance to 5-FU in CRC cells, while *Lactiplantibacillus* (previously *Lactobacillus*) *plantarum*-derived supernatant seemed to sensitize CRC cells to the anti-cancer effects of 5-FU (24-26). In contrast to this, our research group did not detect consistent capecitabine-induced changes in gut microbiota composition and diversity in a relatively small and heterogeneous group of CRC patients (27). Other clinical studies, using different chemotherapeutics, described that chemotherapy treatment affected gut microbiota composition and the abundance of prominent SCFA-producing bacteria such as *Veillonella* and *Prevotella* (14, 28, 29). While most of the previous research focused on the abundance of gut bacteria, more activity-based analyses, such as the measurement of microbial metabolites, would be of special interest in a clinical setting. Even if there was no major effect of capecitabine on taxa abundance in the previous study (27), metabolic activity and the production of relevant metabolites might have been changed in these patients, with possible clinical implications.

In mice, Ferreira *et al.* showed that oral administration of SCFA and particularly butyrate could counteract 5-FU-induced intestinal mucositis (30). However, there is currently no knowledge of the role of gut microbiota-derived SCFA and BCFA during 5-FU-based chemotherapy in a clinical setting. The present research aims to fill this gap of knowledge and investigates the effect of three cycles of capecitabine on fecal SCFA and BCFA levels and their associations with tumor response, nutritional status, physical performance, chemotherapy-induced toxicity, as well as systemic inflammation in patients with metastatic or unresectable CRC. Based on previous studies and the described beneficial effects of SCFA, it might be expected that fecal SCFA levels would reduce during capecitabine. In addition, it is hypothesized that higher SCFA levels would be associated with better tumor response, a less fragile nutritional status, increased physical performance, less toxicity, and reduced systemic inflammation. Furthermore, associations between fecal SCFA and BCFA levels and the abundance of microbial taxa are explored in this patient population.

Methods

Study design and patient inclusion

This prospective longitudinal multicenter cohort study was conducted in four hospitals in the Netherlands (Maastricht University Medical Center (MUMC+), Catharina Hospital Eindhoven, Hospital Gelderse Vallei, VieCuri Medical Center) between 2017 – 2020. Patients with metastatic and/or unresectable CRC who were planned for treatment with capecitabine (± intravenous VEGF inhibitor bevacizumab) were eligible for participation. Exclusion criteria were abdominal radiotherapy <2 weeks before inclusion, other systemic therapy <1 month before inclusion, antibiotic use <3 months before inclusion, microsatellite instability (MSI-H), and impaired renal function (creatinine clearance of <30ml/min).

Fecal sample collection

Fecal samples and questionnaires were collected before start of the first capecitabine cycle (T1), during the second week of the third cycle (T2), and after the third cycle (T3) (Figure S1). Each capecitabine cycle consisted of two weeks (days 1-14) oral capecitabine ingestion (2x per day) and one week of rest (days 15-21). Patients were asked to collect the fecal samples at home in preservation-free tubes (*Sarstedt*) and to immediately store them in the freezer. The samples were transported to the hospital in a cooled container (*Sarstedt*) to prevent thawing and stored at -20°C for short-term and at -80°C for long-term storage.

Clinical data collection

At the same timepoints, patients filled in a questionnaire concerning previous use of anti-, pre-, or probiotics, medical history as well as nutritional status (Malnutrition Universal Screening Tool, MUST), and physical performance (Karnofsky Performance Score, KPS). The MUST scores the nutritional status on a scale between 0 (low risk) and 2 or more (high risk). The KPS is a scale between 0-100 (0: dead 100: no physical complaints). The occurrence of chemotherapy-induced toxicity was scored based on the *Common Terminology Criteria for Adverse Events* (CTCAE, version 4.0) (31) and included scores on nausea, vomiting, diarrhea, constipation, peripheral sensory neuropathy, oral mucositis, hand-foot syndrome, fever, hair loss and fatigue (Table S1). Tumor response was evaluated based on CT or MRI scans which were performed before and at the end of three cycles of capecitabine. The tumor size change (%) was calculated as described in Table S2 and included as a continuous variable (with negative values indicating tumor decrease and positive values indicating tumor increase). In addition, RECIST *(Response Evaluation Criteria in Solid Tumours, version 1.1)* was used to categorize tumor response as complete response, partial response, progressive disease, or stable disease (Table S2) (32). Additional data on clinical characteristics, treatment regimen, medical history as well as blood inflammatory parameters (leukocytes, neutrophils, and thrombocytes) before the start of cycle 1 (around T1) and before the start of cycle 4 (around T3) were collected from medical records.

Analysis of fecal levels of SCFA and BCFA

For SCFA/BCFA analysis, 500mg of frozen fecal samples were mixed 1:1 (weight:weight) with PBS (5 minutes) and afterwards centrifuged at 14.000g for 10 minutes. Subsequently, 50µl of supernatant was mixed with 650µl internal standard solution, containing methanol, internal standard (2 mg/ml 2-ethyl butyric acid), and formic acid (20%). The SCFA/BCFA concentrations were determined through gas chromatography-mass spectrometry (GC-MS) *(8890 GC System, Agilent Technologies*) equipped with a PAL3 RSI 85 autosampler (*Agilent*). The temperature settings of the injector port, oven, flame-ionization detector, and mass spectrometer detector were 250 ºC, 200 ºC, 275 ºC, and 225 ºC, respectively. In order to correct for sample consistency, measured SCFA and BCFA concentrations were divided by the sample dry weight (g). In order to assess sample dry weight, samples were weighed, freeze-dried until stable weight-loss had occurred, and weighed again.

Analysis of gut microbiota composition and bacterial abundances

Analysis of the gut microbiota was performed as previously described (27). In short, metagenomic DNA from fecal samples was isolated using the Ambion MagMax™ Total Nucleic Acid Isolation Kit (*Thermo Fisher Scientific*). The manual pre-processing consisted of mechanical disruption with bead-beating, as well as chemical and thermal disruption. This was followed by automated nucleic acid purification with the KingFisher FLEX (*Thermo Fisher Scientific*). Upon PCR-amplification of the 16S ribosomal RNA (rRNA) hypervariable V4 gene-region, amplicons were sequenced on a MiSeq platform, as described by Galazzo *et al.* (33). For preprocessing of the raw sequencing data, a standardized in-house pipeline using the software package DADA2 (R version 4.0.3) was applied (34).

Statistical analysis of SCFA/BCFA levels and clinical variables

Baseline characteristics of the patient population were assessed using SPSS (Version 27, IBM). All other statistical analyses were conducted using R in R Studio (R version 4.0.0) (35). For all statistical tests, including procedures with correction for multiple testing, p-values <0.05 were considered to be statistically significant. For continuous variables, the decision on normality was based on histograms, Q-Q-Plots, and the Shapiro-Wilk test. For normally distributed data, the mean (±SD) is shown, while the median (±IQR) is shown if the assumption of normality was violated. For categorical variables, the number of patients (n) and percentages (%) are shown.

Longitudinal analysis of non-normal or ordinal variables was conducted using Friedman's ANOVA and using complete cases (=patients who have measurements for all three timepoints) only. In case of significant results, post-hoc analyses were performed by means of a paired Wilcoxon signed-rank test with Bonferroni correction using the *rstatix* package (version 0.7.0) (36). For SCFA and BCFA, results were confirmed with linear mixed models by means of the *lmer* function from the *lme4* package (version 1.1-26), using log-transformed (log1p) data, sampling timepoint as fixed effect and patient ID as random effect (37).

For cross-sectional analyses comparing groups based on prior treatment, tumor response (RECIST), or capecitabine dose adjustments, Kruskal-Wallis or Mann-Whitney U test were used, depending on the number of groups to be compared. If the Kruskal-Wallis test showed

significant results, post-hoc analyses were performed using Dunn's test with Bonferroni correction (38).
In order to analyze associations between SCFA/BCFA and clinical variables of interest, Spearman correlation was calculated using the *corr.test* function from the *psych* package (version 2.2.5) and a dataframe with all variables of interest (39). P-values were adjusted for multiple testing by means of False Discovery Rate (fdr) adjustment according to the Benjamini and Hochberg procedure (40). Correlations with an adjusted p-value >0.05 but <0.07 are reported as a trend. Correlations between sample dry weight and diarrhea were calculated with *corr.test*, without fdr adjustment, since only those two variables were included. Visualization of correlations between SCFA/BCFA and clinical or blood inflammatory parameters was done by means of the *corrplot* package (version 0.92), using the correlation matrix from *corr.test* (41). Scatterplots were made for all correlations between SCFA/BCFA and clinical or blood parameters that are reported in the article.

Statistical analysis of gut microbiota data

Spearman correlations between fecal SCFA/BCFA and relative abundances of bacterial taxa on family level were calculated using the *corr.test* function from the *psych* package (version 2.2.5). Taxa present in less than 20% of the samples were filtered out for these analyses. P-values were adjusted for multiple testing by means of fdr adjustment. Correlations with an adjusted p-value >0.05 but <0.07 are reported as a trend. The correlation heatmaps were produced using the *cor_heatmap* function from the *microViz* package (version 0.9.2) and R version 4.1.3 (42). For the correlation heatmaps, all taxa with p-value <0.07 at one of the timepoints were included.

Results

Baseline clinical characteristics of the study population

In total, 44 patients with metastatic or unresectable CRC were included in the current study and completed the baseline sampling at T1. At T2, 38 fecal samples were collected, while 39 fecal samples were collected at sampling timepoint T3. Thirty-seven patients collected fecal samples at all three timepoints (Figure S1).
Of the total group, 45.5% received other systemic therapies before the start of capecitabine (>1 month before inclusion) (Table 1). Prior systemic treatments included CAPOX (capecitabine + oxaliplatin) ± bevacizumab (n=12), capecitabine ± bevacizumab (n=5), FOLFOXIRI (folinic acid + 5-FU + irinotecan + oxaliplatin) ± bevacizumab (n=2) and trifluridine/tipiracil + bevacizumab (n=1). In ten of these patients with prior systemic treatment, chemoradiation was applied. Median time between previous systemic treatment and fecal sample collection was 686 days (IQR=813 days). In addition, 47.7% of the patients used prophylactic or therapeutic antibiotics in the year before inclusion, with a mean time of 113 days (SD=103) between the last antibiotic use and T1 (Table 1). Thirty-two patients (72.7%) were current or past smokers.

Table 1: *Baseline characteristics of the current study population (n=44)*

Clinical characteristics	
Age – Years	median (IQR) 74.5 (13)
Male gender	n (%) 32 (72.7%)
BMI - kg/m^2	mean (SD) 27.2 (5.1)
Co-treatment with bevacizumab (7.5mg/kg)	n (%) 32 (72.7%)
Sidedness tumor	n (%)
Left-sided	31 (70.5%)
Right-sided	12 (27.3%)
Missing	1 (2.3%)
Number of metastatic sites	n (%)
1	12 (27.3%)
2	19 (43.2%)
3	10 (22.7%)
4	2 (4.5%)
7	1 (2.3%)
Colostomy in situ	n (%) 14 (31.8%)
Prior treatments	
Prior systemic treatment	n (%) 20 (45.5%)
Antibiotic use last year	n (%) 21 (47.7%)
Colorectal surgery in the past	n (%) 37 (84.1%)
Type of colorectal surgery	n (%)
Rectum resection	16 (36.4%)
Sigmoid resection	7 (15.9%)
Hemicolectomy left	2 (4.5%)
Extended hemicolectomy left	1 (2.3%)
Hemicolectomy right	7 (15.9%)
Other	1 (2.2%)
Unknown	3 (6.8%)

Tumor response and dose adjustments during three cycles of capecitabine

From the 44 patients included, tumor response according to the RECIST criteria could be evaluated in 42 patients (95.45%). None of these patients showed complete response, while six patients (14.3%) had a partial response. Stable disease was found in 31 patients (73.8%) and five patients (11.9%) showed progressive disease. In 29 out of 43 patients (67.4%), the starting dose of capecitabine was not adjusted during the study period. In six patients (13.9%) the dose needed to be reduced, while it was increased in seven patients (16.3%). In one patient (2.3%) the dose was reduced in cycle 2 due to reduced thrombocytes and back to the starting dose in cycle 3. Reasons for dose reductions were impaired renal function (n=1), hand-foot-syndrome (n=4) or cytopenia (n=1). Dose increases occurred because these

patients started with a reduced dose in cycle 1, which could later be increased due to good tolerance. After completion of the study, 36 patients (81.8%) continued with the fourth cycle of capecitabine.

Nutritional status, physical performance, and the prevalence of chemotherapy-induced toxicity during three cycles of capecitabine

During three cycles of capecitabine, the risk for malnutrition (MUST score) did not change significantly in the current study population (p=0.127) (Table S3). KPS scores, which are patient-reported measures of physical performance status, decreased significantly at T2 (median=80, IQR=20, p=0.02) and T3 (median=80, IQR=29, p=0.021) when compared to T1 (median=90, IQR=15) (Table S3).

Regarding chemotherapy-induced toxicity, Friedman's ANOVA showed that the prevalence of oral mucositis (p<0.001), the hand-foot-syndrome (HFS) (p<0.001), and peripheral sensory neuropathy (p= 0.039) increased during three cycles of capecitabine, as illustrated in Figure 1 and Table S4. Post-hoc analysis revealed that oral mucositis and HFS were significantly more prevalent at T2 and T3 compared to T1 (Figure 1). The increase in peripheral sensory neuropathy was no longer significantly different after post-hoc analysis with Bonferroni correction (Figure 1). The prevalence of nausea (p=0.118), diarrhea (p=0.368), unintended weight loss (p=0.236), constipation (p=0.558), and fatigue (p=0.146) did not significantly increase during capecitabine treatment as compared to baseline (Figure 1 and Table S4).

Levels of valerate and caproate decreased during capecitabine, while levels of the other SCFA and BCFA remained unchanged

In the current study population, fecal levels of acetate were highest across all timepoints (median=478.42mM/g, IQR=242.41mM/g), followed by propionate (median=166.39mM/g, IQR=92.88mM/g), butyrate (median=134.52mM/g, IQR=88.96mM/g), iso-butyrate (median=40.91mM/g, IQR=14.99mM/g), iso-valerate (median=33.74mM/g, IQR=14.4mM/g), valerate (median=14.78mM/g, IQR=16.46mM/g), and caproate (median=3.30mM/g, IQR=7.94mM/g). In general, we observed considerable inter-individual variability of fecal SCFA and BCFA levels. Friedman's ANOVA indicated that fecal concentrations of the SCFA valerate (x^2=10.74, p=0.005) and caproate (x^2= 8.842, p=0.012) decreased significantly during three cycles of capecitabine. Post-hoc analysis with Bonferroni correction showed that valerate concentrations were significantly different between T1 and T3 ($p_{adjusted}$=0.001), while caproate concentrations reduced significantly between T1 and T2 ($p_{adjusted}$= 0.008) (Figure 2). Fecal levels of the SCFA acetate (x^2=1.513, p=0.469), propionate (x^2=1.135, p=0.567) and butyrate (x^2=0.162, p=0.922) as well as of the BCFA iso-butyrate (x^2=0.676, p=0.713) and iso-valerate (x^2=1.401, p=0.496) were not significantly different between T1, T2 and T3 (Figure 2).

These results were confirmed using linear mixed models based on log transformed data, also indicating a significant reduction of valerate and caproate during capecitabine (95% confidence intervals: valerate: -0.404; -0.081 and caproate: -0.444; -0.027) and no reduction of the other SCFA and BCFA (Table S5).

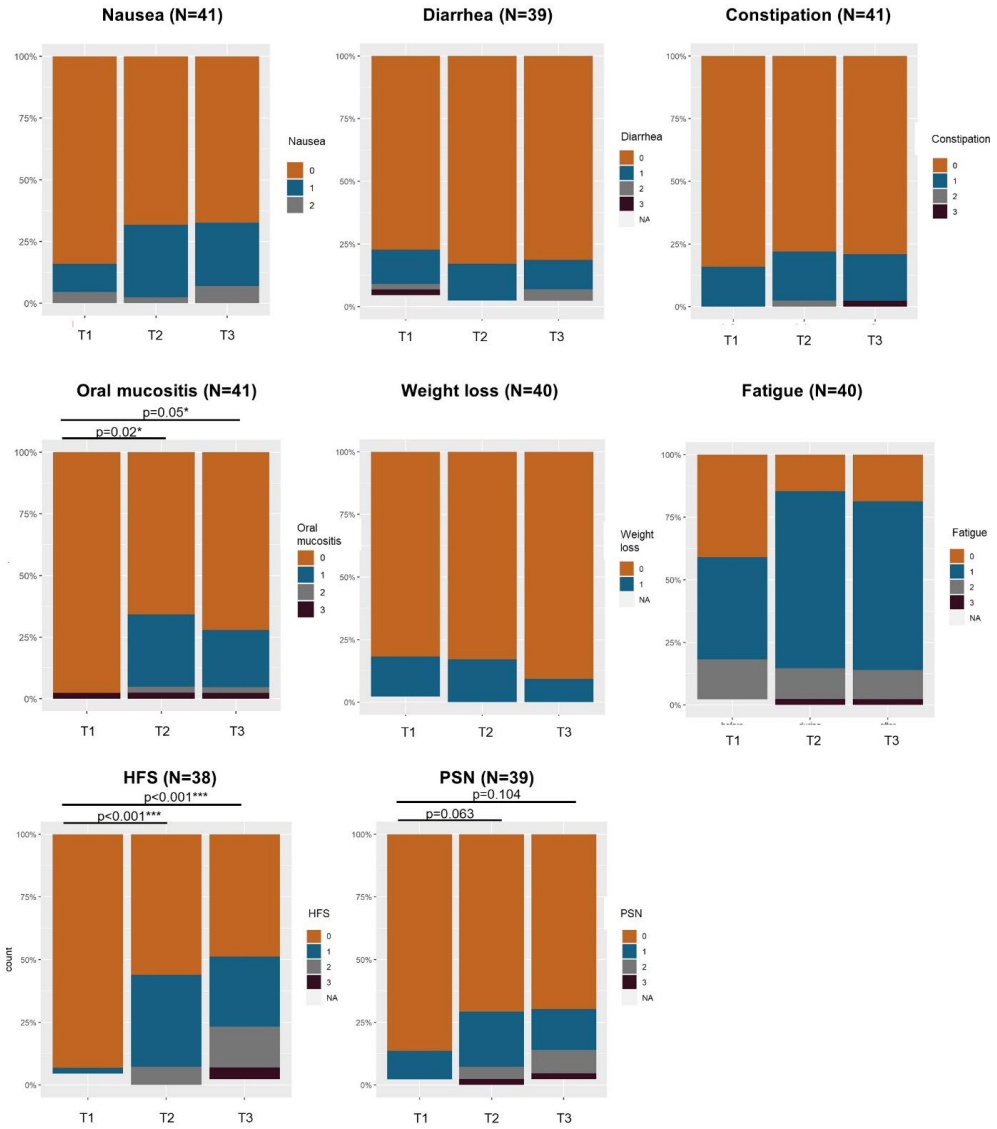


Figure 1: *Symptoms related to chemotherapy-induced toxicity before (T1), during (T2), and after (T3) three cycles of capecitabine. The number of complete cases (=individuals who have values for all three timepoints) are given per variable. Results from post-hoc analysis (adjusted p-values) are indicated for variables that showed significant differences according to Friedman's ANOVA. HFS=hand-foot-syndrome. PSN=Peripheral sensory neuropathy. NA=missing values*

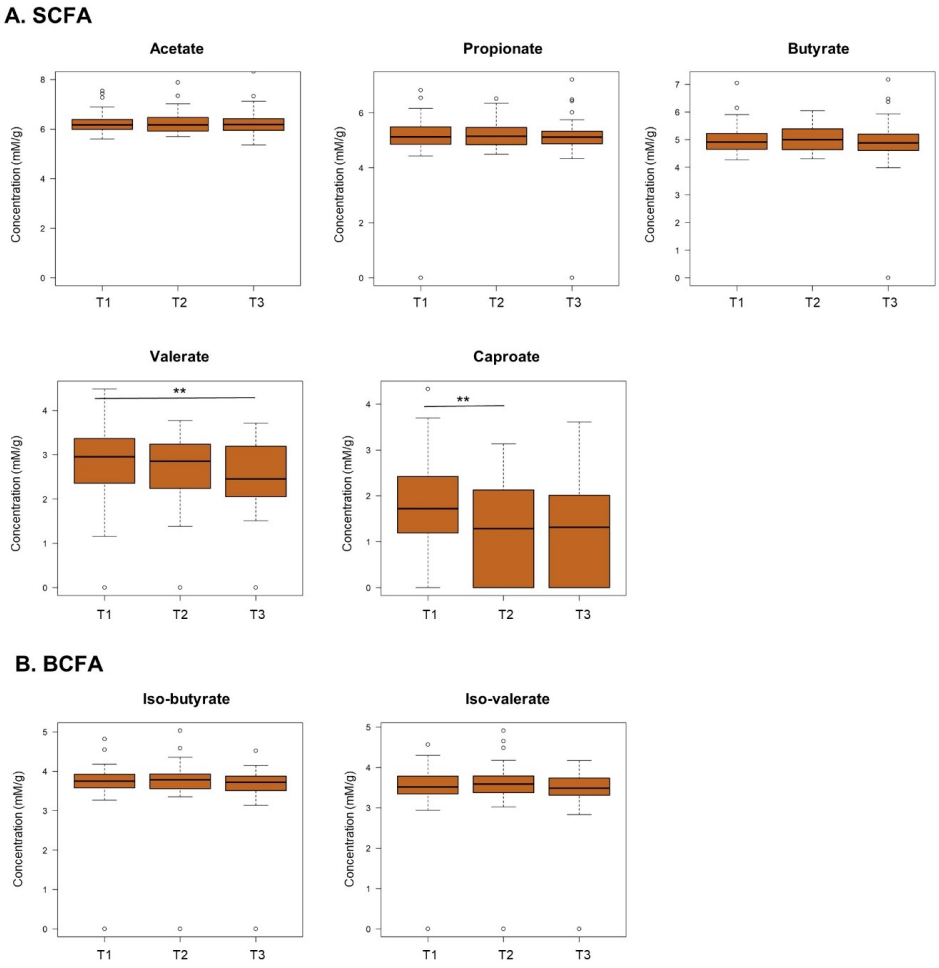


Figure 2: *Changes in fecal SCFA and BCFA concentrations (mM/g dry weight; log-transformed (log1p)) during three cycles of capecitabine. Significant differences according to paired Wilcoxon signed-rank test with Bonferroni correction are indicated with asterisks (**=p<0.01).*

Prior treatment, bevacizumab co-treatment, or the necessity for dose adjustments had no major impact on fecal levels of SCFA and BCFA

Cross-sectional analyses revealed that neither prior chemotherapy, nor antibiotic administration before T1 caused statistically significant differences in fecal SCFA or BCFA concentrations compared to patients without prior treatment (Table S6). In addition, co-treatment with bevacizumab did not impact fecal levels of SCFA or BCFA at sampling timepoints T2 and T3 in the present study population (Table S6). Concerning capecitabine dose adjustments, it was found that fecal SCFA and BCFA levels at T1, T2, and T3 did not differ between patients with or without dose adjustments during three cycles of capecitabine

(Table S6). Similarly, fecal SCFA and BCFA levels were not different between patients who did or did not continue with the fourth cycle of capecitabine after the study period (Table S6).

Baseline BCFA iso-butyrate was associated with tumor response

It was hypothesized that higher fecal SCFA levels would be associated with a better tumor response during three cycles of capecitabine. Cross-sectional analysis with the Kruskal-Wallis test indicated that fecal levels of all SCFA as well as of the BCFA iso-valerate were similar among patients with progressive disease, stable disease, or partial response at all timepoints (Table S6). However, fecal levels of the BCFA iso-butyrate were found to be significantly different between these groups (p=0.014) at baseline (T1). Post hoc analysis by means of Dunn's test with Bonferroni correction showed that iso-butyrate was significantly lower in the feces of patients with partial response compared to patients with stable disease ($p_{adjusted}$=0.017) or progressive disease ($p_{adjusted}$=0.043) (Figure S2).
Furthermore, correlation analysis revealed that fecal levels of iso-butyrate were positively correlated with tumor size change (%) (rho=0.550, $p_{adjusted}$=0.005, Figures 3 and S3) at T1. Fecal levels of iso-valerate tended to be associated with tumor size change (%) at this timepoint (rho=0.421, $p_{adjusted}$=0.060, Figures 3 and S3). Fecal SCFA and BCFA concentrations at T2 or T3 were not associated with tumor size change.

Fecal SCFA and BCFA were not significantly correlated with nutritional status, physical performance or chemotherapy-induced toxicity

It was hypothesized that higher SCFA levels would be associated with better nutritional status (as assessed by the MUST score), increased physical performance (as assessed by KPS), as well as with less chemotherapy-induced toxicity.
Correlation analysis revealed that none of the SCFA or BCFA concentrations were significantly associated with the MUST or KPS scores at T1, T2, or T3 in the present study population. There was a statistically non-significant trend towards a positive association between iso-valerate and MUST at T2 (rho=0.433, $p_{adjusted}$=0.066, Figures 3 and S3). In addition, no statistically significant correlations were found between fecal SCFA and BCFA concentrations and chemotherapy-induced toxicity during (T2) or after (T3) three cycles of capecitabine.
In order to evaluate the reliability of patient-reported diarrhea scores, we also investigated whether patient-reported diarrhea was associated with the sample dry weight as assessed in our laboratories. At T1 and T3, a higher score for diarrhea was significantly associated with lower sample dry weight (T1: rho= -0.391, p=0.010; T3: rho= -0.489, p=0.002, Figure S4). This negative correlation was also present, but not statistically significant at T2 (rho= -0.318, p=0.055, Figure S4).

Baseline SCFA correlated with blood neutrophil counts

Furthermore, it was hypothesized that higher fecal levels of SCFA would be associated with reduced systemic inflammation, which would be reflected in reduced levels of the blood counts of leukocytes, neutrophils, and thrombocytes (in 10^9/l). Correlations with blood inflammatory parameters were tested at T1 and T3 only since no blood was drawn in close proximity to T2.

At T1, higher fecal levels of acetate (rho=0.469, $p_{adjusted}$=0.021), as well as propionate (rho=0.428, $p_{adjusted}$=0.043) were significantly correlated with an increased count of blood neutrophils (Figures 3 and S3). In addition, also butyrate tended to be positively correlated with neutrophils (rho=0.405, $p_{adjusted}$=0.061, Figures 3 and S3), but this association did not reach statistical significance. Additionally, there was a non-significant trend towards a positive correlation between acetate and leukocytes (rho=0.378, $p_{adjusted}$=0.061, Figures 3 and S3) at T1. At T3, we did not identify significant correlations between fecal SCFA or BCFA and blood inflammatory parameters.

Associations between SCFA, BCFA, and bacterial abundances

In a subgroup of patients (n=32, 89 samples) we also related fecal levels of SCFA and BCFA to the relative abundance of bacterial families, as assessed by 16S rRNA V4 amplicon sequencing. At T1, iso-valerate correlated significantly and positively with Anaerovoracaceae ($p_{adjusted}$=0.039). In addition, there was a positive correlation between iso-butyrate and Erysipelotrichaceae, which was not statistically significant ($p_{adjusted}$=0.057) (Figure 4). At T2, butyrate tended to be positively correlated with Veillonellaceae ($p_{adjusted}$=0.068), while propionate tended to be negatively correlated with Oscillospiraceae ($p_{adjusted}$=0.068) (Figure 4). Most associations between SCFA/BCFA and the relative abundance of bacterial taxa were identified at T3 (Figure 4). Again, Oscillospiraceae tended to be negatively associated with propionate ($p_{adjusted}$=0.055) and at this timepoint also with acetate ($p_{adjusted}$=0.067). Furthermore, statistically significant negative associations were found between propionate and Ruminococcaceae ($p_{adjusted}$=0.022), Desulfovibrionaceae ($p_{adjusted}$=0.029), Barnesiellaceae ($p_{adjusted}$=0.045), and Defluviitaleaceae ($p_{adjusted}$=0.024) as well as a non-significant association between propionate and Rikenellaceae ($p_{adjusted}$=0.067). Fecal levels of butyrate were negatively correlated to the relative abundance of Methanobacteriaceae ($p_{adjusted}$=0.036).

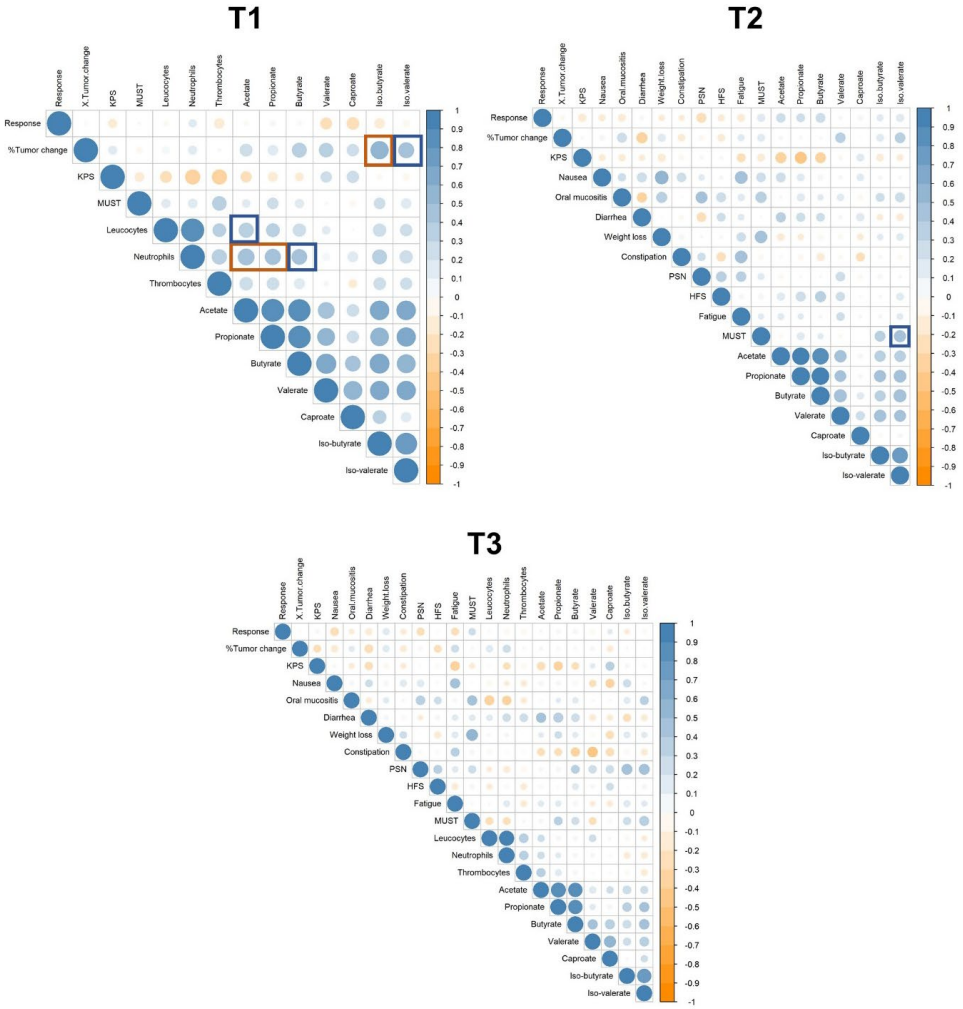


Figure 3: *Spearman correlations between SCFA, BCFA, and clinical as well as blood inflammatory parameters at different sampling timepoints (T1, T2, T3). Significance was assessed for associations involving SCFA/BCFA only, not for potential relations between the other parameters. Significant correlations (p<0.05) are marked with orange boxes, and correlations with a trend towards significance (p>0.05 but <0.07) are marked with blue boxes.*

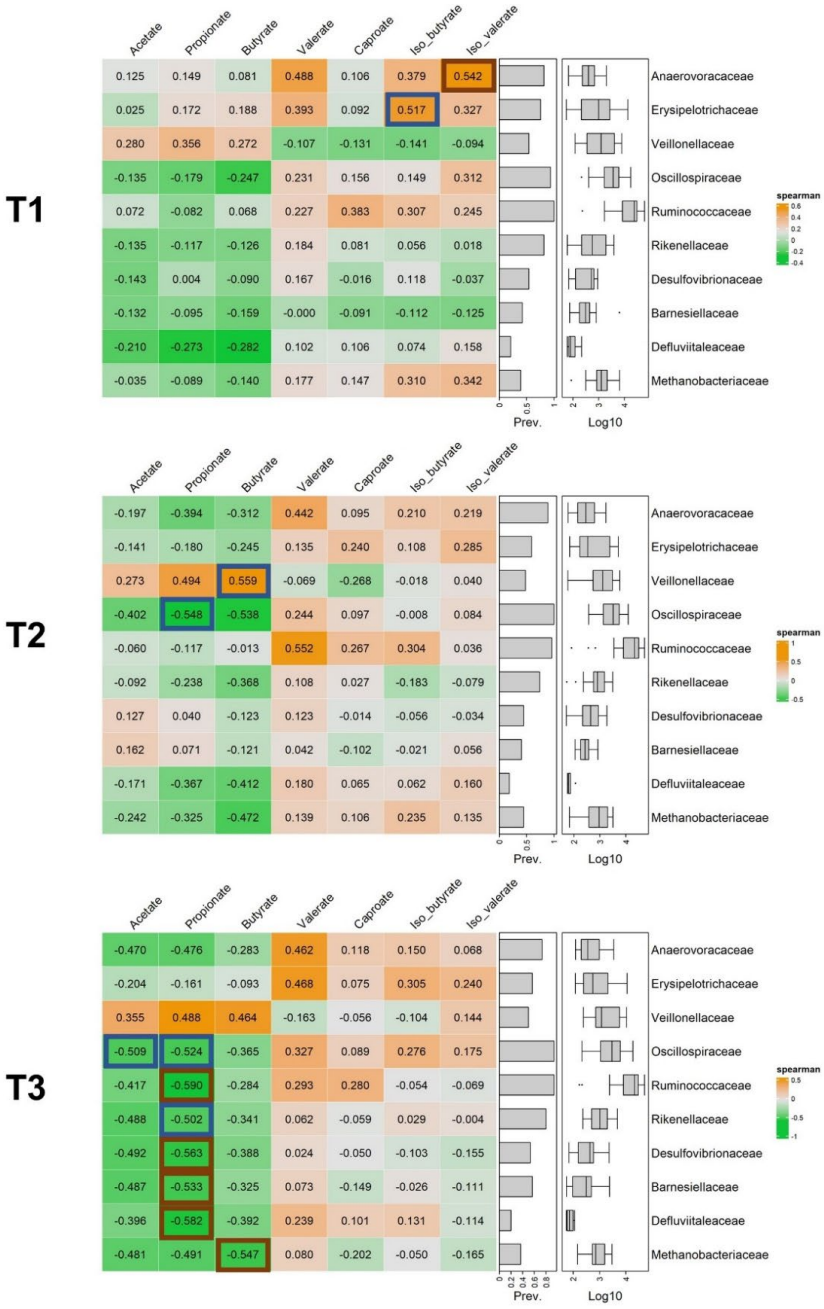


Figure 4: *Correlation heatmaps with Spearman correlation coefficients for the correlations between fecal levels of SCFA and BCFA and the relative abundance of bacterial taxa on family level. All taxa which showed associations with SCFA/BCFA at one of the timepoints (p-value <0.07) were included. Prevalence and log10 abundance are depicted for each taxon. Significant correlations (p<0.05) are marked with orange boxes, correlations with a trend towards significance (p>0.05 but <0.07) are marked with blue boxes*

Discussion

The current study indicated that fecal levels of the SCFA valerate and caproate decreased significantly during three cycles of capecitabine in patients with metastatic or unresectable CRC. Furthermore, we showed that baseline fecal levels of the BCFA iso-butyrate were associated with tumor response. Nutritional status, physical performance as well as chemotherapy-induced toxicity were not statistically significantly associated with SCFA or BCFA. Concerning systemic inflammation, it was found that baseline SCFA correlated positively with blood neutrophil counts. Lastly, fecal levels of SCFA and BCFA were associated with relative abundance of different bacterial families at the three timepoints under investigation.

Interestingly, we identified a reduction of fecal valerate and caproate levels during capecitabine treatment, while concentrations of the more common SCFA acetate, propionate, and butyrate remained stable. A possible explanation is that acetate, propionate, and butyrate are produced by a wider range of bacterial species (17). Consequently, SCFA production could potentially be taken over by other gut bacteria that fill the niche if the abundance of dominant SCFA-producers would change during capecitabine treatment. A similar mechanism has been already described for antibiotics (43). On the other hand, our results suggest an effect of capecitabine treatment on fecal levels of valerate and caproate. Concentrations of these SCFA are generally lower compared to the other SCFA, and their potential physiological roles are currently poorly understood. Valerate is produced by some *Clostridium* species via different mechanisms and has been shown to inhibit the growth of the pathogenic *Clostridioides difficile* (17, 44). In addition, Hinnebusch *et al.* showed that valerate, next to propionate and butyrate, caused histone hyperacetylation and growth inhibition in human carcinoma cells (45). This suggests that the observed decrease during capecitabine treatment could also be of relevance for tumor response. The exact cause(s) of this valerate reduction is unknown, but one possible explanation is that capecitabine might impact the abundance of valerate-producing bacteria. Alternatively, capecitabine might interfere with pathways or intermediate metabolites involved in valerate metabolism (46, 47). In any case, the molecular interactions between capecitabine, valerate, and caproate require further investigation, for instance by future *in vitro* incubation experiments.

Since a significant proportion of the patients in our study population received chemotherapy (> 1 month) or antibiotic treatment (>3 months) before inclusion, we also assessed whether this had an impact on fecal SCFA/BCFA levels. In contrast to previous literature describing considerable chemotherapy-induced changes in gut microbiota composition (e.g. (2, 28, 29, 48)) and detrimental effects of antibiotics (49), we did not identify significant differences between those groups. This suggests that the chosen wash-out periods were sufficient to prevent the confounding effects of prior treatments. However, the complexity of the current study population should be taken into consideration. In view of extensive and complex medical histories, we cannot rule out that previous therapies disturbed gut barrier function and thereby SCFA/BCFA absorption into the blood. Impaired absorption might lead to increased fecal excretion, while actual production might be constant or even reduced. Due to a lack of adequate and non-invasive alternatives, fecal SCFA and BCFA are used as markers for the luminal SCFA/BCFA content in this study and should be interpreted accordingly.

Additionally, we hypothesized that, if higher fecal SCFA would reflect higher production, it would be associated with better tumor response. This was expected based on the earlier described anti-carcinogenic effects of particularly butyrate (11, 50), but also valerate and propionate (45). While butyrate is the preferred energy substrate for normal colonocytes, cancer cells preferably consume glucose. Consequently, butyrate is accumulated in cancer cells and can act as an HDAC inhibitor there, modulating cell proliferation, apoptosis, and differentiation (50). Surprisingly, we did not find an association between SCFA and tumor response in our patient cohort. On the other hand, the baseline values of the BCFA iso-butyrate were significantly lower in patients who showed partial response (at least -30% tumor size change) and were also correlated with tumor size change. This suggests that baseline BCFA levels should be further evaluated as potential factor to predict tumor response in these patients.
Furthermore, it might be hypothesized that increased fecal BCFA could be a sign of increased amino acid catabolism in these patients, since BCFA can also be produced by gut bacteria through branched-chain amino acid digestion (16, 17). In patients with advanced CRC, increased amino acid catabolism could potentially be caused by cancer cachexia. Cancer cachexia is a multifactorial metabolic syndrome, which is characterized by increased protein degradation and loss of muscle mass and also negatively affects treatment outcomes (51, 52). Therefore, it might be beneficial to also include markers of cancer cachexia (e.g. exact weight loss, body composition) in future studies and to further explore the association between baseline BCFA and tumor response. More knowledge on this association and the potential predictive value of baseline BCFA could be of great relevance to identify patients who are at risk for a suboptimal tumor response already before start of the treatment.

In the current research population, we did not identify statistically significant correlations between fecal levels of SCFA/BCFA and nutritional status, physical performance, or chemotherapy-induced toxicity. This was unexpected regarding the known beneficial effects of SCFA (4, 9) and not in line with a previous study reporting an association between the SCFA-producing *Eubacterium hallii* and fatigue (53). In contrast to the earlier described anti-inflammatory effects of SCFA (e.g. (6, 9)), higher fecal SCFA were associated with increased concentrations of blood inflammatory markers in our patient population, which could be caused by disturbed SCFA absorption, as described above.

As a next step, we investigated associations between SCFA, BCFA, and the abundance of bacterial taxa. Interestingly, iso-butyrate, which seemed to have negative effects on tumor response in our patient population, tended to be associated with the relative abundance of Erysipelotrichaceae at T1. It has been previously described that this family was enriched in CRC and might be associated with lipid metabolism and inflammation (54).

There are some methodological limitations inherent to the current study, which should be taken into account when interpreting the results. First, the current sample size is relatively small and fecal SCFA/BCFA levels varied consistently between patients. Consequently, the current study should be seen as a pilot study, providing first indications concerning the role of SCFA/BCFA during capecitabine treatment. Furthermore, it should be noted that the patients harbored diverse and complex medical histories as well as different living environments and dietary habits, which could have confounding effects on SCFA and BCFA levels and might also contribute to the observed large heterogeneity. Particularly dietary fiber intake is

considered to have a relevant role here because non-digestible carbohydrates are the precursors of SCFA (9). Another potential confounding factor that was not assessed in the current research is gut transit time (55). In addition, it should be noted that fecal and blood samples were not always collected on the same day, since blood sample analysis was part of standard care and depended on individual treatment schedules.

To the best of our knowledge, this is the first clinical study evaluating fecal SCFA and BCFA in patients with metastatic or unresectable CRC during treatment with capecitabine. The current study provides first indications that SCFA and BCFA might be of relevance during treatment with capecitabine and should also be considered in future studies. By exploring various correlations in a clinical setting, we provide a set of different points of attention for future studies and hope to stimulate a new understanding of the role of SCFA/BCFA during chemotherapy.
However, the gut microbiota also produces numerous other metabolites with diverse functions, for instance phenolic acids, secondary bile acids, or polyamines (17). Therefore, future research should also investigate the net metabolic output as well as the metabolic capacity of the gut microbiota by metabolomics or metagenomic sequencing. Since the gut microbiota is not an isolated organism but a whole ecosystem with numerous interactions, it will be pivotal, but challenging, to elucidate the complex and diverse mutual interactions between gut bacteria, their metabolites, and chemotherapy.
Furthermore, our data suggest that more attention should be given to valerate and caproate. Although only present in low concentrations, these SCFA could potentially have relevant physiological roles, especially in dysbiotic and pro-inflammatory conditions during chemotherapy.
Similarly, the role of BCFA in tumor response and underlying molecular mechanisms should be explored further. In line with this, future research could examine whether baseline BCFA could be used to predict tumor response to capecitabine, as suggested by our results. Furthermore, the association between SCFA and systemic inflammation in CRC needs further investigation. To assess possible malabsorption of SCFA, blood SCFA concentrations should also be assessed in future studies. Likewise, it might be beneficial to include markers of gastrointestinal inflammation (e.g. fecal calprotectin) and gut transit time (55, 56).

Conclusions

Altogether, the present study provided the first indications for a role of SCFA and BCFA during treatment with capecitabine as well as implications and recommendations for further research. More knowledge on the exact roles of these gut microbiota-derived metabolites will contribute to the evidence-based design of interventions targeting the gut microbiota and/or SCFA/BCFA production during chemotherapy.

List of abbreviations

5-FU	5-fluorouracil
BCFA	Branched-chain fatty acids
CRC	Colorectal cancer
CTCAE	Common Terminology Criteria for Adverse Events
GC-MS	Gas chromatography – mass spectrometry
HDAC	Histone deacetylases
ICTRP	International Clinical Trial Registry Platform
KPS	Karnofsky Performance Score
mCRC	Metastatic colorectal cancer
MUMC+	Maastricht University Medical Center
MUST	Malnutrition Universal Screening Tool
RECIST	Response Evaluation Criteria in Solid Tumours
SCFA	Short-chain fatty acids

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of the azM/UM. Written informed consent was received from each patient. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice.

Consent for publication

Not applicable

Availability of data and materials

Sequencing data were submitted to Qiita and deposited in the European Nucleotide Archive (ENA). The accession code is: ERP143365. Additional data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

JZ and MLS have received research funding from Danone Nutricia Research, outside the submitted work. RA, JdVG and MLS have received institutional research funding from Servier, outside the submitted work. JdVG has served as a consultant for Amgen, AstraZeneca, MSD, Pierre Fabre, and Servier, all outside the submitted work. KV acknowledges support in funding from the Dutch Province of Limburg. All other authors have no competing interests.

Funding

This work was financially supported by the Stichting Jules Coenegracht Sr. The funding organization had no influence on the study design, collection, analysis, and interpretation of data or manuscript writing.

Authors' contributions

RA, MLS, JdVG, and JP designed the study. RA, and LH coordinated the inclusion and follow-up. JdVG, LVI, GJC, AB, and JHMJV consulted patients for inclusion. JZ, RA, AH, LH and JW collected patient data and samples. KV and JP facilitated the laboratory analyses (SCFA/BCFA and gut microbiota composition). JZ and AH performed statistical and bioinformatic data analysis, supported by KV and JP. JZ, AH, JP, KV, and MLS interpreted the data. JZ wrote the manuscript. All authors participated in the discussion and revision and approved the final version of the manuscript.

Acknowledgments

First of all, we are grateful to all patients who participated in this study. In addition, we thank all medical oncologists and research nurses from MUMC+, Catharina Hospital, VieCuri Medical Centre, and Hospital Gelderse Vallei who assisted with patient inclusion and sample collection, particularly Nicol Pepels-Aarts, Kim Puts-van der Burgt, Marjan Laven, Ramon Bax, Eva de Jong, Alina van de Vendel, Janneke van den Brink, Ilona van Rooij-Tieleman and Wendy Heuts. Furthermore, we wish to acknowledge Jessica Verhoeven and Sanne Verbruggen for supporting the SCFA/BCFA analyses, and Christel Driessen and Wesley Nix for helping us with the 16S rRNA amplicon sequencing.

References

1. Alexander JL, Wilson ID, Teare J, Marchesi JR, Nicholson JK, Kinross JM. Gut microbiota modulation of chemotherapy efficacy and toxicity. Nat Rev Gastroenterol Hepatol. 2017;14(6):356-65.
2. Aarnoutse R, Ziemons J, Penders J, Rensen SS, de Vos-Geelen J, Smidt ML. The Clinical Link between Human Intestinal Microbiota and Systemic Cancer Therapy. Int J Mol Sci. 2019;20(17).
3. Wong SH, Yu J. Gut microbiota in colorectal cancer: mechanisms of action and clinical applications. Nat Rev Gastroenterol Hepatol. 2019;16(11):690-704.
4. van der Beek CM, Dejong CHC, Troost FJ, Masclee AAM, Lenaerts K. Role of short-chain fatty acids in colonic inflammation, carcinogenesis, and mucosal protection and healing. Nutr Rev. 2017;75(4):286-305.
5. Ganapathy V, Thangaraju M, Prasad PD, Martin PM, Singh N. Transporters and receptors for short-chain fatty acids as the molecular link between colonic bacteria and the host. Curr Opin Pharmacol. 2013;13(6):869-74.
6. Louis P, Hold GL, Flint HJ. The gut microbiota, bacterial metabolites and colorectal cancer. Nat Rev Microbiol. 2014;12(10):661-72.
7. Corrêa-Oliveira R, Fachi JL, Vieira A, Sato FT, Vinolo MA. Regulation of immune cell function by short-chain fatty acids. Clin Transl Immunology. 2016;5(4):e73.
8. Bach Knudsen KE, Lærke HN, Hedemann MS, Nielsen TS, Ingerslev AK, Gundelund Nielsen DS, et al. Impact of Diet-Modulated Butyrate Production on Intestinal Barrier Function and Inflammation. Nutrients. 2018;10(10).
9. Morrison DJ, Preston T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes. 2016;7(3):189-200.
10. Encarnação JC, Pires AS, Amaral RA, Gonçalves TJ, Laranjo M, Casalta-Lopes JE, et al. Butyrate, a dietary fiber derivative that improves irinotecan effect in colon cancer cells. J Nutr Biochem. 2018;56:183-92.
11. Kim HJ, An J, Ha EM. Lactobacillus plantarum-derived metabolites sensitize the tumor-suppressive effects of butyrate by regulating the functional expression of SMCT1 in 5-FU-resistant colorectal cancer cells. J Microbiol. 2022;60(1):100-17.
12. Lee CS, Ryan EJ, Doherty GA. Gastro-intestinal toxicity of chemotherapeutics in colorectal cancer: the role of inflammation. World J Gastroenterol. 2014;20(14):3751-61.
13. Lin XB, Farhangfar A, Valcheva R, Sawyer MB, Dieleman L, Schieber A, et al. The role of intestinal microbiota in development of irinotecan toxicity and in toxicity reduction through dietary fibres in rats. PLoS One. 2014;9(1):e83644.
14. Motoori M, Yano M, Miyata H, Sugimura K, Saito T, Omori T, et al. Randomized study of the effect of synbiotics during neoadjuvant chemotherapy on adverse events in esophageal cancer patients. Clin Nutr. 2017;36(1):93-9.
15. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nat Rev Gastroenterol Hepatol. 2017;14(8):491-502.
16. Taormina VM, Unger AL, Schiksnis MR, Torres-Gonzalez M, Kraft J. Branched-Chain Fatty Acids-An Underexplored Class of Dairy-Derived Fatty Acids. Nutrients. 2020;12(9).
17. Oliphant K, Allen-Vercoe E. Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. Microbiome. 2019;7(1):91.
18. Ran-Ressler RR, Khailova L, Arganbright KM, Adkins-Rieck CK, Jouni ZE, Koren O, et al. Branched chain fatty acids reduce the incidence of necrotizing enterocolitis and alter gastrointestinal microbial ecology in a neonatal rat model. PLoS One. 2011;6(12):e29032.
19. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab. 2009;9(4):311-26.
20. Reigner B, Blesch K, Weidekamm E. Clinical pharmacokinetics of capecitabine. Clin Pharmacokinet. 2001;40(2):85-104.
21. Maier L, Pruteanu M, Kuhn M, Zeller G, Telzerow A, Anderson EE, et al. Extensive impact of non-antibiotic drugs on human gut bacteria. Nature. 2018;555(7698):623-8.
22. Zimmermann M, Zimmermann-Kogadeeva M, Wegmann R, Goodman AL. Mapping human microbiome drug metabolism by gut bacteria and their genes. Nature. 2019;570(7762):462-7.

23. Sougiannis AT, VanderVeen BN, Enos RT, Velazquez KT, Bader JE, Carson M, et al. Impact of 5 fluorouracil chemotherapy on gut inflammation, functional parameters, and gut microbiota. Brain Behav Immun. 2019;80:44-55.
24. Yu T, Guo F, Yu Y, Sun T, Ma D, Han J, et al. Fusobacterium nucleatum Promotes Chemoresistance to Colorectal Cancer by Modulating Autophagy. Cell. 2017;170(3):548-63.e16.
25. Zhang S, Yang Y, Weng W, Guo B, Cai G, Ma Y, et al. Fusobacterium nucleatum promotes chemoresistance to 5-fluorouracil by upregulation of BIRC3 expression in colorectal cancer. J Exp Clin Cancer Res. 2019;38(1):14.
26. An J, Ha EM. Combination Therapy of Lactobacillus plantarum Supernatant and 5-Fluouracil Increases Chemosensitivity in Colorectal Cancer Cells. J Microbiol Biotechnol. 2016;26(8):1490-503.
27. Aarnoutse R, Ziemons J, de Vos-Geelen J, Valkenburg-van Iersel L, Wildeboer ACL, Vievermans A, et al. The Role of Intestinal Microbiota in Metastatic Colorectal Cancer Patients Treated With Capecitabine. Clin Colorectal Cancer. 2022;21(2):e87-e97.
28. Li J, Li J, Lyu N, Ma Y, Liu F, Feng Y, et al. Composition of fecal microbiota in low-set rectal cancer patients treated with FOLFOX. Ther Adv Chronic Dis. 2020;11:2040622320904293.
29. Kong C, Gao R, Yan X, Huang L, He J, Li H, et al. Alterations in intestinal microbiota of colorectal cancer patients receiving radical surgery combined with adjuvant CapeOx therapy. Sci China Life Sci. 2019;62(9):1178-93.
30. Ferreira TM, Leonel AJ, Melo MA, Santos RR, Cara DC, Cardoso VN, et al. Oral supplementation of butyrate reduces mucositis and intestinal permeability associated with 5-Fluorouracil administration. Lipids. 2012;47(7):669-78.
31. Common Terminology Criteria for Adverse Events (CTCAE) U.S.: National Institutes of Health _ National Cancer Institute; 2010 [Available from: https://ctep.cancer.gov/protocoldevelopment/electronic_applications/ctc.htm.
32. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer. 2009;45(2):228-47.
33. Galazzo G, van Best N, Benedikter BJ, Janssen K, Bervoets L, Driessen C, et al. How to Count Our Microbes? The Effect of Different Quantitative Microbiome Profiling Approaches. Front Cell Infect Microbiol. 2020;10:403.
34. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13(7):581-3.
35. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.: R Core Team; 2020 [Available from: https://www.R-project.org/.
36. Kassambara A. rstatix: Pipe-Friendly Framework for Basic Statistical Tests. R package version 0.7.0. 2021 [Available from: https://CRAN.R-project.org/package=rstatix.
37. Bates D, Maechler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software. 2015;67(1):1-48.
38. Ogle DH, Wheeler P, Dinno A. FSA: Fisheries Stock Analysis. R package version 0.8.32 2021 [Available from: https://github.com/droglenc/FSA.
39. Revelle W. psych: Procedures for Personality and Psychological Research, Northwestern University, Evanston, Illinois, USA 2022 [Available from: https://CRAN.R-project.org/package=psych Version=2.2.5.
40. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal statistical society: series B (Methodological). 1995;57(1):289-300.
41. Taiyun Wei, Simko V. R package "corrplot": Visualization of a Correlation Matrix (Version 0.84) 2017 [Available from: https://github.com/taiyun/corrplot.
42. Barnett D, Arts I, Penders J. microViz: an R package for microbiome data visualization and statistics. Journal of Open Source Software. 2021;6(63).
43. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature. 2012;489(7415):220-30.
44. McDonald JAK, Mullish BH, Pechlivanis A, Liu Z, Brignardello J, Kao D, et al. Inhibiting Growth of Clostridioides difficile by Restoring Valerate, Produced by the Intestinal Microbiota. Gastroenterology. 2018;155(5):1495-507.e15.
45. Hinnebusch BF, Meng S, Wu JT, Archer SY, Hodin RA. The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. J Nutr. 2002;132(5):1012-7.
46. Barker HA, D'Ari L, Kahn J. Enzymatic reactions in the degradation of 5-aminovalerate by Clostridium aminovalericum. J Biol Chem. 1987;262(19):8994-9003.

47. Bornstein BT, Barker HA. The energy metabolism of Clostridium kluyveri and the synthesis of fatty acids. J Biol Chem. 1948;172(2):659-69.
48. Papanicolas LE, Sims SK, Taylor SL, Miller SJ, Karapetis CS, Wesselingh SL, et al. Conventional myelosuppressive chemotherapy for non-haematological malignancy disrupts the intestinal microbiome. BMC Cancer. 2021;21(1):591.
49. Maier L, Goemans CV, Wirbel J, Kuhn M, Eberl C, Pruteanu M, et al. Unravelling the collateral damage of antibiotics on gut bacteria. Nature. 2021;599(7883):120-4.
50. Koh A, De Vadder F, Kovatcheva-Datchary P, Bäckhed F. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. Cell. 2016;165(6):1332-45.
51. Fearon KC, Glass DJ, Guttridge DC. Cancer cachexia: mediators, signaling, and metabolic pathways. Cell Metab. 2012;16(2):153-66.
52. Fearon K, Arends J, Baracos V. Understanding the mechanisms and treatment options in cancer cachexia. Nat Rev Clin Oncol. 2013;10(2):90-9.
53. Hajjar J, Mendoza T, Zhang L, Fu S, Piha-Paul SA, Hong DS, et al. Associations between the gut microbiome and fatigue in cancer patients. Sci Rep. 2021;11(1):5847.
54. Kaakoush NO. Insights into the Role of Erysipelotrichaceae in the Human Host. Front Cell Infect Microbiol. 2015;5:84.
55. Procházková N, Falony G, Dragsted LO, Licht TR, Raes J, Roager HM. Advancing human gut microbiota research by considering gut transit time. Gut. 2022.
56. Burri E, Beglinger C. The use of fecal calprotectin as a biomarker in gastrointestinal disease. Expert Rev Gastroenterol Hepatol. 2014;8(2):197-210.

Supplementary tables

Table S1: *Scoring of toxicity according to CTCAE criteria. Patients were asked to select the score describing the current situation at the time of fecal sample collection*

Symptom	Score	Explanation	Symptom	Score	Explanation
Nausea	0	Not at all	Unintentional weight loss past 3-6 months	0	Not at all (less than 5%)
	1	Less appetite		1	Moderate weight loss (between 5 and 10%)
	2	Less food intake		2	Severe weight loss (between 10 and 20%)
	3	Insufficient food intake		3	Very severe weight loss (>20%)
Vomiting	0	Not at all	Constipation	0	Not at all
	1	1-2 times per 24 hours		1	Occasional complaints
	2	3-5 times per 24 hours		2	Persistent complaints
	3	6 or more times per 24 hours		3	Necessary to remove stool by hand
	4	life threatening, acute intervention needed		4	Life threatening
Fever	0	Not at all	Peripheral sensory neuropathy	0	Not at all
	1	38°C – 39°C		1	Minimal complaints
	2	39,1°C – 40°C		2	Moderate complaints
	3	More than 40°C, less than 24 hours		3	Severe complaints
	4	More than 40°C, more than 24 hours		4	Life threatening
Diarrhea (patients without stoma)	NA	Not applicable	Diarrhea (Patients with stoma)	NA	Not applicable
	0	Not at all		0	Not at all
	1	Compared to normal an increase of <4 times stool per day		1	Compared to normal a minimal increase
	2	Compared to normal an increase of 4-6 times stool per day		2	Compared to normal a moderate increase

	3	Compared to normal an increase of >7 times stool per day		3	Compared to normal a severe increase
	4	Life threatening, acute intervention needed		4	Life threatening
Fatigue	0	Not at all	Hand-foot syndrome	0	Not at all
	1	Fatigue which decreases after rest		1	Skin changes or skin inflammation without pain
	2	Fatigue which does not decrease after rest: limited possibility to normal daily functioning		2	Skin changes with pain: with limited possibility to normal daily functioning
	3	Fatigue which does not decrease after rest: limited possibility to self-care		3	Severe skin changes with pain with limited possibility to self-care
Oral mucositis	0	Not at all	Hair loss	0	Not at all
	1	No complaints or mild complaints		1	Hair loss <50%
	2	Moderate pain		2	Hair loss >50%
	3	Severe pain			
	4	Life threatening			

Table S2: *Calculation of the %tumor change before and after three cycles of capecitabine and classification of tumor response according to the RECIST categories*

Calculation of %tumor size change	
SLD before	Sum longest diameter of the target lesion before start of treatment with capecitabine
SLD after	Sum longest diameter of the target lesion after three cycles of capecitabine
Formula	$\frac{(SLD\ after - SLD\ before)}{SLD\ before} x\ 100\%$
RECIST categories	
Complete response	Disappearance of all target lesions
Partial response	At least 30% decrease in the sum of the target lesions
Progressive disease	At least 20% increase in the sum of target lesions
Stable disease	Small changes that did not meet above criteria

Table S3: *MUST scores and Karnofsky Performance Scores (KPS) before (T1), during (T2) and after (T3) three cycles of capecitabine. Percentages are calculated based on valid measurements (missings are excluded).*

	T1 n(%)	**T2** n(%)	**T3** n(%)
MUST – low risk	34 (77.3%)	34 (82.9%)	39 (92.8%)
MUST – medium risk	7 (15.9%)	6 (14.6%)	1 (2.4%)
MUST – high risk	3 (6.8%)	1 (2.4%)	2 (4.8%)
	T1 median (IQR)	**T2** median (IQR)	**T3** median (IQR)
KPS	90 (15)	80 (20)	80 (20)

Table S4: *Number of patients suffering from chemotherapy-induced toxicities before (T1), during (T2) and after (T3) three cycles of capecitabine. Percentages are calculated based on valid measurements (missings are excluded)*

Gastrointestinal complications			
	T1 n(%)	**T2** n(%)	**T3** n(%)
Nausea			
Grade 0	37 (84.1%)	28 (68.3%)	29 (67.4%)
Grade 1	5 (11.4%)	12 (29.3%)	11 (25.6%)
Grade 2	2 (4.5%)	1 (2.4%)	3 (7.0%)
Diarrhea			
Grade 0	34 (80.9%)	34 (85.0%)	35 (83.3%)
Grade 1	6 (14.3%)	6 (15.0%)	5 (11.9%)
Grade 2	1 (2.4%)	-	2 (4.8%)
Grade 3	1 (2.4%)	-	-
Constipation			
Grade 0	37 (84.1%)	32 (78.0%)	34 (79.1%)
Grade 1	7 (15.9%)	8 (19.5%)	8 (18.6%)
Grade 2	-	1 (2.4%)	1 (2.3%)
Oral mucositis			
Grade 0	43 (97.7%)	27 (65.9%)	31 (72.1%)
Grade 1	-	12 (29.3%)	10 (23.3%)
Grade 2	-	1 (2.4%)	1 (2.3%)
Grade 3	1 (2.3%)	1 (2.4%)	1 (2.3%)
Weight loss			
Grade 0	36 (83.7%)	34 (82.9%)	39 (90.7%)
Grade 1	7 (16.3%)	7 (17.1%)	4 (9.3%)
Peripheral sensory neuropathy			
Grade 0	38 (88.4%)	29 (70.7%)	30 (71.4%)
Grade 1	5 (11.6%)	9 (22.0%)	7 (16.7%)
Grade 2	-	2 (4.9%)	4 (9.5%)
Grade 3	-	1 (2.4%)	1 (2.4%)
Hand-foot-syndrome			
Grade 0	41 (97.6%)	23 (56.1%)	21 (50.0%)
Grade 1	1 (2.4%)	15 (36.6%)	12 (28.6%)
Grade 2	-	3 (7.3%)	7 (16.7%)
Grade 3	-	-	2 (4.8%)
Fatigue			
Grade 0	18 (41.9%)	6 (14.6%)	8 (18.6%)
Grade 1	18 (41.9%)	29 (70.7%)	29 (67.4%)
Grade 2	7 (16.3%)	5 (12.2%)	5 (11.6%)
Grade 3	-	1 (2.4%)	1 (2.3%)

Table S5: *Results from the linear mixed model testing the fixed effect of sampling timepoint, correcting for random effects produced by longitudinal sampling within patients*

SCFA	Estimate	Std. Error	t-value	2.5% - 97.5%
Acetate	0.00096	0.033	0.029	-0.065 - 0.067
Propionate	-0.06031	0.096	-0.629	-0.249 - 0.128
Butyrate	-0.1150	0.083	-1.388	-0.278 - 0.048
Valerate	-0.2423	0.082	-2.955	-0.404 – (-0.081)*
Caproate	-0.2362	0.106	-2.232	-0.444 – (-0.027)*

BCFA	Estimate	Std. Error	t-value	2.5% - 97.5%
Iso-butyrate	-0.1346	0.133	-1.012	-0.397 - 0.127
Iso-valerate	-0.05961	0.095	-0.627	-0.247 - 0.127

Table S6: *Cross-sectional differences in SCFA and BCFA concentrations*

Dose adjustments during T1 – T3 (groups: no dose adjustments, dose reduction, dose increase, dose increase and reduction)									
Kruskal Wallis Test	**T1**			**T2**			**T3**		
	X^2	**df**	**p-value**	**X^2**	**df**	**p-Value**	**X^2**	**df**	**p-Value**
Acetate	3.6827	3	0.2978	1.7386	3	0.6284	5.3489	3	0.148
Propionate	3.296	3	0.3482	0.29991	3	0.96	4.5232	3	0.2102
Butyrate	2.9675	3	0.3967	0.76836	3	0.857	3.7203	3	0.2933
Valerate	1.378	3	0.7107	1.6757	3	0.6424	4.0907	3	0.2518
Caproate	1.7971	3	0.6156	6.227	3	0.1011	2.4859	3	0.4779
Iso-butyrate	2.7853	3	0.4259	2.6389	3	0.4507	2.2016	3	0.5316
Iso-valerate	4.1181	3	0.249	2.217	3	0.5286	2.9868	3	0.3937
Tumor response (groups: progressive disease, stable disease, partial response)									
Kruskal Wallis Test	**T1**			**T2**			**T3**		
	X^2	**df**	**p-value**	**X^2**	**df**	**p-value**	**X^2**	**df**	**p-value**
Acetate	0.506	2	0.777	2.566	2	0.277	0.059	2	0.971
Propionate	0.448	2	0.799	2.322	2	0.313	0.148	2	0.928
Butyrate	2.087	2	0.352	3.216	2	0.200	0.306	2	0.858
Valerate	4.633	2	0.099	0.316	2	0.854	0.343	2	0.842
Caproate	3.397	2	0.183	0.418	2	0.811	1.062	2	0.588

Iso-butyrate	8.544	2	0.014*	0.779	2	0.677	0.454	2	0.797
Iso-valerate	2.184	2	0.336	1.380	2	0.501	0.602	2	0.740

*significant result of Kruskal Wallis test, subsequently a post-hoc test Dunn's test with Bonferroni correction was performed:
stable disease vs. partial response: p.adjusted=0.017
stable disease vs. progressive disease: p.adjusted=1.000
partial response vs. progressive disease: p.adjusted=0.043

Therapy continuation (groups: continuation capecitabine (4th cycle), no continuation capecitabine)

Mann Whitney U Test	**T1**		**T2**		**T3**	
	W	**p-value**	**W**	**p-value**	**W**	**p-value**
Acetate	133	0.834	82	1.0	101	0.955
Propionate	155	0.356	97	0.557	118	0.471
Butyrate	116	0.760	93	0.675	108.5	0.726
Valerate	114	0.711	86	0.897	133	0.192
Caproate	102	0.437	75	0.753	78.5	0.425
Iso-butyrate	166	0.193	76.5	0.812	112.5	0.612
Iso-valerate	155	0.348	84.5	0.948	130	0.235

Systemic treatment before T1 (groups: no previous systemic treatment, previous systemic treatment)

Mann Whitney U Test	**W**	**p-value**
Acetate	237	0.953
Propionate	230	0.825
Butyrate	242	0.972
Valerate	276	0.406
Caproate	276.5	0.393
Iso-butyrate	273	0.443
Iso-valerate	285.5	0.289

Antibiotic use last year (>3 months before inclusion, groups: no antibiotic use, antibiotic use)

Mann Whitney U Test	**W**	**p-value**
Acetate	242	1.00
Propionate	245	0.944
Butyrate	249	0.871
Valerate	274	0.456
Caproate	197	0.298

Iso-butyrate	211	0.481
Iso-valerate	268	0.541

Table S6 continued: *Cross-sectional differences in SCFA and BCFA concentrations*

Co-treatment with bevacizumab (groups: bevacizumab co-treatment, no bevacizumab co-treatment)				
Mann Whitney U Test	**T2**		**T3**	
	W	**p-value**	**W**	**p-value**
Acetate	154	0.660	197	0.098
Propionate	133	0.832	176	0.327
Butyrate	128	0.708	180.5	0.260
Valerate	131	0.778	173	0.376
Caproate	95.5	0.123	137	0.805
Iso-butyrate	147.5	0.816	135	0.759
Iso-valerate	142	0.960	113	0.311

Supplementary figures

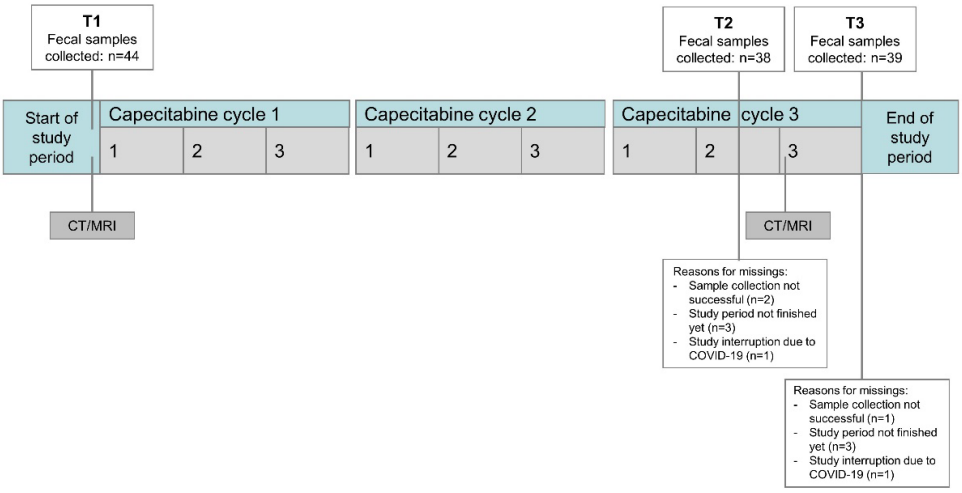


Figure S1: *Overview of study period and sampling timepoints.*

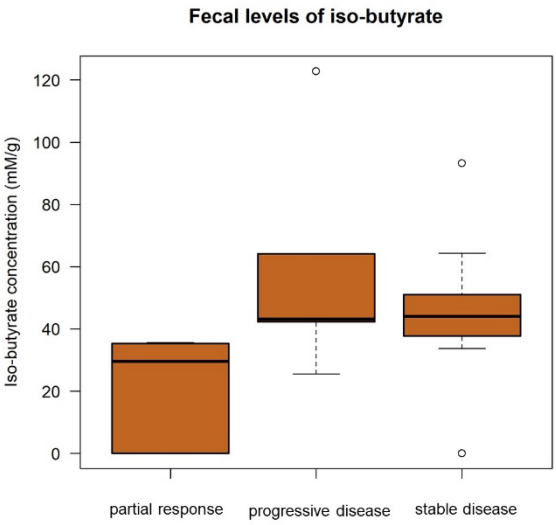
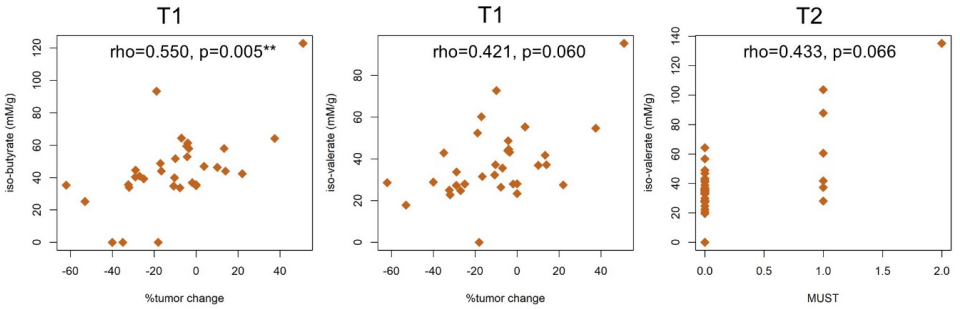


Figure S2: *Fecal levels of iso-butyrate at T1 were significantly lower in patients showing partial response compared to patients with stable disease ($p_{adjusted}$=0.017) or progressive disease ($p_{adjusted}$=0.043)*

A. Clinical parameters



B. Blood inflammatory parameters

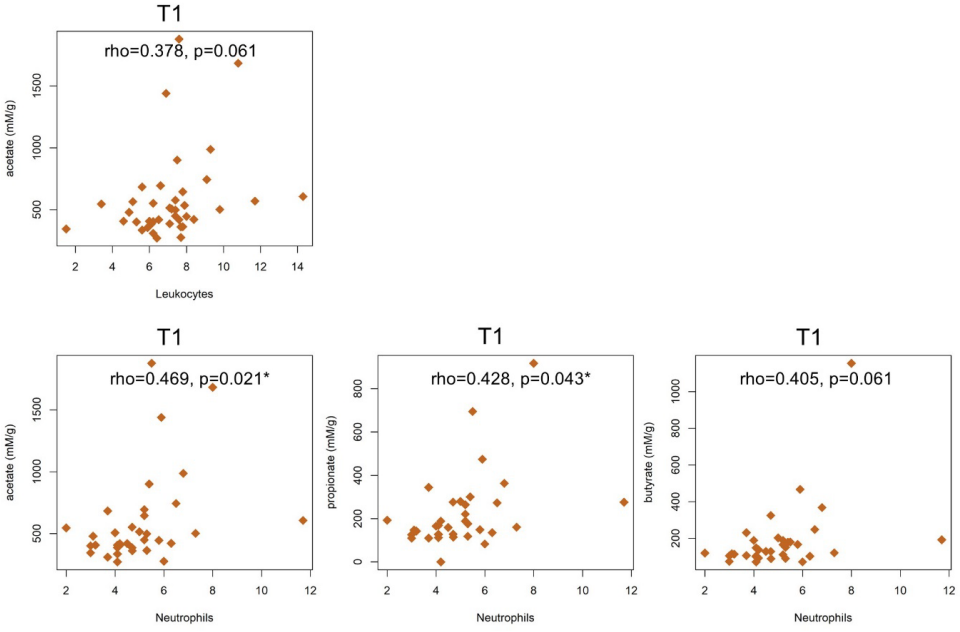


Figure S3: *Scatterplots for correlations between SCFA/BCFA and clinical as well as blood inflammatory parameters. Statistical significance is indicated with asterisks: *=p<0.05; **=p<0.01*

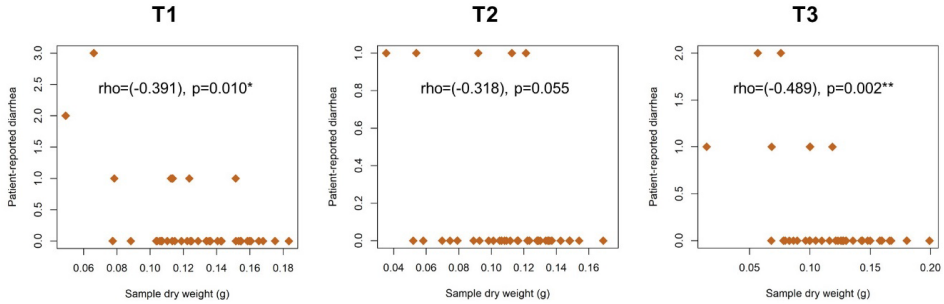


Figure S4: *Scatterplots for correlations between sample dry weight and patient-reported diarrhea. Statistical significance is indicated with asterisks: *=p<0.05; **=p<0.01*

Chapter 5

Prebiotic fiber mixtures counteract the manifestation of gut microbial dysbiosis induced by the chemotherapeutic 5 - Fluorouracil (5-FU) in a validated *in vitro* model of the colon

Janine Ziemons, Lars E. Hillege, Romy Aarnoutse, Judith de Vos-Geelen, Liselot Valkenburg-van Iersel, Jasper Mastenbroek, Robin van Geel, David J. M. Barnett, Sander S. Rensen, Ardy van Helvoort, Lotte H. J. Dopheide, Guus Roeselers, John Penders, Marjolein L. Smidt* & Koen Venema*

** Shared last authorship*

Submitted

Chapter 5

is currently under embargo

Chapter 6

Changes in intestinal microbiota in post-menopausal estrogen receptor positive breast cancer patients treated with (neo)adjuvant chemotherapy

Romy Aarnoutse, **Janine Ziemons**, Lars E. Hillege, Judith de Vos-Geelen, Maaike de Boer, Saskia M.P. Bisschop, Birgit E.P.J. Vriens, Jeroen Vincent, Agnes J. van de Wouw, Giang N. Le, Koen Venema, Sander S. Rensen, John Penders, Marjolein L. Smidt

NPJ Breast Cancer. 2022 Jul 29;8(1):89.
DOI: 10.1038/s41523-022-00455-5.

Abstract

This clinical study explored the associations between the intestinal microbiota, chemotherapy toxicity, and treatment response in postmenopausal estrogen receptor positive breast cancer patients. Estrogen receptor positive postmenopausal breast cancer patients were prospectively enrolled in a multicenter cohort study and treated with 4 cycles of (neo)adjuvant adriamycin, cyclophosphamide (AC) followed by 4 cycles of docetaxel (D). Patients collected a fecal sample and completed a questionnaire before treatment, during AC, during D, and after completing AC-D. Chemotherapy toxicity and tumor response were determined. Intestinal microbiota was analyzed by amplicon sequencing of the 16S rRNA V4 gene-region. In total, 44 patients, including 18 neoadjuvant patients, were included, and 153 fecal samples were collected before AC-D (*n*=44), during AC (*n*=43), during D (*n*=29), and after AC-D treatment (*n*=37), 28 participants provided all four samples. In the whole group, observed species richness reduced during treatment (*p*=0.042). The abundance of Proteobacteria, unclassified Enterobacterales, *Lactobacillus*, *Ruminococcaceae NK4A214 group*, *Marvinbryantia*, *Christensenellaceae R7 group*, and *Ruminococcaceae UCG-005* changed significantly over time. Patients with any grade diarrhea during docetaxel treatment had a significantly lower observed species richness compared to patients without diarrhea. In the small group neoadjuvant treated patients, pathologic response was unrelated to baseline intestinal microbiota richness, diversity and composition. While the baseline microbiota was not predictive for pathologic response in a rather small group of neoadjuvant treated patients in our study, subsequent shifts in microbial richness, as well as the abundance of specific bacterial taxa, were observed during AC-D treatment in the whole group and the neoadjuvant group.

Introduction

Breast cancer is the most common cancer in women worldwide (1). Despite recent developments in systemic therapy, classical chemotherapeutic agents such as adriamycin, cyclophosphamide (AC), and docetaxel (D) remain the backbone of (neo)adjuvant chemotherapy regimes in postmenopausal estrogen receptor positive (ER+) breast cancer patients. Besides reducing tumor load in the neoadjuvant setting and improving disease free- and overall survival, AC-D treatment may induce toxicity, which impacts the quality of life and may require dose reductions. The most common non-hematological toxicities during adriamycin and cyclophosphamide treatment are oral mucositis, fatigue, alopecia, nausea, and vomiting (2-4). Docetaxel treatment shows a comparable toxicity profile with the addition of diarrhea and peripheral sensory neuropathy (2-4).

In order to reduce toxicity and optimize treatment outcome, factors need to be identified that impact the individual response to and safety profile of AC-D. During the last decade, evidence on the interaction between systemic cancer therapies and the human intestinal microbiota has rapidly expanded (5, 6). The intestinal microbiota is an ecosystem that harbors trillions of intestinal microorganisms, consisting of bacteria, archaea, fungi, protozoa, and viruses. It is well-established that crosstalk occurs between intestinal microbiota and the human host. This crosstalk is essential for the maintenance of immune function, homeostasis, and metabolism of dietary components and medication, including chemotherapeutic agents (7). In case of dysbiosis, intestinal microbiota can instigate carcinogenesis or affect systemic cancer therapy (8).

Although interactions between AC-D and microbiota have not been studied in postmenopausal ER+ breast cancer patients, *in vitro* and mouse studies indicate that significant interactions occur between the intestinal microbiota and cyclophosphamide, adriamycin, and docetaxel (9-16). In mice, cyclophosphamide induces translocation of Gram-positive intestinal bacteria, including *Enterococcus hirae, Lactobacillus johnsonii,* and *Lactobacillus murinus,* to mesenteric lymph nodes and the spleen. These bacteria, as well as *Barnesiella intestinihominis,* trigger an immune response and increase cyclophosphamide efficacy (9, 11, 15). Furthermore, pre-clinical evidence has demonstrated an interaction between intestinal microbiota and adriamycin (12, 16). Rigby *et al.* (2016) concluded that the intestinal microbiota is necessary for adriamycin-induced intestinal damage and repair, but not for jejunal epithelial apoptosis (14). Limited pre-clinical evidence exists for an interaction between docetaxel and intestinal microbiota (13). Flórez *et al.* (2016) determined the susceptibility profiles of lactic acid bacteria and bifidobacteria to multiple chemotherapeutics and found that adriamycin perturbs the intestinal microbiota (10). Conversely, all tested members of the intestinal microbiota showed resistance to high doses of cyclophosphamide and docetaxel. However, these *in vitro* tests did not take into account the potential effect of in vivo transformation to more toxic compounds.

Despite the availability of the previously described pre-clinical evidence, no clinical studies with longitudinal microbiota sampling have yet explored the interaction between AC-D and the intestinal microbiota regarding chemotherapy toxicity and tumor response in postmenopausal ER+ breast cancer patients (17). We hypothesize that the intestinal microbiota changes during

AC-D treatment, and that the intestinal microbiota is associated with chemotherapy toxicity and tumor response in postmenopausal ER+ breast cancer patients.

Results

Baseline characteristics

In total, 44 patients were included (Figure 1). At baseline, mean age was 59 years. Mean BMI was 26 kg/m^2. Nine percent of the patients reported 5-10% weight loss during the previous 3-6 months before inclusion. Most patients were diagnosed with early-stage breast cancer.

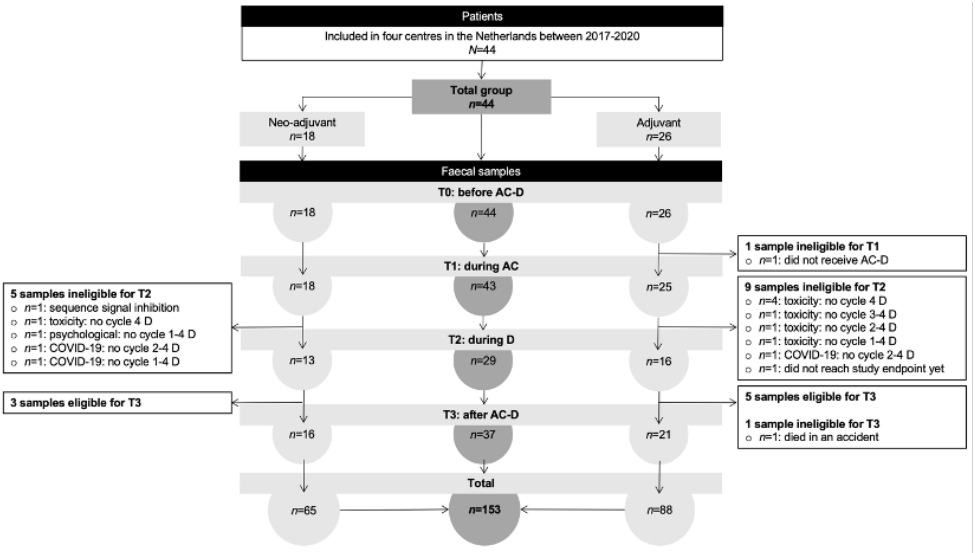


Figure 1: Flow chart *The flow chart presents the number of patients included and the number of fecal samples collected by those patients during the study period. Multiple patients who did not collect a fecal sample at T2 were able to collect a fecal sample at T3. In total, 44 patients collected 153 fecal samples at four time points. 28 participants provided all four samples. The total group is presented in the middle. On the left and right sides, the total group is subdivided into neoadjuvant and adjuvant groups.*

In the year prior to inclusion, 27% of the patients used therapeutic antibiotics with a median use of seven days. None of the patients used therapeutic antibiotics within the three months prior to inclusion. The mean time between the last therapeutic antibiotic dose and baseline fecal sample collection was 31 weeks (range 15-52 weeks). Twelve (46%) adjuvant treated patients received prophylactic cefazolin at the start of the operation. In addition, four (14%) of these twelve patients also received prophylactic amoxicillin/clavulanic acid for five days after the operation. The mean time between the operation and baseline fecal sample collection was 50 days. One patient used prebiotics in the year prior to inclusion. None of the patients used probiotics or nutritional supportive drinks in the year prior to inclusion. At baseline,

neoadjuvant patients had higher clinical tumor stages and higher Karnofsky Performance Scores compared to adjuvant treated patients (Table 1 and Supplementary Table 1).

Table 1: *Clinical characteristics of the total study population (N=44) at baseline including the comparison between adjuvant and neoadjuvant treated patients.*

Baseline characteristics	Total *n*=44	Adjuvant *n*=26	Neoadjuvant *n*=18	*p*-value
Age - Years				
Mean (SD)	59 (6)	59 (6)	58 (5)	0.478
BMI - kg/m^2				
Median (IQR)	26 (5)	26 (4)	26 (7)	0.943
Weight loss past 3-6 months - in kg				
<5%	40 (91)	24 (92)	16 (89)	
5-10%	4 (9)	2 (8)	2 (11)	1.000
Clinical tumour stage - No (%) [1]				
Stage I	17 (40)	15 (58)	2 (12)	
Stage II	23 (54)	11 (42)	12 (71)	
Stage III	3 (7)	0 (0)	3 (18)	**0.001**
Tumour-type - No (%)				
Invasive carcinoma of no special type (NST)	33 (75)	17 (65)	16 (89)	
Lobular	8 (18)	6 (23)	2 (11)	
Mucinous	2 (5)	2 (8)	0 (0)	
Unknown	1 (2)	1 (4)	0 (0)	0.089
Therapeutic antibiotic use last year - No. (%)	12 (27)	8 (31)	4 (22)	0.733
Weeks between collection T0 fecal sample and last therapeutic antibiotic treatment				
Mean (SD)	31 (13)	29 (12)	33 (15)	0.713
Karnofsky Performance Score - No (%)*				
70-80	9 (21)	7 (27)	2 (11)	
90-100	35 (79)	19 (73)	16 (89)	**0.006**
MUST-score - No (%)				
Low risk	38 (86)	22 (85)	16 (89)	
Medium risk	6 (14)	4 (15)	2 (11)	
High risk	0 (0)	0 (0)	0 (0)	0.688
Oral contraception use past	34 (77)	19 (73)	15 (83)	0.489

Clinical characteristics of patients during the course of AC-D treatment

During the course of AC-D treatment, patients had an increased risk of malnutrition (p<0.001). The MUST-score improved in the period between T2 and T3 (p=0.005). BMI remained stable over time (p=0.338) (Supplementary Table 2).

Between T0-T1, 21% of the patients used antibiotics; 38% between T1 and T2 and 5% between T2 and T3. Most administered antibiotics included amoxicillin/clavulanic acid, nitrofurantoin, and ciprofloxacin. None of the patients used prebiotics, probiotics, or nutritional supportive drinks during the course of AC-D treatment. In contrast to prophylactic antibiotic use prior to T0 fecal sample collection, antibiotic administration during AC-D treatment was not different between adjuvant and neoadjuvant treated patients (Supplementary Table 3).

Dose intensity was high, with a median of 94% of the chemotherapy dosage administered during AC-D treatment (Supplementary Table 4).

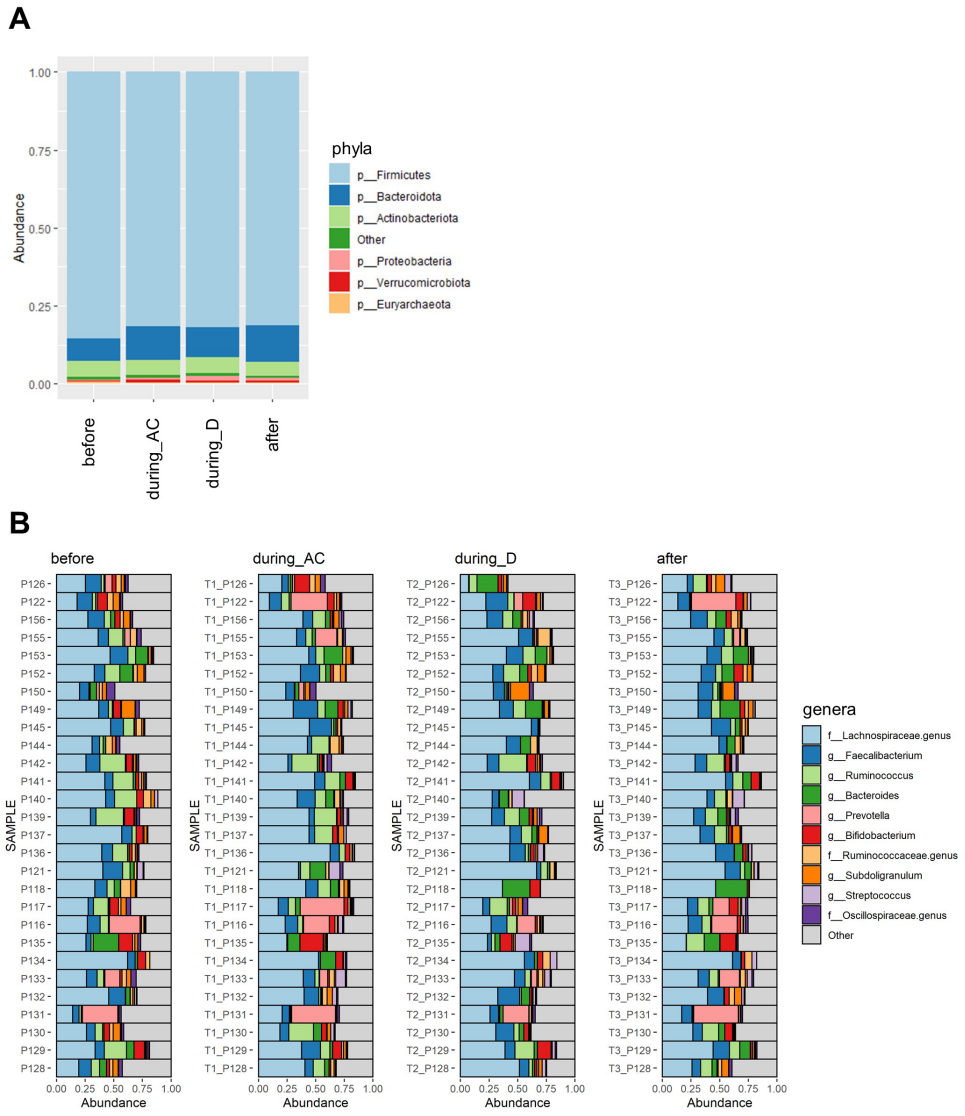


Figure 2: Relative abundances of microbiota before, during, and after chemotherapy
A: *Relative abundances of different phyla before AC-D (n=44), during AC (n=43), during D (n=29), and after AC-D treatment (n=37).* ***B:*** *Composition plot of individual samples (of participants who provided all four samples, n=28) indicating changes in relative abundance of most common genera over the course of AC-D treatment.*

Intestinal microbiota composition of the total study population

In total, 153 fecal samples were collected. Fecal samples were collected before AC-D (*n*=44), during AC (*n*=43), during D (*n*=29), and after AC-D treatment (*n*=37). 28 participants provided all four samples (Figure 1).
In the total study population, Firmicutes was the most abundant phylum, followed by Bacteroidetes and Actinobacteria (Figure 2A). Figure 2B indicates changes in the relative abundance of the most common genera.

Differences in microbiota richness, diversity and composition during the course of AC-D

Observed species richness reduced significantly during AC-D treatment (*p*=0.042) (Figure 3A and Supplementary Table 5). Pairwise comparison with Bonferroni correction of all samples revealed a significant decrease in observed species richness between T0-T3 (*p*=0.003; *n*=37) (Figure 3B and Supplementary Table 6).
Additional analyses were performed to assess the influence of exposure to therapeutic antibiotics before and during the course of AC-D treatment on α-diversity. Observed species richness and Shannon index before AC-D, during AC, during D, and after D were not different between patients with or without therapeutic antibiotic use up to one year until three months prior to T0 (Supplementary Table 7A).
Antibiotic administration between T0 and T1 was negatively correlated with observed species richness (*p*=0.002) and Shannon index (*p*=0.003) at T1 (Supplementary Table 8). Cumulative therapeutic and prophylactic antibiotic use from the year prior to baseline fecal sample collection until the index sample, was not correlated with lower α-diversity at T1, T2 or T3 (Supplementary Table 9).

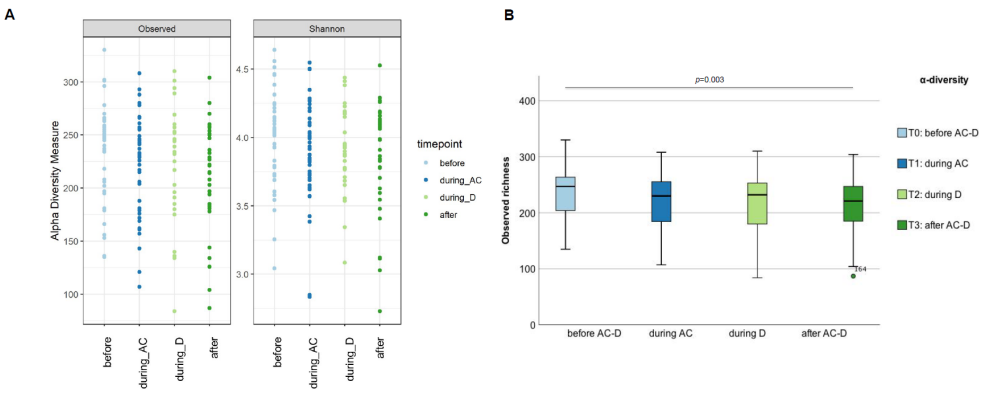


Figure 3: Microbiota diversity before, during, and after chemotherapy A: *Changes in α-diversity measures of the 28 participants who provided all four samples before AC-D, during AC, during D, and after AC-D treatment, measured in terms of observed species richness (p=0.042; n=28) and Shannon index (p=0.206; n=28) (Supplementary Table 5).* ***B:*** *Pairwise comparison (Wilcoxon signed-rank sum test with Bonferroni correction) of all samples before AC-D (n=44), during AC (n=43), during D (n=29), and after AC-D treatment (n=37) revealed significant differences in observed species richness between T0-T3 (p=0.003; n=37) (Supplementary Table 6). The boxplot in figure 3B shows the medians, IQR's, minimum, maximum, and an outlier.*

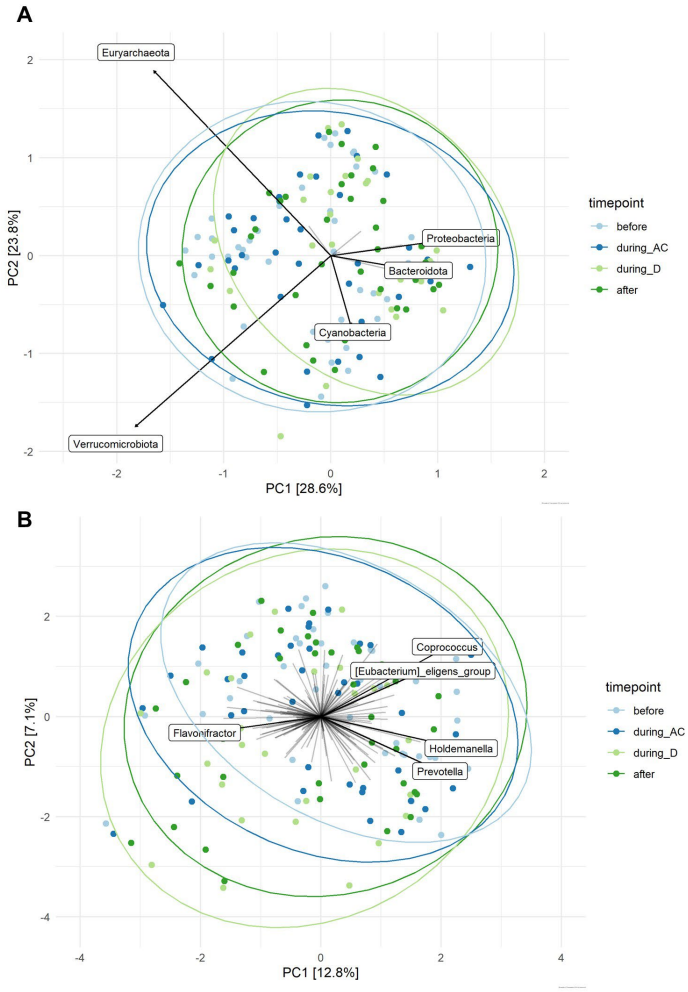


Figure 4: Ordination plots *Ordination plots derived from unconstrained Principal Components Analysis (PCA) based on the Aitchison distance, showing overall composition of the microbial community at phylum (A) and genus level (B) before AC-D (n=44), during AC (n=43), during D (n=29), and after AC-D treatment (n=37). Taxa that were present in less than 5 samples were excluded for this analysis. Data were transformed using centre-log-ratio transformation. Names are given for taxa, which contributed most to overall microbial variation.*

Principal Component Analysis (PCA) showed large heterogeneity in individual microbial community structures. PERMANOVA revealed that there was no statistically significant association between overall microbial community structure at phylum (p=0.086) and genus (p=0.102) level and the different sampling time points (Figure 4). In line with these PERMANOVA results, dbRDA indicated no effect of the sampling timepoint on microbial community structure after partial out the effect of patient ID (genus level: variance=2.0006, p=1.0, phylum level: variance=2.2747, p=1.0).

Furthermore, we identified no consistent significant differences in microbial community structure between patients with or without cumulative therapeutic and prophylactic antibiotic use before or during AC-D (Supplementary Figure 1).
At phylum level, ANCOM-II analysis identified that Proteobacteria were differently abundant during the course of AC-D (Figure 5A). This significant change over time was confirmed by Friedman's ANOVA (p=0.006). More specifically, pairwise comparison indicated that the abundance of Proteobacteria increased during D, and decreased after AC-D treatment.
Furthermore, according to differential abundance analysis using ANCOM-II, eight genera were differently abundant during the course of AC-D (Figure 5B). Except for *Turicibacter* and *Intestinibacter*, Friedman's ANOVA using $\log^{10}(1+x)$ abundance confirmed these results and indicated significant changes for unclassified Enterobacterales (p<0.001), *Lactobacillus* (p=0.004), *Ruminococcaceae NK4A214 group* (p<0.001), *Marvinbryantia* (p=0.020), *Christensenellaceae R7 group* (p=0.008), and *Ruminococcaceae UCG-005* (p<0.001).
Unclassified Enterobacterales and *Lactobacillus* increased during AC-D treatment. After AC-D treatment, unclassified Enterobacterales decreased (p<0.001). Abundances of the *Ruminococcaceae NK4A214 group*, the *Christensenellaceae R7 group*, *Ruminococcaceae UCG-005*, and *Marvinbryantia* decreased during AC-D treatment (Figure 5 and Supplementary Table 10). More information on longitudinal changes of bacterial abundances in individual patients can be found in Supplementary Figures 2 and 3.
Of note, patients who received antibiotics between T0 and T1 had significantly lower levels of *Christensenellaceae R7 group* at T1 (p=0.001). Furthermore, patients who received antibiotics between T1 and T2 had significantly lower levels of *Marvinbryantia* at T2 (p=0.028).

Associations of microbiota richness, diversity and composition with chemotherapy toxicity

The most common CTCAE toxicities are reported in Supplementary Figure 4 and Supplementary Table 11 and 12. During docetaxel (T2), 19% experienced grade 1 diarrhea and 19% grade 2. Observed species richness and Shannon index at T2, as well as T3, were negatively correlated with diarrhea at T2. Patients with any grade diarrhea during D (T2) had a significantly lower level of observed species richness at T2 compared to patients without diarrhea (p=0.039). Patients with any grade diarrhea after AC-D treatment (T3) had a lower Shannon index at T3 compared to patients without diarrhea (p=0.006). Diarrhea at T3 was negatively correlated with the levels of *Ruminococcaceae UCG-005 (*p=0.027*) and the Ruminococcaceae NK4A214 group (*p=0.033*)*. Nausea at T3 was negatively correlated with observed species richness (p=0.048) and Shannon index (p=0.029). There were no correlations between oral mucositis, hand foot syndrome or peripheral sensory neuropathy with observed species richness or Shannon index at the different time points.
PERMANOVA showed that microbial community structure on both phylum and genus level during AC (T1) and during D (T2) was not associated with diarrhea, nausea, oral mucositis, hand-foot syndrome or peripheral sensory neuropathy (Supplementary Table 13).
Diarrhea was not correlated to previous therapeutic antibiotics, perioperative prophylactic antibiotic administration, or antibiotic exposure during the course of AC-D treatment.

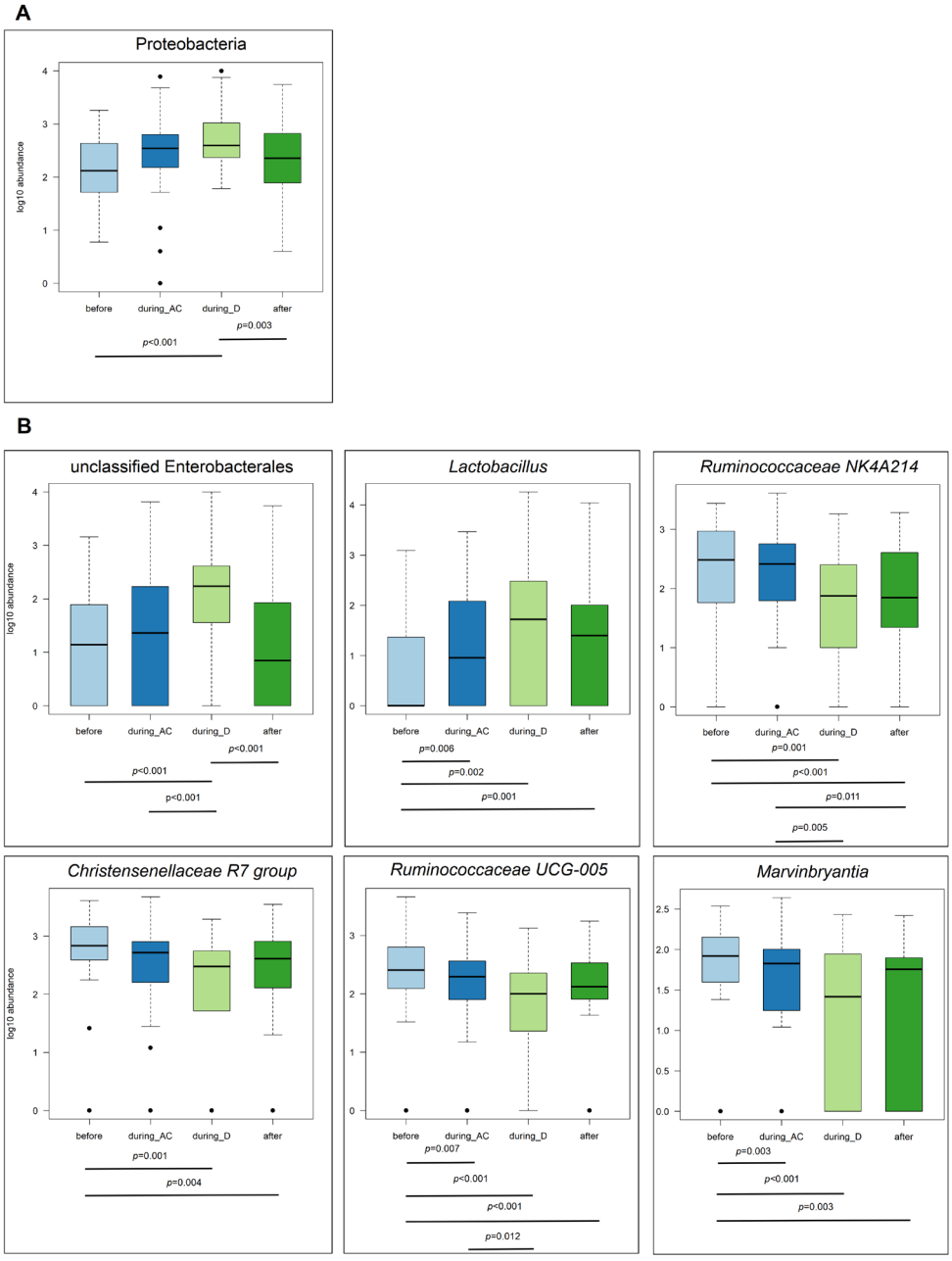


Figure 5: Differential abundant taxa during the course of AC-D *Log[10] abundance of taxa with significant differential abundance before AC-D (n=44), during AC (n=43), during D (n=29), and after AC-D treatment (n=37). P-values below boxplots indicate significant differential abundances analysed with a pairwise Wilcoxon signed-rank sum test (Supplementary Table 10). A: Phylum level. B: Genus level. The boxplots show the medians, IQRs, minimum, maximum, and outliers.*

Associations between pathologic response and intestinal microbiota richness, diversity and composition in patients treated with neoadjuvant AC-D

In total, 18 patients received neoadjuvant chemotherapy. The clinical characteristics of the neoadjuvant subgroup are presented in Supplementary Tables 14, 15 and 16.
Response measured after AC-D according to EUSOMA could not be determined in one patient with occult breast cancer (cTxN2). After AC-D treatment one patient (6%) achieved pathologic complete response (pCR), six patients (35%) presented with <10% remaining tumor cells, four patients (24%) with 10-50% remaining tumor cells, and six patients (35%) with >50% remaining tumor cells. Accordingly, ten patients were classified as low-responders and seven as high-responders (Supplementary Table 17). Baseline characteristics were not different between low and high-responders (Supplementary Table 18). No differences in clinical characteristics were observed between low and high responders after AC-D (Supplementary Table 19). Before AC-D, during AC, during D, and after AC-D, both α-diversity measures were not significantly different between low and high-responders (Supplementary Table 20). PERMANOVA revealed that there was no statistically significant association between baseline microbial community structure and response after AC-D at phylum (p=0.073) and genus (p=0.130) level. There were no differences in bacterial abundances at baseline between low and high-responders.

Influence of surgery and perioperative use of prophylactic antibiotics

In order to address the potential confounding effects of breast cancer surgery and the perioperative use of prophylactic antibiotics, we also analyzed intestinal microbiota composition in the adjuvant (n=26) and neoadjuvant group (n=18) separately. In contrast to the adjuvant group, patients treated in the neoadjuvant setting did not receive surgery and/or prophylactic antibiotics yet.
Baseline α-diversity measures were not significantly different between patients receiving neoadjuvant or adjuvant chemotherapy (observed species richness: p=0.543; Shannon index: p=0.254). PCA and PERMANOVA showed that microbial community structure was significantly different between patients treated in the adjuvant or neoadjuvant setting respectively (p=0.037 on phylum level and p=0.048 on genus level, Supplementary Figure 5). However, ANCOM-II analysis showed that only abundance of the genus *Dialister* was found to be higher in adjuvant patients (Supplementary Figure 6).
In addition, we examined whether the longitudinal changes in bacterial abundances that were identified in the whole group, could be replicated when analyzing neoadjuvant and adjuvant patients separately. Differential abundance analysis using the ANCOM-II workflow, confirmed significant changes in abundance of Proteobacteria, unclassified Enterobacterales and *Lactobacillus* during AC-D in the neoadjuvant group, while the other taxa did not change significantly. In the adjuvant group, similar to the neoadjuvant group, the abundance of unclassified Enterobacterales changed significantly during AC-D. In addition, other genera (*Intestinibacter, Clostridium sensu stricto, Turicibacter*) also changed in abundance during AC-D in the adjuvant group.
As described earlier, 46% (n=12) of the adjuvant treated patients received perioperative prophylactic antibiotics before inclusion, while neoadjuvant patients were not treated with prophylactic antibiotics. Therefore, the influence of prophylactic antibiotic administration was

investigated in more detail within the group of adjuvant treated patients. Baseline α-diversity measures were not significantly different between adjuvant treated patients with or without perioperative prophylactic antibiotic administration (observed species richness: p=0.667, Shannon index: p=0.155, Supplementary Table 7B). PCA and PERMANOVA indicated that baseline microbial community structure was associated with perioperative use of prophylactic antibiotics on genus level (p=0.026), but not on phylum level (p=0.838, Supplementary Figure 5).

Discussion

This longitudinal pilot study examined the associations between adriamycin, cyclophosphamide and docetaxel (AC-D) treatment and intestinal microbiota, as well as the associations between the intestinal microbiota, chemotherapy toxicity, and tumor response in ER+ and HER2- postmenopausal breast cancer patients. Our study showed that during AC-D treatment observed species richness reduced and the abundance of specific microbial taxa changed. In addition, diarrhea was associated with lower α-diversity. In addition, we did not detect associations between pathologic response and baseline microbiota richness, diversity, and composition in a small group of neoadjuvant treated patients.

Concerning the observed changes in microbiota richness, diversity, and composition in postmenopausal breast cancer patients, no comparable longitudinal clinical studies during AC-D treatment are available. To our knowledge, only the studies of Yulzari *et al*. (2020) (18) and Terrisse *et al.* (2021) (19) are comparable to our study exploring the role of intestinal microbiota in breast cancer patients treated with chemotherapy. Yulzari *et al.* (2020) (18) collected fecal samples of 28 breast cancer patients prior to the start of (neo)adjuvant adriamycin, cyclophosphamide and paclitaxel (P) to study metabolic changes during chemotherapy. However, they did not analyze longitudinal microbiota changes.

In the context of α-diversity, our results show that observed species richness significantly reduced during the course of AC-D with the lowest levels one month after the last docetaxel administration. Previous studies of Montassier *et al* (2015) (20) and Galloway-Peña *et al.* (2017) (21), in patients with acute myeloid leukemia or Non-Hodgkin lymphoma respectively, observed similar α-diversity reductions during different chemotherapy regimens. In general, a lower microbial α-diversity is associated with diseases of metabolic and immunologic origin (22). As a next step, the consequences of reduced α-diversity warrant further investigation, for example by studying microbial functions and long-term clinical associations between dysbiosis, chemotherapy toxicity, tumor response or recurrence free survival.

Beside the reduction of α-diversity, the abundance of specific microbial taxa changed during the course of AC-D treatment. In our small study population, a general trend was observed, in which genera of the *Ruminococcaceae NK4A214 group*, *Christensenellaceae R7 group*, *Ruminococcaceae UCG-005*, and *Marvinbryantia* decreased during AC-D and recovered again after AC-D treatment. The abundance of Proteobacteria, unclassified Enterobacterales, and *Lactobacillus* significantly increased during AC-D treatment. After AC-D treatment, Proteobacteria and unclassified Enterobacterales significantly decreased, reaching levels comparable to baseline.
Our results suggest that the *Ruminococcaceae NK4A214 group*, *Christensenellaceae R7 group*, *Ruminococcaceae UCG-005*, and *Marvinbryantia* might be more sensitive to the effect of AC-D. Many bacteria within the family of Ruminococcaceae are able to produce short-chain fatty acids (SCFA) by degrading polysaccharides. SCFA positively influence intestinal homeostasis and are known to be involved in immunologic and metabolic functions (23, 24). Therefore, reduction of these bacteria during chemotherapy might contribute to the manifestation of intestinal inflammation and dysregulated homeostasis.

In contrast to the genera that decreased during AC-D, Proteobacteria, and specifically unclassified Enterobacterales, increased during chemotherapy and decreased after AC-D treatment. This could be explained by AC-D-induced intestinal inflammation in combination with facultative anaerobic properties of these bacteria. It has been demonstrated that cyclophosphamide and adriamycin are able to disrupt and impair the intestinal barrier, which resulted in the translocation of bacteria via the intestinal wall causing systemic inflammation in mice (13, 14, 25, 26). In addition, the Enterobacterales order includes familiar pathogens such as *Salmonella, Escherichia coli* and *Shigella*, which are known to be associated with intestinal inflammation. Furthermore, Enterobacterales includes facultative anaerobic bacteria, which means that these bacteria have a growth advantage when the blood flow increases due to intestinal inflammation. This systemic inflammation, accompanied with higher blood flow, may contribute to increased levels of unclassified Enterobacterales and its phylum, Proteobacteria. Subsequently, the bloom of Proteobacteria/Enterobacterales at the expense of genera from the Ruminococcaceae family might promote further intestinal inflammation during AC-D.

The largest differences in differentially abundant taxa were observed during docetaxel treatment. Previous research has demonstrated that docetaxel and its metabolites are mainly (75%) eliminated via the feces, while cyclophosphamide is mainly excreted via the kidneys (up to 70%) (27, 28). Due to high exposure of the gastro-intestinal tract to docetaxel and its metabolites, we hypothesize that the direct effect of docetaxel on the intestinal microbiota is more evident compared to the direct effect of cyclophosphamide. As discussed above, it is expected that cyclophosphamide might have a more indirect immune-mediated effect on the intestinal microbiota (9, 15).

Recently, Terrisse *et al.* (2021) published a comparable French study, where the intestinal microbiota of 63 patients who received (neo)adjuvant eight cycles of anthracycline or anthracycline-taxane based therapy were analyzed with metagenomic shotgun sequencing (29). Compared to baseline, richness increased after chemotherapy, which was in contrast with our study, where observed species richness significantly decreased after chemotherapy. Furthermore, the study of Terrisse *et al.* observed at species level that chemotherapy increased the abundance of *Methanobrevibacter smithii*, *Dorea formicigenerans,* and *Ruminococcus torques* and that chemotherapy tended to reduce the species of *Clostridium asparagiforme, Bacteroides uniformis,* and *Eggerthella lenta.* We were not able to identify these microbiota shifts on species level in our study, because taxa were annotated on genus level. However, the shifts described by Terrisse *et al*. do not correspond with our findings on genus level, where *Ruminococcaceae UCG-005 and Ruminococcaceae NK4A214* decreased after chemotherapy. These discrepancies could be due to some methodological differences between the two studies, limiting comparability. First of all, in the French study patients were not homogenous concerning tumor subtype and systemic cancer therapy scheme. In the French study, up to 24% of the breast cancer patients had triple negative breast cancer, while in our study only ER+ patients were included. 60% of the French study patients received additional endocrine therapy before the last fecal sample collection, and 31% of the patients received HER2-directed therapy. In addition, sampling time points were different with samples before and after chemotherapy in the study of Terrisse *et al.* and four sampling time points in our study. With respect to the potential interaction between the estrogen metabolism and the intestinal microbiota via microbial β-glucuronidase, no distinction was made between pre- or

postmenopausal women in the French study, while we only included postmenopausal women. In line with this, Zhu *et al.* (2018) indicated microbial differences between postmenopausal breast cancer patients and postmenopausal controls but not between premenopausal breast cancer patients and premenopausal controls, potentially indicating that the intestinal microbiota behaves differently in postmenopausal breast cancer patients (30). Despite these differences in methodological design, these two small studies together form an important basis for further research in this field.

Concerning chemotherapy toxicity, we detected that patients with any grade of diarrhea during docetaxel treatment had significantly lower observed species richness compared to patients without diarrhea. Furthermore, diarrhea was not correlated to antibiotic use prior to AC-D treatment or during AC-D treatment. This makes the assumption stronger that patients suffered from AC-D-induced diarrhea. In addition, lower performance scores, as well as increased toxicity levels during AC-D treatment, further confirm the systemic inflammatory effects of AC-D treatment. Limited clinical studies confirmed a decrease in microbial richness and its association with diarrhea in patients undergoing chemotherapy (31). It might be speculated that patients with lower microbial richness have a higher risk to develop diarrhea or vice versa. However, the exact mechanism by which AC-D-induced diarrhea occurs should be examined further, for example using the TIMER (translocation, immunomodulation, metabolism, enzymatic degradation, and reduced diversity) model that was recently proposed by Alexander *et al.* (2017) (32).

In our small group of neoadjuvant treated patients, we did not detect associations between pathologic response and baseline intestinal microbiota richness, diversity and composition. This is in contrast to the results from Terrisse *et al.* (2021), who explored the associations between anthracycline, taxane-based and/or hormone therapy and the intestinal microbiota in breast cancer patients. In both pre and post chemotherapy fecal samples specific microbiota were associated with either a worse prognosis (lymph node positive patients and TNM staging >1) or a more favorable prognosis (lymph node negative patients and/or TNM stage 1) (19). Goubet *et al.* (2018) observed a longer survival in patients with non-small cell lung cancer and ovarian cancer with an *Enterococcus hirae* and *Barnesiella intestinihominis* specific interferon gamma-mediated tumor response (11). Comparability between our study and the study of Goubet *et al.* (2018) is limited since species-level differences could not be analyzed in our study. In addition, it concerns other cancer types and the effects of adriamycin and docetaxel were not taken into account by Goubet *et al*. (2018). Furthermore, our results were based on a relatively small sample size of 18 patients. Therefore, these observations should be interpreted carefully and warrant further investigation in a larger study population. In addition, since pathologic response on neoadjuvant chemotherapy is not a useful surrogate endpoint in ER+, HER2- breast cancer (33), evaluating recurrence-free survival of these patients would be of interest in future research into associations between the intestinal microbiota and treatment efficacy.

In the present cohort, patients were included in both the adjuvant and neoadjuvant setting. Although the group of neoadjuvant patients was relatively small in our cohort, we could confirm the observed significant increase in the abundance of Proteobacteria, unclassified Enterobacterales and *Lactobacillus* during the course of AC-D treatment in the neoadjuvant group. However, we observed some clinical differences between adjuvant and neoadjuvant

patients, for instance, higher clinical tumor stages and higher Karnofsky Performance Scores in neoadjuvant patients. In addition, analysis of the intestinal microbiota indicated differences in microbiota community structure between these groups. Longitudinal changes in abundance, which were observed in the whole group, could not be confirmed in both subgroups. Consequently, we cannot rule out that breast cancer surgery in the adjuvant group might have a confounding effect on our results. Due to these potential confounding factors, it is likely that the intestinal microbiota behaves differentially in these two subgroups, despite the fact that patients receive the same AC-D treatment. Therefore, it would be beneficial to perform similar future studies in neoadjuvant patients only, in order to exclude breast cancer surgery as a confounding factor. However, the fact that the abundance of unclassified Enterobacterales changed consistently among all groups, does suggest that overgrowth of these bacteria could be a common phenomenon during chemotherapy and requires further evaluation.

Next to breast cancer surgery, it is widely described that antibiotic exposure can disturb the intestinal microbiota. For this purpose, additional in-depth analyses were performed to examine the potential influence of antibiotic administration on our results. The effect of antibiotic administration in the total group was mainly observed in adjuvant patients receiving perioperative prophylactic antibiotics. Therefore, we also analyzed the influence of perioperative administration of prophylactic antibiotics on intestinal microbiota composition and diversity, and we identified significant effects on baseline microbial community structure. As a consequence, perioperative prophylactic antibiotic administration might have a confounding effect on our results. As described above, this could be prevented in future studies by including only patients receiving neoadjuvant chemotherapy. Of note, cumulative antibiotic use (therapeutic or prophylactic) before or during AC-D did not indicate differences in microbiota composition at T3. This could be partly explained by time and the interaction with other microbiota-modulating factors such as docetaxel treatment. Consequently, the primary observed prophylactic antibiotic effect might diminish or disappear over time (34). In addition, as discussed earlier, the direct effect of docetaxel on the intestinal microbiota (27) might be more evident compared to the effect of (earlier) prophylactic antibiotic administration. None of the patients used prebiotics, probiotics or nutritional supportive drinks during the course of AC-D treatment. This means that the differences in microbiota richness, diversity and composition are not attributable to these microbiota-modulating agents.

There are several limitations and strengths of this study. The main limitation is the small sample size. It is not possible to draw conclusions concerning causal relationships, or to predict therapy outcomes, based on this small cohort. Consequently, the current study should be seen as a pilot study providing insights into the feasibility of a study with longitudinal microbiota sampling in patients during AC-D. Our results provide early indications that there might be an interaction between the intestinal microbiota and AC-D. Furthermore, our results indicate that patients treated in the adjuvant or neoadjuvant setting should be analyzed separately in future studies.
In addition, another limitation is the use of pCR, as it is known that pCR is not a useful surrogate endpoint for the specific breast cancer subtype studied (ER+/HER2-) (33). Besides that, it was only possible to conduct chemotherapy response measurements in the subgroup of neoadjuvant treated patients. As a consequence, the group size was reduced from 44 to 18 patients. In addition, response measurement based on residual tumor is not possible in adjuvant treated patients, since adjuvant patients will be subjected to tumor resection first. To

circumvent this, increased samples sizes and other response measurements should be used, for instance, recurrence free survival or progression free survival (35).
Unfortunately, no analysis of bacterial metabolites (e.g. SCFA), has been performed. Insights into the levels of SCFA would provide more knowledge on the intestinal bacterial activities involved in the regulation of the host's immune system and metabolism, as well as their associations with cancer treatment (36). Besides that, co-medication, diet, and surgery-related factors, such as type, route, and duration of antibiotic administration, might form alternative explanations for our findings.
Lastly, sequencing of the V4 region rather than larger segments (e.g. V3-V4 regions) has some limitations, but also advantages. Although regions such as V3-V4 span a longer segment of the 16S rRNA gene, the overlap between forward and reverse reads are shorter, and in particular with amplicon sequence variant calling this has been shown to result in spurious inflation of the ASV diversity (37). With 250bp paired-end sequencing, the overlap between forward and reverse reads is (near to) complete when sequencing the V4 region and consequently the sequencing errors are significantly reduced. Moreover, in contrast to what might be expected based upon the longer region, a recent extensive comparison showed that analysis of the V3-V4 results in a less accurate taxonomic assignment when compared to the V4 region (38). However, in silico analyses showed that short-read sequencing of hypervariable regions cannot achieve the same level of taxonomic resolution as can be achieved by sequencing of the entire 16S rRNA gene (39). Full-length 16S rRNA gene sequencing allows better discrimination between closely related species (40). To sequence even longer regions other platforms such as MinION nanopore or PacBio have recently been used. However, these platforms generate read data with significantly lower nucleotide accuracy than the Illumina platform due to random base-calling errors. This has recently been overcome by a novel technology developed by Loop Genomics, which enables long-read sequencing by utilizing an existing Illumina short-read sequencer combined with a unique molecule barcoding technology. This method was not yet widely applied at the time of initiating our lab analyses (41).

One unique advantage of this study is its relatively homogenous study population. To make the group as homogeneous as possible, we only included postmenopausal women to exclude the effect of physiologically higher estrogen levels in premenopausal patients. To exclude the effect of HER2-targeted therapy, HER2 receptor positive patients were not included. Based on the expected differences between the groups described above, our results are not directly generalizable to premenopausal patients, HER2+, or triple negative breast cancer patients. Another strength of this study is the longitudinal design, including the collection of fecal samples at four different time points. In addition, in-depth analyses of antibiotic administration have been performed to reveal potential confounding effects of antibiotic administration.

As described above, our study might be seen as a pilot study, providing guidance for the design of future studies in this field of research. Future research could focus on the role and function of the bacteria that increased or decreased during chemotherapy treatment. This could be done with quantitative assessment of microbial metabolites. Also, full length 16S rRNA gene sequencing or high-throughput whole metagenomic shotgun sequencing, including the possibility to determine bacterial metabolic capacity will be highly relevant to further establish the microbiota composition, including its microbial functions and their associations with chemotherapy toxicity. In addition, to overcome the influence of a small

sample size on tumor response measurement in the neoadjuvant treated group, larger breast cancer cohorts should be recruited. Finally, it will be highly relevant to compare our results with upcoming studies that address the link between intestinal microbiota and chemotherapy in breast cancer patients (e.g. NCT03586297 and NCT04138979) to see whether the chemotherapy-induced patterns are similar among different breast cancer subtypes and if it is possible to identify key species susceptible to chemotherapy.

In conclusion, this is the first clinical study with longitudinal fecal sampling in breast cancer patients that explored the associations between adriamycin, cyclophosphamide, and docetaxel treatment and the intestinal microbiota, as well as the impact of the intestinal microbiota on chemotherapy toxicity and tumor response in ER+ and HER2- postmenopausal breast cancer patients. We reported shifts in intestinal microbiota richness and composition during AC-D treatment. Our findings provide important insights into an association between chemotherapy and intestinal microbiota in postmenopausal ER+ and HER2- breast cancer patients. Our results emphasize the necessity to further explore chemotherapy-induced microbiota changes and potential metabolic and immunologic consequences in breast cancer patients.

Methods

Patients

Between November 2017 and February 2020, breast cancer patients were prospectively enrolled in four Dutch hospitals. Eligible patients were postmenopausal women with histologically proven ER+ (≥10%), and human epidermal growth factor receptor-2 (HER-2) negative breast cancer (42) starting with (neo)adjuvant chemotherapy. Exclusion criteria included distant metastasis, previous chemotherapy and therapeutic antibiotics within three months prior to AC-D treatment.
The study was approved by the Medical Ethics Committee azM/UM (METC 17-4-075). The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. Each patient provided written informed consent.

Treatment

During the study period, patients received four cycles of adriamycin (A), 60 mg/m^2 i.v. and cyclophosphamide (C) 600 mg/m^2 i.v. on day 1, in either a two-weekly (dose dense, dd) or three-weekly cycle. AC treatment was followed by four cycles of docetaxel (D), 100 mg/m^2 i.v. on day 1, in a three-weekly cycle. Patients received chemotherapy in neoadjuvant or adjuvant setting according to standard care using the Dutch guideline for systemic breast cancer treatment (43). These guidelines are largely in line with the ESMO and ASCO guidelines. Corticosteroid administration was provided according to local protocols. In general, 8 mg dexamethasone once a day was provided during each adriamycin/cylophosphamide cycle at day 1 (i.v.), day 2 (oral) and day 3 (oral). During each docetaxel cycle 8 mg oral dexamethasone was provided twice a day on the day before, during and after the first day of the cycle. These administrations were uniformly done across all the patients.

Fecal sample and data collection

Patients collected a fecal sample and completed a questionnaire at four time points: before the start of AC-D (T0), during the second week of the fourth cycle AC (T1), during the second week of the fourth cycle D (T2), and one month after the last dose D (T3) (Supplementary Figure 7). Samples were immediately stored in the freezer and transported to the hospital in a cooled container (Sarstedt) (44). In the hospital, samples were stored immediately at -20°C and subsequently at -80°C for long-term storage. Patient characteristics were registered, including chemotherapy dose reductions, prophylactic and therapeutic antibiotic use, prebiotic/probiotic use and the use of nutritional supportive drinks. Therapeutic antibiotic treatment included treatment between one year and three months prior to T0 fecal sample collection. Prophylactic cefazolin administration at the start of the breast cancer operation and prophylactic amoxicillin/clavulanic acid administration after the operation was summarized as perioperative prophylactic antibiotic use. Nutritional status was assessed with the Malnutrition Universal Screening Tool (MUST).

Clinical characteristics assessed by the questionnaires.

Questionnaire were taken from the patient at 4 time points:
T0: Before administration of first chemotherapy treatment
T1: 1-2 weeks after administration of 4th chemotherapy treatment
T2: 1-2 weeks after administration 16th chemotherapy treatment
T3: 4 weeks after administration of 16th and last chemotherapy

Questionnaire T0-T3:

- Birth month/year
- Fill-in date
- Current weight
- Current length
- Malnutrition Universal Screening Tool (MUST) score: based on following questions:
    - Are you feeling ill at this moment?
    - Do you have a normal appetite?
    - Did you eat bad the past 5 days?
    - Do you think that you could eat bad for more than 5 days?
- Karnofsky Performance Score (KPS): Scale from 0 to 100
    - 0 = deceased
    - 100 = No complaints, no symptoms of illness
- Did you collect feces today?
    - If no, what date did you collect feces?
- CTCAE score (Common Terminology Criteria for Adverse Events)
    - Nausea (scale; 0-3)
    - Vomit (scale; 0-4)
    - Inflammation of the mouth (scale; 0-4)
    - Diarrhea (patients without stoma) (scale; not applicable, 0-4)
    - Diarrhea (patients with stoma) (scale; not applicable, 0-4)
    - Unintentional weight loss past 3-6 months (scale; 0-3
    - Constipation (scale; 0-4)
    - Fever (scale; 0-4)
    - Changed feeling (deaf, irritation, tingling) (scale; 0-4)
    - Hand-feet complaints (scale; 0-3)
    - Fatigue (scale; 0-3)
    - Hair loss (scale; 0-2)

Additional questions in questionnaire T0:

- Past treatment with chemotherapy (when, name of therapy, number of treatments)
- Antibiotics use past year (when, name, number of days)
- Prednison (steroids) use past year
- Prednison (steroids) use past month
- Use of oral contraception
- Past use of contraception (stop date; month & year, total amount of years used)
- Use of Intra Uterine Device (IUD) (type)
- Past use of IUD (type, date of removal, total amount of years used)
- Diabetes (type)
- Smoking (years, number of cigarettes in a day)
- Past smoking (years, number of cigarettes in a day, stop date)
- Past abdominal surgery (what type)
- Crohn's disease
- Colitis Ulcerosa

Additional questions in questionnaire T1-T3:

- How many of the next tablets did you use since filling in the previous questionnaire (before start of chemotherapy):
    - Metoclopramide (primperan) (number of tablets)
    - Granisetron (kytril) (number of tablets)
    - Diarrhea inhibitors (Imodium/loperamide) (number of tablets)
    - Antibiotics (If yes, name, and duration)
    - Prednison/dexamethasone

Response measurement

In neoadjuvant patients, pathologic tumor response after neoadjuvant AC-D treatment was assessed using the scoring system according to European Society of Breast Cancer Specialists (EUSOMA). High-responders were defined as EUSOMA 1 and EUSOMA 2 (i). Low-responders were defined as EUSOMA 2 (ii), EUSOMA 2 (iii), and EUSOMA 3 (45). The complete definition of the EUSOMA scoring system is presented below.

EUSOMA 1: Complete pathological response

- **(i)**: no residual carcinoma.
- (**ii)**: no residual invasive carcinoma but DCIS present.

EUSOMA 2: Partial response to therapy.

- **(i):** minimal residual disease/near total effect (e.g. only a few loose tumor cells or tumor cells located in small groups).
- **(ii):** evidence of response to therapy but with 10-50% of tumor remaining.
- **(iii):** >50% of tumor cellularity remains evident, when compared to the previous core biopsy sample, although some features of response to therapy are present (e.g. fibrosis).

EUSOMA 3: No response: no evidence of response to therapy.

Chemotherapy toxicity measurement

Toxicity was scored with Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 (46) .The following aspects were scored: diarrhea, peripheral sensory neuropathy, hand-foot syndrome, fatigue, nausea, oral mucositis, vomiting, alopecia and constipation. For binary toxicity analysis, patients with toxicity were defined as having toxicity scores ≥ grade 1.

Fecal microbiota analyses

Metagenomic DNA was isolated using the Ambion MagMax™ Total Nucleic Acid Isolation Kit (*Thermo Fisher Scientific*). We performed a manual pre-processing procedure followed by automated nucleic acid purification with the KingFisher FLEX (*Thermo Fisher Scientific*). In more detail, in order to extract metagenomic DNA, 250 mg of the frozen fecal samples was homogenized in phosphate buffered saline (PBS) and centrifuged for 1 minute at 900 rpm. For cell lysis, a combination of chemical, mechanical and thermal disruption was used. A lysis buffer containing 1M Tris-HCl, 0.5M EDTA, 5M sterile NaCl and SDS (final concentration 4%)

was filled into bead tubes of the Ambion MagMax™ Total Nucleic Acid Isolation Kit *(Thermo Fisher Scientific)* and mixed with 175µl supernatant of feces in PBS. Mechanical disruption consisted of a bead-beating procedure using the Fastprep™ Homogenizer *(*5,5ms for 3x1min; resting 1 min in between, *MP Biomedicals)*. Samples were subsequently incubated for 15 minutes at 95°C with gentle shaking. After centrifugation for five minutes at 11000 rpm, supernatant was filled in an Eppendorf tube. Afterwards, a second round of bead beating and incubation was performed and supernatants were pooled and stored at -20°C until further analysis. 200µl of the supernatants were introduced into a KingFisher 96-wells deep well plate *(Thermo Fisher Scientific)*, together with bead mix of the Ambion MagMax™ Total Nucleic Acid Isolation Kit (*Thermo Fisher Scientific*), isopropanol, and lysis buffer. Other plates were filled with wash buffers, elution buffer (+RNAse), and 96-tips for DW magnets *(Thermo Fisher Scientific)*. Afterwards, the prepared plates were introduced into the KingFisher system and the DNA isolation was performed according to the manufacturer's standard protocol *(Thermo Fisher Scientific).* After removal of the plates from the system, the plate containing purified nucleic acids was incubated for 15 minutes at 37°C for degradation of RNA.

Subsequently, the V4 hypervariable region of the 16S rRNA gene was amplified in triplicate using the 515F/806R barcoded primer pair described previously (47). Pooled amplicons from the triplicate reactions were purified using AMPure XP purification (Agencourt) according to the manufacturer's instructions and eluted in 25 µl 1 × low TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Quantification of amplicons was subsequently performed by the Quant-iT PicoGreen dsDNA reagent kit (Invitrogen) using a Victor3 Multilabel Counter (*Perkin Elmer, Waltham, USA*). Amplicons were mixed in equimolar concentrations to ensure equal representation of each sample and sequenced on an Illumina MiSeq instrument (MiSeq Reagent Kit v3, 2 × 300 cycles, 10% PhiX) to generate paired-end reads of 250 bases (~25.000 reads/sample) (48). All basic 16S rRNA gene sequencing statistics are presented in Supplementary Table 21.
(47)
Bioinformatic analysis of the sequencing data was performed using R (version 4.0.3) (49). For the pre-processing, a standardized in-house pipeline using the software package DADA2 was applied (50). The pre-processing consisted of the following steps: reads filtering, identification of sequencing errors, dereplication, and removal of chimeric sequences.

In order to assign taxonomy, the SILVA 138 database and DECIPHER's IDTAXA algorithm (51) were used to annotate to the genus level. Data were expressed as amplicon sequence variants (ASVs). Decontam was used with the "either" setting, which combines the two statistical methods prevalence and frequency for the identification of contamination in marker-gene and metagenomics data (52). Contaminant ASVs identified by decontam, were filtered out, together with ASVs present in less than 5% of all samples and those with a total abundance of less than 0.001%. After filtering, 816 taxa remained in the analysis. The final file was saved in the phyloseq format (53).

Statistical analysis of clinical data

Baseline characteristics, longitudinal clinical data, statistical tests for α-diversity measures, and abundances of phyla and genera of interest were analyzed in IBM SPSS version 26. For continuous data, normality was tested using the Shapiro-Wilk test. Depending on whether the

variable was normally distributed or not, an unpaired t-test or the non-parametric Mann-Whitney U test was applied. Levene's test was used to test for equal variances. For categorical variables, the non-parametric Chi-square test was performed. In case of low frequencies of binary variables, a Fisher's exact test was used.
For longitudinal analysis with two time points of quantitative variables, a paired sample t-test or the non-parametric Wilcoxon signed-rank sum test was used. For longitudinal analysis with four time points, repeated-measures ANOVA or Friedman's ANOVA were used for normally and non-normally distributed data, respectively. For repeated measures ANOVA, Greenhouse-Geisser correction was used when the assumption of sphericity was not met.
Longitudinal significant results were subjected to a post hoc Wilcoxon signed-rank sum tests with Bonferroni correction. After Bonferroni correction, *p*-values below 0.0125 indicated significance. Spearman's rho (r_s) correlation coefficient was used to assess the relation between ordinal and continuous data. Two-tailed tests were used and in general *p*-values below 0.05 were considered statistically significant.

Statistical analysis of intestinal microbiota data

Non-rarefied data were used for diversity analysis. Both α-diversity indices, including observed species richness and Shannon index, which is a measure of microbial diversity, were calculated on ASV level, using the phyloseq package (53). Testing the assumptions of normality and homogeneity of variance, and subsequent statistical testing was performed as described in the clinical data analysis section. The R packages, phyloseq (53), vegan (54), microbiome (55), dplyr (56), ggplot2 (57) and microViz (58) were used for ordination and visualization of taxonomic composition. Taxa present in less than 5 samples were filtered out for all analyses. Unconstrained ordination was performed using Principal Component Analysis (PCA) based on Aitchison distances at genus and phylum level (58). Homogeneity of multivariate dispersions was evaluated by means of the microViz package and was similar in all cases. Permutational analysis of variance (PERMANOVA), by means of the dist_permanova function from the microViz package, (58) was used to analyse longitudinal changes in overall microbiota composition (based on Aitchison distances). Since this analysis does not account for the clustered nature of the data, i.e., the correlated measurements within subjects, we additionally performed a distance-based redundancy analysis (dbRDA) using Aitchison distance by means of the capscale function from the vegan package [27]. Patient ID was defined as variable to be partialled out in order to account for the correlated data. In addition, PERMANOVA was used for cross-sectional analyses to assess the association between diarrhea, nausea, oral mucositis, hand-foot-syndrome, peripheral sensory neuropathy with overall microbiota composition. Within the neoadjuvant subgroup, PERMANOVA was used to analyze the association between treatment response and overall microbiota composition (58). Differential abundance analysis, investigating changes of individual taxa abundance on phylum and genus level during the course of AC-D treatment, was conducted using the workflow of ANCOM v.2.1 (random intercept model for repeated measures) which accounts for the underlying compositional structure, sparseness of microbiota data and random effects caused by longitudinal data (59). We set $\alpha < 0.05$ at 70% (W) of comparisons as threshold for significance. Structural zeros were not considered as differentially abundant taxa. For the purpose of visualization, bacterial relative abundance were transformed into $\log^{10}(1+x)$ abundance by means of the microbiome package (55). Non-

parametric tests based on $\log^{10}(1+x)$ abundance were used to confirm the ANCOM-II results in SPSS.

Data Availability Statement

Sequencing data were submitted to Qiita and deposited in the European Nucleotide Archive (ENA) (60). The accession code is: ERP136994. Additional data generated during and/or analyzed for the current study are available from the corresponding author on reasonable request.

Acknowledgements

We thank the patients who participated in this study. Medical oncologists and research nurses from Catharina Hospital, VieCuri Medical Centre, Elkerliek Hospital and Maastricht University Medical Centre+ assisted with patient inclusion and sample collection. Especially, we are grateful to Marjan Laven, Ramon Bax, Sanne Achten, Eva de Jong, Angelie van de Bosch, Ilona van Rooij - Tieleman, Wendy Heuts. Monique Vercoulen, Minie Rutten and Lida Tilet. We wish to acknowledge Christel Driessen and Wesley Nix for their support in the laboratory analyses. We also thank Renée Granzier for providing support for data analysis. Finally, we would like to make a special acknowledgement to David Barnett. We are grateful for both his support in data analysis and his expertise and contribution as a Native English speaker. We thank the Kootstra Talent Fellowship who partly financially supported this study.

Competing interests

JdVG has served as a consultant for Amgen, AstraZeneca, MSD, Pierre Fabre and Servier. All outside the submitted work. JdVG, MLS and RA have received institutional research funding from Servier. All outside the submitted work. MdB received research funding of Roche, Novartis, Pfizer, Eisai, and AstraZeneca outside this submitted work. MdB received a travel grant of Roche outside this submitted work. The other authors declare no competing financial interest. The Kootstra Talent Fellowship partly financially supported this study. KV acknowledges support in funding from the Dutch Province of Limburg. All authors declare no competing non-financial interests.

Author contributions

RA, MLS, JdVG, MdB, SSR and JP designed the study. RA coordinated the inclusion and follow-up. MdB, BEPJV, JV, AJvdW consulted patients for inclusion, RA, LH, JZ and SMPB collected patient data and samples. RA and JZ performed the laboratory analyses. NGL and JZ performed bioinformatic data analysis of microbiota data guided by JP. RA conducted statistical analysis of clinical data, α-diversity measures, and differential abundant taxa. RA, JZ, JP, KV and SSR interpreted the data. RA, LH, and JZ wrote the manuscript. All authors participated in discussion and revision and approved the final version of the manuscript.

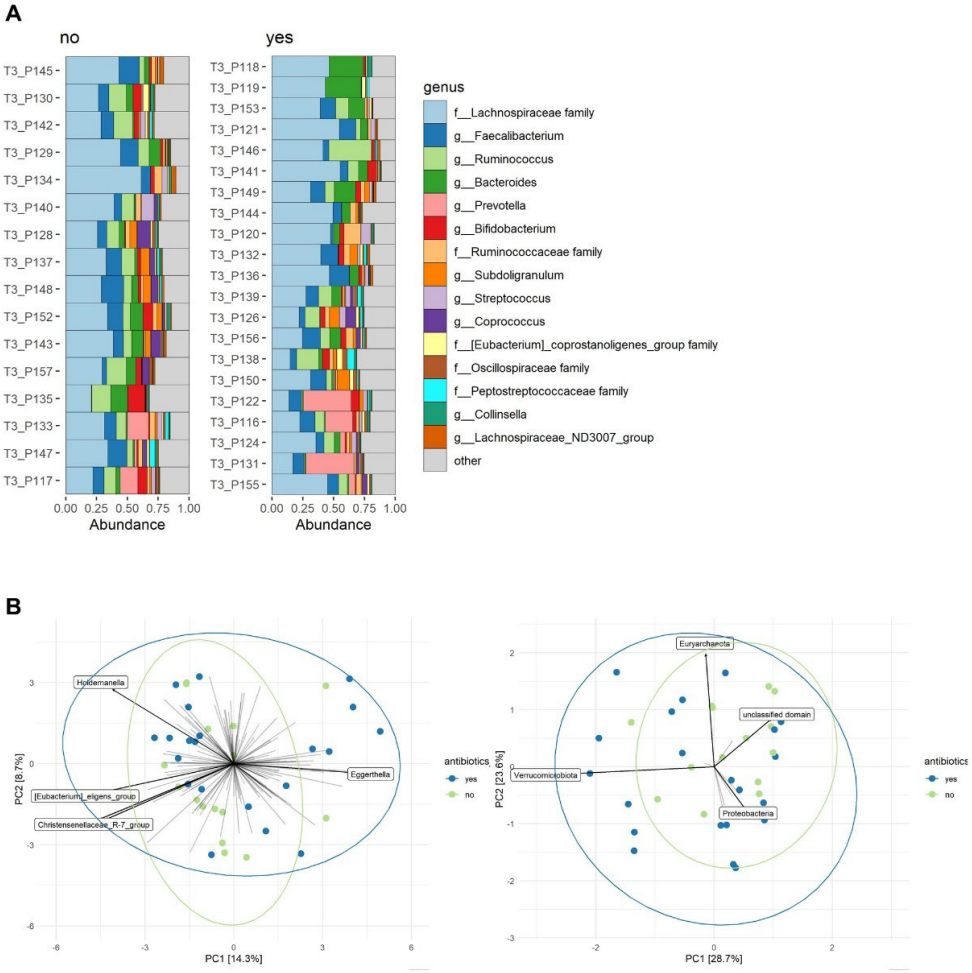
References

1. Winters S, Martin C, Murphy D, Shokar NK. Breast Cancer Epidemiology, Prevention, and Screening. Prog Mol Biol Transl Sci. 2017;151:1-32.
2. Bear HD, Anderson S, Brown A, Smith R, Mamounas EP, Fisher B, et al. The effect on tumor response of adding sequential preoperative docetaxel to preoperative doxorubicin and cyclophosphamide: preliminary results from National Surgical Adjuvant Breast and Bowel Project Protocol B-27. J Clin Oncol. 2003;21(22):4165-74.
3. Eiermann W, Pienkowski T, Crown J, Sadeghi S, Martin M, Chan A, et al. Phase III study of doxorubicin/cyclophosphamide with concomitant versus sequential docetaxel as adjuvant treatment in patients with human epidermal growth factor receptor 2-normal, node-positive breast cancer: BCIRG-005 trial. J Clin Oncol. 2011;29(29):3877-84.
4. Puglisi F, Mansutti M, Aprile G, Minisini AM, Di Loreto C, Bazzocchi M, et al. Tumor shrinkage evaluation during and after preoperative doxorubicin and cyclophosphamide followed by docetaxel in patients with breast cancer. Anticancer Res. 2004;24(4):2487-93.
5. Garajova I, Balsano R, Wang H, Leonardi F, Giovannetti E, Deng D, et al. The role of the microbiome in drug resistance in gastrointestinal cancers. Expert Rev Anticancer Ther. 2021;21(2):165-76.
6. Zimmermann M, Zimmermann-Kogadeeva M, Wegmann R, Goodman AL. Mapping human microbiome drug metabolism by gut bacteria and their genes. Nature. 2019;570(7762):462-7.
7. Sommer F, Backhed F. The gut microbiota--masters of host development and physiology. Nat Rev Microbiol. 2013;11(4):227-38.
8. Helmink BA, Khan MAW, Hermann A, Gopalakrishnan V, Wargo JA. The microbiome, cancer, and cancer therapy. Nat Med. 2019;25(3):377-88.
9. Daillere R, Vetizou M, Waldschmitt N, Yamazaki T, Isnard C, Poirier-Colame V, et al. Enterococcus hirae and Barnesiella intestinihominis Facilitate Cyclophosphamide-Induced Therapeutic Immunomodulatory Effects. Immunity. 2016;45(4):931-43.
10. Florez AB, Sierra M, Ruas-Madiedo P, Mayo B. Susceptibility of lactic acid bacteria, bifidobacteria and other bacteria of intestinal origin to chemotherapeutic agents. Int J Antimicrob Agents. 2016;48(5):547-50.
11. Goubet AG, Daillere R, Routy B, Derosa L, P MR, Zitvogel L. The impact of the intestinal microbiota in therapeutic responses against cancer. C R Biol. 2018;341(5):284-9.
12. Lehouritis P, Cummins J, Stanton M, Murphy CT, McCarthy FO, Reid G, et al. Local bacteria affect the efficacy of chemotherapeutic drugs. Sci Rep. 2015;5:14554.
13. Miko E, Kovacs T, Sebo E, Toth J, Csonka T, Ujlaki G, et al. Microbiome-Microbial Metabolome-Cancer Cell Interactions in Breast Cancer-Familiar, but Unexplored. Cells. 2019;8(4):293.
14. Rigby RJ, Carr J, Orgel K, King SL, Lund PK, Dekaney CM. Intestinal bacteria are necessary for doxorubicin-induced intestinal damage but not for doxorubicin-induced apoptosis. Gut Microbes. 2016;7(5):414-23.
15. Viaud S, Saccheri F, Mignot G, Yamazaki T, Daillere R, Hannani D, et al. The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. Science. 2013;342(6161):971-6.
16. Westman EL, Canova MJ, Radhi IJ, Koteva K, Kireeva I, Waglechner N, et al. Bacterial inactivation of the anticancer drug doxorubicin. Chem Biol. 2012;19(10):1255-64.
17. Aarnoutse R, Ziemons J, Penders J, Rensen SS, de Vos-Geelen J, Smidt ML. The Clinical Link between Human Intestinal Microbiota and Systemic Cancer Therapy. Int J Mol Sci. 2019;20(17):4145.
18. Uzan-Yulzari A, Morr M, Tareef-Nabwani H, Ziv O, Magid-Neriya D, Armoni R, et al. The intestinal microbiome, weight, and metabolic changes in women treated by adjuvant chemotherapy for breast and gynecological malignancies. BMC Med. 2020;18(1):281.
19. Terrisse S, Derosa L, Iebba V, Ghiringhelli F, Vaz-Luis I, Kroemer G, et al. Intestinal microbiota influences clinical outcome and side effects of early breast cancer treatment. Cell Death Differ. 2021;28(9):2778-96.
20. Montassier E, Gastinne T, Vangay P, Al-Ghalith GA, Bruley des Varannes S, Massart S, et al. Chemotherapy-driven dysbiosis in the intestinal microbiome. Aliment Pharmacol Ther. 2015;42(5):515-28.
21. Galloway-Pena JR, Smith DP, Sahasrabhojane P, Ajami NJ, Wadsworth WD, Daver NG, et al. The role of the gastrointestinal microbiome in infectious complications during induction chemotherapy for acute myeloid leukemia. Cancer. 2016;122(14):2186-96.
22. Mosca A, Leclerc M, Hugot JP. Gut Microbiota Diversity and Human Diseases: Should We Reintroduce Key Predators in Our Ecosystem? Front Microbiol. 2016;7:455.

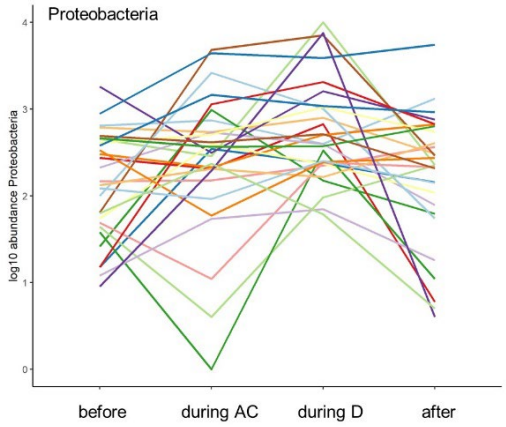
23. Calderon-Perez L, Gosalbes MJ, Yuste S, Valls RM, Pedret A, Llaurado E, et al. Gut metagenomic and short chain fatty acids signature in hypertension: a cross-sectional study. Sci Rep. 2020;10(1):6436.
24. Jiang W, Wu N, Wang X, Chi Y, Zhang Y, Qiu X, et al. Dysbiosis gut microbiota associated with inflammation and impaired mucosal immune function in intestine of humans with non-alcoholic fatty liver disease. Sci Rep. 2015;5:8096.
25. Yang J, Liu KX, Qu JM, Wang XD. The changes induced by cyclophosphamide in intestinal barrier and microflora in mice. Eur J Pharmacol. 2013;714(1-3):120-4.
26. Zeng MY, Inohara N, Nuñez G. Mechanisms of inflammation-driven bacterial dysbiosis in the gut. Mucosal Immunol. 2017;10(1):18-26.
27. Bruno R, Riva A, Hille D, Lebecq A, Thomas L. Pharmacokinetic and pharmacodynamic properties of docetaxel: results of phase I and phase II trials. Am J Health Syst Pharm. 1997;54(24 Suppl 2):S16-9.
28. de Jonge ME, Huitema AD, Rodenhuis S, Beijnen JH. Clinical pharmacokinetics of cyclophosphamide. Clin Pharmacokinet. 2005;44(11):1135-64.
29. Terrisse S, Derosa L, Iebba V, Ghiringhelli F, Vaz-Luis I, Kroemer G, et al. Intestinal microbiota influences clinical outcome and side effects of early breast cancer treatment. Cell Death Differ. 2021.
30. Zhu J, Liao M, Yao Z, Liang W, Li Q, Liu J, et al. Breast cancer in postmenopausal women is associated with an altered gut metagenome. Microbiome. 2018;6(1):136.
31. Secombe KR, Coller JK, Gibson RJ, Wardill HR, Bowen JM. The bidirectional interaction of the gut microbiome and the innate immune system: Implications for chemotherapy-induced gastrointestinal toxicity. Int J Cancer. 2019;144(10):2365-76.
32. Alexander JL, Wilson ID, Teare J, Marchesi JR, Nicholson JK, Kinross JM. Gut microbiota modulation of chemotherapy efficacy and toxicity. Nat Rev Gastroenterol Hepatol. 2017;14(6):356-65.
33. von Minckwitz G, Untch M, Blohmer JU, Costa SD, Eidtmann H, Fasching PA, et al. Definition and impact of pathologic complete response on prognosis after neoadjuvant chemotherapy in various intrinsic breast cancer subtypes. J Clin Oncol. 2012;30(15):1796-804.
34. Langdon A, Crook N, Dantas G. The effects of antibiotics on the microbiome throughout development and alternative approaches for therapeutic modulation. Genome Med. 2016;8(1):39.
35. Guan X, Ma F, Sun X, Li C, Li L, Liang F, et al. Gut Microbiota Profiling in Patients With HER2-Negative Metastatic Breast Cancer Receiving Metronomic Chemotherapy of Capecitabine Compared to Those Under Conventional Dosage. Front Oncol. 2020;10:902.
36. Laborda-Illanes A, Sanchez-Alcoholado L, Dominguez-Recio ME, Jimenez-Rodriguez B, Lavado R, Comino-Mendez I, et al. Breast and Gut Microbiota Action Mechanisms in Breast Cancer Pathogenesis and Treatment. Cancers (Basel). 2020;12(9):2465.
37. Liu PY, Wu WK, Chen CC, Panyod S, Sheen LY, Wu MS. Evaluation of Compatibility of 16S rRNA V3V4 and V4 Amplicon Libraries for Clinical Microbiome Profiling. bioRxiv. 2020.
38. Liu P-Y, Wu W-K, Chen C-C, Panyod S, Sheen L-Y, Wu M-S. Evaluation of Compatibility of 16S rRNA V3V4 and V4 Amplicon Libraries for Clinical Microbiome Profiling. 2020.
39. Johnson JS, Spakowicz DJ, Hong BY, Petersen LM, Demkowicz P, Chen L, et al. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nat Commun. 2019;10(1):5029.
40. Matsuo Y, Komiya S, Yasumizu Y, Yasuoka Y, Mizushima K, Takagi T, et al. Full-length 16S rRNA gene amplicon analysis of human gut microbiota using MinION nanopore sequencing confers species-level resolution. BMC Microbiol. 2021;21(1):35.
41. Jeong J, Yun K, Mun S, Chung WH, Choi SY, Nam YD, et al. The effect of taxonomic classification by full-length 16S rRNA sequencing with a synthetic long-read technology. Sci Rep. 2021;11(1):1727.
42. Wolff AC, Hammond MEH, Allison KH, Harvey BE, Mangu PB, Bartlett JMS, et al. Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update. J Clin Oncol. 2018;36(20):2105-22.
43. Richtlijnendatabase Borstkanker [Internet]. Federatie Medisch Specialisten. 2021. Available from: https://richtlijnendatabase.nl/richtlijn/borstkanker/algemeen.html.
44. Aarnoutse R, de Vos-Geelen J, Penders J, Boerma EG, Warmerdam F, Goorts B, et al. Study protocol on the role of intestinal microbiota in colorectal cancer treatment: a pathway to personalized medicine 2.0. Int J Colorectal Dis. 2017;32(7):1077-84.
45. Oncoline-IKNL. Dutch breast cancer guideline. the Netherlands; 2012.
46. Institute NIoHNC. Common Terminology Criteria for Adverse Events (CTCEA). U.S.: Department of Health and Human Services; 2010.

47. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. Isme j. 2012;6(8):1621-4.
48. Galazzo G, Tedjo DI, Wintjens DSJ, Savelkoul PHM, Masclee AAM, Bodelier AGL, et al. Fecal Microbiota Dynamics and their Relation to Disease Course in Crohn's Disease. J Crohns Colitis. 2019;13(10):1273-82.
49. Team RC. R: A language and environment for statistical computing. R Foundation for Statistical Computing 2020 [
50. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13(7):581-3.
51. Wright ES. Using DECIPHER v2.0 to Analyze Big Biological Sequence Data in R. The R Journal. 2016;8(1):352-9.
52. Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome. 2018;6(1):226.
53. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One. 2013;8(4):e61217.
54. Jari Oksanen FGB, Michael Friendly, Roeland Kindt, Pierre Legendre, Dan McGlinn, Peter R. Minchin, R. B. O'Hara, Gavin L. Simpson, Peter Solymos, M. Henry H. Stevens, Eduard Szoecs and Helene Wagner. vegan: Community Ecology Package 2019 [Available from: https://CRAN.R-project.org/package=vegan.
55. Lahti L, Ernst FGM, Huang R, Shetty S, Borman T, Braccia DC, et al. microbiome R package 2020 [Available from: http://microbiome.github.io.
56. Wickham H, François R, Henry L, Müller K. dplyr: A Grammar of Data Manipulation. R package version 1.0. 2 (2020). 2020.
57. Wickham H. ggplot2: elegant graphics for data analysis: springer; 2016.
58. Barnett DJM, Arts ICW, Penders J. microViz: an R package for microbiome data visualization and statistics. The Journal of Open Source Software. 2021;6(63):3201.
59. Mandal S, Van Treuren W, White RA, Eggesbø M, Knight R, Peddada SD. Analysis of composition of microbiomes: a novel method for studying microbial composition. Microbial ecology in health and disease. 2015;26(1):27663.
60. Gonzalez A, Navas-Molina JA, Kosciolek T, McDonald D, Vazquez-Baeza Y, Ackermann G, et al. Qiita: rapid, web-enabled microbiome meta-analysis. Nat Methods. 2018;15(10):796-8.

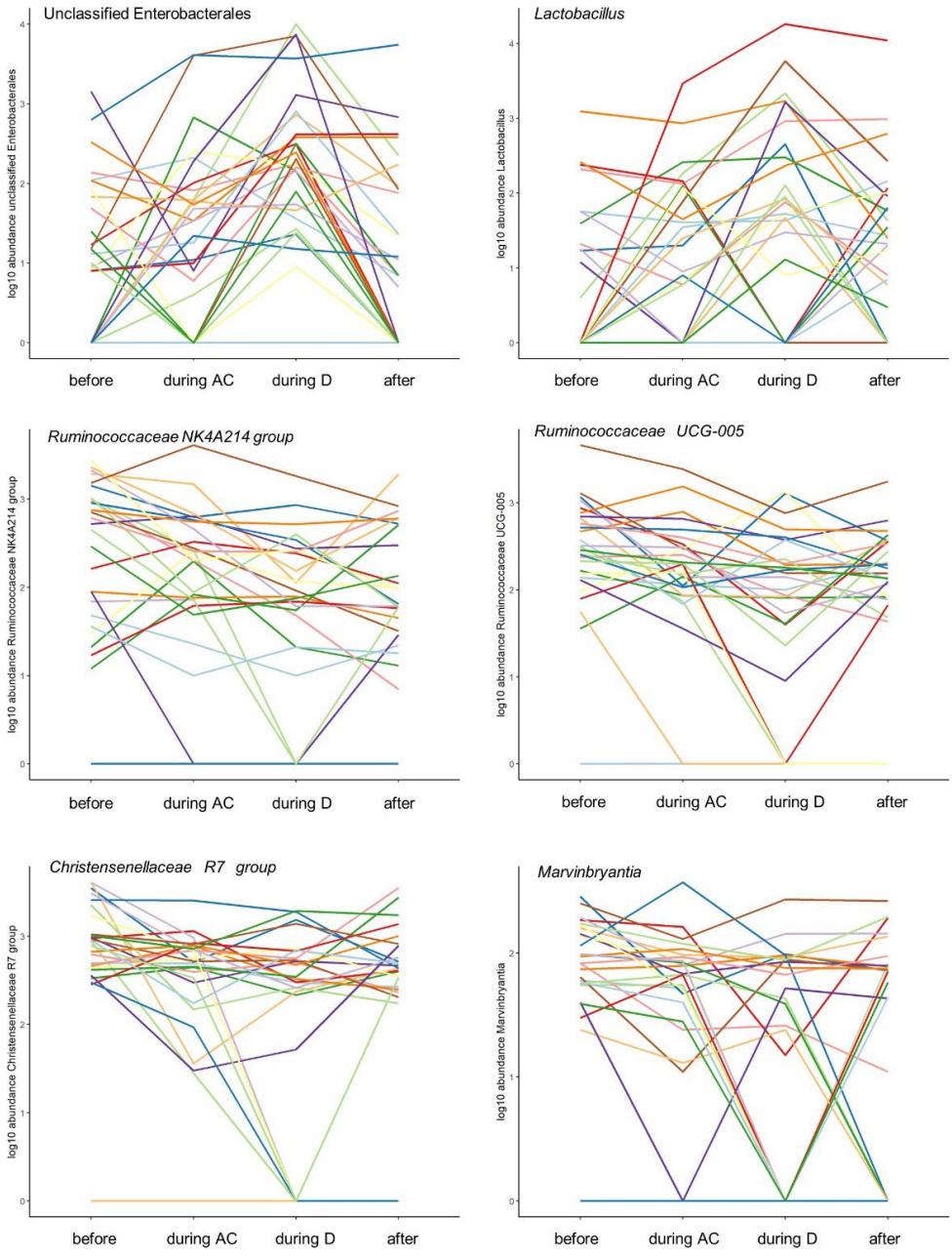
Supplementary Figures



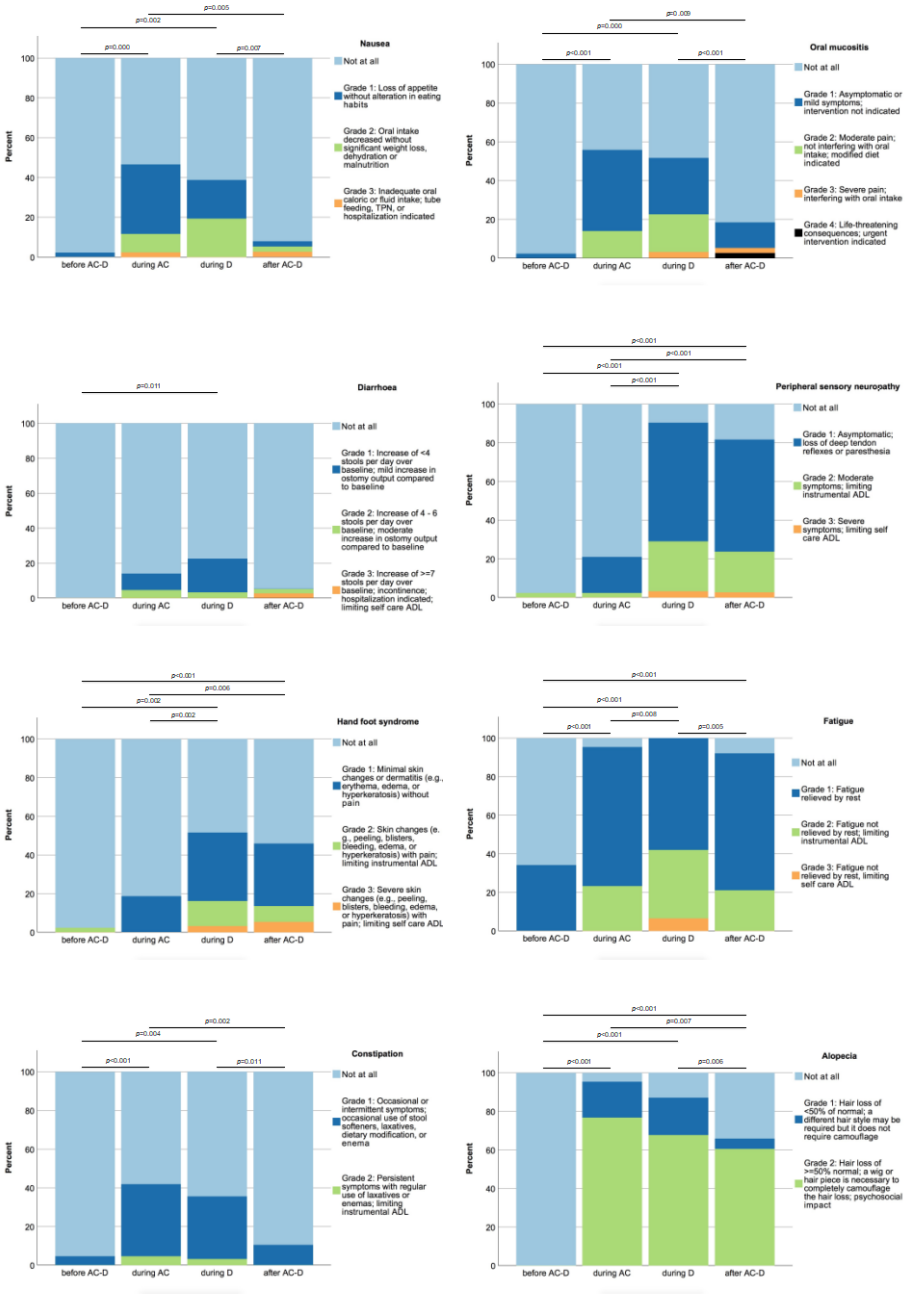
Supplementary Figure 1: A: *Relative abundance of the 15 most common genera at sampling timepoint T3 among patients without previous antibiotic use ("no", n=16) or with previous antibiotic use before or during AC-D ("yes", n=21).* ***B:*** *Ordination plots derived from unconstrained Principal Components Analysis (PCA) based on the Aitchison distance, showing differences in intestinal microbiota composition at T3 between patients with (**blue**, n=21) or without (**green**, n=16) previous antibiotic use before or during AC-D. PERMANOVA showed that there were no statistically significant differences at phylum (p=0.280) and genus level (p=0.522).*



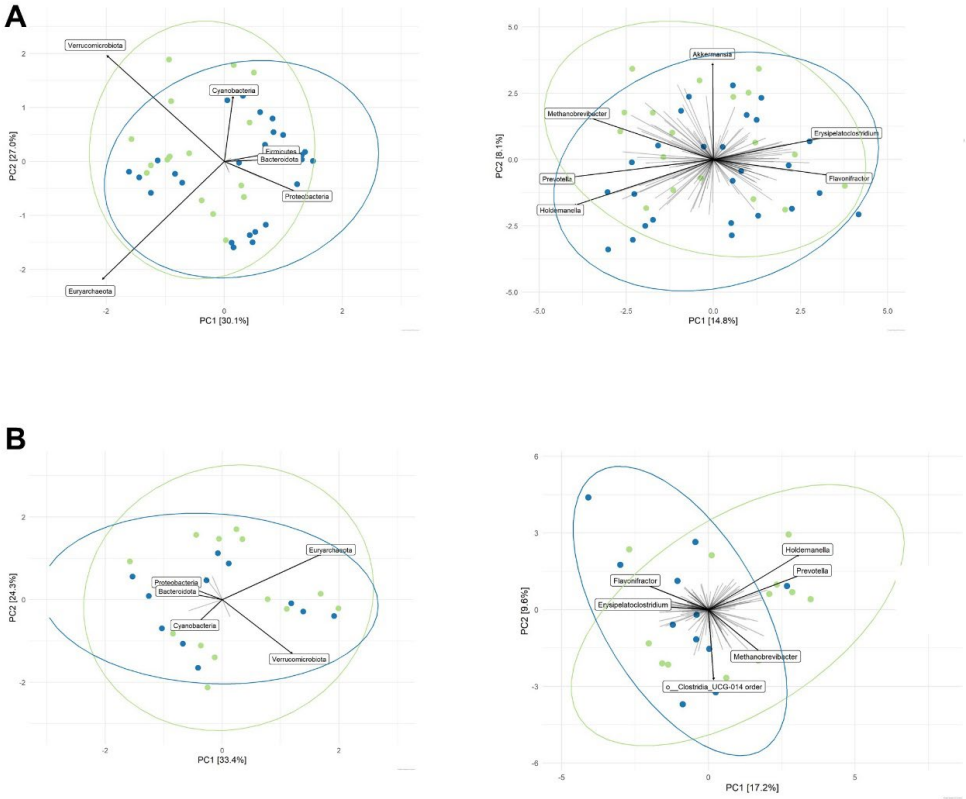
Supplementary Figure 2: *Longitudinal changes of Proteobacteria abundance in individual patients. Only complete cases (n=28) are displayed. Each line represents an individual patient.*



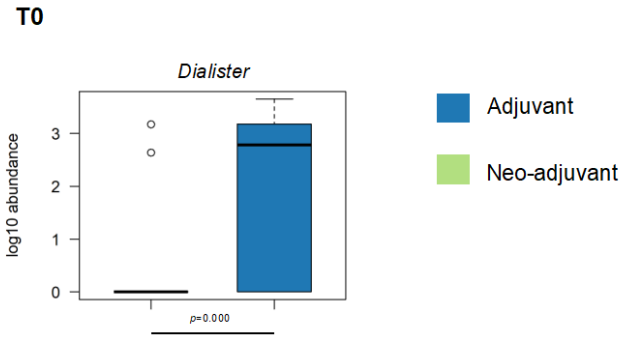
Supplementary Figure 3: *Longitudinal changes of abundance of different genera in individual patients. Only complete cases (n=28) are displayed. Each line represents an individual patient.*



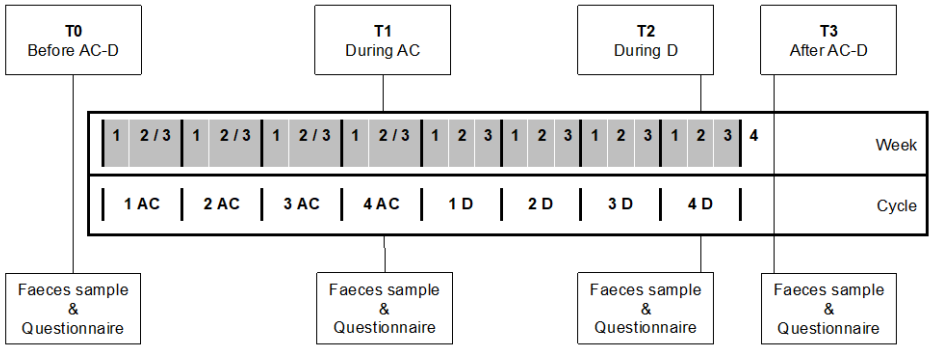
Supplementary Figure 4: *Stacked bar charts presenting percentage toxicity grades before AC-D, during AC, during D, and after AC-D. Friedman tests indicated that nausea, oral mucositis, diarrhea, peripheral sensory neuropathy, hand foot syndrome, fatigue, constipation and alopecia changed significantly over time. Presented p-values indicate significant differences between the different time points revealed by the Wilcoxon test with Bonferoni correction (See table S11 for numbers of patients per grade classification).*



Supplementary Figure 5: A: *Ordination plots derived from unconstrained Principal Components Analysis (PCA) based on the Aitchison distance, showing differences in baseline (T0) microbiota composition between adjuvant (**blue**, n=26) and neo-adjuvant patients (**green**, n=18) at phylum and genus level.* ***B:*** *Ordination plots derived from unconstrained Principal Components Analysis (PCA) based on the Aitchison distance at phylum and genus level, showing differences in baseline (T0) microbiota composition between adjuvant patients who received perioperative prophylactic antibiotics (**blue**, n=12) and adjuvant patients who did not receive perioperative prophylactic antibiotics (**green**, n=14). For all plots, taxa that were present in less than 5 samples were excluded for this analysis. Data were transformed using centre-log-ratio transformation. Names are given for taxa, which contributed most to overall microbial variation.*



Supplementary Figure 6: *Baseline log[10] abundance of the genus Dialister in adjuvant and neo-adjuvant patients. ANCOM-II analysis identified this genus to be differentially abundant among adjuvant (n=26) and neo-adjuvant (n=18) patients, which was confirmed by a Mann-Whitney-U Test (p<0.001).*



Supplementary Figure 7: Study design. *Patients collected a fecal sample and completed a questionnaire at four time points: T0 was collected before the start of the AC-D, T1 during week 2 of cycle 4 AC, T2 during week 2 of cycle 4 D, and T3 one month after the dose D. During the study period, patients received four cycles adriamycin (A), 60 mg/m² i.v. and cyclophosphamide (C) 600 mg/m² i.v. on day 1, in either a two-weekly (dose dense, dd) or three-weekly cycle. AC was followed by four cycles of docetaxel (D), 100 mg/m² i.v. on day 1, in a three-weekly cycle.*

Supplementary Tables

Supplementary Table 1: *Clinical characteristics of the total study population at baseline including the comparison between adjuvant and neoadjuvant treated patients.*

Baseline characteristics	Total (*n*=44)	Adjuvant (*n*=26)	Neoadjuvant (*n*=18)	*p*-value
Focality - No. (%)				
Unifocal tumor	33 (75)	19 (73)	14 (78)	
Multifocal tumor	10 (23)	7 (27)	3 (17)	
Unknown	1 (2)	0 (0)	1 (6)	0.714
cT stage - No. (%)*				
1	20 (46)	17 (65)	3 (17)	
2	17 (39)	7 (27)	10 (56)	
3	4 (9)	2 (8)	2 (11)	
4	2 (5)	0 (0)	2 (11)	
Unknown	1 (2)	0 (0)	1 (6)	**0.003**
cG grade - No. (%)*				
1	9 (21)	7 (27)	2 (11)	
2	22 (50)	10 (39)	12 (67)	
3	9 (21)	6 (23)	3 (17)	
Unknown	4 (9)	3 (12)	1 (6)	0.638
cN stage - No. (%)*				
0	34 (77)	23 (89)	11 (61)	
1	7 (16)	3 (12)	4 (22)	
2	1 (2)	0 (0)	1 (6)	
3	2 (5)	0 (0)	2 (11)	**0.016**
cT size - in mm				
Median (IQR)	22 (16)	19 (12)	28 (16)	**0.012**
(y)pT size -				
Median (IQR)	20 (11)	21 (14)	15 (13)	
25%-75%	15-26	15-29	13-25	0.257
MIB1%				
Median (IQR)	15 (25)	20 (20)	10 (24)	
25%-75%	5-30	10-30	5-29	0.492
Ki-67%				
Mean (SD)	26 (20)	27 (17)	25 (25)	
Range	2-75	5-60	2-75	0.850
OK-type - No (%)				
Lumpectomy	20 (46)	12 (46)	8 (44)	
Mastectomy	23 (52)	14 (54)	9 (50)	
Unknown	1 (2)	0 (0)	1 (6)	0.954
ER- No (%)				
Negative	0 (0)	0 (0)	0 (0)	
Positive	44 (100)	26 (100)	18 (100)	-
ER %				
Median (IQR)	100 (0)	100 (0)	100 (5)	
25%-75%	100-100	100-100	95-100	0.263
PR- No (%)				
Negative	18 (41)	9 (35)	9 (50)	
Positive	26 (59)	17 (65)	9 (50)	0.307

PR %				
Median (IQR)	25 (79)	30 (75)	12 (91)	
25%-75%	1-80	5-80	0-92	0.745
DM-type II - No. (%)*				
No	39 (89)	23 (89)	16 (89)	
Yes	5 (11)	3 (12)	2 (11)	1.000
Prior systemic treatment - No. (%)				
No	44 (100)	26 (100)	18 (100)	
Yes	0 (0)	0 (0)	0 (0)	-
Days therapeutic antibiotic use last year				
Median (IQR)	7 (4)	6 (3)	9 (-)	
25-75%	5-9	5-8	7- -	0.286
Days from operation				
Mean (SD)	50 (23)	50 (23)		
Range	18-93	18-93	-	-
Prophylactic antibiotic use during operation - No. (%)				
No	14 (54)	14 (54)	18 (100)	
Yes	12 (46)	12 (46)	0 (0)	**<0.001**
Oral contraception use - years				
Median (IQR)	12 (13)	10 (15)	15 (19)	
25%-75%	8-21	5-20	10-29	0.104
Years between T0 fecal sample and last oral contraception use				
Mean (SD)	20 (13)	22 (14)	17 (12)	
Range	0.1-49.3	0.2-49	0.1-39	0.274
Years between T0 fecal sample and last hormone IUD use				
Mean (SD)	7 (5)	5 (5)	9 (5)	
Range	1-15	1-12	5-15	0.229

**Percentages do not add up to 100% due to rounding.*

Supplementary Table 2: *Longitudinal clinical characteristics of the total study population*

Clinical characteristics	T0 before AC-D	T1 during AC	T2 during D	T3 after AC-D	*p*-value	Pairwise comparison
Karnofsky Performance Score - No (%)*						**T0 vs T1 < 0.001**
20-30	0 (0)	0 (0)	1 (3)	0 (0)	**<0.001**	**T0 vs T2 < 0.001**
40-50	0 (0)	0 (0)	2 (6)	1 (3)		**T0 vs T3 < 0.001**
60-70	3 (7)	9 (22)	13 (40)	11 (29)		T1 vs T2 = 0.051
80-90	22 (50)	31 (74)	11 (41)	23 (61)		T1 vs T3 = 0.531
100	19 (43)	2 (5)	3 (9)	3 (8)		**T2 vs T3 = 0.005**
MUST-score - No (%)	38 (86)	34 (79)	22 (67)	32 (84)	**0.023**	T0 vs T1 = 0.117 T0 vs T2 = 0.022 T0 vs T3 = 0.739
Low risk	6 (14)	5 (12)	6 (18)	6 (16)		**T1 vs T2 < 0.001**
Medium risk	0 (0)	4 (9)	5 (15)	0 (0)		T1 vs T3 = 0.531
High risk						**T2 vs T3 = 0.005**
BMI - kg/m^2	26 (5)	26 (5)	27 (5)	26 (4)	0.338	T0 vs T1 = 0.024 T0 vs T2 = 0.265 T0 vs T3 = 0.777
Median (IQR)	24-29	23-28	24-28	24-28		T1 vs T2 = 0.470 T1 vs T3 = 0.330
25%-75%						T2 vs T3 = 0.048

**Percentages do not add up to 100% due to rounding.*
MUST: Malnutrition Universal Screening Tool.
Wilcoxon signed-rank sum test with Bonferroni correction was used for pairwise comparison. After Bonferroni correction p-values below 0.0125 indicated significance.

Supplementary Table 3: *Longitudinal clinical data - therapeutic and prophylactic antibiotic use during the course of AC-D treatment of the total study population including the comparison between adjuvant and neoadjuvant treated patients.*

Antibiotic use	Total	Adjuvant	Neoadjuvant	*p*-value
Between T0-T1 Antibiotic use - No (%)				
No	33 (79)	17 (68)	16 (94)	
Yes	9 (21)	8 (32)	1 (6)	0.060
Between T1-T2 Antibiotic use - No (%)				
No	18 (62)	10 (63)	8 (62)	
Yes	11 (38)	6 (38)	5 (39)	1.000
Between T2-T3 Antibiotic use - No (%)				
No	35 (95)	20 (95)	15 (94)	
Yes	2 (5)	1 (5)	1 (6)	1.000

Supplementary Table 4: *Therapy adjustments of the total study population during the course of AC-D*

Therapy adjustments	T1 during AC	T2 during D	T3 overall
Therapy adjustments overall - No (%)			
No	37 (86)	18 (42)	14 (33)
Yes	6 (14)	25 (58)	29 (67)
Type therapy adjustments overall - No (%)			
Stop	0 (0)	16 (37)	12 (28)
Reduction, delay, and/or switch	6 (14)	9 (21)	17 (39)
% dose received overall			
Median (IQR)	100 (0)	88 (25)	94 (13)
25%-75%	100-100	75-100	88-100

N=43 since one patient did not start with AC-D.
During AC, only one patient received 95% of the planned dosses.

Supplementary Table 5: *Longitudinal α-diversity measures of participants who provided all four samples (n=28)*

α-diversity measures	T0 before AC-D	T1 during AC	T2 during D	T3 after AC-D	ANOVA *p*-value
Observed richness					
Mean (SD)	240 (48)	229 (40)	218 (57)	217 (47)	
Range	136-330	157-308	84-310	87-304	*p*=0.042
Shannon					
Mean (SD)	4.0 (0.3)	3.9 (0.3)	3.9 (0.3)	3.9 (0.3)	
Range	3.3-4.6	3.4-4.5	3.1-4.4	3.1-4.5	*p*=0.206

Changes in α-diversity measures of the 28 participants who provided all four samples before AC-D, during AC, during D, and after AC-D treatment, measured in terms of observed species richness (p=0.042; n=28) and Shannon index (p=0.206; n=28). Repeated measure ANOVA was performed to test differences in α-diversity measures over time.

Supplementary Table 6: *Longitudinal α-diversity measures of all samples of the total population*

α-diversity measures	T0 Before AC-D (n=44)	T1 During AC (n=43)	T2 During D (n=29)	T3 after AC-D (n=37)	Pairwise comparison
Observed richness *Median (IQR) 25%-75%*	247 (61) 203-264	230 (75) 181-256	232 (78) 178-255	221 (64) 185-249	T0 vs T1 = 0.038 T0 vs T2 = 0.029 **T0 vs T3 = 0.003** T1 vs T2 = 0.284 T1 vs T3 = 0.088 T2 vs T3 = 0.657
Shannon index *Median (IQR) 25%-75%*	4.03 (0.4) 3.8-4.2	3.92 (0.5) 3.7-4.2	3.90 (0.5) 3.7-4.2	3.99 (0.5) 3.7-4.1	T0 vs T1 = 0.137 T0 vs T2 = 0.090 T0 vs T3 = 0.099 T1 vs T2 = 0.611 T1 vs T3 = 0.429 T2 vs T3 = 0.569

Wilcoxon signed-rank sum test with Bonferroni correction was used for pairwise comparison. After Bonferroni correction p-values below 0.0125 indicated significance.

Supplementary Table 7A: *α-diversity measures in patients with or without therapeutic antibiotics 1 year prior to T0*

α-diversity measures	Total (*n*=44)	- Antibiotics (*n*=32)	+ Antibiotics (*n*=12)	*p*-value
T0 Observed richness				
Mean (SD)	234 (45)	230 (48)	244 (39)	
Range	135-330	135-330	166-301	0.388
T1 Observed richness				
Mean (SD)	222 (47)	221 (46)	225 (52)	
Range	107-308	121-293	107-308	0.802
T2 Observed richness				
Mean (SD)	217 (57)	216 (48)	222 (78)	
Range	84-310	134-294	84-310	0.789
T3 Observed richness				
Mean (SD)	213 (48)	215 (41)	206 (66)	
Range	87-304	104-280	87-304	0.606
T0 Shannon index				
Mean (SD)	4.0 (0.3)	4.0 (0.36)	4.0 (0.29)	
Range	3.0-4.6	3.0-4.6	3.6-4.5	0.856
T1 Shannon index				
Mean (SD)	3.9 (0.4)	3.9 (0.35)	3.9 (0.43)	
Range	2.8-4.5	2.8-4.5	2.8-4.5	0.996
T2 Shannon index				
Mean (SD)	3.9 (0.3)	3.9 (0.27)	4.0 (0.45)	
Range	3.1-4.4	3.3-4.4	3.1-4.4	0.413
T3 Shannon index				
Median (IQR)	4.0 (0.5)	4.0 (0.36)	4.0 (0.66)	
25-75%	3.7-4.1	3.8-4.1	3.5-4.1	0.671

Supplementary Table 7A: *α-diversity measures in patients with or without therapeutic antibiotics 1 year prior to T0*

α-diversity measures	**Total (*n*=26)**	**- Antibiotics (*n*=14)**	**+ Antibiotics (*n*=12)**	***p*-value**
T0 Observed richness				
Median (IQR)	248 (62)	239 (61)	249 (71)	
25-75%	205-267	204-264	186-258	0.667
T0 Shannon index	4.0			
Mean (SD)	(0.25)	4.0 (0.29)	3.8 (0.29)	
Range	3.5-4.5	3.9-4.2	3.7-4.0	0.155

Supplementary Table 8: *correlation between antibiotic administration during AC-D treatment and α-diversity*

α-diversity measures	**antibiotics during T0-T1 (*n*=9, 21%)**		**antibiotics during T1-T2 (*n*=11, 38%)**		**antibiotics during T2-T3 (*n*=2, 5%)**	
	Correlation coefficient	***p*-value**	**Correlation coefficient**	***p*-value**	**Correlation coefficient**	***p*-value**
T1 Observed species richness	-0.457	**0.002**	NA	NA	NA	NA
T2 Observed species richness	-0.296	0.126	-0.221	0.250	NA	NA
T3 Observed species richness	-0.091	0.597	-0.092	0.640	-0.078	0.645
T1 Shannon index	-0.452	**0.003**	NA	NA	NA	NA
T2 Shannon index	-0.312	0.105	-0.195	0.310	NA	NA
T3 Shannon index	-0.233	0.171	-0.018	0.926	<0.001	1.000

Spearman's rho (r_s) correlation coefficient was used to assess the relation between antibiotic administration and α-diversity measures

NA: not applicable

Supplementary Table 9: *correlation between cumulative antibiotic administration and α-diversity*

α-diversity measures	**Cumulative antibiotics until T1 (*n*=17, 40%)**		**Cumulative antibiotics until T2 (*n*=17, 59%)**		**Cumulative antibiotics until T3 (*n*=21, 57%)**	
	Correlation coefficient	***p*-value**	**Correlation coefficient**	***p*-value**	**Correlation coefficient**	***p*-value**
T1 Observed species richness	-0.178	0.253	NA	NA	NA	NA
T2 Observed species richness	-0.084	0.666	-0.088	0.650	NA	NA
T3 Observed species richness	-0.026	0.878	-0.089	0.651	-0.158	0.349
T1 Shannon index	-0.241	0.119	NA	NA	NA	NA
T2 Shannon index	-0.025	0.897	-0.075	0.698	NA	NA
T3 Shannon index	-0.198	0.239	-0.080	0.684	-0.225	0.181

Spearman's rho (r_s) correlation coefficient was used to assess the relation between cumulative antibiotic administration and α-diversity measures.

NA: not applicable

Cumulative antibiotic use was defined as: cumulative therapeutic and prophylactic antibiotic use from the year prior to baseline fecal sample collection until the index sample

Supplementary Table 10: *Longitudinal differential abundant taxa of the total study population*

Taxa	T0 before AC-D (*n*=44)	T1 during AC (*n*=43)	T2 during D (*n*=29)	T3 after AC-D (*n*=37)	*p*-value	Pairwise comparison
Proteobacteria						T0 vs T1 = 0.023
Median (IQR)	2.12 (0.94)	2.54 (0.69)	2.60 (0.67)	2.36 (0.98)	**0.006**	**T0 vs T2 < 0.001**
25%-75%	1.70-2.63	2.18-2.87	2.36-3.03	1.84-2.82		T0 vs T3 = 0.074 T1 vs T2 = 0.043 T1 vs T3 = 0.667 **T2 vs T3 = 0.003**
unclassified Enterobacterales						T0 vs T1 = 0.122
Median (IQR)	1.15 (1.91)	1.36 (2.28)	2.24 (1.24)	0.85 (1.95)	**<0.001**	**T0 vs T2 < 0.001** T0 vs T3 = 0.877
25%-75%	0.00-1.91	0.00-2.28	1.49-2.74	0.00-1.95		**T1 vs T2 < 0.001** T1 vs T3 = 0.117 **T2 vs T3 < 0.001**
Lactobacillus						**T0 vs T1 = 0.006**
Median (IQR)	0.00 (1.39)	0.95 (2.10)	1.72 (2.57)	1.40 (2.04)	**0.004**	**T0 vs T2 = 0.002** **T0 vs T3 = 0.001**
25%-75%	0.00-1.39	0.00-2.10	0.00-2.57	0.00-2.04		T1 vs T2 = 0.024 T1 vs T3 = 0.245 T2 vs T3 = 0.174
Rumino-coccaceae NK4A214 group						T0 vs T1 = 0.132
Median (IQR)	2.48 (1.25)	2.41 (0.97)	1.88 (1.91)	1.85 (1.36)	**<0.001**	**T0 vs T2 = 0.001** **T0 vs T3 < 0.001** **T1 vs T2 = 0.005**
25%-75%	1.72-2.97	1.79-2.76	0.50-2.41	1.30-2.66		**T1 vs T3 = 0.011** T2 vs T3 = 0.927
Intestinibacter						T0 vs T1 = 0.013
Median (IQR)	2.00 (1.44)	1.54 (2.47)	2.23 (1.90)	2.46 (1.26)	0.347	T0 vs T2 = 0.200 T0 vs T3 = 0.219
25%-75%	1.21-2.65	0.00-2.47	1.17-3.07	1.65-2.90		T1 vs T2 = 0.041 **T1 vs T3 = 0.002** T2 vs T3 = 0.716
Marvinbryantia						
Median (IQR)	1.92 (0.56)	1.83 (0.91)	1.41 (1.95)	1.76 (1.91)	**0.020**	**T0 vs T1 = 0.003** **T0 vs T2 < 0.001** **T0 vs T3 = 0.003**
25%-75%	1.59-2.15	1.11-2.02	0.00-1.95	0.00-1.91		T1 vs T2 = 0.041 T1 vs T3 = 0.140 T2 vs T3 = 0.685

Christensenel-laceae R7 group						
Median (IQR)	2.83 (0.60)	2.72 (0.74)	2.48 (1.93)	2.61 (1.24)	**0.008**	T0 vs T1 = 0.013 **T0 vs T2 = 0.001** **T0 vs T3 = 0.004** T1 vs T2 = 0.387 T1 vs T3 = 0.489 T2 vs T3 = 0.548
25%-75%	2.57-3.17	2.17-2.91	0.86-2.79	1.71-2.95		
Ruminococcac-eae UCG-005						
Median (IQR)	2.41 (0.73)	2.30 (0.73)	2.00 (1.31)	2.12 (0.65)	**<0.001**	**T0 vs T1 = 0.007** **T0 vs T2 < 0.001** **T0 vs T3 < 0.001** **T1 vs T2 = 0.012** T1 vs T3 = 0.105 T2 vs T3 = 0.317
25%-75%	2.09-2.81	1.87-2.60	1.16-2.47	1.90-2.54		
Turicibacter						
Median (IQR)	1.42 (2.27)	0.95 (1.79)	1.89 (1.68)	1.45 (2.26)	0.069	T0 vs T1 = 0.098 T0 vs T2 = 0.062 T0 vs T3 = 0.962 T1 vs T2 = 0.015 T1 vs T3 = 0.066 T2 vs T3 = 0.067
25%-75%	0.00-2.27	0.00-1.79	0.85-2.52	0.00-2.26		

Friedman's ANOVA was used to test for changes in abundance over time.
Wilcoxon signed-rank sum test with Bonferroni correction was used for pairwise comparison. After Bonferroni correction p-values below 0.0125 indicated significance.

Supplementary Table 11: *Longitudinal CTCAE in grade of the total study population*

Toxicity grade	T0 before AC-D	T1 during AC	T2 during D	T3 after AC-D	*p*-value	Pairwise comparison
Nausea - No. (%)*						**T0 vs T1 < 0.001**
0	43 (98)	23 (54)	19 (61)	35 (92)		**T0 vs T2 = 0.002**
1	1 (2)	15 (35)	6 (19)	1 (3)	**<0.001**	T0 vs T3 = 0.197
2	0 (0)	4 (9)	6 (19)	1 (3)		T1 vs T2 = 1.000
3	0 (0)	1 (2)	0 (0)	1 (3)		**T1 vs T3 = 0.005**
						T2 vs T3 = 0.007
Vomiting - No (%)					0.392	T0 vs T1 = 0.317 T0 vs T2 = 0.317 T0 vs T3 = 0.317 T1 vs T2 = 1.000 T1 vs T3 = 1.000 T2 vs T3 = 1.000
0	44 (100)	42 (98)	30 (97)	37 (97)		
1	0 (0)	1 (2)	1 (3)	1 (3)		
Oral mucositis - No (%)*						**T0 vs T1 < 0.001**
0	43 (98)	19 (44)	15 (48)	31 (82)		**T0 vs T2 < 0.001**
1	1 (2)	18 (42)	9 (29)	5 (13)	**<0.001**	T0 vs T3 = 0.024
2	0 (0)	6 (14)	6 (19)	0 (0)		T1 vs T2 = 0.227
3	0 (0)	0 (0)	1 (3)	1 (3)		**T1 vs T3 = 0.009**
4	0 (0)	(0)	0 (0)	1 (3)		**T2 vs T3 < 0.001**
Diarrhea - No (%)*						T0 vs T1 = 0.023
0	44 (100)	37 (86)	24 (77)	36 (95)		**T0 vs T2 = 0.011**
1	0 (0)	4 (9)	6 (19)	0 (0)	**0.005**	T0 vs T3 = 0.180
2	0 (0)	2 (5)	1 (3)	1 (3)		T1 vs T2 = 0.527
3	0 (0)	0 (0)	0 (0)	1 (3)		T1 vs T3 = 0.666
						T2 vs T3 = 0.014
Constipation - No. (%)*						**T0 vs T1 < 0.001**
0	42 (96)	25 (58)	20 (65)	34 (90)		**T0 vs T2 = 0.004**
1	2 (5)	16 (37)	10 (32)	4 (11)	**<0.001**	T0 vs T3 = 0.157
2	0 (0)	2 (5)	1 (3)	0 (0)		T1 vs T2 = 0.197
						T1 vs T3 = 0.002
						T2 vs T3 = 0.011
Fever - No (%)*						T0 vs T1 = 0.102
0	44 (100)	40 (93)	27 (87)	36 (95)		T0 vs T2 = 0.063
1	0 (0)	2 (5)	2 (7)	2 (5)	0.072	T0 vs T3 = 0.157
2	0 (0)	0 (0)	2 (7)	0 (0)		T1 vs T2 = 1.000
3	0 (0)	0 (0)	0 (0)	0 (0)		T1 vs T3 = 0.414
4	0 (0)	1 (2)	0 (0)	0 (0)		T2 vs T3 = 0.102
Peripheral sensory neuropathy – No (%)						T0 vs T1 = 0.021
0	43 (98)	34 (79)	3 (10)	7 (18)	**<0.001**	**T0 vs T2 < 0.001**
1	0 (0)	8 (19)	19 (61)	22 (58)		**T0 vs T3 < 0.001**
2	1 (2)	1 (2)	8 (26)	8 (21)		**T1 vs T2 < 0.001**
3	0 (0)	0 (0)	1 (3)	1 (3)		**T1 vs T3 < 0.001**
						T2 vs T3 = 0.132
Hand foot syndrome –	43 (98)	35 (81)	15 (48)	20 (54)	**<0.001**	T0 vs T1 = 0.083 **T0 vs T2 = 0.002** **T0 vs T3 < 0.001**

No. (%)*	0 (0)	8 (19)	11 (36)	12 (32)		**T1 vs T2 = 0.002**
0	1 (2)	0 (0)	4 (13)	3 (8)		**T1 vs T3 = 0.006**
1	0 (0)	0 (0)	1 (3)	2 (5)		T2 vs T3 = 0.323
2						
3						
Fatigue - No (%)*						**T0 vs T1 < 0.001**
0	29 (66)	2 (5)	0 (0)	3 (8)		**T0 vs T2 < 0.001**
1	15 (34)	31 (72)	18 (58)	27 (71)	**<0.001**	**T0 vs T3 < 0.001**
2	0 (0)	10 (23)	11 (36)	8 (21)		**T1 vs T2 = 0.008**
3	0 (0)	0 (0)	2 (7)	0 (0)		T1 vs T3 = 0.593
						T2 vs T3 = 0.005
Alopecia - No (%)*						**T0 vs T1 < 0.001**
0	44 (100)	2 (5)	4 (13)	13 (34)	**<0.001**	**T0 vs T2 < 0.001**
1	0 (0)	8 (19)	6 (19)	2 (5)		**T0 vs T3 < 0.001**
2	0 (0)	33 (77)	21 (68)	23 (61)		T1 vs T2 = 0.163
						T1 vs T3 = 0.007
						T2 vs T3 = 0.006

**Percentages do not add up to 100% due to rounding.*
Friedman's ANOVA was used to indicate differences in chemotherapy toxicity during the course of AC-D Wilcoxon signed-rank sum test with Bonferroni correction was used for pairwise comparison. After Bonferroni correction p-values below 0.0125 indicated significance.

Supplementary Table 12: *Longitudinal clinical characteristics of the total study population (N=44) - bone marrow toxicity*

Bone marrow toxicity	T0 before AC-D (*n*=44)	T1 during AC (*n*=43)	T2 during D (*n*=29)	T3 after AC-D (*n*=37)	*p*-value	Pairwise comparison
Hemoglobin – in µ/L					**<0.001**	**T0 vs T1 < 0.001** **T0 vs T2 < 0.001** **T0 vs T3 < 0.001** T1 vs T2 = 0.030 T1 vs T3 = 0.464 T2 vs T3 = 0.374
Median (IQR)	8.4 (0.7)	7.3 (0.9)	7.1 (0.7)	6.9 (0.7)		
25%-75%	8.1-8.8	6.7-7.6	6.7-7.4	6.7-7.4		
Thrombocytes - in 10^9/l					**0.012**	T0 vs T1 = 0.111 T0 vs T2 = 0.280 T0 vs T3 = 0.465 T1 vs T2 = 0.308 T1 vs T3 = 0.082 T2 vs T3 = 0.030
Median (IQR)	272 (92)	323 (158)	331 (108)	317 (132)		
25%-75%	255-347	250-405	259-367	260-392		
Leucocytes – in 10^9/l					0.151	T0 vs T1 = 0.621 T0 vs T2 = 0.256 T0 vs T3 = 0.455 T1 vs T2 = 0.993 T1 vs T3 = 0.092 T2 vs T3 = 0.041
Median (IQR)	7.2 (3.1)	6.9 (7.4)	7.9 (6.2)	6.8 (3.6)		
25%-75%	5.9-9.0	4.6-12	5.3-11.5	5.2-8.8		
Neutrophils – in 10^9/l					0.190	T0 vs T1 = 0.350 T0 vs T2 = 0.104 T0 vs T3 = 0.600 T1 vs T2 = 0.801 T1 vs T3 = 0.056 T2 vs T3 = 0.016
Median (IQR)	4.6 (2.5)	4.6 (7.4)	6.0 (6.6)	4.3 (2.0)		
25%-75%	3.3-5.8	2.9-10.3	3.5-10.1	3.4-5.4		

Friedman's ANOVA was used to indicate differences in bone marrow toxicity during the course of AC-D Wilcoxon signed-rank sum test with Bonferroni correction was used for pairwise comparison. After Bonferroni correction p-values below 0.0125 indicated significance.

Supplementary Table 13: *β-diversity and chemotherapy toxicity of the total study population*

	T1 during AC (*n*=43)		**T2 during D (*n*=29)**	
	Genus	**Phylum**	**Genus**	**Phylum**
Any grade CTCAE	*p*-value	*p*-value	*p*-value	*p*-value
Diarrhea	0.4656	0.6536	0.1584	0.3597
Nausea	0.9138	0.6290	0.6312	0.2483
Oral mucositis	0.4268	0.6718	0.8789	0.4593
Hand foot syndrome	0.8237	0.9645	0.8926	0.3600
Peripheral sensory neuropathy	0.8630	0.9962	0.6169	0.4959

PERMANOVA showed that microbial community structures on both phylum and genus level during AC and during D were not associated with toxicity.

Supplementary Table 14: *Longitudinal clinical characteristics of neoadjuvant treated patients*

Clinical characteristics	**T0 before AC-D**	**T1 during AC**	**T2 during D**	**T3 after AC-D**	***p*-value**	**Pairwise comparison**
Karnofsky Performance Score - No (%)*						**T0 vs T1 < 0.001** **T0 vs T2 = 0.001** **T0 vs T3 = 0.001** T1 vs T2 = 0.031 T1 vs T3 = 0.480 **T2 vs T3 = 0.013**
40-50	0 (0)	0 (0)	1 (7)	0 (0)	**<0.001**	
60-70	0 (0)	3 (18)	6 (43)	4 (25)		
80-90	5 (28)	13 (77)	6 (43)	12 (76)		
100	13 (72)	1 (6)	1 (7)	0 (0)		
MUST-score - No (%)*						T0 vs T1 = 0.157 T0 vs T2 = 0.129 T0 vs T3 = 0.180 T1 vs T2 = 0.450 T1 vs T3 = 1.000 T2 vs T3 = 0.083
Low risk	16 (89)	14 (78)	11 (73)	12 (75)	0.294	
Medium risk	2 (11)	2 (11)	2 (13)	4 (25)		
High risk	0 (0)	2 (11)	2 (13)	0 (0)		
BMI - kg/m^2					0.332	T0 vs T1 = 0.494 T0 vs T2 = 0.865 T0 vs T3 = 0.755 T1 vs T2 = 0.753 T1 vs T3 = 0.348 T2 vs T3 = 0.154
Median (IQR)	26 (7)	27 (7)	27 (6)	27 (5)		
25%-75%	24-31	23-30	24-29	24-29		

** Percentages do not add up to 100% due to rounding*
Wilcoxon signed-rank sum test with Bonferroni correction was used for pairwise comparison. After Bonferroni correction p-values below 0.0125 indicated significance.

Supplementary Table 15: *Longitudinal CTCAE in grade of neo-adjuvant treated patients*

Toxicity grade	T0 before AC-D	T1 during AC	T2 during D	T3 after AC-D	*p*-value	Pairwise comparison
Nausea - No. (%)						T0 vs T1 = 0.025
0	18 (100)	13 (72)	7 (50)	16 (100)		T0 vs T2 = 0.014
1	0 (0)	5 (28)	5 (36)	0 (0)	**0.001**	T0 vs T3 = 1.000
2	0 (0)	0 (0)	2 (14)	0 (0)		T1 vs T2 = 0.096
3	0 (0)	0 (0)	0 (0)	0 (0)		T1 vs T3 = 0.046
						T2 vs T3 = 0.014
Vomiting - No (%)						
0	18 (100)	18 (100)	14 (100)	16 (100)	-	-
1	0 (0)	0 (0)	0 (0)	0 (0)		
Oral mucositis - No (%)*						**T0 vs T1 = 0.003**
0	18 (100)	8 (44)	7 (50)	14 (88)		T0 vs T2 = 0.015
1	0 (0)	8 (44)	4 (29)	2 (13)	**0.002**	T0 vs T3 = 0.157
2	0 (0)	2 (11)	3 (21)	0 (0)		T1 vs T2 = 0.603
3	0 (0)	0 (0)	0 (0)	0 (0)		T1 vs T3 = 0.035
4	0 (0)	0 (0)	0 (0)	0 (0)		T2 vs T3 = 0.014
Diarrhea - No (%)*						T0 vs T1 = 0.180
0	18 (100)	16 (89)	10 (71)	16 (100)		T0 vs T2 = 0.046
1	0 (0)	1 (6)	4 (29)	0 (0)	**0.019**	T0 vs T3 = 1.000
2	0 (0)	1 (6)	0 (0)	0 (0)		T1 vs T2 = 0.083
3	0 (0)	0 (0)	0 (0)	0 (0)		T1 vs T3 = 0.317
						T2 vs T3 = 0.046
Constipation - No. (%)						**T0 vs T1 = 0.008**
0	18 (100)	11 (61)	10 (71)	16 (100)	**0.019**	T0 vs T2 = 0.046
1	0 (0)	7 (39)	4 (29)	0 (0)		T0 vs T3 = 1.000
2	0 (0)	0 (0)	0 (0)	0 (0)		T1 vs T2 = 0.705
						T1 vs T3 = 0.025
						T2 vs T3 = 0.046
Fever - No (%)*						T0 vs T1 = 0.180
0	18 (100)	16 (89)	12 (86)	16 (100)		T0 vs T2 = 0.157
1	0 (0)	1 (6)	0 (0)	0 (0)	0.194	T0 vs T3 = 1.000
2	0 (0)	0 (0)	2 (14)	0 (0)		T1 vs T2 = 0.785
3	0 (0)	0 (0)	0 (0)	0 (0)		T1 vs T3 = 0.180
4	0 (0)	1 (6)	0 (0)	0 (0)		T2 vs T3 = 0.157
Peripheral sensory neuropathy – No (%)*						T0 vs T1 = 0.102
0	18 (100)	15 (83)	2 (14)	3 (19)		**T0 vs T2 = 0.001**
1	0 (0)	2 (11)	9 (64)	10 (63)	**<0.001**	**T0 vs T3 = 0.001**
2	0 (0)	1 (6)	3 (21)	3 (19)		T1 vs T2 = 0.013
3	0 (0)	0 (0)	0 (0)	0 (0)		**T1 vs T3 = 0.009**
						T2 vs T3 = 0.655
Hand foot syndrome –	18 (100)	14 (78)	8 (57)	11 (69)	**0.014**	T0 vs T1 = 0.046 T0 vs T2 = 0.024

No. (%)*	0 (0)	4 (22)	3 (21)	3 (19)		T0 vs T3 = 0.039 T1 vs T2 = 0.063 T1 vs T3 = 0.336 T2 vs T3 = 0.157
0	0 (0)	0 (0)	3 (21)	1 (6)		
1	0 (0)	0 (0)	0 (0)	1 (6)		
2						
3						
Fatigue - No (%)*						**T0 vs T1 < 0.001**
0	14 (78)	0 (0)	0 (0)	2 (13)		**T0 vs T2 = 0.001**
1	4 (22)	12 (67)	8 (57)	11 (69)	**<0.001**	**T0 vs T3 = 0.002**
2	0 (0)	6 (33)	6 (43)	3 (19)		T1 vs T2 = 0.317
3	0 (0)	0 (0)	0 (0)	0 (0)		T1 vs T3 = 0.102 T2 vs T3 = 0.034
Alopecia - No (%)*						**T0 vs T1 < 0.001**
0	18 (100)	2 (11)	0 (0)	7 (44)	**<0.001**	**T0 vs T2 = 0.001**
1	0 (0)	5 (28)	5 (36)	2 (13)		T0 vs T3 = 0.005 T1 vs T2 = 0.705
2	0 (0)	11 (61)	9 (64)	7 (44)		T1 vs T3 = 0.132 T2 vs T3 = 0.034

** Percentages do not add up to 100% due to rounding*
Wilcoxon signed-rank sum test with Bonferroni correction was used for pairwise comparison. After Bonferroni correction p-values below 0.0125 indicated significance.

Supplementary Table 16: *Longitudinal clinical data - therapy adjustments of neoadjuvant treated patients (n=18)*

Therapy adjustments	**T1 during AC**	**T2 during D**	**T3 overall**
Therapy adjustments overall – No (%)			
No	14 (78)	11 (61)	8 (44)
Yes	4 (22)	7 (39)	10 (56)
Type therapy adjustments overall – No (%)			
Stop	0 (0)	4 (22)	4 (22)
Reduction, delay, and/or switch	4 (22)	3 (17)	6 (33)
% dose received overall			
Median (IQR)	100 (0)	100 (20)	100 (10)
25%-75%	100-100	80-100	90-100

Supplementary Table 17: *Response to AC-D of the neoadjuvant treated patients*

Response to AC-D	Total	N
After AC-D response tumour pathology category - No. (%)		
Complete pathologic response	1 (6)	
EUSOMA 2 (i)	6 (35)	17
EUSOMA 2 (ii)	4 (24)	
EUSOMA 2 (iii)	6 (35)	
After AC-D pathologic response*		
Low responders	10 (59)	17
High responders	7 (41)	

** High-responders (<10% remaining tumour cells) were defined as EUSOMA 1 and EUSOMA 2 (i). Low-responders (≥10 remaining tumour cells) were defined as EUSOMA 2 (ii), EUSOMA 2 (iii), and EUSOMA 3.*

Supplementary Table 18: *Clinical characteristics of the neoadjuvant study population at baseline. Response measured after AC-D according to EUSOMA*

Baseline characteristics	Total *n*=18	High responders *n*=7	Low responders *n*=10	*p*-value
Age - Years				
Mean (SD)	58 (5)	60 (5)	57 (5)	0.199
Range	49-71	55-71	49-65	
BMI - kg/m^2				
Mean (SD)	28 (6)	27 (4)	29 (6)	0.429
Range	20-42	23-33	23-42	
Weight loss past 3-6 months - in kg				
<5%	16 (89)	6 (86)	10 (100)	-
5%-10%	2 (11)	1 (14)	0 (0)	
Focality - No. (%)*				
Unifocal tumour	14 (78)	6 (86)	8 (80)	1.000
Multifocal tumour	3 (17)	1 (14)	2 (20)	
Unknown	1 (6)	0 (0)	0 (0)	
cT stage - No. (%)*				
1	3 (17)	2 (29)	1 (10)	
2	10 (56)	2 (29)	8 (80)	0.325
3	2 (11)	1 (14)	1 (10)	
4	2 (11)	2 (29)	0 (0)	
Unknown	1 (6)	0 (0)	0 (0)	
cG grade - No. (%)*				
1	2 (11)	1 (14)	1 (10)	
2	12 (67)	4 (57)	7 (70)	0.621
3	3 (17)	2 (29)	1 (10)	
Unknown	1 (6)	0 (0)	1 (0)	
cN stage - No. (%)*				
0	11 (61)	4 (57)	7 (70)	
1	4 (22)	2 (29)	2 (20)	0.665
2	1 (6)	0 (0)	0 (0)	
3	2 (11)	1 (14)	1 (10)	
cT size - in mm				
Median (IQR)	28 (16)	24 (20)	29 (16)	0.812
25%-75%	22-38	20-40	22-38	
MIB1%				
Median (IQR)	10 (24)	50 (-)	10 (17)	0.190
25%-75%	5-29	25- -	4-20	
Ki-67%				
Median (IQR)	25 (25)	50 (-)	10 (24)	0.190
25%-75%	5-30	25- -	4-28	

Tumour-type - No (%)				
Invasive carcinoma of no special type (NST)	16 (89)	7 (100)	8 (80)	0.485
Lobular	2 (11)	0 (0)	2 (20)	
Mucinous	0 (0)	0 (0)	0 (0)	
Unknown	0 (0)	0 (0)	0 (0)	
ER- No (%)				
Negative	0 (0)	0 (0)	0 (0)	-
Positive	18 (100)	7 (100)	10 (100)	
ER %				
Median (IQR)	100 (5)	100 (5)	100 (6)	0.962
25%-75%	95-100	95-100	94-100	
PR- No (%)*				
Negative	9 (50)	4 (57)	4 (40)	0.637
Positive	9 (50)	3 (43)	6 (60)	
PR %				
Median (IQR)	12 (91)	8 (80)	33 (95)	0.417
25%-75%	0-92	0-80	1-96	
Radiotherapy received any time - No. (%)*				0.537
No	3 (17)	2 (29)	1 (10)	
Yes	15 (83)	5 (71)	9 (90)	
Karnofsky Performance Score - No (%)*				0.935
80-90	5 (28)	2 (28)	3 (30)	
100	13 (72)	5 (71)	7 (70)	
MUST-score - No (%)				
Low risk	16 (89)	6 (86)	10 (100)	0.232
Medium risk	2 (11)	1 (14)	0 (0)	
High risk	0 (0)	0 (0)	0 (0)	
DM-type II - No. (%)*				
No	16 (89)	6 (86)	9 (90)	1.000
Yes	2 (11)	1 (14)	1 (10)	
Prior systemic treatment - No. (%)				
No	18 (100)	7 (100)	10 (100)	-
Yes	0 (0)	0 (0)	0 (0)	
Therapeutic antibiotic use last year - No. (%)*				1.000
No	14 (78)	6 (86)	8 (80)	
Yes	4 (22)	1 (14)	2 (20)	
Days therapeutic antibiotic use last year				-
Median (IQR)	9 (-)		9 (-)	
25%-75%	7- -	-	7- -	

**Percentages do not add up to 100% due to rounding.*

Supplementary Table 19: *Clinical characteristics after neoadjuvant chemotherapy. Response measured after AC-D according to EUSOMA*

Clinical characteristics	Total *n*=18	High responders *n*=7	Low responders *n*=10	*p*-value
OK-type - No (%)*				
Lumpectomy	8 (44)	3 (43)	5 (50)	1.000
Mastectomy	9 (50)	4 (57)	5 (50)	
Unknown	1 (6)	0 (0)	0 (0)	
pG grade - No. (%)*				
1	4 (22)	1 (14)	3 (30)	
2	5 (28)	2 (29)	3 (30)	0.251
3	1 (6)	1 (14)	0 (0)	
Unknown	8 (44)	3 (43)	4 (40)	
pN stage - No. (%)*				
0	9 (50)	4 (57)	5 (50)	
1	6 (33)	3 (43)	3 (30)	0.690
2	2 (11)	0 (0)	1 (10)	
3	0 (0)	0 (0)	0 (0)	
Unknown	1 (6)	0 (0)	1 (10)	
pT size - in mm				
Mean (SD)	21 (14)	19 (15)	22 (13)	0.638
Range	0-48	0-47	6-48	
pT stage - No. (%)*				
0	1 (6)	1 (14)	0 (0)	
1	9 (50)	4 (57)	5 (50)	
2	7 (39)	2 (29)	5 (50)	0.232
3	0 (0)	0 (0)	0 (0)	
4	0 (0)	0 (0)	0 (0)	
Unknown	1 (6)	0 (0)	0 (0)	
T2 Tumour reduction in %				
Mean (SD)	36 (29)	39 (39)	33 (21)	0.682
Range	-20-100	-20-100	-5-73	

**Percentages do not add up to 100% due to rounding.*

Supplementary Table 20: *α-diversity measures of the neoadjuvant study population. Response measured after AC-D according to EUSOMA*

α-diversity measures	Total *n*=18	High responders *n*=7	Low responders *n*=10	*p*-value
T0 Observed richness				
Mean (SD)	238 (52)	232 (70)	242 (41)	
Range	136-330	136-330	153-302	0.708
T1 Observed richness				
Mean (SD)	233 (34)	232 (45)	234 (29)	
Range	169-293	172-293	169-266	0.907
T2 Observed richness				
Mean (SD)	218 (48)	211 (31)	225 (57)	
Range	136-294	175-239	136-294	0.674
T3 Observed richness				
Mean (SD)	218 (48)	212 (36)	210 (48)	
Range	136-294	178-257	126-260	0.931
T0 Shannon index				
Mean (SD)	4.1 (0.4)	3.9 (0.6)	4.2 (0.3)	
Range	3.0-4.6	3.0-4.6	3.6-4.6	0.238
T1 Shannon index				
Mean (SD)	4.0 (0.3)	4.0 (0.3)	4.1 (0.3)	
Range	3.4-4.5	3.4-4.2	3.6-4.5	0.669
T2 Shannon index				
Mean (SD)	3.9 (0.3)	3.9 (0.2)	3.9 (0.30)	
Range	3.5-4.4	3.7-4.2	3.5-4.4	0.999
T3 Shannon index				
Median (IQR)	3.9 (0.4)	3.8 (0.3)	4.1 (0.5)	
25-75%	3.7-4.1	3.7-4.0	3.7-4.1	0.456

Differences in α-diversity between high and low responders measured at T2 according to EUSOMA were analyzed with an unpaired t-test.

High-responders (<10% remaining tumor cells) were defined as EUSOMA 1 and EUSOMA 2 (i). Low-responders (≥10 remaining tumor cells) were defined as EUSOMA 2 (ii), EUSOMA 2 (iii), and EUSOMA

Supplementary Table 21: *Basic 16S rRNA gene sequencing statistics of the gut bacterial microbiota using the Illumina MiSeq instrument*

Item	Data
Amplified region	515F-806R
Primer sequence 515F	5‘-GTGCCAGCMGCCGCGGTAA-3‘
Primer sequence 806R	5‘-GGACTACHVGGGTWTCTAAT-3‘
Number of samples	153
Total raw reads	18.890.872
Mean reads per sample	123.470
Total sequences per sample	
Minimum	51.119
Maximum	176.490
Mean %GC	53
Sequence length	251
Sequences flagged as poor quality	0

Chapter 7

The gut microbiota and the metabolic aspects of cancer cachexia

Janine Ziemons, Marjolein Smidt, Steven Olde Damink, Sander S. Rensen

Best Practice & Research: Clinical Endocrinology & Metabolism. 2021 Feb; 35(3):101508.
DOI: 10.1016/j.beem.2021.101508

Abstract

Cancer cachexia is a metabolic syndrome characterized by unintended weight loss and muscle wasting. It has a strong negative impact on survival. Its underlying mechanisms involve systemic inflammation and insulin resistance, which are known to be influenced by the gut microbiota. Preclinical studies support a role for the gut microbiota in cancer cachexia by demonstrating that cachectic mice display: 1) various gut microbiota composition changes; 2) increased gut permeability and translocation of pro-inflammatory microbial compounds; 3) muscle atrophy-related processes linked to gut microbiota properties; 4) positive effects of microbiota-modulating interventions. Data on the relationships between gut microbiota, insulin resistance, and hepatic/adipose tissue metabolism in cachexia models are lacking. Nevertheless, the available data and existing evidence for the impact of gut microbiota on metabolic aberrations in human obesity urge for exploration of its role in human cancer cachexia. We provide practical recommendations and discuss the challenges for such future clinical studies.

1. Introduction - Cancer cachexia and its metabolic consequences

1.1 What is cachexia?

Cancer cachexia is a devastating metabolic syndrome affecting ~50-80% of all cancer patients, depending on the tumor type (1). It is characterized by pronounced weight loss which is considered to be primarily due to loss of skeletal muscle and adipose tissue (2). Until the 1980s, cachexia was mainly attributed to anorexia and/or increased energy expenditure. However, it has now been firmly established that conventional nutritional support does not reverse cachexia symptoms (3), demonstrating that nutrient deficiency is not its root cause.

Cancer cachexia is associated with poor prognosis, and directly contributes to at least 20% of all cancer-related mortality by affecting chest, diaphragm, and cardiac muscle function as a result of tissue loss and compromised contractility (4). Moreover, cancer cachexia limits therapeutic options because it enhances the toxic side effects of chemotherapy (5). On top of this, chemotherapy can also induce cachexia. In Europe, an estimated one million people suffer from cancer cachexia (6).

The international consensus definition of cancer cachexia is based on body weight loss over the last six months, the presence of low muscle mass (sarcopenia), and/or a low body mass index (7, 8). Cachexia should not be considered merely a terminal illness. Symptoms of cachexia may already occur before the diagnosis in a so-called pre-cachectic phase characterized by subtle metabolic changes accompanied by mild systemic inflammation and/or mild anorexia (7). Pre-cachexia may develop into cachexia with clinically evident weight loss. Patients with refractory cachexia display a permanently altered metabolism, unresponsiveness to anti-cancer therapy, and a life expectancy of less than three months (7). The symptoms and clinical presentation of cancer cachexia can vary substantially (9), complicating its diagnosis in early stages.

Despite the widely accepted importance of cancer cachexia for cancer-related morbidity, mortality, and treatment options, cachexia is currently not managed actively because of a lack of adequate evidence for therapeutic targets. Indeed, the mechanistic basis of cancer cachexia is not yet fully established, complicating the development of effective interventions. Several pathophysiological drivers are thought to underlie its development, including local and systemic inflammation, altered energy metabolism, anorexia, malabsorption, and neuro-endocrine changes (7, 8). Systemic inflammation is often considered to be the key driving force (10), but the identification of tumor-derived pro-inflammatory factors related to human cachexia is limited to IL-6 and TNF-alpha. Moreover, neutralizing TNF-alpha antibodies are not effective in preventing cachexia, and the side-effects of anti-IL-6 treatment with respect to infection risk prevent its clinical application. In view of this, more knowledge of factors that are causally involved in the pathogenesis of cancer cachexia is urgently required. Recent animal data indicate that gut bacteria are promising candidate mediators of the metabolic changes observed in cachexia. In this review, we will summarize these preclinical data and highlight the steps that are required to translate these findings into impact for patients' survival and/or quality of life.

2. Gut microbiota alterations associated with cancer cachexia

Obviously, an important first step towards assessing the role of the gut microbiota in cancer cachexia is a description of the gut microbiota profile that is typical of it. In the following section, we will describe the recent evidence concerning cachexia-associated shifts in gut microbiota composition and diversity.

Bindels *et al.* repeatedly demonstrated changes in gut microbiota composition and diversity in mice with leukemia (BaF3 mice) or colon cancer (C26 mice) (11-13). Both models display muscle atrophy and loss of fat mass and are used as mouse models of cancer cachexia.
In 2012, it was shown that cecal levels of Lactobacilli were reduced in BaF3 mice when compared to control mice, while the total bacterial content as well as levels of *Bacteroides spp*. were similar between these groups (13). Within the *Lactobacillus* genus, *Lactobacillus johnsonii/gasseri* and *Lactobacillus reuteri* were significantly decreased, but *Lactobacillus murinus/animalis* were not affected. Interestingly, the authors also described a negative correlation between abundance of Lactobacilli and expression of atrophy markers in the gastrocnemius muscle. However, it should be noted that PCR and denaturing gradient gel electrophoresis (DGGE) and no sequencing approaches were used to assess bacterial abundances in this early study.
A later study using Illumina sequencing supported the occurrence of profound shifts in gut microbiota composition of cachectic BaF3 and C26 mice (11). In both models, microbial diversity was significantly decreased when compared to control mice. Furthermore, the cachexia-associated microbiota profile was characterized by decreased abundance of bacteria belonging to the Clostridiales order, Clostridia class, Lactobacillaceae family and *Lactobacillus* genus. This was accompanied by increased abundance of the Bacteroidetes phylum, Enterobacteriales order, the Enterobacteriaceae family, as well as the genus *Parabacteroides.* The cachexia-associated increased abundance of Enterobacteriaceae was confirmed in a later study with cachectic C26 mice (12).
In view of this, Pötgens *et al.* (2018) investigated this bacterial group in more detail and discovered that the species *Klebsiella oxytoca* was commonly increased in tumor-bearing mice with cachexia, independently of anorexia (14). In these mice, increased abundance of Proteobacteria was accompanied by a reduction of Firmicutes, particularly of Ruminococcaceae and Lachnospiraceae. The authors suggested that the observed *Klebsiella oxytoca* expansion might evolve due to an interplay between decreased abundance of Ruminococcaceae and Lachnospiraceae, reduced PPAR-γ, and a reduced pool of cecal regulatory T cells. Furthermore, *Klebsiella oxytoca* was suggested to act as gut pathobiont in cancer cachexia, since administration of this species increased bacterial translocation and deteriorated gut barrier function in cachectic mice. However, it did not affect tumor growth or systemic features of cachexia, such as body weight loss and expression of muscle atrophy markers.
An interesting recent study addressed the important question whether gut microbiota changes are related to the cancer or to cachexia. C26 mice were treated with blocking activin receptor ligands, which partly prevented muscle loss (15). Whereas the cancer affected gut microbiota

diversity and composition, alleviation of cachexia by the anti-activin treatment had only minor and inconsistent effects on the gut microbiota at the phylum, family, and genus level.

As in most disease areas, the focus in cancer cachexia microbiota studies has been on gut bacteria. However, one recent study described changes in the fungal population (mycobiota) during the development of cancer cachexia in mice (16). It was reported that experimental Lewis lung carcinoma-induced cachexia was associated with reduced abundance of Mucoromycota and increased abundance of Sordariomycetes, Saccharomycetaceae and Malassezia. Some of these fungi are known to have metabolic characteristics that could directly contribute to cachexia development. In this context, Rhyzopus oryzae, a Mucoromycota species, was proposed as probiotic candidate based on its chitosan content as well as its ability to produce various antioxidants and organic acids with a positive effect on glucose and lipid metabolism as well as inflammation.

All in all, these pre-clinical studies using mouse models of cancer cachexia provide important insights into potential characteristics of a cachexia-associated gut microbiota profile. Nevertheless, it is worth mentioning that the current scientific evidence on the role of the gut microbiota in cancer cachexia is solely based on animal models and that translatability of these cachexia models to the human situation might be limited. Three main limitations of animal studies in cancer cachexia should be taken into consideration. The first relates to the fact that cancer cachexia is a highly complex and multifactorial syndrome that develops over a longer period of time. Current microbiota data are mostly based on mouse models with aggressive, rapidly growing tumors. It is likely that the rapid deterioration of the condition of these mice leads to metabolic and inflammatory processes characteristic of refractory cachexia, rather than modelling pre-cachectic or cachectic stages that may still be treatable. Second, the major influence of diet and other environmental factors on gut microbiota composition is well established. Therefore, the gut microbiota of vegetarian mice, housed under experimental conditions, is expected to be significantly different from the gut microbiota of a human individual with a varied diet and living in a community. Third, the recent data provided by Pekkala *et al.* (15), underline the necessity to further evaluate whether the observed gut microbiota alterations arise due to cancer cachexia or as a consequence of the cancer itself, by using cancer models that differ in the development and/or severity of cachexia. These limitations emphasize the urgent need for well-designed clinical studies with cachectic cancer patients to elucidate cachexia-associated gut microbiota changes in humans.

3. Associations between the gut microbiota and metabolic aspects of cancer cachexia

Whereas cancer cachexia and obesity are characterized by fundamentally opposite changes in body weight, their pathogenesis shares important underlying mechanisms that provoke profound metabolic perturbations. In particular, systemic inflammation, altered skeletal muscle metabolism, insulin resistance, as well as deranged food intake and body weight regulation are key factors in both conditions. Interestingly, these metabolic disturbances have previously

been connected to various roles of the gut microbiota in the context of human obesity (17), leading to the assumption that the gut microbiota also affects these hallmarks in patients with cancer cachexia. Below, we will summarize some of the existing evidence that links metabolic aspects of cancer cachexia to gut microbiota characteristics (see also Figure 1). We will focus on systemic inflammation and skeletal muscle wasting since they are the most defining and best studied aspects of cachexia.

3.1 Systemic inflammation and gut permeability

Systemic inflammation is considered to be a hallmark of cancer cachexia and probably acts as driving force behind many metabolic alterations observed in cachectic patients (1, 8). In a study of 122 newly diagnosed cancer patients, plasma levels of several inflammatory markers (e.g. TGF-β, IL-8, IL-6) and absolute neutrophil counts as well as expression of inflammation-associated genes (e.g. angiotensin II) were found to be increased in cachectic or pre-cachectic cancer patients, compared to non-cachectic cancer patients (18). Similarly, increased serum levels of the acute phase protein lipopolysaccharide binding protein (LBP) and of the pro-inflammatory cytokine IL-6 have been demonstrated in cachectic lung cancer and colorectal cancer patients (12). In these patients, LBP levels have been described as predictive for mortality as well as for the presence of cachexia and associated metabolic features (e.g. appetite, body weight loss, performance status, and quality of life) (12). Previous research already identified various molecular interactions between gut bacteria and these elements of the immune system (19). For instance, we have reported that markers of systemic and intestinal inflammation correlated with relative abundance of specific bacterial groups in overweight subjects (20).

One important link between gut bacteria and the development and manifestation of systemic inflammation is the function of the intestinal barrier. The intestinal barrier separates the internal milieu from the lumen, and its adequate function is crucial for avoiding bacterial translocation into the blood stream, which might elicit an inflammatory response. Previous research described that the gut microbiota is able to modulate gut barrier function (21). In line with this, Bindels *et al.* (2018) reported several alterations of the intestinal architecture as well as increased gut permeability in C26 mice (12). Expression of various molecular markers of gut barrier function (e.g. Tjp1, Ocln, Muc2, Cldn2), cell renewal (e.g. Lgr5, Klf4) and gut immunity (e.g. Cd3g, Foxp3) were decreased in C26 mice compared to control (sham-injected) mice. This was accompanied by changes in gut microbiota composition (increased abundance of Enterobacteriaceae), increased plasma LBP levels, as well as activation of the TLR4 pathway. Stimulation of TLR4 by endotoxin triggers the expression of pro-inflammatory cytokines (22). Interestingly, administration of an anti-IL-6 antibody improved many of the cachexia-associated disturbances (12). IL-6 has been proposed as important mediator in cancer cachexia, as reviewed elsewhere (2, 10). Similarly, Puppa *et al.* (2011) indicated that gut permeability and plasma endotoxin concentrations increased simultaneously with the development and progression of cancer cachexia in $APC^{Min/+}$ mice (23).
In line with these results obtained in animal studies, Jiang *et al.* (2014) showed increased intestinal permeability and more bacterial translocation, quantified by detection of bacterial DNA in serum, in cachectic gastric cancer patients, in comparison to non-cachectic patients (24).

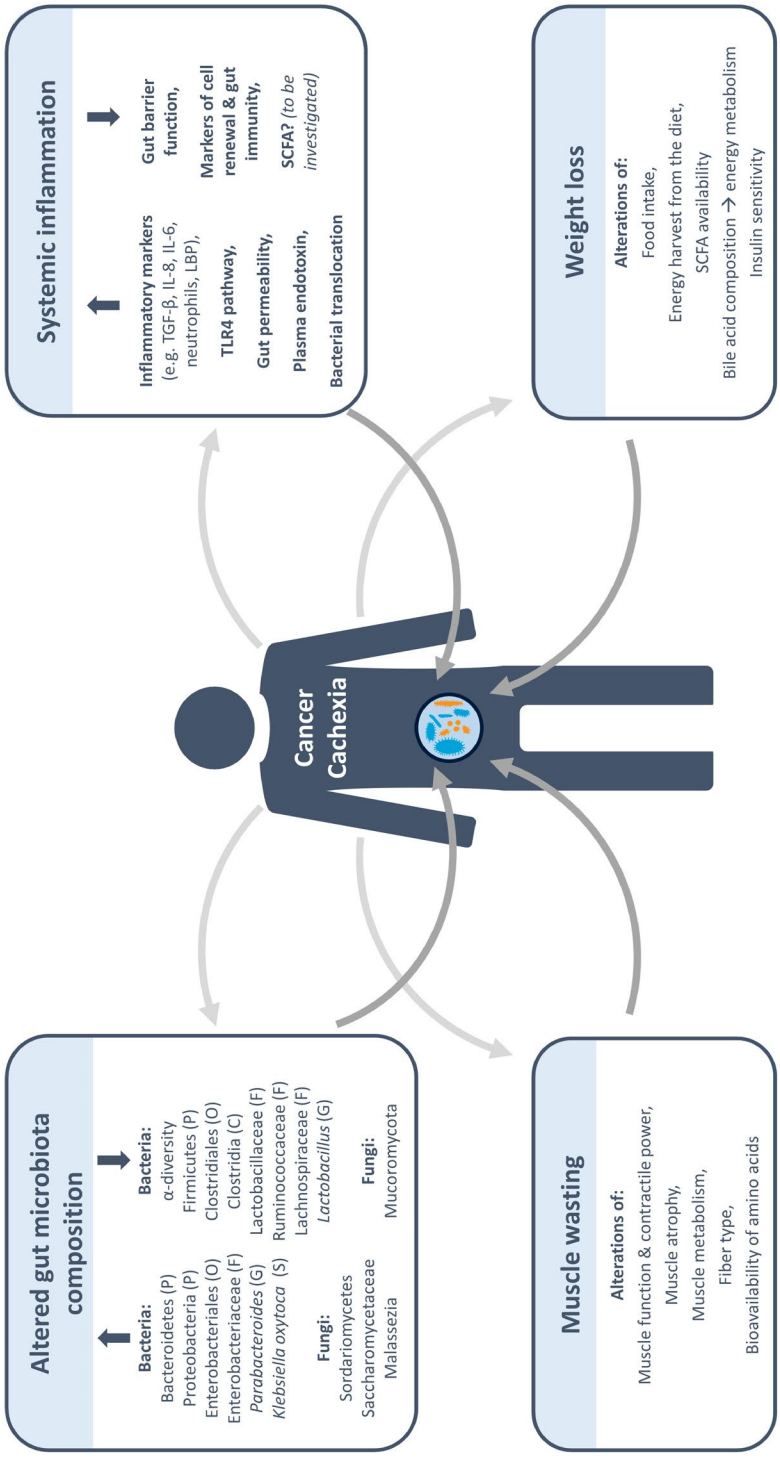


***Figure 1:** Schematic overviewof interction between the gut microbiota and different metabolic aspects of cancer cachexia*

Next to structural microbial compounds, microbiota-derived metabolites such as short-chain fatty acids (SCFA) have been shown to modulate inflammation. Although the role of SCFA in cancer cachexia remains to be investigated, the molecular mechanisms described for these metabolites are likely to be of importance in this context. SCFA such as butyrate, propionate, and acetate are produced by microbial fermentation of non-digestible carbohydrates and are considered to fulfill crucial roles in colonic and metabolic health. For instance, several studies showed that SCFA and particularly butyrate appear to be essential for gut barrier function by serving as primary energy source for colonocytes and by enhancing expression of tight junction proteins and mucins (25-27).
Since a significant proportion of the SCFA is absorbed into the blood stream (28), they can directly interact with the immune system. Previous research showed that SCFA modulate several inflammatory pathways by inhibition of histone deacetylases and by interaction with several G protein coupled receptors (29). Likewise, butyrate administration has been shown to inhibit activation of NF-κB, a key player in coordinating the expression of a wide variety of genes involved in immune responses (30, 31). Moreover, butyrate suppressed endotoxin-induced production of IL-8, TNF-α, IL-6, and IL-12 and promoted the production of anti-inflammatory IL-10 in cell lines (31). However, it should be noted that the scientific literature about the immune modulating effect of SCFA contains some controversies, since SCFA might also have pro-inflammatory effects in certain circumstances (29).
In view of these numerous interactions between the gut microbiota, their metabolites, and the immune system, it is likely that microbiota-mediated immune modulation plays a role in systemic inflammation in cancer cachexia.

3.2 Muscle wasting and muscle metabolism

Muscle wasting or ‘sarcopenia’ is an integral aspect of cachexia. Several studies have reported associations between the gut microbiota and cachexia-related muscle properties, although most of them were not done in the context of cachexia.
One study in the field of chronic inflammation-related cachexia established a link between specific gut bacteria and muscle wasting. In several mouse models of chronic intestinal inflammation, colonization of the gut by an *Escherichia coli* strain was shown to prevent skeletal muscle atrophy by activating the insulin-like growth factor 1/phosphatidylinositol 3-kinase/AKT pathway mediated by the NLRC4 inflammasome (32). The intervention studies of Bindels (11-14, 33) also support the impact of gut microbiota on skeletal muscle metabolism and atrophy, although the effects are generally small and limited to proxies of muscle wasting such as expression of E3 ligases or autophagy factors, as discussed in the next section.

One of the most direct links between the gut microbiota and muscle physiology was provided by a recent study by Nay *et al.*, who treated mice for 21 days with broad-spectrum antibiotics and extensively studied muscle function after treatment as well as after reseeding with bacteria from non-treated controls (34). Depletion of bacteria did not affect muscle wet weight or expression of E3 ligases or proteins involved in protein synthesis, inflammation, or autophagy. Nevertheless, running tests revealed that mice treated with antibiotics were exhausted earlier. In line, ex vivo tests of their intrinsic muscle contractile properties showed significantly earlier reduction of contractile power of extensor digitorum longus muscle in an artificial stimulation protocol. Importantly, these differences disappeared after restoration of

the microbiota. Mechanistically, this was suggested to be attributable to the impact of gut microbiota metabolites on glucose metabolism and muscle glycogen availability. In support of this, microbiota-derived phenolic compounds were recently found to promote glucose uptake in cultured differentiated human skeletal muscle myotubes in a dose-dependent manner, with the strongest effect for sulfated isovanillic acid (35, 36).

The impact of gut microbiota on exercise performance has also been associated with their influence on muscle fiber type. For instance, the well-known probiotic *Lactobacillus plantarum* has been shown to promote the slow and oxidative type I muscle phenotype associated with high muscle endurance as well as grip strength, while improving muscle mass in mice (37). Furthermore, gut microbiota transplantation from obese versus lean pigs into germfree mice has been shown to affect fiber size, proportions of slow-contracting versus fast-contracting fibers, and lipogenesis in the gastrocnemius muscle (38). Gut microbiota effects on muscle fatty acid metabolism have further been shown in the context of obesity studies, where germfree mice were found to display increased fatty acid breakdown in muscle secondary to the impact of gut microbiota on AMP-activated protein kinase and peroxisomal proliferator-activated receptor coactivator-1alpha (39).

An additional mechanism by which the gut microbiota could affect muscle protein synthesis and breakdown relates to their impact on the bioavailability of amino acids (40). Whereas gut microbiota can utilize several amino acids originating from both alimentary and endogenous proteins, they can also provide amino acids to the host. This could have significant implications in the context of cachexia, which is characterized by protein catabolism and altered systemic amino acid levels. Furthermore, elevated concentrations of certain amino acids, in particular the aromatic and branched-chain amino acids, are known to contribute to insulin resistance (40), a prominent feature of cachexia. Moreover, several amino acids released by gut bacteria can serve as precursors for the synthesis of SCFA (40), which are likely to play a role in cachexia, as discussed above.

Collectively, these data highlight several mechanisms by which the gut microbiota affects muscle properties that are fundamentally altered in cachexia, underscoring its potential impact on the most defining pathological aspect of cachexia.

3.3 Insulin sensitivity, food intake, and body weight regulation

As described above, cancer cachexia is characterized by unintentional weight loss. The link between gut microbiota composition and body weight has been well described in the context of obesity, and it is suggested that the obesity-associated microbiota might have increased capacity for energy harvest from the diet in the form of SCFA (41, 42). Next to this, the production of SCFA by gut bacteria has been shown to promote body weight gain via regulation of food intake, insulin sensitivity, and substrate metabolism (43). Furthermore, gut bacteria affect bile acid composition, which, in turn, alters energy homeostasis and glucose/lipid metabolism, predominantly by activating farnesoid X receptor and TGR5 signaling in a variety of tissues (44). These data from the obesity field pave the way for the question whether a cachexia-associated microbiota with altered bile acid signaling potential, decreased capacity for energy harvest, and detrimental impact on appetite exists. In support

of this, patients with anorexia nervosa have been reported to display a significantly altered gut microbiota composition compared to healthy controls (45). Amongst others, abundance of Enterobacteriaceae was increased in these patients, similar to the results from studies in cachectic mice.

4. Interventions targeting the gut microbiota in cancer cachexia

Further evidence for the role of the gut microbiota in cancer cachexia arises from studies indicating positive effects of microbiota-modulating interventions. There are different strategies for targeting the gut microbiota, including probiotics, which are live microorganisms that, when administered in adequate amounts, confer a health benefit to the host (46). An alternative strategy of gut microbiota modulation is the use of prebiotic fibers which are fermented by the intestinal bacteria, leading to the formation of SCFA. Prebiotics can positively influence gut microbiota composition by stimulating growth and/or activity of beneficial intestinal bacteria (47). Finally, synbiotics contain a mixture of live microorganisms and substrate(s) selectively utilized by host microorganisms (48).

4.1 Synbiotics

In the context of cancer cachexia, it has been shown that a synbiotic containing inulin-type fructans and *Lactobacillus reuteri* was able to counteract the observed disturbances of gut microbiota composition in cachectic mice (11). Furthermore, the intervention had positive effects on cancer progression, morbidity score, and survival. Several cachectic features such as loss of muscle mass, skeletal muscle expression of Cathepsin L and LC3, and expression of several markers of intestinal permeability and immune function slightly improved upon synbiotic administration.

4.2 Probiotics

It has also been reported that restoration of the decreased *Lactobacilli* levels in cachectic mice by means of *Lactobacillus* administration successfully improves some, but not all cachectic features (13). Body weight gain, cancer progression, and loss of fat mass remained unaffected. However, *Lactobacillus* supplementation successfully reduced the levels of several blood inflammatory markers (IL-4, Mcp-1, G-CSF, IL-6). Furthermore, the expression of muscle atrophy markers was lowered due to the probiotic treatment. These positive effects were only observed upon supplementation with *Lactobacillus reuteri 100-23* and *Lactobacillus gasseri 311476* but not with *Lactobacillus acidophilus*, indicating that the positive effects on inflammation and muscle atrophy might be strain-specific.
In line with these results, Varian *et al.* showed that cachectic mice treated with a probiotic (*Lactobacillus reuteri*) had less signs of muscle atrophy compared to untreated mice, with a larger mean muscle fiber cross-sectional area (49). This study also reported that *Lactobacilli* supplementation reduced blood neutrophil counts as well as intestinal tumor burden. Interestingly, in another experimental setting, the same treatment also protected wild type mice without neoplasms from age-associated sarcopenia. The authors also observed that

probiotic-treated mice had a larger thymus and higher expression of the FoxN1 protein, which helps to control production of T lymphocytes. This might point to another link between gut microbiota modulation and immune homeostasis. On the contrary, administration of *Faecalibacterium prausnitzii* did not influence gut permeability, tumor mass, or the expression of markers of gut barrier function in cachectic mice (12). Finally, the probiotic *Bacillus coagulans GBI-30, 6086* has been reported to reduce exercise-induced muscle damage and to increase recovery in humans when supplemented with 20g of casein protein (50), providing some evidence that a probiotics approach is feasible and effective in man.

4.3 Prebiotics

A study in leukemic mice evaluated whether non digestible carbohydrates with prebiotic properties could modulate the development of cachexia. The study revealed that the two compounds under investigation (pectic oligosaccharides, inulin) differentially affected the gut microbiota and SCFA profile and that pectic oligosaccharides might contribute to adipose tissue sparing (33). However, supplementation of these prebiotics to cachectic mice did not affect tibialis or gastrocnemius muscle weight. In a different model of murine neuroblastoma-associated cachexia, it was shown that prebiotic oligosaccharide supplementation consisting of dextrin and guar gum did not affect gut permeability or triceps surae muscle weight, while having significant impact on the fecal microbiome (51).

5. Ongoing clinical trials and future perspectives

5.1 Current clinical trials focusing on gut microbiota in cachexia

Although there is increasing evidence for an important role of the gut microbiota in different types of cancers (52), currently nothing is known about gut microbiota aberrations in cachectic cancer patients specifically. However, the high interest in the role of the gut microbiota in human cachexia is reflected in the fact that several clinical trials on this topic are registered in the clinicaltrials.gov database. In one study, the effects of a specific fraction from fermented soy milk on gut microbiota and risk/severity of cachexia is studied in 40 pancreatic cancer patients who undergo chemotherapy (53). The primary outcome of this study is a change in cachexia severity after 12 weeks of treatment and secondary outcomes include the effect on the skeletal muscle index. Another ongoing study aims to investigate the composition and activity of the gut microbiota in patients with acute myeloid leukemia (AML) in relation to important cachexia parameters (54). In this study, correlations between the gut microbiota, cachectic hallmarks, and gut permeability as well as microbial compounds and metabolites will be established.

Our department is also performing microbiota studies in cachectic and non-cachectic patients with pancreatic cancer, breast cancer, ovarian cancer, lung cancer, and colorectal cancer (55, 56). Preliminary qPCR data after inclusion of 54 patients suggested that the relative abundance of *Akkermansia muciniphila*, Lactobacilli, and butyrate producers was not significantly different between cachectic and non-cachectic individuals (55). SCFA levels were consistently lower in cachectic individuals, but the differences only showed a trend towards significance. We are currently investigating cachexia-associated changes in gut microbiota

composition and SCFA levels in a larger cohort, and also study gut microbiota dynamics in relation to therapy-related toxicity, body composition alterations, as well as treatment outcome in cancer patients during chemotherapy.

5.2 Points of attention for future clinical studies on the role of the gut microbiota in cachexia

Several challenges to the successful conduction of these clinical studies can be identified, both at the level of the microbiota characterization and at the level of cachexia phenotyping.

With respect to cachexia phenotyping, it is important to realize that diagnosing cachexia is not as easy as it may seem (7). Involuntary weight loss of ≥5% in the preceding six months is the most widely used diagnostic criterion (7). One major challenge in this regard lies in obtaining reliable data on body weight evolution. Body weight is usually well documented at the time of diagnosis, but patient-reported body weight six months earlier is frequently absent or inaccurate. In addition, it should be recognized that body weight measurements do not differentiate between skeletal muscle and fat tissue mass and may therefore underestimate lean body mass loss in overweight patients and in those who gained weight because of a growing tumor, edema, or accumulation of ascites (57). This underscores the need for including objective, quantitative, and accurate body composition measurements in future microbiota studies with cachectic patients. In this context, computed-tomography (CT)-based body composition analysis is of high interest because CT-scans of cancer patients are routinely available as part of their diagnosis, staging, and treatment response evaluation. CT-based body composition analysis is not influenced by tumor load, edema, or ascites, and can be performed unbiased, yielding quantitative and reproducible data. Furthermore, individual tissue compartments (e.g., skeletal muscle mass, adipose tissue, and bone) can be distinguished and analyzed simultaneously (58). The cross-sectional muscle area at the third lumbar vertebra (L3) has been shown to be a good estimate of whole-body muscularity when corrected for stature (expressed in the so-called skeletal muscle index, SMI). This L3-SMI has good prognostic value for patient survival (58). Repeated microbiota sampling in combination with assessment of body composition using CT-can analysis enables the investigation of links between cachexia-associated metabolism and gut microbiota changes in patients before and during treatment.

Researchers analyzing the microbiota in cancer cachexia face the same challenges that have been identified in the context of other diseases, in particular with respect to optimization and standardization of sample collection. We previously described an exemplary study procedure which allows easy and hygienic sample collection at home without too high burden for the patient (56). Moreover, some additional points of attention should be mentioned. First, it is difficult to disentangle the effects of the tumor versus the cachexia status on the microbiota. This may be particularly important for gastrointestinal cancers, where the tumor is in close proximity to the gut bacteria. For colorectal and gastric cancer, cancer-associated shifts of gut microbiota composition have been linked to prognosis and treatment response (59, 60) but the potential effect of cachexia have not been taken into account in these studies so far. Second, in pancreatic cancer, which is often studied because it has a high prevalence of cachexia, exocrine insufficiency is likely to affect microbiota composition and activity because

of its impact on the secretion of bile acids, bicarbonate, and digestive enzymes. Together, this argues for studies on the relationship between cancer cachexia and the gut microbiota in non-gastrointestinal cancers such as lung cancer, which also has a relatively high cachexia prevalence. Third, analysis of the relationship between the gut microbiota and treatment-induced cachexia might be confounded by the direct detrimental impact of many systemic anti-cancer therapies on the gut microbial ecosystem (61). As such, characterization of the gut microbiota in treatment-naïve patients, followed by longitudinal microbiota sampling and CT-based cachexia monitoring during treatment is recommended.

Another challenge is the regular use of antibiotics in cancer patients. In view of the detrimental and potentially long-lasting effects of antibiotics on the microbial community, antibiotic use should be excluded or well documented (62).

Finally, gut microbiota composition is known to be strongly influenced by several environmental factors, such as diet and the living environment. With respect to the design of microbiota studies in cancer patients, it might be beneficial to include the (household) partners of patients as healthy controls since they generally live in the same environment and share dietary habits, which are important confounders in microbiota studies that are hard to control for.

6. Summary

There are many indications supporting the hypothesis that the gut microbiota potentially plays an important role in the progression of cancer cachexia. This evidence is predominantly derived from studies with cachectic mice and from research in the context of obesity, the other end of the body weight spectrum that features many similar metabolic alterations. The link between cancer cachexia and the gut microbiota is not surprising given that the gut microbiota and its metabolites are known to influence many metabolic aspects that are deranged in cancer cachexia. In currently reported preclinical studies, most attention has been paid to the impact of the gut microbiota on body weight, muscle properties, and pro-inflammatory cytokines, while there is less information on the relationships between the gut microbiota and food intake, adipose tissue metabolism, and insulin resistance, all important aspects of cancer cachexia. It will be important to collect more information on these drivers of cachexia in future studies.

Unfortunately, there is a lack of human data in the current literature. This is likely to be related to the short time between diagnosis and treatment of cancer, limiting the opportunities for longitudinal studies of treatment naïve patients, and to the relative lack of attention to cachexia management in oncology in general. As such, an important first step would be to gain insight into cachexia-associated gut microbiota composition shifts in patients with various cancer types. It will be important for these studies to perform a comprehensive phenotyping of cachexia parameters, ideally including CT-based body composition analysis as well as functional muscle tests and the assessment of insulin sensitivity. Sample collection and handling should be standardized and performed according to state-of-the-art procedures to enable high quality microbiota analyses, including metagenomic sequencing, assessment of absolute abundances, transcriptomics, proteomics, and metabolomics. These studies should involve longitudinal microbiota sampling in a sufficiently high number of patients considering the many confounding factors (e.g. BMI, diet, living environment, antibiotics). Such investigations will be the prelude to follow-up studies addressing causality of microbiota alterations in cachexia and the design of microbiota-targeting interventions in cachectic cancer patients. Such novel treatment approaches are urgently required given the current lack of effective treatment despite the considerable impact of cancer cachexia on the survival and quality of life of cancer patients.

Research Agenda

- A comprehensive description of the cachexia-associated gut microbiota profile across different tumor types is urgently required
- Studies with preclinical models of cancer cachexia should address the interrelationships between the gut microbiota and its metabolites with food intake, hepatic and adipose tissue metabolism, and insulin resistance
- The associations between microbiota properties and specific body composition features characteristic of human cancer cachexia need to be documented
- The effect of microbiota-modulating interventions on the progression of cancer cachexia should be investigated to establish causality in the human setting

References

1. Argiles JM, Busquets S, Stemmler B, Lopez-Soriano FJ. Cancer cachexia: understanding the molecular basis. Nat Rev Cancer. 2014;14(11):754-62.
2. Fearon KC, Glass DJ, Guttridge DC. Cancer cachexia: mediators, signaling, and metabolic pathways. Cell Metab. 2012;16(2):153-66.
3. van de Worp WRPH, Schols AMWJ, Theys J, van Helvoort A, Langen RCJ. Nutritional Interventions in Cancer Cachexia: Evidence and Perspectives From Experimental Models. Front Nutr. 2020;7.
4. Porporato PE. Understanding cachexia as a cancer metabolism syndrome. Oncogenesis. 2016;5:e200.
5. Prado CM, Antoun S, Sawyer MB, Baracos VE. Two faces of drug therapy in cancer: drug-related lean tissue loss and its adverse consequences to survival and toxicity. Curr Opin Clin Nutr Metab Care. 2011;14(3):250-4.
6. von Haehling S, Anker SD. Prevalence, incidence and clinical impact of cachexia: facts and numbers-update 2014. Journal of cachexia, sarcopenia and muscle. 2014;5(4):261-3.
7. Fearon K, Strasser F, Anker SD, Bosaeus I, Bruera E, Fainsinger RL, et al. Definition and classification of cancer cachexia: an international consensus. Lancet Oncol. 2011;12(5):489-95.
8. Fearon K, Arends J, Baracos V. Understanding the mechanisms and treatment options in cancer cachexia. Nat Rev Clin Oncol. 2013;10(2):90-9.
9. Lodewick TM, van Nijnatten TJ, van Dam RM, van Mierlo K, Dello SA, Neumann UP, et al. Are sarcopenia, obesity and sarcopenic obesity predictive of outcome in patients with colorectal liver metastases? HPB : the official journal of the International Hepato Pancreato Biliary Association. 2015;17(5):438-46.
10. Miller J, Laird BJA, Skipworth RJE. The immunological regulation of cancer cachexia and its therapeutic implications. Journal of Cancer Metastasis and Treatment. 2019;5.
11. Bindels LB, Neyrinck AM, Claus SP, Le Roy CI, Grangette C, Pot B, et al. Synbiotic approach restores intestinal homeostasis and prolongs survival in leukaemic mice with cachexia. Isme j. 2016;10(6):1456-70.
12. Bindels LB, Neyrinck AM, Loumaye A, Catry E, Walgrave H, Cherbuy C, et al. Increased gut permeability in cancer cachexia: mechanisms and clinical relevance. Oncotarget. 2018;9(26):18224-38.
13. Bindels LB, Beck R, Schakman O, Martin JC, De Backer F, Sohet FM, et al. Restoring specific lactobacilli levels decreases inflammation and muscle atrophy markers in an acute leukemia mouse model. PLoS One. 2012;7(6):e37971.
14. Pötgens SA, Brossel H, Sboarina M, Catry E, Cani PD, Neyrinck AM, et al. Klebsiella oxytoca expands in cancer cachexia and acts as a gut pathobiont contributing to intestinal dysfunction. Sci Rep. 2018;8(1):12321.
15. Pekkala S, Keskitalo A, Kettunen E, Lensu S, Nykänen N, Kuopio T, et al. Blocking Activin Receptor Ligands Is Not Sufficient to Rescue Cancer-Associated Gut Microbiota—A Role for Gut Microbial Flagellin in Colorectal Cancer and Cachexia? Cancers. 2019;11(11):1799.
16. Jabes DL, de Maria YNLF, Aciole Barbosa D, Santos KBNH, Carvalho LM, Humberto AC, et al. Fungal Dysbiosis Correlates with the Development of Tumor-Induced Cachexia in Mice. J Fungi. 2020;6(4):364.
17. Sonnenburg JL, Bäckhed F. Diet-microbiota interactions as moderators of human metabolism. Nature. 2016;535(7610):56–64.
18. Penafuerte CA, Gagnon B, Sirois J, Murphy J, MacDonald N, Tremblay ML. Identification of neutrophil-derived proteases and angiotensin II as biomarkers of cancer cachexia. Br J Cancer. 2016;114(6):680-7.
19. Blander JM, Longman RS, Iliev ID, Sonnenberg GF, Artis D. Regulation of inflammation by microbiota interactions with the host. Nat Immunol. 2017;18(8):851-60.
20. Verdam FJ, Fuentes S, de Jonge C, Zoetendal EG, Erbil R, Greve JW, et al. Human intestinal microbiota composition is associated with local and systemic inflammation in obesity. Obesity (Silver Spring). 2013;21(12):E607-15.
21. Natividad JM, Verdu EF. Modulation of intestinal barrier by intestinal microbiota: pathological and therapeutic implications. Pharmacol Res. 2013;69(1):42-51.
22. Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. Cytokine. 2008;42(2):145-51.
23. Puppa MJ, White JP, Sato S, Cairns M, Baynes JW, Carson JA. Gut barrier dysfunction in the Apc(Min/+) mouse model of colon cancer cachexia. Biochim Biophys Acta. 2011;1812(12):1601-6.

24. Jiang Y, Guo C, Zhang D, Zhang J, Wang X, Geng C. The altered tight junctions: an important gateway of bacterial translocation in cachexia patients with advanced gastric cancer. J Interferon Cytokine Res. 2014;34(7):518-25.
25. van der Beek CM, Dejong CHC, Troost FJ, Masclee AAM, Lenaerts K. Role of short-chain fatty acids in colonic inflammation, carcinogenesis, and mucosal protection and healing. Nutr Rev. 2017;75(4):286-305.
26. Bach Knudsen KE, Lærke HN, Hedemann MS, Nielsen TS, Ingerslev AK, Gundelund Nielsen DS, et al. Impact of Diet-Modulated Butyrate Production on Intestinal Barrier Function and Inflammation. Nutrients. 2018;10(10).
27. Suzuki T, Yoshida S, Hara H. Physiological concentrations of short-chain fatty acids immediately suppress colonic epithelial permeability. Br J Nutr. 2008;100(2):297-305.
28. Neis EP, van Eijk HM, Lenaerts K, Olde Damink SW, Blaak EE, Dejong CH, et al. Distal versus proximal intestinal short-chain fatty acid release in man. Gut. 2019;68(4):764-5.
29. Corrêa-Oliveira R, Fachi JL, Vieira A, Sato FT, Vinolo MA. Regulation of immune cell function by short-chain fatty acids. Clin Transl Immunology. 2016;5(4):e73.
30. Segain JP, Raingeard de la Blétière D, Bourreille A, Leray V, Gervois N, Rosales C, et al. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. Gut. 2000;47(3):397-403.
31. Lee C, Kim BG, Kim JH, Chun J, Im JP, Kim JS. Sodium butyrate inhibits the NF-kappa B signaling pathway and histone deacetylation, and attenuates experimental colitis in an IL-10 independent manner. Int Immunopharmacol. 2017;51:47-56.
32. Schieber AMP, Lee YM, Chang MW, Leblanc M, Collins B, Downes M, et al. Disease tolerance mediated by microbiome E. coli involves inflammasome and IGF-1 signaling. Science. 2015;350(6260):558–63.
33. Bindels LB, Neyrinck AM, Salazar N, Taminiau B, Druart C, Muccioli GG, et al. Non Digestible Oligosaccharides Modulate the Gut Microbiota to Control the Development of Leukemia and Associated Cachexia in Mice. PLoS One. 2015;10(6):e0131009.
34. Nay K, Jollet M, Goustard B, Baati N, Vernus B, Pontones M, et al. Gut bacteria are critical for optimal muscle function: a potential link with glucose homeostasis. American Journal of Physiology-Endocrinology and Metabolism. 2019.
35. Houghton MJ, Kerimi A, Mouly V, Tumova S, Williamson G. Gut microbiome catabolites as novel modulators of muscle cell glucose metabolism. FASEB J. 2019;33(2):1887–98.
36. Honors MA, Kinzig KP. The role of insulin resistance in the development of muscle wasting during cancer cachexia. J Cachexia Sarcopenia Muscle. 2012;3(1):5-11.
37. Chen Y-M, Wei L, Chiu Y-S, Hsu Y-J, Tsai T-Y, Wang M-F, et al. Lactobacillus plantarum TWK10 Supplementation Improves Exercise Performance and Increases Muscle Mass in Mice. Nutrients. 2016;8(4):205.
38. Yan H, Diao H, Xiao Y, Li W, Yu B, He J, et al. Gut microbiota can transfer fiber characteristics and lipid metabolic profiles of skeletal muscle from pigs to germ-free mice. Sci Rep. 2016;6:31786.
39. Bäckhed F, Manchester JK, Semenkovich CF, Gordon JI. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci USA. 2007;104(3):979–84.
40. Neis EPJG, Dejong CHC, Rensen SS. The role of microbial amino acid metabolism in host metabolism. Nutrients. 2015;7(4):2930–46.
41. Scheithauer TP, Dallinga-Thie GM, de Vos WM, Nieuwdorp M, van Raalte DH. Causality of small and large intestinal microbiota in weight regulation and insulin resistance. Mol Metab. 2016;5(9):759-70.
42. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006;444(7122):1027-31.
43. Canfora EE, Jocken JW, Blaak EE. Short-chain fatty acids in control of body weight and insulin sensitivity. Nat Rev Endocrinol. 2015;11(10):577-91.
44. Vitek L, Haluzik M. The role of bile acids in metabolic regulation. J Endocrinol. 2016;228(3):R85-96.
45. Borgo F, Riva A, Benetti A, Casiraghi MC, Bertelli S, Garbossa S, et al. Microbiota in anorexia nervosa: The triangle between bacterial species, metabolites and psychological tests. PLoS One. 2017;12(6):e0179739.
46. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 2014;11(8):506-14.
47. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus

statement on the definition and scope of prebiotics. Nat Rev Gastroenterol Hepatol. 2017;14(8):491-502.
48. Swanson KS, Gibson GR, Hutkins R, Reimer RA, Reid G, Verbeke K, et al. The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of synbiotics. Nat Rev Gastroenterol Hepatol. 2020;17(11):687-701.
49. Varian BJ, Gourishetti S, Poutahidis T, Lakritz JR, Levkovich T, Kwok C, et al. Beneficial bacteria inhibit cachexia. Oncotarget. 2016;7(11):11803-16.
50. Jäger R, Shields KA, Lowery RP, De Souza EO, Partl JM, Hollmer C, et al. Probiotic Bacillus coagulans GBI-30, 6086 reduces exercise-induced muscle damage and increases recovery. PeerJ. 2016;4:e2276.
51. Obermüller B, Singer G, Kienesberger B, Klymiuk I, Sperl D, Stadlbauer V, et al. The Effects of Prebiotic Supplementation with OMNi-LOGiC® FIBRE on Fecal Microbiome, Fecal Volatile Organic Compounds, and Gut Permeability in Murine Neuroblastoma-Induced Tumor-Associated Cachexia. Nutrients. 2020;12(7):2029.
52. Helmink BA, Khan MAW, Hermann A, Gopalakrishnan V, Wargo JA. The microbiome, cancer, and cancer therapy. Nat Med. 2019;25(3):377–88.
53. MS-20 on Gut Microbiota and Risk/Severity of Cachexia in Pancreatic Cancer Patients - Full Text View - ClinicalTrials.gov. 2021.
54. Investigation of the Gut Microbiota in Patients With Acute Myeloid Leukemia - Full Text View - ClinicalTrials.gov. 2021.
55. Ubachs J, Ziemons J, van Eijk H, Kruitwagen R, Van Gorp T, Penders J, et al. Abstracts of the 11th International Conference on Cachexia, Sarcopenia and Muscle Wasting, Maastricht, The Netherlands, 7–9 December 2018. Abstract 2-25: Wiley-Blackwell; 2018. p. 1121.
56. Aarnoutse R, de Vos-Geelen J, Penders J, Boerma EG, Warmerdam F, Goorts B, et al. Study protocol on the role of intestinal microbiota in colorectal cancer treatment: a pathway to personalized medicine 2.0. Int J Colorectal Dis. 2017;32(7):1077-84.
57. Kim EY, Lee HY, Kim YS, Park I, Ahn HK, Cho EK, et al. Prognostic significance of cachexia score assessed by CT in male patients with small cell lung cancer. Eur J Cancer Care. 2018;27(1):.
58. Martin L, Birdsell L, MacDonald N, Reiman T, Clandinin MT, McCargar LJ, et al. Cancer Cachexia in the Age of Obesity: Skeletal Muscle Depletion Is a Powerful Prognostic Factor, Independent of Body Mass Index. J Clin Oncol. 2013;31(12):1539–47.
59. Tilg H, Adolph TE, Gerner RR, Moschen AR. The Intestinal Microbiota in Colorectal Cancer. Cancer Cell. 2018;33(6):954-64.
60. Dias-Jácome E, Libânio D, Borges-Canha M, Galaghar A, Pimentel-Nunes P. Gastric microbiota and carcinogenesis: the role of non-Helicobacter pylori bacteria - A systematic review. Rev Esp Enferm Dig. 2016;108(9):530-40.
61. Aarnoutse R, Ziemons J, Penders J, Rensen SS, de Vos-Geelen J, Smidt ML. The Clinical Link between Human Intestinal Microbiota and Systemic Cancer Therapy. Int J Mol Sci. 2019;20(17).
62. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature. 2012;489(7415):220–30.

Chapter 8

Gut microbiota and short-chain fatty acid alterations in cachectic cancer patients

Jorne Ubachs*, **Janine Ziemons***, Zita Soons, Romy Aarnoutse, David P. J. van Dijk, John Penders, Ardy van Helvoort, Marjolein L. Smidt, Roy F.P.M. Kruitwagen, Lieke Baade-Corpelijn, Steven W.M. Olde Damink, Sander S. Rensen

** Shared first authorship*

Journal of Cachexia Sarcopenia Muscle. 2021 Dec;12(6):2007-2021.
DOI: 10.1002/jcsm.12804

Abstract

Background

Cancer cachexia is characterized by a negative energy balance, muscle and adipose tissue wasting, insulin resistance, and systemic inflammation. Due to its strong negative impact on prognosis and its multifactorial nature that is still not fully understood, cachexia remains an important challenge in the field of cancer treatment. Recent animal studies indicate that the gut microbiota is involved in the pathogenesis and manifestation of cancer cachexia, but human data are lacking. The present study investigates gut microbiota composition, short chain fatty acids (SCFA), and inflammatory parameters in human cancer cachexia.

Methods

Fecal samples were prospectively collected in patients (nN=107) with pancreatic cancer, lung cancer, breast cancer, or ovarian cancer. Household partners (nN=76) of the patients were included as healthy controls with similar diet and environmental conditions. Patients were classified as cachectic if they lost >5% body weight in the last six months. Gut microbiota composition was analyzed by sequencing of the 16S rRNA V4 gene-region. Fecal SCFA levels were quantified by gas chromatography. Fecal calprotectin was assessed with ELISA. Serum CRP and leukocyte counts were retrieved from medical records.

Results

Cachexia prevalence was highest in pancreatic cancer (66.7%), followed by ovarian cancer (25%), lung cancer (20.8%), and breast cancer (17.3%). Microbial α-diversity was not significantly different between cachectic cancer patients (N=33), non-cachectic cancer patients (N=74), or healthy controls (N=76) (species richness p=0.31, Shannon effective index p=0.46). Community structure (β-diversity) tended to differ between these groups (p=0.053), although overall differences were subtle and no clear clustering of samples was observed. Proteobacteria (p<0.001), an unknown genus from the Enterobacteriaceae family (p<0.01), and *Veillonella* (p<0.001) were more abundant among cachectic cancer patients. *Megamonas* (p<0.05) and *Peptococcus* (p<0.001) also showed differential abundance. Fecal levels of all SCFA tended to be lower in cachectic cancer patients, but only acetate concentrations were significantly reduced (p<0.05). Fecal calprotectin levels were positively correlated with the abundance of *Peptococcus*, unknown Enterobacteriaceae, and *Veillonella*. We also identified several correlations and interactions between clinical and microbial parameters.

Conclusions

This clinical study provided the first insights into the alterations of gut microbiota composition and SCFA levels that occur in cachectic cancer patients, and how they are related to inflammatory parameters. These results pave the way for further research examining the role of the gut microbiota in cancer cachexia and its potential use as therapeutic target.

Introduction

Cancer-induced cachexia is one of the greatest challenges in the field of cancer treatment. This metabolic syndrome, affecting 50-80% of all cancer patients depending on the tumor type, has severe negative consequences for physical functioning, quality of life, and survival (1, 2). Cancer cachexia has a multifactorial background and is characterized by an ongoing loss of skeletal muscle mass that cannot be fully reversed by conventional nutritional support. Its pathophysiology is characterized by a negative protein and energy balance driven by a combination of reduced food intake and abnormal metabolism (1, 3).

During the last decade, it has been shown that crosstalk between commensal bacteria and the human host is essential for the maintenance of homeostasis. More specifically, the gut microbiota has been demonstrated to modulate energy harvest from the diet, systemic inflammation, gut barrier function, and insulin sensitivity, which are metabolic features found to be altered in cancer cachexia (4, 5). In light of these findings, it is not surprising that recent animal data indicate that the gut microbiota might be involved in the pathogenesis of cancer cachexia (6, 7). For example, Bindels *et al.* repeatedly demonstrated that cachexia was associated with profound changes in gut microbiota composition and diversity in mouse models of leukemia and colon cancer (5, 8, 9). Importantly, different approaches to modulate the intestinal microbiota have been shown to affect experimental cancer cachexia. *Lactobacillus* supplementation successfully reduced pro-inflammatory cytokine levels and muscle atrophy in mice (9, 10). Similarly, a synbiotic approach consisting of inulin-type fructans and *Lactobacillus reuteri* was able to counteract microbial aberrations associated with cancer cachexia and improved gut barrier integrity as well as immune function (8). In addition, this synbiotic intervention reduced muscle wasting and prolonged survival in tumor-bearing mice (8). Other multi-nutrient interventions including amongst others prebiotic oligosaccharides have also been shown to diminish features of cancer cachexia in mice (11).

Mechanistically, the gut microbiota has been shown to influence muscle metabolism by modulating amino acid availability and through the impact of microbial metabolites on glucose metabolism and muscle glycogen availability (7). Important microbial metabolites exerting systemic effects are bile acids, branched chain fatty acids (BCFA), and short chain fatty acids (SCFA), which are produced by macronutrient degradation and released into the blood (12). The SCFA acetate, propionate, butyrate, and valerate are generated by microbial fermentation of dietary fibers and are closely linked to metabolic health (13). Butyrate is particularly important for the maintenance of the intestinal epithelium since it serves as fuel for colonocytes and because it promotes expression of tight junction proteins that fortify the epithelial barrier (13). Furthermore, SCFA have potent anti-inflammatory and immune-modulatory effects by activating G-protein coupled cell surface receptors and by inhibiting the action of histone deacetylases (14).

Despite the promising pre-clinical data mentioned above, human studies addressing the potential relationship between the gut microbiota and cancer cachexia are currently lacking. We present the first clinical study that explores the gut microbiota, SCFA, and inflammatory parameters in cachectic cancer patients.

Methods

Study population

For this cross-sectional case-control study, patients with pancreatic cancer, lung cancer, breast cancer, or ovarian cancer were recruited via the outpatient clinics of the Maastricht University Medical Centre (MUMC+) between April 2016 and May 2019. Patients were included at the time of diagnosis, before start of systemic therapy or surgery. By enrolling patients with different tumor types, we were able to study cancers with a generally high prevalence of cachexia (pancreas/lung/ovarian) versus those with a low prevalence (breast), as well as gastro-intestinal (pancreas) versus non-gastro-intestinal (lung/breast/ovarian) cancers. In order to be eligible, patients had to be older than 18 years and should have a recent cancer diagnosis based on radiology, pathology, or cytology. Exclusion criteria were the use of systemic glucocorticoids <4 weeks before inclusion or antibiotics <3 months before inclusion as well as chemotherapy or radiotherapy before sampling. Patients with an additional distinct cancer type, except for basocellular carcinoma of the skin, were excluded. Partners or relatives >18 years and sharing a household with the patient were included as healthy controls. They were included on the assumption to have similar diet- and lifestyle habits, thereby partly controlling for environmental effects on the gut microbiota. Exclusion criteria were the same as for patients. In total, 107 cancer patients and 76 healthy controls were included. The study was approved by the local medical ethics committee of the MUMC under number 15-4-022 and was conducted according to the Declaration of Helsinki, and its revisions. All participants and enrolled partners gave written informed consent.

Collection of stool samples and clinical data

Stool samples were collected at one timepoint before treatment. Subjects received a plastic container *(SKL, the Netherlands)* as well as a stool collection device *(Tag Hemi VOF, the Netherlands)* to ensure hygienic sampling. Prior to a hospital visit, samples were collected at home by the participants and stored in the refrigerator. Upon arrival in the hospital, samples were aliquoted in 2ml screw cap tubes and stored at -80°C until further analysis.
In addition, clinical information concerning sex, age, BMI, and weight loss in the past six months was collected by means of a questionnaire. Patients were subsequently classified as cachectic (>5% weight loss in the last six months) or non-cachectic (≤%5 weight loss in the last six months). BMI-adjusted weight loss was categorized using the grading system as described by Martin *et al.* (15). In short, they identified five distinct grades (0-4) of BMI-adjusted weight loss, which were associated with significantly different survival (grade 0: longest survival; grade 4: shortest survival). Additional laboratory parameters (C-reactive protein (CRP), leukocyte counts) were assessed in the context of routine care and were retrieved from the patient's medical records, if available (CRP: N=35, 19 cachectic and 16 non-cachectic; leukocyte counts: N=51, 21 cachectic and 30 non-cachectic).

Fecal microbiota analysis

Metagenomic DNA from fecal samples was extracted by a combination of repeated bead-beating and column-based DNA purification using protocol Q of the International Human

Microbiome Standards (IHMS) consortium (16). In short, 200mg of frozen feces were homogenized with 1.0ml ASL lysis buffer *(Qiagen, Germany)* in 2ml tubes containing 0.3g of Ø 0.1mm sterile zirconia beads *(BioSpec, USA)*. Cell lysis was obtained by incubation at 95°C and repeated mechanical disruption using the Fastprep Homogenizer (*MP Biomedicals, USA*). Subsequently, DNA isolation was performed using the QIAamp DNA Stool kit according to the IHMS protocol. DNA was eluted in a final volume of 200µl and DNA concentration was measured using a spectrophotometer *(DeNovix, USA)*. Generation of amplicon libraries and sequencing was performed as previously described (17). Briefly, the V4 hypervariable region of the 16S rRNA gene was PCR amplified from each DNA sample in duplicate. Pooled amplicons from the duplicate reactions were purified using AMPure XP purification *(Agencourt, Massachusetts, USA)* according to the manufacturer's instructions and quantified by Quant-iT PicoGreen dsDNA reagent kit *(Invitrogen, USA)*. Amplicons were mixed in equimolar concentrations to ensure equal representation of each sample and sequenced on an Illumina MiSeq instrument using the V3 reagent kit.

Analysis of fecal SCFA and BCFA concentrations

Fecal levels of SCFA (acetate, propionate, butyrate, valerate) and BCFA (iso-butyrate, iso-valerate) were assessed in a subgroup of 165 participants of whom sufficient fecal material was available. This subgroup consisted of 94 cancer patients (30 cachectic and 64 non-cachectic) and 71 healthy controls. Within the group of cancer patients, there were 40 patients with breast cancer, 30 with lung cancer, 21 with pancreatic cancer and 3 patients with ovarian cancer.

Fecal levels of SCFA and BCFA were quantified by direct-injection gas chromatography using a Shimadzu GC2025 gas chromatograph *(Shimadzu Corporation, Kyoto, Japan)* equipped with a flame ionization detector (18, 19). Samples were prepared based on an established protocol (20) (see supplementary methods for a detailed description). SCFA and BCFA levels were corrected for dry weight. For this purpose, 500mg of frozen feces were dried in a vacuum dryer *(Eppendorff, Germany)* for five hours.

Assessment of fecal calprotectin

Fecal calprotectin levels were assessed in a subgroup of 168 individuals of whom sufficient fecal material was available, amongst which 30 were cachectic cancer patients, 68 non-cachectic cancer patients, and 70 healthy controls. We excluded one non-cachectic lung cancer patient from further analysis, since the calprotectin value (829,0 µg/g) was more than ten-fold higher compared to the rest of the population, without any clinical explanation.

100mg feces was weighed into a 15ml tube and 4.9ml extraction buffer (0.1 M Tris, 0.15 M NaCl, 1.0 M urea, 10 mM $CaCl_2 \cdot 2H_2O$, 0.1 M citric acid, 0.5% bovine serum albumin, pH 8.0) was added (21). After 90 minutes of mixing, 1ml of suspension was centrifuged at 10,000g for five minutes at 4°C, and 700µl supernatant was transferred into a fresh tube and stored at −80°C. Calprotectin concentrations were measured using a commercially available human fecal calprotectin enzyme linked immunosorbent assay (ELISA) (lower detection limit 2.56 µg/g) *(Hycult Biotech, the Netherlands)*. Fecal calprotectin concentrations are expressed in micrograms of calprotectin per gram of feces.

Statistical analysis of gut microbiota data

Please consult the supplementary methods for a more detailed description of the data analysis.

Preprocessing

Data demultiplexing, quality and length filtering, merging of paired reads, and clustering into Operational Taxonomic Units (OTUs) at 97% sequence identity was performed using the Integrated Microbial Next Generation Sequencing platform (IMNGS, www.imngs.org) (22). All downstream analyses were conducted in the R statistical computing environment (version 4.0.2) (23).

Microbial richness, diversity and community structure

Selection of an appropriate method and subsequent normalization of OTU count tables was done as previously described (24), using variant stabilization by the R-package DESeq2 (25). Calculation of alpha diversity indices (Observed species richness, Shannon effective index) and beta-diversity (generalized UniFrac) was performed using the Rhea pipeline (26).

Bacterial abundances

To examine potential differences in the relative abundance of bacterial genera between cachectic cancer patients, non-cachectic cancer patients, and controls, all OTUs were combined that were taxonomically assigned to the same genera or phyla. 111 different genera were detected in the dataset. After filtering for a prevalence threshold of at least ten counts in at least one sample and a presence threshold (one count) in at least ten samples, 94 genera were obtained. Count-tables were normalized using variant stabilization by DESeq2 (25). We used size factor correction to account for differences in sequencing depth between the samples. DESeq2 was also applied to test for differential abundance of genera and phyla. First, all groups were analyzed at once in a likelihood ratio test (LRT). Further pairwise comparisons were done using a Wald-test to identify genera or phyla that showed changes in abundance across the specific groups. Results are reported as log2 fold changes and associated adjusted p-values of the LRT (BH-correction for the number of taxa and in addition the number of groups for the Wald-test). Dendrograms were obtained by hierarchical clustering using Ward's method where 1-Pearson's correlation was used as the distance measure. Composition plots were obtained by transforming the normalized phyla abundances to relative data and next plotting the mean relative abundances per groups using the R package ggplot2 (geom_bar). Correlation analysis was performed using the R-package PerformanceAnalytics (27). Differential co-occurrence networks were estimated using the R-package MDiNE for 1000 Monte Carlo iterations (28).

Statistical analysis of clinical data, SCFA levels, and inflammatory parameters

Statistical analysis was performed using R version 4.0.0 (23). Depending on whether variables were normally distributed or not, means (±SD) or medians (±IQR) are reported and two-sided Mann Whitney U, one-way ANOVA, or the Kruskal Wallis test was applied to assess differences between groups. If the Kruskal-Wallis test revealed significant differences, Dunn’s multiple comparison test was used for post-hoc analysis and p-values were adjusted with the Benjamini-Hochberg method (29). To investigate correlations between different variables,

Kendall's tau was used. For assessing potential differences in the distribution of cachectic vs. non-cachectic patients over disease stages, a Chi square test was performed. P-values <0.05 were considered statistically significant.

Results

Baseline characteristics of the study population and prevalence of cancer cachexia

In total, 107 cancer patients and 76 healthy controls participated in the study. Twenty-seven patients were diagnosed with pancreatic cancer, 52 patients with breast cancer, 24 patients with lung cancer and four patients with ovarian cancer. The majority of patients with breast cancer (96%) or lung cancer (46%) had local disease, whereas the majority of pancreatic cancer patients had lymph nodes involved (59%) or metastatic disease (14%) (Table S2a).
Thirty-three patients (30.8%) had >5% weight loss in the past six months and were classified as cachectic. The prevalence of cancer cachexia varied per cancer type, with the highest prevalence in pancreatic cancer (66.7%), followed by ovarian cancer (25%), lung cancer (20.8%), and breast cancer (17.3%). In cachectic as well as non-cachectic cancer patients, most patients had local disease (48% in cachectic and 74% in non-cachectic patients). Lymph node involvement and metastatic disease were more common in the cachectic group, although the distribution of cachectic and non-cachectic patients over the different disease stages did not differ significantly (Table S2b).
While age (p=0.287) and BMI (p=0.055) were not significantly different, weight loss during the past six months differed markedly between groups (p<0.001) and was highest in cachectic cancer patients (8.0±3.0% vs. 0.0±1.7% for non-cachectic patients, Table 1).

Table 1: *Clinical characteristics of the study population. Variables with a normal distribution are presented as mean ± SD, variables which were not normally distributed are presented as median ± IQR.*

	Cachectic (N=33)	**Non-cachectic (N=74)**	**Healthy controls (N=76)**
Age (years)			
mean ± SD	65.3 (±12.1)	61.9 (±10.4)	62.9 (±9.4)
BMI (kg/m^2)			
median IQR	24.3 (±4.8)	25.5 (±5.1)	26.5 (±4.1)
Weight loss (%)			
median ± IQR	8.0 (±3.0)	0.0 (±1.7)	0.0 (±0.0)
Females	N=20 (60.6%)	N=61 (82.4%)	N=22 (28.9%)
Cancer type			
- Pancreatic cancer	N=18 (54.5%	N=9 (12.2%)	
- Breast cancer	N=9 (27.3%))	N=43 (58.1%)	
- Lung cancer	N=5 (15.2%)	N=19 (25.7%)	
- Ovarian cancer	N=1 (3.0%)	N=3 (4.1%)	

Similar microbial diversity and community structure in cachectic and non-cachectic patients

First, we determined α-diversity, reflecting the within-sample taxonomic diversity. The observed species richness as well as the Shannon effective index, both measures of microbial α-diversity, were not significantly different in cachectic cancer patients versus non-cachectic cancer patients or healthy controls (Figure 1).

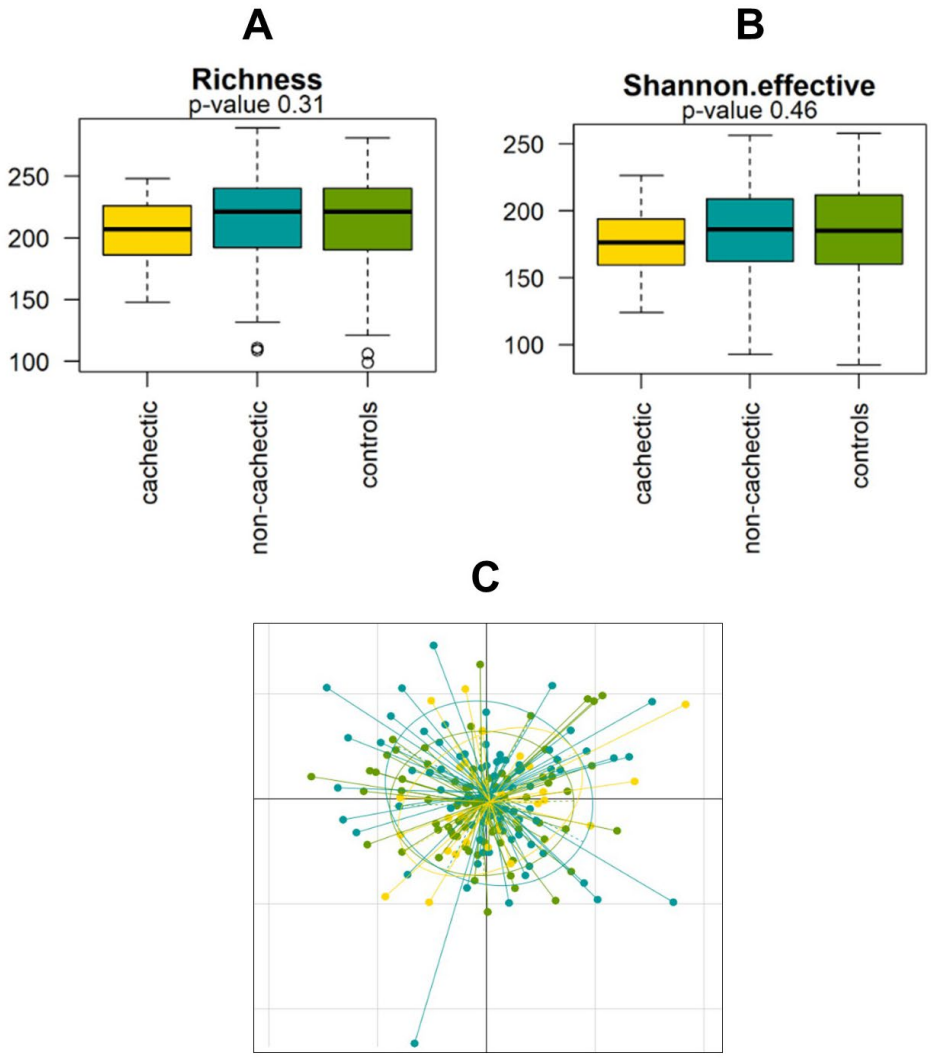


Figure 1: *Microbial richness and diversity in cachectic cancer patients (yellow, N=33), non-cachectic cancer patients (blue, N=74), and healthy control subjects (green, N=76). A: Observed species richness and B: Shannon effective index, both indices of α-diversity, were similar between the groups, C: The NMDS plot showed no clear clustering of samples from cachectic cancer patients, non-cachectic cancer patients, or healthy controls.*

Next, (dis)similarities in microbial community structure (β-diversity) using generalized Unifrac distances were assessed. Whereas PERMANOVA revealed borderline significant differences in microbial community structure (p=0.053), the NMDS plot demonstrated that these differences were subtle and no clear clustering of samples was apparent (Figure 1C). Dendrograms also showed no distinct clustering patterns based on cachexia status or BMI, and revealed high inter-individual variability in all study groups (Figure S1).

Distinct gut microbiota composition in cachectic cancer patients

Firmicutes were the most abundant bacterial phylum in all groups, followed by Bacteroidetes, Actinobacteria, and Proteobacteria (Figure 2A). Bacteria belonging to the phylum of Proteobacteria were significantly more abundant in cachectic cancer patients (median log2 abundance=9.5, IQR=3.0) when compared to non-cachectic patients (median log2 abundance=9.0, IQR=1.2) and healthy controls (median log2 abundance=8.8, IQR=1.2) (p<0.001) (Figure 2B).

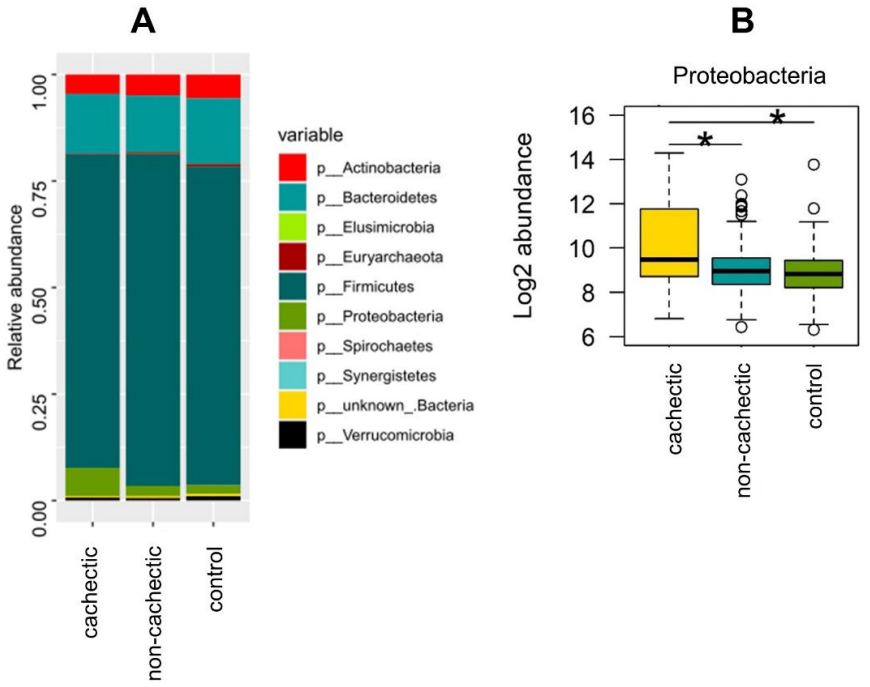


Figure 2: Microbiota composition on phylum level. A: *Relative abundances of all phyla present in the study population,* ***B:*** *Log2 abundance of Proteobacteria, statistically significant differences according to the Wald test (α=0.05) are marked with asterisks. Proteobacteria were significantly elevated in cachectic cancer patients compared to non-cachectic cancer patients and healthy controls.*

On the genus level, the abundances of *Megamonas, Peptococcus, Veillonella,* and an unknown genus from the Enterobacteriaceae family were found to be significantly different in

cachectic cancer patients (Figure 3). For *Megamonas* and *Peptococcus*, all medians were zero. While these genera were present in only few samples within the cachectic group, they were more often detected in the non-cachectic and control groups (*Megamonas*: $p<0.05$, *Peptococcus*: $p<0.001$). With a median log2 abundance of 5.4 (IQR=10.0), unknown Enterobacteriaceae were much more abundant in cachectic cancer patients compared to non-cachectic cancer patients (median=0.69, IQR=2.1) and healthy controls (median=0.73, IQR=1.8) ($p<0.01$). Similarly, log2 abundance of *Veillonella* was highest in cachectic cancer patients (median=3.2, IQR=3.5) and significantly lower in non-cachectic cancer patients (median=2.3, IQR=2.7) and healthy controls (median=1.7, IQR=3.4) ($p<0.001$). No significant differences were found between non-cachectic cancer patients and healthy controls.
Since >5% weight loss might have different clinical relevance in underweight, normal weight, or overweight individuals, we also analyzed differential genera abundance in different categories of BMI adjusted weight loss, yielding similar results (Figure S2).

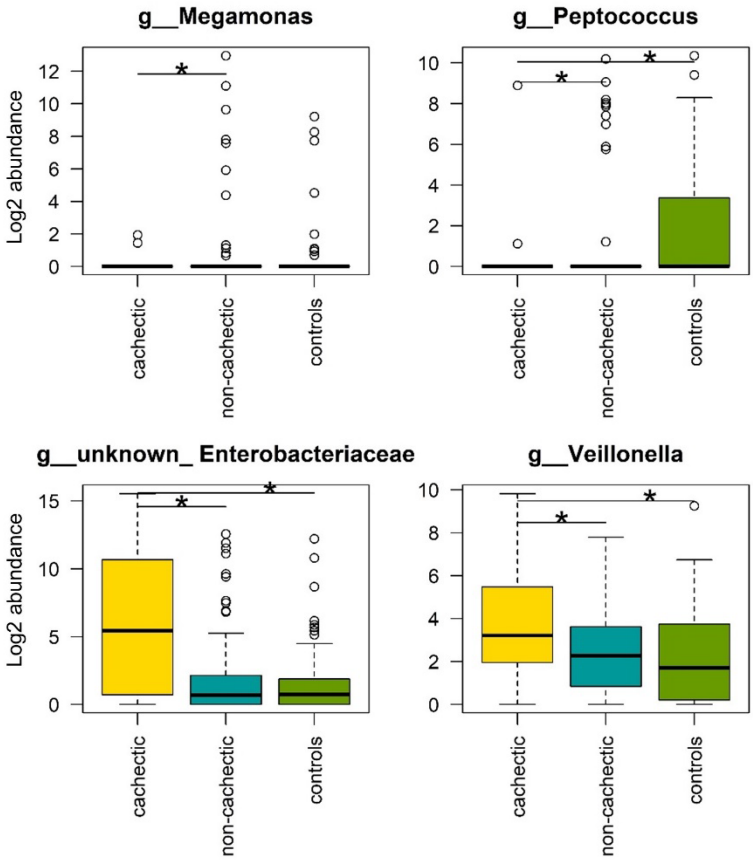


Figure 3: *Genera with altered abundance in cachectic versus non-cachectic cancer patients and/or healthy controls. The log2 abundance of genera which differed significantly between the groups is depicted. Statistically significant differences according to the Wald test (α=0.05) are marked with asterisks.*

Additionally, we analyzed differential abundances in pancreatic cancer and lung cancer separately, since cancer cachexia was most prevalent in these two cancer types. These analyses revealed that *Enterococcus*, *Lactobacillus*, unknown Enterobacteriaceae, and *Veillonella* showed differential abundance between cachectic cancer patients, non-cachectic cancer patients, and healthy controls (Figure S3).

Lower fecal acetate levels in cachectic cancer patients

Total fecal SCFA concentrations tended to be lower in cachectic cancer patients (median=38.6mM/g, IQR=27.0mM/g) compared to non-cachectic cancer patients (median=48.8mM/g, IQR=56.1mM/g) and healthy controls (median=52.1mM/g, IQR=51.4mM/g) (p=0.08, Figure 4). The same pattern, with a tendency towards lower levels in the cachectic group, could also be observed when analyzing the different SCFA separately (Figure 4).

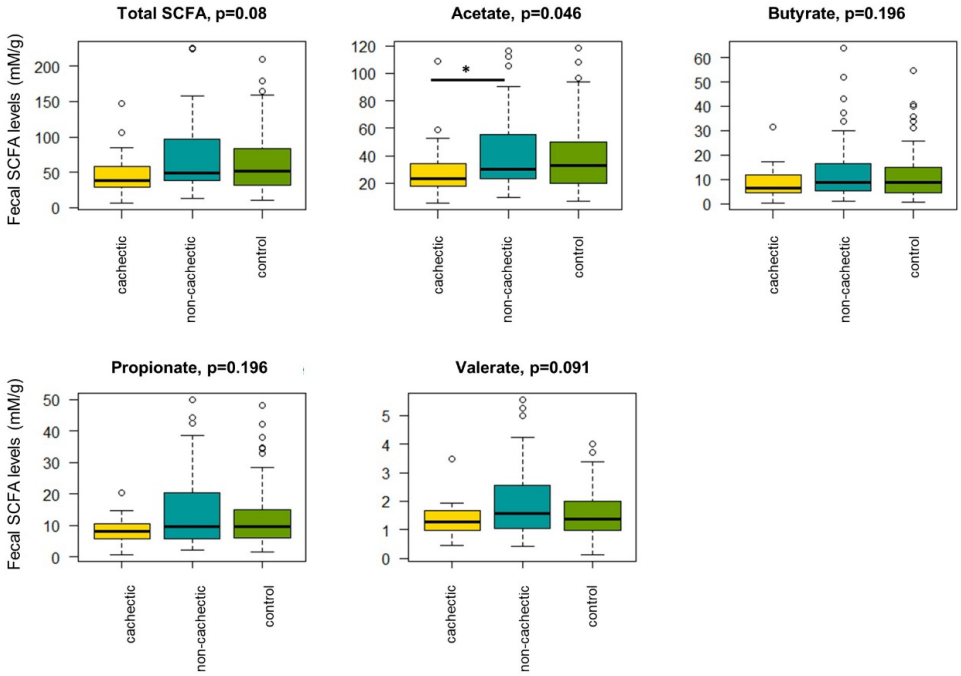


Figure 4: *Fecal levels of total SCFA and acetate, butyrate, propionate, and valerate separately. Acetate levels were found to be reduced in cachectic cancer patients (N=30) compared to non-cachectic cancer patients (N=64) and healthy controls (N=71). P-values from Kruskal Wallis test are shown.*

Acetate concentrations were significantly lower in cachectic cancer patients ($p<0.05$). Post-hoc analysis with Benjamini-Hochberg correction revealed a significant difference between cachectic and non-cachectic cancer patients ($p=0<0.05$), but not between cachectic cancer patients and healthy controls ($p=0.059$) or non-cachectic cancer patients and healthy controls ($p=0.62$). Fecal concentrations of propionate, butyrate, and valerate were consistently, but not

significantly lower in cachectic versus non-cachectic cancer patients or healthy controls (Figure 4).
We also compared the groups of cachectic cancer patients, non-cachectic cancer patients, and healthy controls in pancreatic cancer and lung cancer separately, since cancer cachexia was most prevalent in these cancer types. Interestingly, acetate concentrations were only significantly lower within the group of pancreatic cancer patients, while there were no differences in patients with lung cancer. In addition, total fecal SCFA and butyrate concentrations tended to be reduced in cachectic pancreatic cancer patients (Figure S4).
Fecal levels of the BCFA iso-butyrate (p=0.608) and iso-valerate (p=0.543) were similar in cachectic and non-cachectic cancer patients and healthy controls (Figure S5).

Similar fecal calprotectin levels in cachectic and non-cachectic cancer patients

Fecal levels of calprotectin, a marker of intestinal inflammation, were not significantly elevated in cachectic cancer patients (median=51.6µg/g, IQR=121.2), compared to non-cachectic cancer patients (median=32.1µg/g, IQR=37.5) and healthy controls (median=33.5µg/g, IQR=52.1) (p=0.2, Figure 5).
CRP levels (p=0.32, N=35) and leukocyte counts (p=0.66, N=51) were also not significantly different between cachectic and non-cachectic cancer patients (CRP: median=7.0mg/l, IQR=14.5 in cachectic cancer patients vs. median=5.0mg/l, IQR=9.5 in non-cachectic cancer patients; leukocyte counts: median=7.7x10^9/l, IQR=3.4 in cachectic cancer patients vs. median=7.9x10^9/l, IQR=3.4 in non-cachectic cancer patients).
While there was a strong positive correlation between CRP and leukocyte counts (τ=0.52, p<0.001), there were no associations between fecal calprotectin and CRP (τ=0.14, p=0.3) or fecal calprotectin and leukocyte counts, respectively (τ=0.05, p=0.6). SCFA levels were also not associated with any of these inflammatory parameters (Table S1).

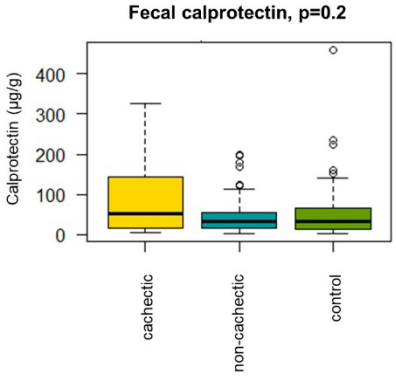


Figure 5: *Fecal levels of calprotectin were not different in cachectic cancer patients (N=30) compared to non-cachectic cancer patients (N=68) or healthy controls (N=70).*

Correlations and co-occurrences between bacterial taxa, SCFA, calprotectin, and clinical parameters

Next, we performed correlation analysis using the parameters that were found to be significantly different in cachectic versus non-cachectic cancer patients according to the previous analyses (Figure 6). We also included fecal calprotectin since we were interested in associations between fecal calprotectin and abundance of specific bacterial taxa. BMI was included as it was almost significant (p=0.055).

We found that fecal acetate concentrations were positively correlated with BMI and negatively correlated with weight loss. In addition, fecal acetate was negatively associated with the abundance of *Peptococcus* and unknown Enterobacteriaceae (Figure 6). Fecal calprotectin levels were positively correlated with the abundance of *Peptococcus*, unknown Enterobacteriaceae, and *Veillonella.* Furthermore, we identified strong positive correlations between weight loss and unknown Enterobacteriaceae and *Veillonella,* respectively. Besides, there was a positive correlation between the abundance of unknown Enterobacteriaceae and *Veillonella (*Figure 6*).*

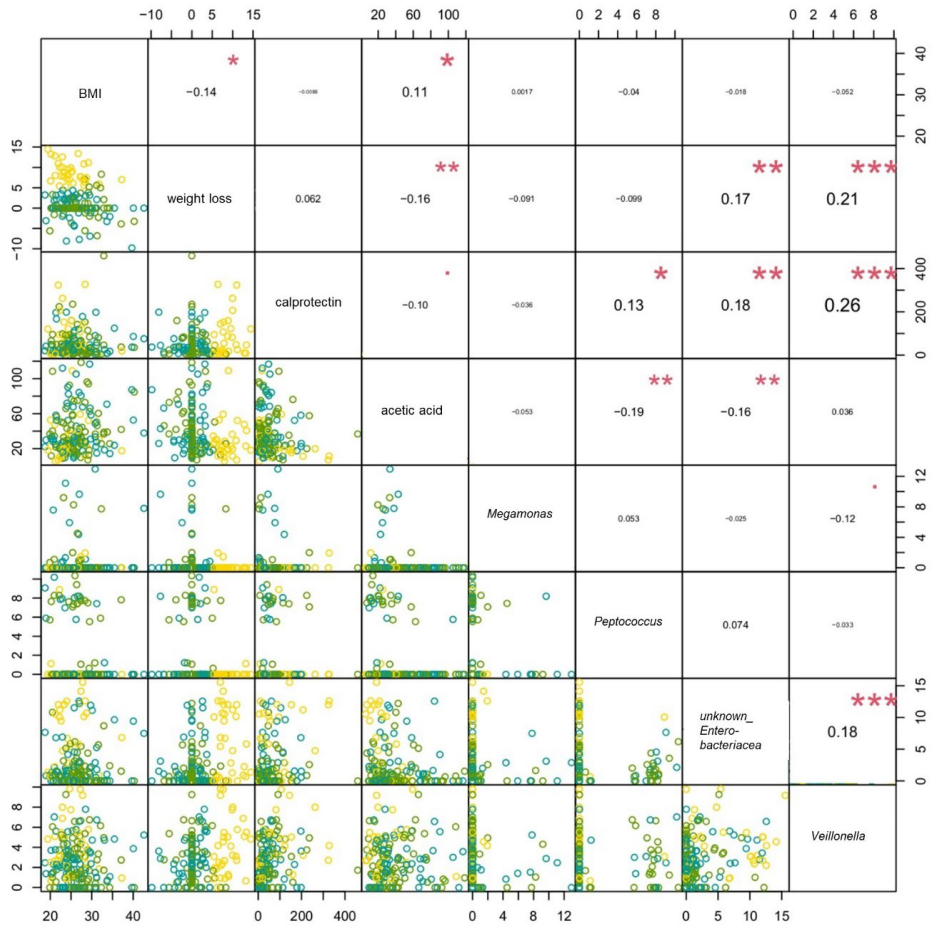


Figure 6: *Correlation analysis between the significant variables from differential analyses of bacterial taxa and SCFA as well as relevant clinical parameters in a pairwise comparison. Factors under investigation are depicted in the diagonal line. The relationships of abundances of four bacterial taxa, acetic acid, calprotectin, BMI, and weight loss were estimated using Kendall's tau correlation coefficients (τ). In the upper panels, significant correlations are indicated with asterisks (*** = p<0.001, ** = p<0.01, * = p<0.05). In the lower panels, scatterplots of pairwise correlations are shown. Yellow dots represent cachectic cancer patients, blue dots depict non-cachectic cancer patients, and green dots indicate healthy controls.*

Estimated co-occurrences confirmed the interaction between unknown Enterobacteriaceae and *Veillonella*, but showed that the direction of the interaction depended on the group. Within the group of cachectic cancer patients, there was a negative association, potentially indicating competition between these bacteria. While there was a positive interaction in the group of non-cachectic cancer patients, there was no interaction in healthy controls (Figure 7).

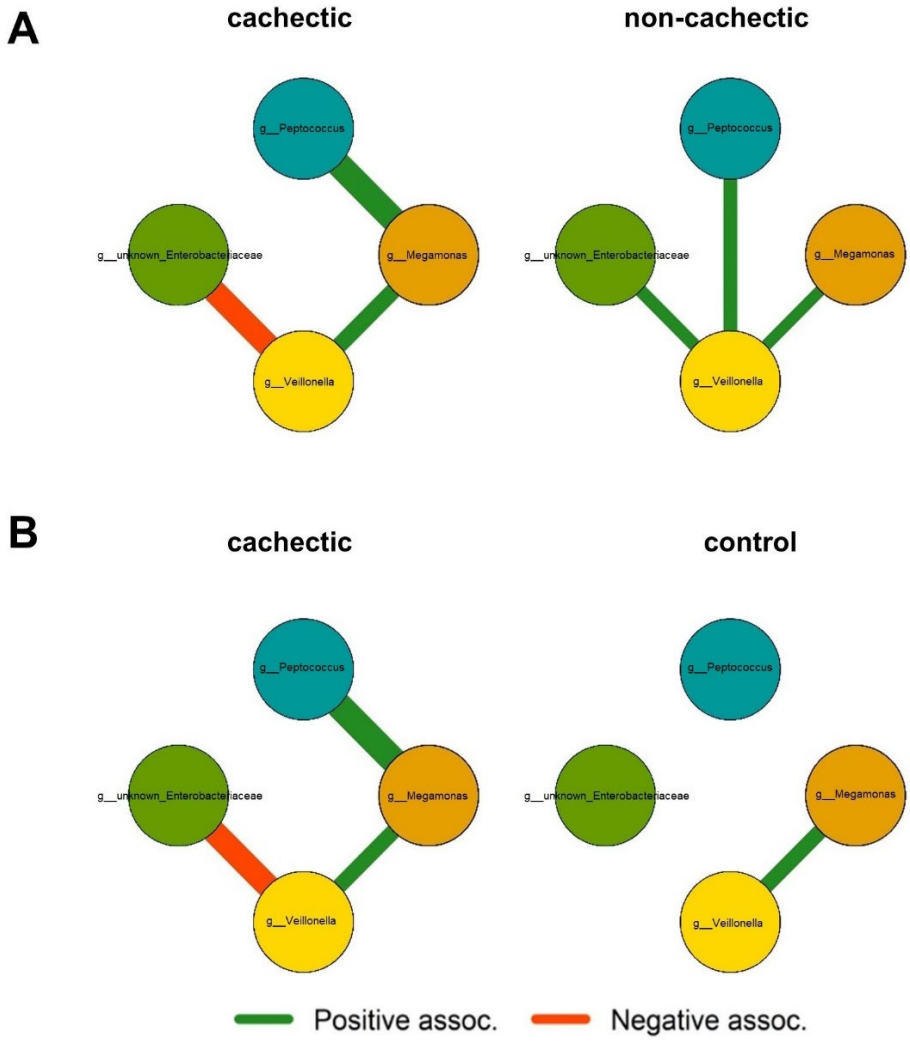


***Figure 7**: Estimated co-occurrence of Megamonas, Peptococcus, Veillonella, and an unknown genus from the Enterobacteriaceae family in cachectic cancer patients vs. non-cachectic cancer patients (A) and cachectic cancer patients vs. healthy controls (B). Green lines represent a positive association, while orange marks a negative association between these genera. An edge is displayed if the 90% credibility interval does not contain zero.*

Discussion

This study is the first to provide insights into gut microbiota composition, SCFA levels, and their relationship with inflammatory parameters in the context of human cancer cachexia. We found that overall microbial diversity (α-diversity) and community structure (β-diversity) were not altered in patients with cancer cachexia. However, Proteobacteria, an unknown genus from the Enterobacteriaceae family, and *Veillonella* were found to be more abundant among cachectic cancer patients, and these genera were strongly positively correlated with weight loss. Conversely, the genera *Megamonas* and *Peptococcus* showed higher abundance in non-cachectic patients. In addition, fecal acetate levels were lower in cachectic cancer patients. Whereas fecal calprotectin, a marker of intestinal inflammation, correlated strongly with the abundance of specific gut bacteria, it was not significantly elevated in cachectic cancer patients.

Based on pre-clinical data showing reduced microbial diversity and altered community structure in cachectic mice (8, 30), we expected that α- and β-diversity would be reduced in cachectic cancer patients. Furthermore, a species-rich and diverse microbial community is generally considered to be more healthy and decreased microbial diversity has been linked to several disease states (31). However, we found no differences in overall microbial diversity and no community pattern that could be linked to the cachectic phenotype in our study population.

Nevertheless, differential abundances of certain bacterial taxa were observed in cachectic cancer patients compared to non-cachectic cancer patients and healthy controls, that corroborate previously reported animal data. Specifically, in line with results from Pötgens *et al.* (2018), who described an expansion of Proteobacteria at the expense of Firmicutes in cachectic C26 mice (32), the abundance of Proteobacteria was found to be increased in cachectic cancer patients in the current study. In addition, previous studies repeatedly described an increased abundance of Enterobacteriaceae, also belonging to the phylum of Proteobacteria, in cachectic mice (5, 8, 32). We confirm that these cachexia-associated increased levels of Enterobacteriaceae are also characteristic of human cancer cachexia.
The facultative anaerobic Enterobacteriaceae are considered to be well adapted to survive in environments with high levels of oxidative stress and have been shown to be associated with inflammation (33, 34). Interestingly, a shift towards more aerotolerant taxa such as Enterobacteriaceae has also been found in other diseases sharing metabolic and inflammatory features with cancer cachexia, including Crohn's disease (33) and anorexia (35). Another interesting finding of our study was the co-abundance of unknown Enterobacteriaceae and *Veillonella*, which were both increased in cachectic cancer patients. These genera were also significantly correlated with weight loss, confirming their relevance in the context of cancer cachexia. However, it remains to be investigated why the direction of the relationship was different in cachectic versus non-cachectic cancer patients. It might be speculated that the increased abundance of unknown Enterobacteriaceae and *Veillonella* in cachectic cancer patients leads to competition between those bacteria.
Megamonas and *Peptococcus*, both belonging to the Firmicutes phylum, were only present in a few samples within the cachectic group. This might correspond to the earlier described

decreased levels of Firmicutes in cachectic mice (32), but the total prevalence of these genera in the current study population was too low to draw definite conclusions.

According to our knowledge, this is the first clinical study investigating fecal SCFA concentrations in cachectic cancer patients. SCFA are interesting metabolites in the context of cancer cachexia since they exert anti-inflammatory effects by interacting with the immune system and by improving gut barrier integrity (14, 36). Of note, gut barrier integrity has also been shown to be debilitated in cachectic mice (5). In addition, it was recently shown that acetate and butyrate were reduced in the caecal content of cachectic mice (30). In line with this, we observed an overall trend towards lower SCFA concentrations in cachectic cancer patients, although this was only significant for acetate. Acetate is considered to fulfill crucial physiological roles and has been linked to body weight regulation, energy expenditure, lipid metabolism, and insulin sensitivity (37). Since it has been demonstrated that acetate affects muscle and adipose tissue metabolism (37), it might be speculated that altered acetate levels could influence cachexia-associated metabolic disturbances in these target tissues.

We found no associations between fecal SCFA and inflammatory parameters (fecal calprotectin, CRP, leukocyte counts), which would be expected based on the anti-inflammatory potential of SCFA. Similarly, we hypothesized that levels of fecal calprotectin would be increased in cachectic cancer patients, since it has been shown to reflect inflammation in the gastrointestinal tract and is useful as biomarker for inflammatory bowel disease and other inflammatory conditions (38). It was also unexpected that we could not detect differences in CRP levels and leukocyte counts between cachectic and non-cachectic cancer patients. However, since these inflammatory markers could only be determined in 35 and 51 patients, respectively, and because CRP was elevated (>5mg/l) in most of them, the relevance of these values in the current study might be limited. Of note, fecal calprotectin strongly correlated with unknown Enterobacteriaceae and *Veillonella* and to a lesser extent with *Peptococcus* abundance. The association with unknown Enterobacteriaceae and *Veillonella* supports the earlier described hypothesis that these bacteria are well adapted to live in a pro-inflammatory environment.

There are several limitations inherent to this study. First, the prevalence of cancer cachexia varied depending on the cancer type. Consequently, the group of cachectic cancer patients mainly consisted of pancreatic cancer patients, while the majority of the non-cachectic group had breast cancer. Therefore, differences between cachectic and non-cachectic cancer patients might be related to the cancer type, next to the presence of cachexia. However, we could partly confirm our results when analyzing the different cancer types separately, despite the limitations of small group sizes in these analyses. Nevertheless, future studies should use larger and more homogeneous patient cohorts in order to study crosstalk and mechanistic interactions between gut microbiota and metabolic target tissues in human cancer cachexia in more detail. For instance, it would be beneficial to evaluate differences between cachectic and non-cachectic cancer patients within one cancer type to rule out the confounding effect of the tumor type.
Besides the cancer type, also the stage of the disease might have influenced microbiota composition. Since lymph node involvement and metastatic disease were more common among cachectic cancer patients, we cannot rule out that the observed microbial disturbances in cachectic cancer patients might also be associated with more advanced stages. In view of

the fact that cancer cachexia is considered to be a feature of especially advanced disease stages, it would be highly relevant to compare cachectic and non-cachectic cancer patients with metastatic disease in future studies. In the metastatic setting, also the role of therapy-induced cachexia will be important to investigate, since a recent metabolomics study indicated distinct metabolic derangements of cancer-induced cachexia and chemotherapy-induced cachexia (39). Since chemotherapy is known to also affect gut bacteria, the gut microbiota might play a prominent role in this type of cachexia (40).
In addition, the definition of cancer cachexia was solely based on self-reported weight loss, which might be unreliable and does not take other hallmarks of cancer cachexia (e.g. inflammation and muscle wasting) into account (41). In addition, it should be recognized that this cachexia definition does not differentiate between loss of skeletal muscle or fat mass and may therefore underestimate lean body mass loss in overweight patients and in those who gained weight because of a tumor, edema, or accumulation of ascites (42). Consequently, future studies should use more objective, quantitative methods to assess cachexia status, for instance computed tomography (CT)-based body composition analysis and should also assess systemic inflammation in these patients. Another possibility to control for obesity as factor masking relevant weight loss is the classification of patients into different categories of BMI-adjusted weight loss (Figure S2) (15).

In summary, we have shown that cachexia is associated with specific alterations in gut microbiota composition and fecal SCFA concentrations in cancer patients. These insights represent a pivotal first step and underscore the need to evaluate whether the gut microbiota can be used as therapeutic target in cancer cachexia. In view of the limited effectiveness of the current treatment approaches and the considerable impact of cancer cachexia on the patient's prognosis and quality of life, such innovative anti-cachexia strategies are urgently required.

Acknowledgements

We gratefully thank all volunteers who participated in this study. Furthermore, we are thankful to C. Driessen, M. Oomen and A. van Bijnen for performing laboratory analyses and E. Katoen, E.M.P.H. de Jong-Vrancken, C. Starren-Goessens, C.M.R. Haekens and J.M.J. Lipsch-Crijns for the inclusion of patients. We also thank H. van Eijk and J. Bastings for laboratory support.

Conflict of interest

RA and MLS has received institutional research funding from Servier, all outside the submitted work. DPJvD was supported by the Netherlands Organization for Scientific Research (NWO Grant 022.003.011). AvH is employed by Danone Nutricia Research. All other authors have no conflict of interest.

Funding

Danone Nutricia Research contributed financially to the analysis of stool samples.

Author contributions

JU, DPJvD, and SSR designed the study. JU and DPJvD coordinated inclusion, sample collection, and study administration. JU, JZ, RA, DPJvD, and LC included patients, MLS, RFPM, and SWMOD supported inclusion. Laboratory analyses were conducted by JU, JZ, and RA. JP and AvH provided resources for laboratory analyses. Bioinformatic and statistical analysis was performed by ZS and JZ. JU, JZ, ZS, RA, JP, AvH, and SSR were involved in data interpretation. JZ and SSR drafted the manuscript. All authors participated in discussion and revision and approved the final version of the manuscript.

References

1. Argiles, J.M., et al., Cancer cachexia: understanding the molecular basis. Nat Rev Cancer, 2014. 14(11): p. 754-62.
2. von Haehling, S. and S.D. Anker, Prevalence, incidence and clinical impact of cachexia: facts and numbers-update 2014. J Cachexia Sarcopenia Muscle, 2014. 5(4): p. 261-3.
3. Fearon, K., J. Arends, and V. Baracos, Understanding the mechanisms and treatment options in cancer cachexia. Nat Rev Clin Oncol, 2013. 10(2): p. 90-9.
4. Tremaroli, V. and F. Backhed, Functional interactions between the gut microbiota and host metabolism. Nature, 2012. 489(7415): p. 242-9.
5. Bindels, L.B., et al., Increased gut permeability in cancer cachexia: mechanisms and clinical relevance. Oncotarget, 2018. 9(26): p. 18224-18238.
6. Bindels, L.B. and J.-P. Thissen, Nutrition in cancer patients with cachexia: A role for the gut microbiota? Clinical Nutrition Experimental, 2016. 6: p. 74-82.
7. Ziemons, J., et al., Gut microbiota and metabolic aspects of cancer cachexia. Best Pract Res Clin Endocrinol Metab, 2021: p. 101508.
8. Bindels, L.B., et al., Synbiotic approach restores intestinal homeostasis and prolongs survival in leukaemic mice with cachexia. Isme j, 2016. 10(6): p. 1456-70.
9. Bindels, L.B., et al., Restoring specific lactobacilli levels decreases inflammation and muscle atrophy markers in an acute leukemia mouse model. PLoS One, 2012. 7(6): p. e37971.
10. Varian, B.J., et al., Beneficial bacteria inhibit cachexia. Oncotarget, 2016. 7(11): p. 11803-16.
11. Faber, J., et al., Beneficial immune modulatory effects of a specific nutritional combination in a murine model for cancer cachexia. Br J Cancer, 2008. 99(12): p. 2029-36.
12. Oliphant, K. and E. Allen-Vercoe, Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. Microbiome, 2019. 7(1): p. 91.
13. Morrison, D.J. and T. Preston, Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes, 2016. 7(3): p. 189-200.
14. van der Beek, C.M., et al., Role of short-chain fatty acids in colonic inflammation, carcinogenesis, and mucosal protection and healing. Nutr Rev, 2017. 75(4): p. 286-305.
15. Martin, L., et al., Diagnostic criteria for the classification of cancer-associated weight loss. J Clin Oncol, 2015. 33(1): p. 90-9.
16. Costea, P.I., et al., Towards standards for human fecal sample processing in metagenomic studies. Nat Biotechnol, 2017. 35(11): p. 1069-1076.
17. Caporaso, J.G., et al., Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. Isme j, 2012. 6(8): p. 1621-4.
18. Bakker-Zierikzee, A.M., et al., Effects of infant formula containing a mixture of galacto- and fructo-oligosaccharides or viable Bifidobacterium animalis on the intestinal microflora during the first 4 months of life. Br J Nutr, 2005. 94(5): p. 783-90.
19. Zhao, G., M. Nyman, and J.A. Jönsson, Rapid determination of short-chain fatty acids in colonic contents and faeces of humans and rats by acidified water-extraction and direct-injection gas chromatography. Biomed Chromatogr, 2006. 20(8): p. 674-82.
20. Thiel, R. and M. Blaut, An improved method for the automated enumeration of fluorescently labelled bacteria in human faeces. J Microbiol Methods, 2005. 61(3): p. 369-79.
21. van der Sluijs Veer, G., et al., Time-resolved fluorimetric immunoassay of calprotectin: technical and clinical aspects in diagnosis of inflammatory bowel diseases. Clin Chem Lab Med, 2006. 44(3): p. 292-8.
22. Lagkouvardos, I., et al., IMNGS: A comprehensive open resource of processed 16S rRNA microbial profiles for ecology and diversity studies. Sci Rep, 2016. 6: p. 33721.
23. Team, R.C. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2020; Available from: https://www.R-project.org/.
24. de Wert, L.A., et al., The cutaneous microbiome in hospitalized patients with pressure ulcers. Sci Rep, 2020. 10(1): p. 5963.
25. Love, M.I., W. Huber, and S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol, 2014. 15(12): p. 550.
26. Lagkouvardos, I., et al., Rhea: a transparent and modular R pipeline for microbial profiling based on 16S rRNA gene amplicons. PeerJ, 2017. 5: p. e2836.
27. Brian G. Peterson and P. Carl. PerformanceAnalytics: Econometric Tools for Performance and Risk Analysis. R package version 2.0.4. 2020; Available from: https://CRAN.R-project.org/package=PerformanceAnalytics.

28. McGregor, K., A. Labbe, and C.M.T. Greenwood, MDiNE: a model to estimate differential co-occurrence networks in microbiome studies. Bioinformatics, 2020. 36(6): p. 1840-1847.
29. Ogle, D.H., P. Wheeler, and A. Dinno. FSA: Fisheries Stock Analysis. R package version 0.8.32. 2021; Available from: https://github.com/droglenc/FSA.
30. Pötgens, S.A., et al., Multi-compartment metabolomics and metagenomics reveal major hepatic and intestinal disturbances in cancer cachectic mice. J Cachexia Sarcopenia Muscle, 2021.
31. Lozupone, C.A., et al., Diversity, stability and resilience of the human gut microbiota. Nature, 2012. 489(7415): p. 220-30.
32. Pötgens, S.A., et al., Klebsiella oxytoca expands in cancer cachexia and acts as a gut pathobiont contributing to intestinal dysfunction. Sci Rep, 2018. 8(1): p. 12321.
33. Morgan, X.C., et al., Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biol, 2012. 13(9): p. R79.
34. Menezes-Garcia, Z., et al., Colonization by Enterobacteriaceae is crucial for acute inflammatory responses in murine small intestine via regulation of corticosterone production. Gut Microbes, 2020. 11(6): p. 1531-1546.
35. Borgo, F., et al., Microbiota in anorexia nervosa: The triangle between bacterial species, metabolites and psychological tests. PLoS One, 2017. 12(6): p. e0179739.
36. Bach Knudsen, K.E., et al., Impact of Diet-Modulated Butyrate Production on Intestinal Barrier Function and Inflammation. Nutrients, 2018. 10(10).
37. Hernández, M.A.G., et al., The Short-Chain Fatty Acid Acetate in Body Weight Control and Insulin Sensitivity. Nutrients, 2019. 11(8).
38. Burri, E. and C. Beglinger, The use of fecal calprotectin as a biomarker in gastrointestinal disease. Expert Rev Gastroenterol Hepatol, 2014. 8(2): p. 197-210.
39. Pin, F., et al., Cachexia induced by cancer and chemotherapy yield distinct perturbations to energy metabolism. J Cachexia Sarcopenia Muscle, 2019. 10(1): p. 140-154.
40. Aarnoutse, R., et al., The Clinical Link between Human Intestinal Microbiota and Systemic Cancer Therapy. Int J Mol Sci, 2019. 20(17).
41. Ng, C.D., Errors in body mass index from self-reported data by sex and across waves of Add Health. Ann Epidemiol, 2019. 39: p. 21-25.
42. Kim, E.Y., et al., Prognostic significance of cachexia score assessed by CT in male patients with small cell lung cancer. Eur J Cancer Care (Engl), 2018. 27(1).

Supplementary Tables and Figures

Table S1: *Correlations between SCFA and inflammatory parameters*

	Kendall'st tau (τ)	p-value
Calprotectin - acetate	-0.09	0.09
Calprotectin - propionate	-0.01	0.8
Calprotectin - butyrate	-0.02	0.7
Calprotectin – total SCFA	-0.06	0.3
CRP – acetate	-0.01	1.0
CRP - propionate	-0.01	1.0
CRP - butyrate	-0.06	0.6
CRP – total SCFA	-0.06	0.6
Leukocytes - acetate	0.12	0.2
Leukocytes - propionate	0.13	0.2
Leukocytes - butyrate	0.06	0.6
Leukocytes – total SCFA	0.12	0.3

Table S2a: *Disease stages per cancer type. Information on tumor stages was retrieved from medical records (if available*) and classified as local disease, lymph node involvement, or metastatic disease*

	All cancer patients (N=101)	Pancreatic cancer (N=22)	Lung cancer (N=24)	Breast cancer (N=52)	Ovarian cancer (N=3)
Local disease	N=67 (66%)	N=6 (27%)	N=11 (46%)	N=50 (96%)	N=0 (0%)
Lymph node involvement	N=23 (23%)	N=13 (59%)	N=5 (21%)	N=2 (4%)	N=1 (33%)
Metastatic disease	N=11 (11%)	N=3 (14%)	N=8 (33%)	N=0 (0%)	N=2 (67%)

**Data from 5 pancreatic cancer patients and one ovarian cancer patient could not be retrieved from medical record*

Table S2b: *Disease stages in cachectic and non-cachectic cancer patients. Information on tumor stages was retrieved from medical records (if available*) and classified as local disease, lymph node involvement or metastatic disease*

	All cancer patients (N=101)	**Cachectic (N=29)**	**Non-cachectic (N=72)**
Local disease	N=67 (66%)	N=14 (48%)	N=53 (74%)
Lymph node involvement	N=23 (23%)	N=9 (31%)	N=12 (17%)
Metastatic disease	N=11 (11%)	N=6 (21%)	N=7 (9%)

**Data from 5 pancreatic cancer patients and one ovarian cancer patient could not be retrieved from medical record*

The distribution of cachectic and non-cachectic patients over these disease stages was not significantly different according to Chi square test (p=0.05)

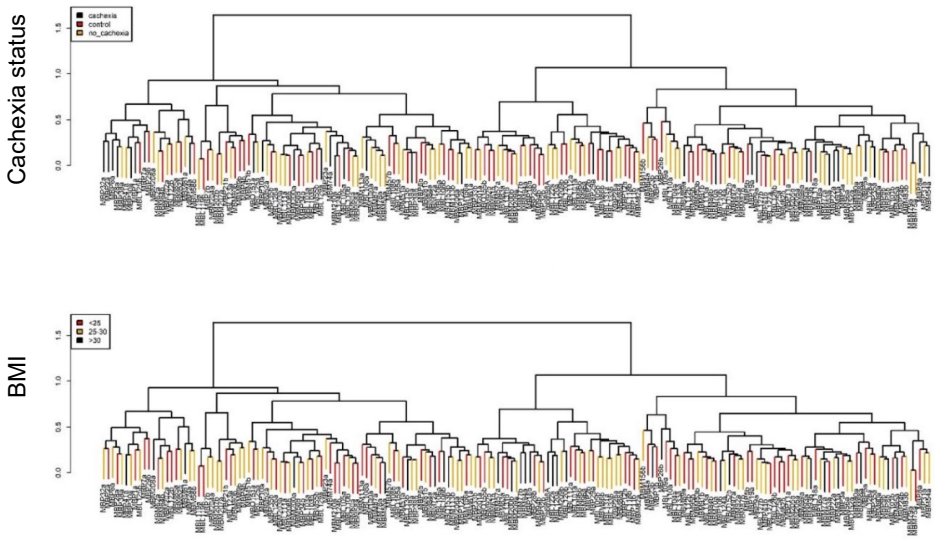


Figure S1: *Dendrograms show no clustering of samples based on cachexia status (upper panel) or BMI (lower panel). We observed high inter-individual heterogeneity.*

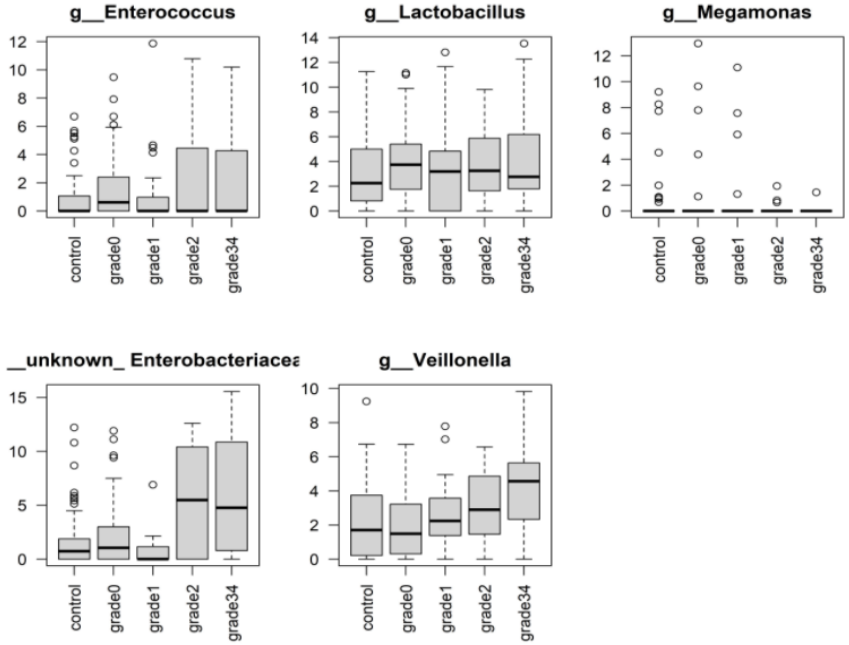


Figure S2: *Genera abundance across different grades of BMI-adjusted weight loss. This categorization into grade 0 – grade 4 was suggested in Martin et al. (2015). Diagnostic criteria for the classification of cancer-associated weight loss. Journal Clinical Oncology, 2015. 33(1): p. 90-9. Each grade was associated with significantly different survival, whereby a gradient of decreasing survival was observed with increasing weight loss and decreasing BMI (grade 0=longest survival; grade 4=shortest survival).*

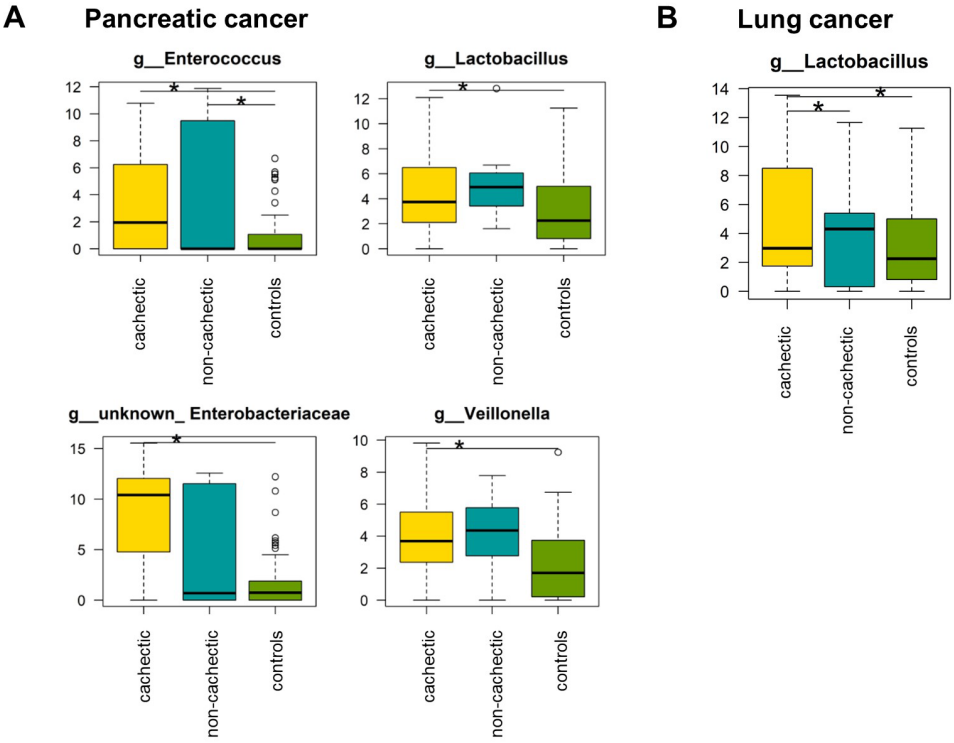


Figure S3: *Log2 abundance of genera which were differentially abundant between cachectic cancer patients, non-cachectic cancer patients, and healthy controls, analyzed for pancreatic cancer and lung cancer patients separately. A: The likelihood ratio test (LRT) indicated significant differential abundance of Enterococcus ($p<0.001$), Lactobacillus ($p<0.01$), unknown Enterobacteriaceae ($p<0.001$) and Veillonella ($p<0.001$) between cachectic and non-cachectic pancreatic cancer patients and healthy controls. Differences which were significantly different according to the Wald test ($\alpha=0.05$) are marked with asterisks. B: LRT indicated that only Lactobacillus ($p<0.001$) showed differential abundance in cachectic and non-cachectic lung cancer patients and healthy controls. Differences which were significantly different according to the Wald test ($\alpha=0.05$) are marked with asterisks.*

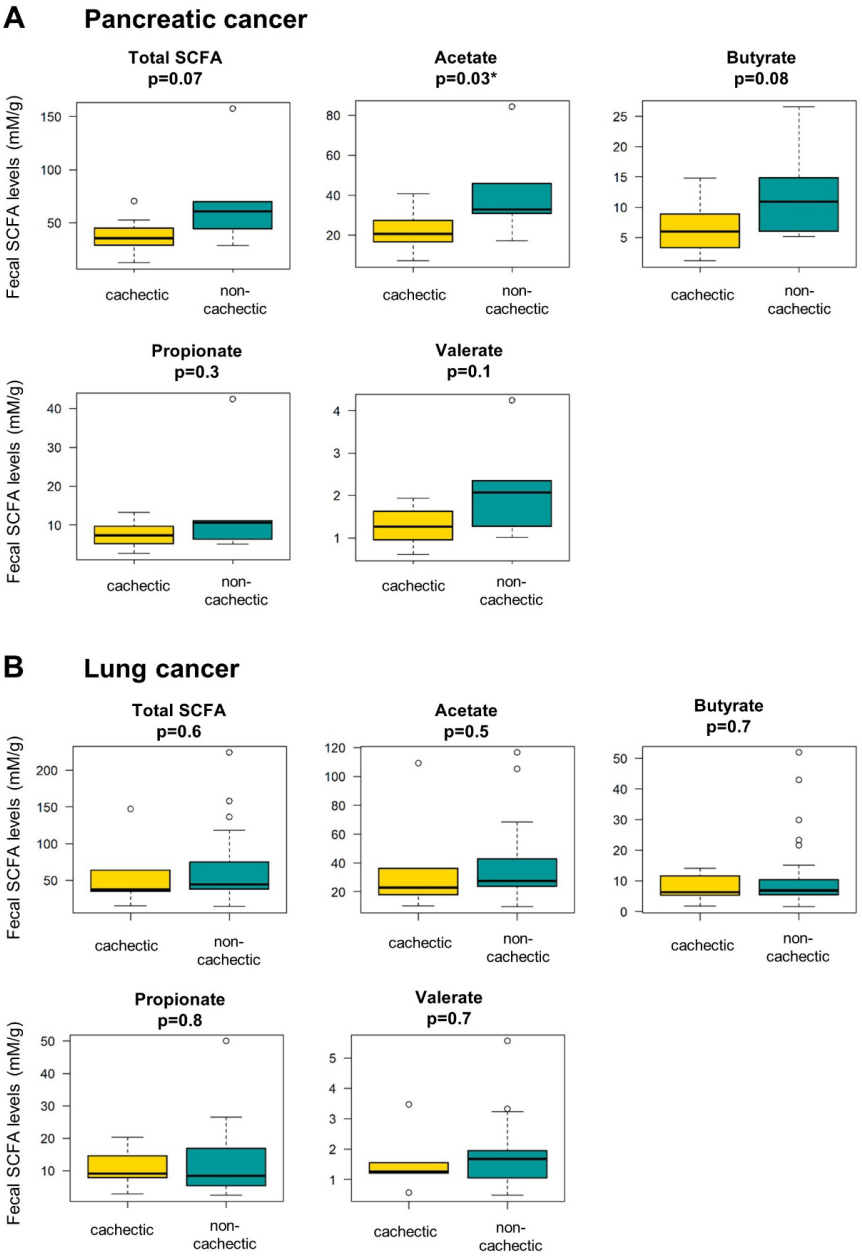


Figure S4: *SCFA levels in patients with pancreatic cancer or lung cancer.* ***A:*** *Acetate levels were significantly lower in cachectic pancreatic cancer patients (N=16) compared to non-cachectic pancreatic cancer patients (N=5). Total SCFA and butyrate concentrations tended to be reduced in cachectic patients.* ***B:*** *Levels of all SCFA were similar in cachectic (N=5) and non-cachectic lung cancer patients (N=25).*

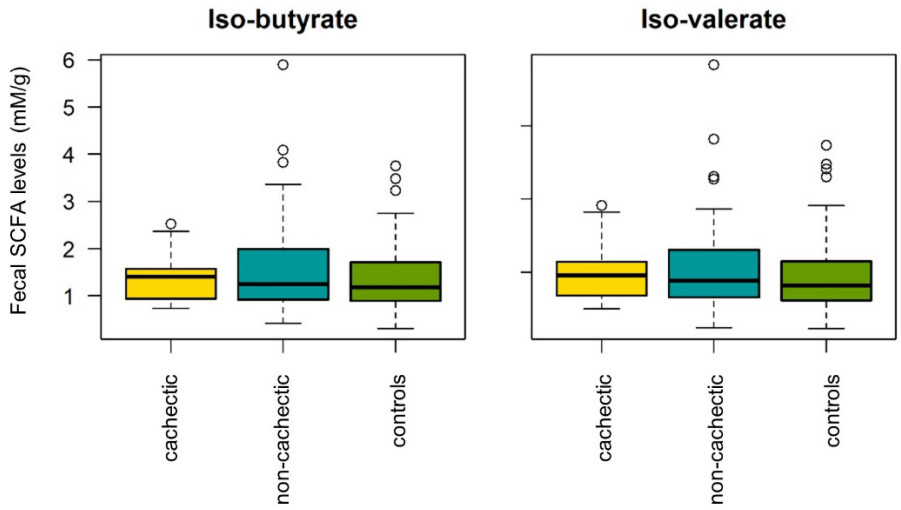


Figure S5: *Fecal levels of the BCFA iso-butyrate and iso-valerate in cachectic cancer patients (N=30), non-cachectic cancer patients (N=64), as well as in healthy controls (N=71). Levels of iso-butyrate (p=0.608) and iso-valerate (p=0.543) were similar in all groups.*

Supplementary Methods

Analysis of fecal SCFA and BCFA concentrations

Fecal levels of SCFA and BCFA were quantified using direct-injection gas chromatography (1, 2). Samples were prepared based on an established protocol (3). Briefly, 3mm glass beads *(Sigma Aldrich, USA)* and 0.5-1.0 g of frozen feces were introduced into a tube. The exact weight of the feces was recorded. A nine-fold volume of ice-cold phosphate buffered saline (PBS) was added and the sample was vortexed for five minutes. To remove glass beads and large particles, samples were centrifuged at 300x g for one minute; the supernatant was centrifuged again for three minutes at 15.000x g at 4°C. 200µl of the supernatant was stored at -80°C for downstream analysis. SCFA and BCFA levels were quantitatively assessed using a Shimadzu GC2025 gas chromatograph *(Shimadzu Corporation, Kyoto, Japan)* equipped with a flame ionisation detector. The sample was injected at 80°C into the column (Stabilwax, 15 m x 0.53 mm, film thickness 1.00 µm; Restek Co., *Bellafonte, PA, USA*) using H_2 as carrier gas (20.7 kPa). New columns were conditioned overnight at 200 °C. After injection of the sample, the oven was heated to 160 °C at a rate of 16 °C/min, followed by heating to 220 °C at 20 °C/min and finally maintained at a temperature of 220 °C for 1.5 min. The temperature of the injector and the detector was 200 °C. After every ten samples, the column was cleared by injection of 0.5 µl formic acid (1 %, by vol.) to avoid memory effects of the column, followed by injection of 0.5 ml standard SCFA mix (1.77 mM acetic acid, 1.15 mM propionic acid, 0.72 mM n-butyric acid, 0.72 mM isobutyric acid, 0.62 mM n-valeric acid 0.62 mM isovaleric acid; *Sigma Aldrich, USA*) to monitor the occurrence of memory effects. SCFA concentrations were determined using 2-ethylbutyric acid as an internal standard.

Statistical analysis of gut microbiota data

Preprocessing

Preprocessing of raw reads was performed using the Integrated Microbial Next Generation Sequencing (IMNGS, www.imngs.org) platform (4). IMNGS is a UPARSE-based analysis pipeline using USEARCH 8.0 for pairing, quality filtering, and OTU clustering (5). UCHIME (with RDP set 15 as a reference database) was used to remove chimeric sequences (6). Taxonomic classification was done by RDP classifier version 2.11 training set 15.8. Sequence alignment was performed by MUSCLE and treeing by Fasttree (7, 8).

Microbial richness, diversity and community structure

Using the Rhea pipeline, observed species richness and the Shannon effective index were calculated as measures of microbial α-diversity and compared using the Kruskal Wallis test (9). To examine the dissimilarity in microbial community structures (β-diversity), generalized UniFrac was used, incorporating phylogenetic distances between organisms (10, 11). Two-dimensional visualization of the multidimensional distance matrix was performed by nonmetric multi-dimensional scaling (NMDS). Differences in diversity of microbial communities were tested by permutational multivariate analysis of variance (PERMANOVA) using the adonis function of the R package vegan (12, 13).

References

1. Bakker-Zierikzee, A.M., et al., Effects of infant formula containing a mixture of galacto- and fructo-oligosaccharides or viable Bifidobacterium animalis on the intestinal microflora during the first 4 months of life. Br J Nutr, 2005. **94**(5): p. 783-90.
2. Zhao, G., M. Nyman, and J.A. Jönsson, Rapid determination of short-chain fatty acids in colonic contents and faeces of humans and rats by acidified water-extraction and direct-injection gas chromatography. Biomed Chromatogr, 2006. **20**(8): p. 674-82.
3. Thiel, R. and M. Blaut, An improved method for the automated enumeration of fluorescently labelled bacteria in human faeces. J Microbiol Methods, 2005. **61**(3): p. 369-79.
4. Lagkouvardos, I., et al., IMNGS: A comprehensive open resource of processed 16S rRNA microbial profiles for ecology and diversity studies. Sci Rep, 2016. **6**: p. 33721.
5. Edgar, R.C., UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods, 2013. **10**(10): p. 996-8.
6. Edgar, R.C., et al., UCHIME improves sensitivity and speed of chimera detection. Bioinformatics, 2011. **27**(16): p. 2194-200.
7. Edgar, R.C., MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res, 2004. **32**(5): p. 1792-7.
8. Price, M.N., P.S. Dehal, and A.P. Arkin, FastTree 2--approximately maximum-likelihood trees for large alignments. PLoS One, 2010. **5**(3): p. e9490.
9. Lagkouvardos, I., et al., Rhea: a transparent and modular R pipeline for microbial profiling based on 16S rRNA gene amplicons. PeerJ, 2017. **5**: p. e2836.
10. Chen, J., et al., Associating microbiome composition with environmental covariates using generalized UniFrac distances. Bioinformatics, 2012. **28**(16): p. 2106-2113.
11. Chen, J. GUniFrac: Generalized UniFrac Distances. R package version 1.1. 2018; Available from: https://CRAN.R-project.org/package=GUniFrac.
12. Anderson, M.J., A new method for non-parametric multivariate analysis of variance. Austral ecology, 2001. **26**(1): p. 32-46.
13. Jari Oksanen, F.G.B., Michael Friendly, Roeland Kindt, Pierre Legendre, Dan McGlinn,, R.B.O.H. Peter R. Minchin, Gavin L. Simpson, Peter Solymos, M. Henry H. Stevens, Eduard Szoecs, and a.H. Wagner. vegan: Community Ecology Package. R package version 2.5-6. 2019; Available from: https://CRAN.R-project.org/package=vegan.

Chapter 9

Discussion and future perspectives

Preamble

The work presented in this thesis indicated how two different aspects of human cancer, more specifically **chemotherapy** and **cancer cachexia**, might challenge the equilibrium of the gut microbiota. Furthermore, it showed that prebiotics represent a promising strategy to **restore or maintain the microbial equilibrium in cancer patients,** as well as to prevent manifestation of a dysbiotic state. Thereby, the current thesis provided pivotal new evidence in this relatively new field of research and paves the way for further studies as well as the design of targeted microbiota-modulating interventions in cancer patients.

Interactions between the gut microbiota and chemotherapy were investigated in the context of two different chemotherapy regimens: 5-Fluorouracil (5-FU)-based chemotherapy and combinational treatment with Adriamycin, Cyclophosphamide and Docetaxel (AC-D). For this purpose, patients with two distinct cancer types were included (colorectal cancer (CRC) and breast cancer).
The role of the gut microbiota in cancer cachexia was examined across four different cancer types (pancreatic cancer, breast cancer, lung cancer, ovarian cancer).
Next to gut microbiota composition and diversity, as assessed by 16S rRNA gene sequencing, this thesis also looked beyond the presence or absence of taxa and examined levels of short-chain fatty acids (SCFA) and branched-chain fatty acids (BCFA), which are gut microbial metabolites with well described health effects. In the following sections, the most important results from the studies presented in this thesis are discussed and integrated into practical recommendations and perspectives for future research.

1. The gut microbiota and chemotherapy

Conventionally, the chemotherapy treatment regimens are highly standardized, and dosages are often calculated based on body surface. Only recently, there is an increasing interest in more personalized approaches. Besides other factors, for instance genetic characteristics of the patient or tumor, the gut microbiota has recently been proposed as another factor influencing chemotherapy efficacy and toxicity (1, 2).
First of all, **Chapter 2** of this thesis provided an overview of the literature concerning the clinical link between the gut microbiota and chemotherapy, immunotherapy and hormonal therapy, because a comprehensive overview of especially clinical studies was lacking at that time (2019). This up-to-date overview supported the hypothesis that the gut microbiota plays an important role during chemotherapy treatment. However, generalization and clinical utility of results were limited because of large differences in study design and sampling timepoints as well as a considerable heterogeneity in patient characteristics (e.g. with respect to cancer type and chemotherapy applied), even within cohorts. In addition, the correction for potential confounders was often inadequate (see *section 3.4* of this discussion for a summary of the most important ones).
In the following chapters **(Chapters 3-6)**, results from our studies concerning interactions between the gut microbiota and chemotherapy were described. While the scope and parameters under investigation differed among the **clinical studies (Chapter 3, 4 and 6),** two outcomes were always central: **tumor response** and **chemotherapy toxicity**. As described

in detail in **Chapter 1**, the gut microbiota has been previously shown to be associated with both (e.g. (3-6)).
In contrast to some earlier studies with very heterogenous patient cohorts (e.g. (7-9)), we chose to analyze the effects of different chemotherapy regimens separately. This approach was chosen because gut microbiota-drug interactions are expected to be very microbe- as well as compound-specific.

1.1 The gut microbiota and 5-FU-based chemotherapy

Chapter 3, 4 and 5 focused on interactions between the gut microbiota and the chemotherapeutic **5-FU**. This compound of interest was chosen based on (amongst others) the preclinical studies from An *et al.* and Sougiannis *et al.*, suggesting that 5-FU treatment does not only affect the gut microbiota, but that gut bacteria might also be able to modulate chemosensitivity (5, 6).
5-FU is an antimetabolite drug that is widely used in distinct solid cancer types, either alone or as part of combination therapies (e.g. FOLFOX or FOLFIRI). **Capecitabine** is an oral prodrug, which is converted to 5-FU by the enzymes cytidine deaminase and thymidine phosphorylase, which are mainly present in tumor tissue (10, 11) (see **Chapter 5** for a detailed description of 5-FU and capecitabine metabolism). In this way, malignant cells can be sustainably exposed to therapeutic drug concentrations, with less toxicity as compared to direct infusion of 5-FU (10, 12). Amongst others, capecitabine is administered as palliative chemotherapy in patients with **metastatic CRC** (12). Patients with metastatic or unresectable CRC, who are treated in the palliative setting represent an interesting study population, because the maintenance of an adequate quality of life is of utmost importance. To achieve this, the optimization of tumor response and decrease of chemotherapy toxicity are important points of attention. Although this study population is very complex, due to the advanced stage and often a prolonged treatment history compared to patients at the initial diagnosis, these patients are expected to benefit most from our results. Additionally, this is a good patient group for the implementation of new interventions, which is the ultimate aim of the current research.

Firstly, we analyzed microbial diversity (α-diversity), community structure (β-diversity), and bacterial abundances on phylum and genus levels before, during, and after three cycles of capecitabine in patients with metastatic or unresectable CRC (**Chapter 3**). In contrast to other studies also focusing on 5-FU-based treatments (13, 14), we did not identify significant differences between pre- and post-chemotherapy samples and no associations between gut microbiota parameters and tumor response. Although no consistent effects could be detected in this relatively small cohort of 33 patients, this pilot study provided a framework and crucial insights into potential challenges for future longitudinal studies in similar complex patient cohorts. Particularly the presence of extensive and diverse medical histories, large inter-individual variability of the gut microbiota, as well as the administration of antibiotics during the study period complicated the detection of capecitabine-induced effects on the gut microbiota.

Secondly, we quantified levels of the gut bacteria-derived metabolites SCFA and BCFA in fecal samples from patients of the same cohort (**Chapter 4**). While gut microbiota composition and diversity were not affected by three cycles of capecitabine **(Chapter 3)**, fecal levels of the

SCFA valerate and caproate decreased significantly. This supports the relevance of looking beyond bacterial abundances and underlines the importance of also analyzing bacterial metabolites, as outlined in *paragraph 3.2* below. Furthermore, the BCFA iso-butyrate was found to be significantly lower in the feces of patients with partial response compared to patients with stable disease or progressive disease. Based on this, it might be hypothesized that iso-butyrate could be a potential target for microbiota-modulating interventions with the aim to improve tumor response. Interestingly, our *in vitro* experiments showed that iso-butyrate was increased due to 5-FU treatment but could be successfully decreased by the addition of prebiotics (**Chapter 5**). However, a causal relationship between iso-butyrate and tumor response has not been detected yet and requires further investigation. For instance, this could be done by treating tumor cells with 5-FU, with and without the addition of iso-butyrate.
Next to tumor response, we also investigated other clinical parameters in more detail in these patients and found that nutritional status, physical performance, and chemotherapy-induced toxicity were not significantly associated with SCFA or BCFA. In view of the well-described anti-inflammatory effects of SCFA (15, 16), correlations with blood inflammatory parameters were also analyzed. Here, it was identified that baseline SCFA correlated positively with blood neutrophil counts, which was contradictory to the expected anti-inflammatory effects. To combine data on gut microbiota composition with the metabolite data, we also described different associations between SCFA/BCFA and abundance of bacterial taxa on family level.

As a follow-up of these two clinical studies, the effects of 5-FU on both gut microbiota composition, as well as SCFA/BCFA levels were also investigated in an *in vitro* setting (**Chapter 5**). In contrast to the high inter-individual variability and various confounding effects, as seen in the clinical studies, this approach enabled us to monitor molecular interactions in a more controlled setting. Using the TNO *in vitro* model of the colon (TIM-2), we could not only identify 5-FU-induced microbiota shifts but could also demonstrate that these shifts could be counteracted by prebiotics via a stimulation of potentially beneficial gut bacteria (e.g. *Lactobacillus*, *Bifidobacterium* and *Anaerostipes*) and inhibition of potentially pathogenic bacteria (e.g. *Klebsiella*, *Enterobacter*).
TIM-2 was the method of choice for these in vitro experiments since it is a validated, dynamic, computer-controlled model, which closely mimics the physiological conditions of the colon. It includes peristaltic movements and a dialysis system for the removal of excess metabolites. Furthermore, it maintains a physiological pH and temperature as well as anaerobic conditions, which is particularly important because most gut bacteria are strictly anaerobic (17).

Altogether, our studies on 5-FU-based chemotherapy produced strong evidence:
(1) that 5-FU-based chemotherapy affects gut microbiota composition and the production of SCFA and BCFA, with potential consequences for tumor response and chemotherapy toxicity and
(2) that prebiotics represent a promising strategy to meet the above-mentioned aim to restore or maintain the microbial equilibrium and to prevent manifestation of a dysbiotic state in cancer patients. Together with the recent publications from LaCourse *et al.* (3) and Spanogiannopoulos *et al.* (18), indicating direct bacterial metabolism of 5-FU and implications for treatment efficacy (see **Chapter 1** and **Chapter 5** for a more detailed description of the results from these studies), our data provide a strong rationale to proceed with further research in this field.

1.2 The gut microbiota and chemotherapy with AC-D

In **Chapter 6** we investigated gut microbiota-chemotherapy interactions in a non-gastrointestinal cancer (breast cancer), where the tumor is not in proximity to the gut microbiota. For this study, 44 postmenopausal breast cancer patients receiving (neo)adjuvant treatment with AC-D were included. In contrast to the patients from the CRC cohort, these patients received chemotherapy in curative setting, either before (neo-adjuvant) or after surgery (adjuvant).

Interestingly, AC-D-induced gut microbiota changes were identified in these patients. While some bacteria (*Ruminococcaceae NK4A214 group*, *Christensenellaceae R7 group*, *Ruminococcaceae UCG-005*, *Marvinbryantia*) responded sensitively to AC-D therapy, resulting in reduced abundances, Proteobacteria, unclassified Enterobacterales and *Lactobacillus* increased during AC-D. These results are of great interest due to different reasons.
Firstly, the Ruminococcaceae genera as well as the *Christensenellaceae R7 group* have been repeatedly reported to be associated with good gut health, probably because of their SCFA producing capability (19, 20).
Secondly, bacteria belonging to the Enterobacterales order are known to have pathogenic and pro-inflammatory properties (21) and seem to play an important role in the cancer-associated gut microbiota. For instance, a genus from the Enterobacteriaceae family, also belonging to Enterobacterales, was found to be elevated in cancer cachexia in our clinical study (**Chapter 8**) as well as in several mice studies (22-24). Furthermore, *E. coli* has been previously shown to be capable of 5-FU metabolism (3, 18). In general, Enterobacteriaceae are considered to be a common feature of gut microbial dysbiosis and to be well adapted to grow under inflammatory circumstances (21). The prevention of Enterobacterales overgrowth might thus be an important strategy to prevent the manifestation of microbial dysbiosis and the associated negative effects on human metabolism and inflammatory state in cancer patients. In view of this, our *in vitro* results **(Chapter 5)**, indicating that prebiotics are able to reduce the growth of different Enterobacterales genera (*Klebsiella*, *Enterobacter*) during 5-FU administration are of great relevance and require further investigation. The inhibiting effects of prebiotics might be (partly) explained by increased production of SCFA, which has been suggested to produce a slightly acidic milieu, which is considered unfavorable for Enterobacteriaceae (25). In line with this, Pötgens *et al.* also proposed a potential negative association between SCFA-producing bacteria and Enterobacterales through a glycolytic switch (24, 26), which further signifies that the microbiota shifts during AC-D, as described above, should not be left untreated.

Concerning chemotherapy toxicity in patients during AC-D treatment, we observed that the occurrence of diarrhea during the Docetaxel cycles seemed to be associated with microbial species richness. However, similar to the results from the CRC cohort **(Chapter 3)**, there was no association between the gut microbiota and tumor response in breast cancer patients during neoadjuvant AC-D.

In general, it should be noted that the gut microbiota in breast cancer is less well explored, as compared to CRC, and results from different studies are contradictory (e.g. (27, 28)). Our

initial results highlight the need for future clinical studies in this patient group and underline that the gut microbiota should also not be neglected in non-gastrointestinal cancers.

2. The gut microbiota and cancer cachexia

Another aspect of human cancer, that was investigated in the context of this thesis, is cancer cachexia. Cancer cachexia is a metabolic syndrome which is characterized by different hallmarks, including systemic inflammation, unintended weight loss, increased gut permeability, muscle wasting, impaired insulin sensitivity, as well as dysregulated food intake (29, 30). Of note, cancer cachexia and chemotherapy are closely interrelated. The presence of cancer cachexia could not only negatively affect tumor response and chemotherapy toxicity, but chemotherapy itself could also contribute to the development or progression of cancer cachexia (31, 32). Furthermore, suboptimal tumor response, high chemotherapy toxicity as well as cancer cachexia all have a considerable negative impact on the patient's prognosis and quality of life and require new strategies to counteract them (Figure 1).

In **Chapter 7**, we reviewed cachexia-associated gut microbiota profiles and associations between gut bacteria and different metabolic hallmarks of cancer cachexia. While most of the research described in this review has been performed in the pre-clinical setting, we carried out one of the first clinical studies in this field, the results of which are presented in **Chapter 8**. For this cross-sectional study, patients suffering from pancreatic-, breast-, lung-, or ovarian cancer were included and classified as cachectic (>5% weight loss) or non-cachectic. As control group, cancer-free partners of the patients were included. Abundance of the pro-inflammatory Proteobacteria, particularly a genus from the Enterobacteriacea family, as well as *Veillonella* were enriched in cachectic cancer patients, compared to non-cachectic cancer patients and controls. On the other hand, the genera *Megamonas* and *Peptococcus* were more abundant in non-cachectic cancer patients. Also in this cohort, we quantified microbiota-derived SCFA and observed that fecal levels of all SCFA tended to be lower in cachectic cancer patients, but only acetate concentrations were significantly reduced. This observation correspondents with the described anti-inflammatory effects of SCFA and the expected inflammation in cachectic patients. However, it should be mentioned in this context that reduced fecal SCFA levels does not necessarily reflect reduced production of these metabolites, but might also indicate increased absorption, as also discussed in **Chapter 4**.
Surprisingly, markers of intestinal inflammation (calprotectin) or systemic inflammation (CRP and leukocyte counts) were not significantly elevated in cachectic cancer patients, possibly because they were only available in a subgroup of patients.

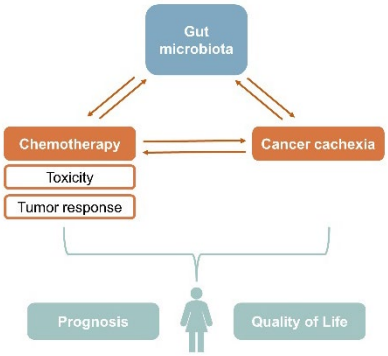


Figure 1: *Mutual relationships between the gut microbiota and the two aspects of human cancer chemotherapy and cancer cachexia, which were investigated in the context of this thesis*

To conclude, we provided first indications that the gut microbiota might also be affected in human cancer cachexia. Due to the close relationships between tumor response, chemotherapy toxicity, and cancer cachexia, it would be desirable to combine all these aspects in future clinical studies.

3. What's next?: Lessons learnt and perspectives for future research

In the last decade, the evidence supporting a role for the gut microbiota in human cancer has rapidly increased. Insights from the work presented in this thesis, together with other research initiatives, provide a sound basis for future research in this field. Therefore, it might be hypothesized that microbiota-based therapeutics as well as microbiota-modulating interventions will be implemented into clinical practice in the upcoming years. However, there are some challenges which are needed to be addressed to advance gut microbiota research and enable successful clinical translation. In the following sections, the most important challenges as well as opportunities will be discussed.

3.1 The combination of clinical and preclinical studies

The clinical studies presented in this thesis **(Chapter 3, 4, 6 and 8)** belong to the first gut microbiota studies in cancer patients and therefore fulfil a pioneering role in this field of research. Due to various reasons, discussed below (*section 3.4*), clinical microbiota studies are highly challenging and imply pitfalls researchers should be aware of. However, in order to sustainably revolutionize the current care for cancer patients, we have to start somewhere, and the work presented here forms an important first step in this journey.

A unique strength of our clinical studies during chemotherapy **(Chapter 3, 4 and 6)** is the longitudinal design, allowing the mapping of gut microbiota shifts over time. Repeated sampling within the same individuals also takes intra-individual temporal variations into account and is therefore considered to be crucial for the reliable quantification of microbial features (33). In addition, comprehensive clinical information was collected, thereby allowing correction for potential confounders (e.g. use of antibiotics or other co-medication, comorbidities or specific dietary habits) as well as to link gut microbial composition and activity to clinical parameters (e.g. tumor response, toxicity, physical performance, nutritional status). Furthermore, the translational character and involvement of fundamental scientists as well as clinicians ensure rapid clinical translation of our results and prevents that research data are lost somewhere between bench and bedside.

A common phenomenon in our clinical studies (**Chapter 3, 4, 6 and 8**) was high inter-individual variability of the gut microbiota, possibly caused by the impact of other environmental and host intrinsic factors (e.g. co-morbidities, medication, diet, living environment, medical history). This complicated the detection of consistent effects across the whole cohort and would require considerably larger cohorts, which is practically challenging in the cancer setting (see also *section 3.4* for a more detailed discussion of challenges and opportunities associated with sample size).

Therefore, we chose to perform more mechanistic *in vitro* experiments to replenish the clinical results. For this purpose, the TIM-2 model was the method of choice, because it uses human gut bacteria as inoculum and closely mimics physiological conditions of the colon (see **Chapter 5** *and section 1.1*). Because of these reasons, it was considered to be superior to other preclinical models (e.g. isolated bacterial species or mice) for our specific purposes.
Cultivation experiments with isolated bacterial species are of great relevance for the detection of new microbes, the characterization of microbe-microbe interactions as well as the exploration of mechanistic gut microbiota – drug interactions (34). Nevertheless, the TIM-2 model was chosen for our experiments, because we predominantly aimed to quantify overall shifts in the microbial equilibrium, rather than the effect of specific bacterial taxa. Furthermore, the use of a whole human-derived microbial consortium enables a more rapid clinical translation of the results.
Previously as well as nowadays, a lot of preclinical microbiota research is also done in mice (e.g. (6, 35, 36)). Although mechanistic animal studies also have an important contribution to gut microbiota research (see e.g. *section 3.3*), they were considered to be inadequate for our purposes. The most important underlying reasons were that mice are herbivores (while most of the patients are omnivores) and are expected to have a distinct drug metabolism (see e.g. (37)).
Nevertheless, the use of the TIM-2 model also has its limitations. Since the model does not encompass host cells, interactions with for instance enterocytes or immune cells cannot be studied. For these purposes other techniques, such as organoids or organs-on-a-chip systems would be more suitable (38). Alternatively, samples collected during the TIM-2 procedure (and containing relevant metabolites) could also be used for further co-incubation with other cell types. Additionally, TIM-2 mimics the colon only, so that the contribution of drug metabolism by liver enzymes cannot be taken into consideration.

To conclude, the combination of clinical studies and innovative, state-of-the-art *in vitro* techniques is a further strength of our multidisciplinary research and enabled us to benefit from the advantages of both settings. To get the complex picture of the gut microbiota as complete as possible, it is surely beneficial to also combine preclinical and clinical research as well as diverse, complementary techniques in future studies. As outlined in **Chapter 1**, diversity is generally a beneficial feature in gut microbiota research. This is also true for the use of techniques since a diversity in methods is expected to significantly contribute to further advancements in this field of research. *In vitro* models, incubation and cultivation experiments, animal studies, organoids, organs-on-a-chip as well as clinical trials all have advantages as well as limitations and should be carefully chosen, depending on the specific research question.

3.2 How to quantify an ecosystem? The different dimensions of the gut microbiota

The quantification of the complexity of a living and dynamic ecosystem is one of the main challenges in gut microbiota research. As microbiota researchers, we have to accept that we could never express all features of this community in simple numbers or figures. However, there are different tools and techniques available to get insights into the world of the gut microbiota. Hereby, it could be focused on different dimensions, by performing composition-based and/or function-based microbiota profiling.

In the last years, sequencing-based techniques for the compositional profiling of microbial taxa in complex ecosystems are rapidly evolving. For the present studies, we applied **16S rRNA gene sequencing (Chapter 3, 4, 5, 6, and 8)**. The 16S rRNA gene is conserved among all bacteria but contains hypervariable regions that differ between taxa, which were targeted by sequencing. Alternatively, shotgun **metagenomic sequencing** (sequencing of all genes present in a sample, including functional genes) should be considered for future research. Hereby, the selection of the appropriate sequencing technique mainly depends on the exact research question and setting, the budget as well as the availability of bioinformatic and computational expertise (39).
While 16S rRNA gene sequencing is less expensive, less prone to the risk of host contamination (because the target gene is only present in bacteria) and produces data that require an only beginner-intermediate level of bioinformatic skills, metagenomic sequencing requires more financial investment and advanced bioinformatic skills (39). Simultaneously, metagenomic sequencing has two major advantages compared to 16S rRNA gene sequencing, namely (1) that it reaches higher resolutions resulting in the classification of taxa down to the species and strain level and (2) that it also produces information concerning the functional capacity of the microbiota in a given sample.
Since our studies had a very explorative nature with the aim to get first insights and to observe shifts in dominant taxa, 16S rRNA gene sequencing was the method of choice here. However, with these first data as a basis, it would be of added value to conduct metagenomic sequencing in future studies. Particularly in the field of gut microbiota-drug interactions this is of great relevance since these interactions might also be species- or strain-specific. For instance, the *preTA* operon involved in 5-FU metabolism was until now identified in certain strains only (18). Another example for strain-specificity is the case of *Eggerthella lenta* and the cardiac glycoside digoxin. Haiser *et al.* showed that only a specific *E.lenta* strain was capable of digoxin reduction, most likely due to presence of the *cgr* operon (40).

From a clinical perspective, **functional activity** of the gut microbiota is also of great interest, since depending on the context, not the presence but the function of a microbe might cause a clinically relevant effect. Therefore, it will be useful to combine composition-based techniques with emerging function-based techniques in future studies.
While metagenomic sequencing encompasses all genes encoded by microbes (predictive functional potential), **metatranscriptomics** provides information on which genes are actually transcribed (active functional potential) (39). As an example, Schirmer *et al.* indicated that the metagenomic abundance of *Facealibacterium prausnitzii* was not predictive of its relative transcriptional activity, indicating the added-value of the complementary use of these two techniques (41). However, metatranscriptomic analyses require a sufficient amount of high-

quality RNA, which can be challenging in the clinical setting due to the presence of RNAses in host samples (42). As a consequence, the use of stabilization reagents for storage of samples is obligatory and already collected samples from previous studies are mostly not usable for metatranscriptomics.
On a next level, **metaproteomics** measures expressed proteins, yielding information on a different dimension, because not all transcripts are actually translated into proteins (42). In the context of gut microbiota-drug interactions, Li *et al.* presented an interesting workflow using ex vivo culturing and metaproteomics to screen compounds against individual microbiomes (43).
Metabolomics directly measures the metabolites present in a sample. This could be applied for the analysis of bacterial as well as drug metabolites and can therefore provide valuable information on bi-directional gut microbiota-drug interactions (42). A potential pitfall of this technique is the differentiation between host-derived and microbiota-derived metabolites, which could be addressed by combining metabolomics data with data on microbiota composition (42).
In addition to these *-omics* analyses, **single cell-targeted approaches** have been proposed to complement future microbiota analyses (44). These approaches are based on cell sorting and enable downstream analyses of individual cells that express a phenotype or function of interest. However, in the context of human cancer, this would require more knowledge on potential phenotypes of interest first.

Although we did not conduct comprehensive metabolite profiling yet, as could be done with metabolomics, we already took the first step into the direction of more function-based analyses and analyzed the microbial metabolites **SCFA and BCFA** in fecal samples from patients **(Chapter 4 and 8)** as well as in samples derived from *in vitro* experiments **(Chapter 5)**. Particularly SCFA gained increased attention in the last years due to their beneficial metabolic, anti-carcinogenic as well as immune-modulatory effects (see e.g. (15, 16, 45)) and were therefore considered to be of great relevance in the context of human cancer.
However, also other bacterial metabolites are of potential interest and should be examined further. It might be assumed that the gut microbiota metabolizes almost all compounds that reach the colon either undigested or as products from previous digestion by the human host. While SCFA are produced mostly through fermentation of dietary carbohydrates and BCFA are derivatives of proteins, also other dietary compounds can be further metabolized by gut bacteria. Amongst others, it has been suggested that gut bacteria also metabolize **phytochemicals**, **bile acids**, and **sulphate** to form metabolites with a potential impact on human health (46-48).
In the cancer setting, ***Lactiplantibacillus*** (previously *Lactobacillus*) ***plantarum*-derived metabolites** have been shown to sensitize 5-FU-resistant CRC cells to the tumor-suppressive effects of butyrate by regulating the functional expression of the SMCT1 transporter (49). However, the exact metabolites responsible for this effect remain to be identified.
Very recently, Tintelnot *et al.* demonstrated that the microbiota-derived tryptophan metabolite indole-3-acetic acid (**3-IAA**) amplified response to chemotherapy against pancreatic ductal adenocarcinoma (PDAC) via accumulation of reactive oxygen species (ROS) and downregulation of autophagy in cancer cells, ultimately reducing their metabolic fitness and proliferation (50). The authors also observed correlations between serum levels of 3-IAA and survival in patients with PDAC (50).

Another recent study indicated that microbiota-mediated **nucleotide synthesis** modulated tumor response to chemoradiotherapy in locally advanced rectal cancer (51). Normally, chemoradiotherapy limits tumor growth by inducing DNA damage to tumor cells. The acquisition of microbiota-derived nucleotides for repair of damaged DNA might thus lead to resistance against anti-proliferative therapy (51).

Consequently, these studies suggest promising candidate molecules, which might be of clinical relevance in the cancer setting and require further investigation. However, it might be assumed that we currently know only a minority of clinically relevant gut microbiota-derived metabolites. Therefore, it will be an important task for gut microbiota research in the upcoming years to identify further potential molecules of interest and to evaluate their usefulness as biomarkers.

Next to abundance of bacterial taxa and their metabolites, **bacterial enzymes** represent another dimension of the gut microbiota. Typically, gut microbes primarily use hydrolytic or reductive reactions to metabolize xenobiotics, while host enzymes catalyze more oxidative and conjugative reactions (52). In the context of human cancer, the bacterial enzyme **β-glucuronidase (GUS)** is of particular interest. GUS enzymes can cleave glucuronides, which are formed in the liver to produce water-soluble substrates from xenobiotics to enable elimination from the body. Different GUS enzymes have been identified in human feces, which are structurally diverse and target different molecules (53). For our research field, the following two already described GUS-catalyzed reactions are of particular interest (Figure 2). Of note, this does not rule out that many more cancer-associated GUS functions remain to be investigated,

1. **The deconjugation of estrogens:** GUS enzymes, more specifically members of the Loop 1 GUS, mini-Loop 1 GUS, and FMN-binding GUS, have been shown to cleave and reactivate estrogen glucuronides, which might result in greater reabsorption of free estrogens into the blood stream (54). In line with this, Flores *et al.* indicated that fecal GUS activity was inversely correlated with fecal estrogen levels, implying that high GUS activity might lead to increased estrogen absorption (55). In combination with the observation that increased blood estrogen levels have been repeatedly linked to an increased breast cancer risk in postmenopausal women, it has been suggested that higher GUS activity could be associated with an increased risk to develop hormone receptor-positive (ER+) breast cancer (56-58). In combination with our results indicating AC-D-induced shifts in gut microbiota composition in postmenopausal women with ER+ breast cancer (**Chapter 6**), this poses the question whether GUS enzymes might also affect response of ER+ tumors during chemotherapy treatment. Consequently, this will be an important question for future studies, since if GUS would modulate tumor response, it might be a potential target to optimize response to chemotherapy in these patients.

2. **Hydrolysis and reactivation of SN-38G:** Next to 5-FU, which has been investigated in the context of this thesis (**Chapter 3,4 and 5**), irinotecan is another important chemotherapeutic agent in the setting of metastatic CRC (59). Previously, increased GUS activity has been linked to severe and dose-limiting gastrointestinal toxicity during irinotecan-based chemotherapy (60-62). Under normal circumstances, SN-38 (the active metabolite of irinotecan) is predominantly detoxified in the liver by the enzyme UGT1A1

through transfer of a glucuronic acid from the cofactor UDP-glucuronic acid to SN-38, resulting into the formation of SN-38G (62). SN-38G is an inactivated and water-soluble metabolite that is secreted via the bile for fecal excretion (62). However, bacterial GUSs present in the intestinal lumen, are able to reactivate SN-38G into the active SN-38 (61). Subsequently, presence of this active and cytotoxic metabolite in the colon triggers gastrointestinal toxicity, mainly in the form of severe diarrhea (62). Interestingly, recent studies showed that particularly Loop 1 GUS enzymes, which are present in bacteria such as *Eubacterium eligens*, *E.coli*, and *Clostridium perfringens*, exert SN-38G-specific activity (63, 64). Abundance of this specific GUS enzymes differs between individuals (63), probably explaining why only part of the patients treated with irinotecan experiences gastrointestinal toxicity. These results suggest that future research should also investigate irinotecan-induced gut microbiota shifts, as well as whether GUS activity and/or the abundance of bacteria harboring Loop 1 GUS enzymes predicts the development of gastrointestinal toxicity. Interestingly, the Enterobacteriaceae family member *E.coli*, which has been shown to be involved in 5-FU metabolism (3, 18) seems to also play a role during irinotecan-based treatment. This further supports our hypothesis that bacteria belonging to the Enterobacteriaceae family might characterize the cancer-associated gut microbiota and might negatively affect different aspects of human cancer (see *section 1.2*). Interventions inhibiting the overgrowth of Enterobacteriaceae, such as our prebiotic intervention (**Chapter 5**), might thus be of great benefit for cancer patients treated with different treatment regimens.

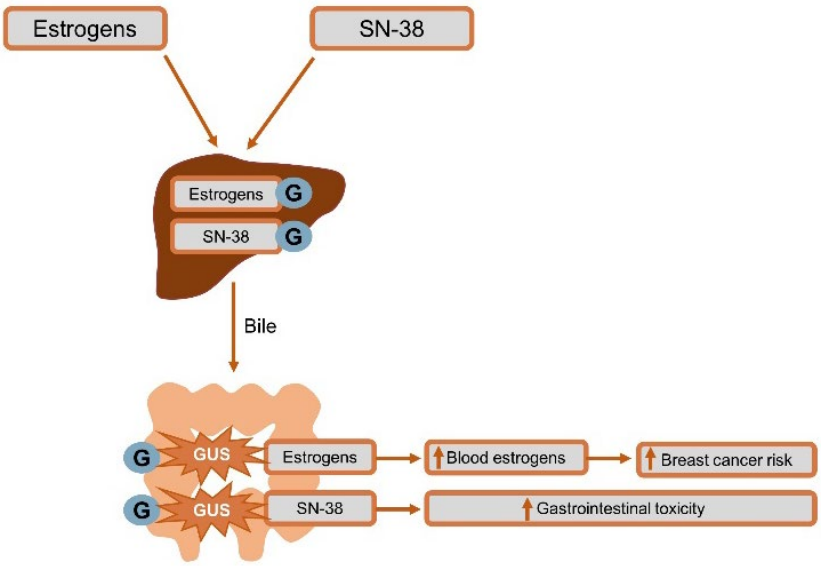


Figure 2: *Schematic representation of two GUS-mediated pathways which are of relevance in the context of human cancer. Blue circles represent added glucuronides, which can be removed by GUS enzymes. (1) GUS activity in the colon leads to estrogen reabsorption into the blood stream and potentially increases the risk to develop ER+ breast cancer in postmenopausal women; (2) GUS activity leads to reactivation of SN-38G into the cytotoxic SN-38, leading to increased gastrointestinal toxicity during irinotecan-based chemotherapy.*

To conclude this paragraph, it needs to be mentioned that many dimensions of the gut microbial equilibrium remain to be investigated before the field can move from basic microbiota research to large-scale microbiota-based precision medicine. Currently, there are several methods available to analyze the different dimensions of the gut microbiota and new methods are rapidly evolving.
Due to the large complexity of the gut microbiota, there is not *one* golden-standard method for future research. On the contrary, the combination of composition-based and function-based techniques as well as the integration of multiple *-omics* approaches is thought to be the most powerful strategy. The application of different techniques on the same cohort is also considered to be beneficial for the robustness of scientific evidence (65). Which methods are chosen for a study is highly dependent on the specific research context and should be well-thought though, taking into consideration research questions and hypotheses, as well as practical and financial issues. In the end, the complementary use of different techniques to cover different dimensions of the gut microbiota will be of great benefit for the advancement of this research field and successful clinical translation of research findings.

3.3 The beginning of a new era: the research field of pharmacomicrobiomics

The work presented in this thesis combines the different research disciplines of oncology, microbiology, and pharmacology, thereby fitting into the research field of pharmacomicrobiomics. To understand what this complex word means, let us start at the beginning.
With the emergence of personalized medicine in the last decades, there was also increasing attention for the influence of the individual genetic profile on drug metabolism. Recently, a large European study demonstrated that genotype-guided treatment based on a 12-gene pharmacogenetic panel significantly decreased the incidence of clinically relevant adverse drug reactions (66). Also in the case of irinotecan metabolism, not only microbial enzymes (GUS, as described in *section 3.2*) affect the occurrence of toxicity, but also genetic variations encoding for the human UGT1A1 enzyme, which couples glucuronic acid to SN-38 in the liver (67).
However, the genetic profile of the patient cannot explain all variability in individual drug responses and the experience of toxicity. Considering that the human body harbors more bacterial than human genes (68), it is likely that the individual gut microbiota profile is another factor interacting with xenobiotic metabolism. And this is where the new research field of pharmacomicrobiomics came into the picture. To support the hypothesis that the gut microbiota plays a role in xenobiotic metabolism, Zhernakova *et al.* performed metagenomic sequencing of fecal samples derived from 1,135 participants and reported that (besides antibiotics), also several other drug categories, for instance proton pump inhibitors (PPI) metformin, statins, and laxatives had a strong impact on the gut microbiome (69). This is in line with the *in vitro* studies of Maier *et al.* and Zimmermann *et al.*, showing an influence of several human-targeted drugs on gut bacteria (70), as well as that bacteria could directly metabolize different drugs (71).

In vitro pharmacomicrobiomics studies as the two described above as well as our own experiments (**Chapter 5**) typically face a common problem: the selection of an appropriate drug concentration which is in line with physiological conditions. Therefore, crucial parameters

which should be taken into consideration in the clinical as well as in the *in vitro* setting are the administration route as well as pharmacokinetics of the drug to be investigated.
Drugs can reach the gut microbiota via different routes. Oral drugs that are incompletely absorbed in the small intestine, can reach the colon directly. Readily absorbed drugs and compounds that are intravenously injected can reach the colon after hepatic metabolism through biliary excretion (52). The current thesis investigated oral (capecitabine, **Chapter 3 and 4**) and intravenously injected drugs (AC-D, **Chapter 6**) as well as 5-FU, which can be administered intravenously or orally as the prodrug capecitabine (**Chapter 5**). Hereby, the 5-FU dosage for the *in vitro* experiments was carefully chosen and described in detail, which is also highly encouraged for future studies in this field.

It should be emphasized that not *either* the gut microbiota *or* human genetics influence drug metabolism. On the contrary, an individuals' drug response is most likely shaped by a complex interplay between the gut microbiome, human genetics as well as exogenous factors (72). In the cancer setting, it is likely to be even more complex due to the interfering effect of the tumor (73). For instance, Zimmermann *et al.* suggested one possible strategy to approach this complexity and presented a physiologically-based pharmacokinetic model to untangle host and microbiota contributions to drug metabolism, if both produce the same metabolites (74). For the future, more and complementary research initiatives to develop similar models will be needed. Hereby, it should be stimulated that proposed models are reproducible and available for use by other research groups. This would considerably increase efficiency and would not only prevent the wasting of research funding, but also that multiple research groups unnecessarily work on the same issues.

The large complexity and variety of factors shaping an individual's drug response poses a great challenge for researchers in this field and will require multidisciplinary approaches, involving (amongst others) the fields of microbiology, oncology, pharmacology, as well as computational and systems biology. Furthermore, distinct techniques and a combination of fundamental and clinical research will be needed to untangle gut microbiota – drug interactions.
Because knowledge concerning the exact roles of specific bacterial strains and associated pathways or genes is very limited at this moment, fundamental and mechanistic research will be an important cornerstone of the pharmacomicrobiomics research of the upcoming years. For instance, the effect of certain gene (variations) could be explored by deletion of the gene of interest in bacterial strains and subsequent incubation with the drug (see e.g. the study of Spanogiannopoulos *et al.* (18)).
Additionally, tumor-bearing mice with a humanized microbiome represent another promising strategy to dissect effects of gut bacteria on anti-tumor efficacy of chemotherapeutics (75). These mechanistic studies will be important to provide evidence on causal relationships between gut bacteria and drug metabolism and to identify potential bacteria, genes, or molecules of interest to be further investigated in the clinical setting.
The different *-omics* techniques, as described in *section 3.2* will also be important tools to unravel gut microbiota-drug interactions. Additionally, the application of machine learning approaches in the field of pharmacomicrobiomics is expected to increase in the nearby future. For instance, one recent example of how data-driven approaches could be used to predict gut microbiota-drug interactions is the study of Algavi & Borenstein, who integrated information

about the chemical properties of drugs and the genomic content of microbes in a machine learning model (76).

However, these state-of-the-art techniques produce a large amount of highly complex data, which require advanced bioinformatic expertise. Simultaneously, bioinformatic analysis is currently often the bottleneck of gut microbiota research, because of a lack of trained personnel (34). This highlights the urgent need for more study programs related to bioinformatics and computational biology, as well as specialized training for young scientists. Especially data obtained from analysis of clinical samples is of great value and should be analyzed with sufficient expertise to generate results of high quality and scientific robustness. The training of next-generation scientists with profound bioinformatic skills is thus considered to be one of the most important action points for the near future in order to advance the research field of pharmacomicrobiomics.

In addition, it will be pivotal to sensitize clinicians to and inform them about the potential role of the gut microbiota during treatment. Clinicians have the best overview of the patient's needs as well as the implementation possibilities for gut microbiota-modulating interventions. Therefore, it is crucial to involve clinicians as early as possible in the design of future studies and to always keep implementation strategies in mind.
Conversely, clinical researchers should feel encouraged to involve fundamental gut microbiota researchers and to incorporate gut microbiota sampling in their clinical studies. Particularly if new drug(combinations) are tested in clinical trials, it would be worthwhile to include the gut microbiota as modulating factor from the beginning.
Together, this will significantly contribute to the rapid clinical translation of research findings and the further progress of the research field of pharmacomicrobiomics. Good communication with clinical practice is thus a further action point for the next years and might be achieved by (amongst others) the integration of pharmacomicrobiomics into the curricula of study programs, publication of microbiota papers in clinical journals, presentations on medical congresses as well as close collaboration between fundamental and clinical scientists at the research sites.

Despite the challenges described above, the investment of money, time, and effort into the evolving research field of pharmacomicrobiomics is expected to be of great benefit, because it has the potential to revolutionize the treatment of cancer. With more insights into factors influencing an individual's' response to chemotherapy, it would be possible to tackle major problems of modern medicine: we could not only prevent that patients experience side effects from a therapy without benefit but could also significantly reduce the necessity for hospitalization and the occurrence of therapy resistance.
To achieve this, targeted microbiota modulation during chemotherapy is supposed to be of increasing importance in the upcoming years. Previous research already identified some potential targets (e.g. Enterobacteriaceae, SCFA, GUS), but further mechanistic fundamental research, followed by large-scale studies are needed to validate and expand these targets. Based on this knowledge, it will be possible to choose the optimal intervention, matching the specific needs of the (sub)population or individual of interest. These specific needs might differ depending on the treatment setting (e.g. curative or palliative, adjuvant or neoadjuvant), the location of the tumor (gastrointestinal or non-gastrointestinal) and the specific bacteria-drug interactions that have been identified for this drug.

To conclude, it might be stated that we are only at the beginning of the era of pharmacomicrobiomics and that there are exciting times ahead. The next years of research will determine whether we can successfully take the step to clinical translation. These are exciting times to be involved in the field of pharmacomicrobiomics!

3.4 Some points of attention for future research

As outlined above, microbiota research is a highly complex research field. Due to the large number of factors interacting with the gut microbiota, which are described in more detail in **Chapter 1**, it is almost impossible to absolutely exclude confounding effects for all of them. As a consequence, no study design is perfect and without shortcomings. However, as researchers with the aim to produce meaningful science output, we should always be aware of potential factors influencing the quality of our research and strive to minimize the impact of them.

This thesis has been written with the aim to contribute to the future implementation of microbiota-based precision medicine into clinical practice. This could be achieved for instance by establishing microbial biomarkers to predict chemotherapy efficacy, followed by targeted gut microbiota modulation. However, the field is still far away from reaching this aim and more high-quality research is urgently required. The work presented here can be seen as pioneering work, which produced some first interesting results, but also (and maybe even more importantly) insights into important points of attention for future research. Therefore, the following parameters should be taken into consideration when designing future microbiota studies.

Diet: The human diet is one of the most important determinants of inter-individual microbiota variation (69, 77, 78). Hereby, different factors play a role, for instance (but not limited to) macronutrient composition, seasonal variations, fiber content or dietary additives (77). Therefore, dietary patterns should also be reported in studies involving gut microbiota analysis. However, the assessment of dietary intake can be quite time-consuming, e.g. when food frequency questionnaires or food diaries are used. This could be challenging in cancer patients and might increase the burden for the patient to participate. Consequently, the exact type and extent of dietary assessment needs to be adapted to the individual research context. However, the investigation of an overall dietary pattern (e.g. vegan or vegetarian or specialized diet) as well as the ingestion of supplements and/or medical nutrition is considered to be the minimum. On the other hand, the strong influence of diet on the gut microbiota also opens unique possibilities. The diet (or ingestion of specific components, such as prebiotics) is under the control of the patient. Consequently, it offers the individual an opportunity to affect his/her own medical condition. Consequently, it would be expected that patients generally show good compliance if microbiota-modulating interventions would be implemented into clinical practice.

Gut transit time and stool consistency: Particularly in the cancer setting, where the prevalence of diarrhea is relatively high, gut transit time and stool consistency can have a considerable impact on gut microbiota composition and activity (79, 80). Therefore, it would be of great benefit for the quality of the research to also include non-invasive markers of stool

consistency, such as the Bristol stool scale (BSS) or fecal dry weight. Especially when total concentrations of microbial metabolites are measured in fecal samples, concentrations should be corrected for stool consistency, as we did in **Chapters 4 and 8** for SCFA and BCFA levels by assessing fecal dry weight.

Temporal fluctuations: In a recent study with 20 Belgian women with longitudinal microbiota sampling, genus abundances showed substantial day-to-day variation around an equilibrium state (81). This might have consequences for data derived from clinical microbiota studies, especially when the sample size is relatively low and/or only one sample per individual is collected. This highlights that it might be beneficial for clinical relevance to include repeated samples per patient. Furthermore, the reporting of sampling day and time is recommended for future studies.

Co-medication: In clinical practice, it cannot be avoided that patients also receive other medication next to the drug of interest. For instance, in **Chapter 3** and **Chapter 6**, we saw strong effects of antibiotic administration during chemotherapy (**Chapter 3**) or perioperatively (**Chapter 6**). In view of the considerable impact of antibiotics (82), as well as the influence of other commonly prescribed drugs on gut microbiota composition (69), the use of all co-medication during the study period should be recorded precisely, as done in our longitudinal clinical studies **(Chapter 3, 4 and 6)**. Furthermore, it should be reflected whether specific drugs should be added as exclusion criteria or not. Since this highly depends on the specific research question and setting, no general recommendations concerning the exclusion of specific drugs can be formulated.

Sample size: Due to its large complexity, considerable inter-individual variation, and the involvement of various confounding factors, clinical gut microbiota studies typically require large sample sizes. Consequently, the inclusion of cohorts with an adequate number of patients might take a prolonged period of time. Our studies (**Chapter 3, 4, 6 and 8**) also showed that larger sample sizes would have been beneficial for the detection of consistent effects. To overcome this limitation and to create sufficiently large cohorts, the concept of *"joining forces"* by collaboration between different research groups is essential.
To enable merging of cohorts from different research sites, gut microbiota researchers should work together to harmonize procedures for sample collection, storage conditions, protocols for nucleic acid extraction as well as primer selection as much as possible. Especially the method of DNA or RNA extraction has been previously shown to produce considerable experimental variability (83). In general, for samples collected at different sites, nucleic acid extraction, sequencing as well as bioinformatic processing should be performed for all samples together and within the same institution, in order to reduce batch effects.
Cross-research group collaboration not only accelerates the production of new and robust evidence but would also increase external validity of research findings and rule out the effects of geographical variation. For reproducibility, replicability as well as generalizability of research findings, it is also beneficial if different research groups can approach the same research question from different perspectives (65). In the context of clinical microbiota research, external validity is of particular high importance because clinical relevance of microbiota-based parameters or interventions increases, if more patients can benefit from it. For instance, our 5-FU *in vitro* results (**Chapter 5**) might be applicable to all patients receiving 5-FU-based treatment regimens. While we are currently focusing on 5-FU-based

chemotherapy in CRC, it would be desirable that other research groups aim to replicate the results in patients with other cancer types, for instance gastric or pancreatic cancer.

Important preconditions to enable fruitful collaborations and increase external validity are:
(1) an open communication and publication of all methods used,
(2) reporting of negative findings as well as
(3) open availability of raw data in public repositories.
In the last years, the awareness and demand for Open Science and FAIR data management are increasing. Although these principles could accelerate the progress of gut microbiota research, their realization is often impaired by regulatory hurdles, differences in privacy legislations or misconduct of individuals. This not only delays the progress of the research field, but also costs a lot of money and time. The further (worldwide) promotion and implementation of Open Science and FAIR data principles is thus also important to stimulate collaboration and the creation of larger cohorts for gut microbiota analyses.

In addition to cross-research group collaboration, close collaboration with clinicians as well as the embedding of gut microbiota sampling into clinical studies, as already discussed in *section 3.3*, will simplify the inclusion of more patients to reach adequate sample sizes. Since not all research institutes have the possibility to closely work together with hospitals, the exchange of patient samples between different institutes should also be facilitated.

Expertise: Education and training of young scientists is one of the most important milestones for the progress of our research field. However, due to an often suboptimal working environment in academia (e.g. due to fixed-term contracts, high performance pressure, shortage of staff or money), many talents are leaving to other sectors. Every leaving scientist, who was trained in gut microbiota research, is also a step back for the research field. Consequently, to advance gut microbiota research, principal investigators need to invest into education of students as well as into the training and personal development of young scientists. Furthermore, it will be of increasing importance to create a pleasant working atmosphere, to make expertise to stay in the team.

4. Conclusion: Human cancer and the gut microbiota equilibrium

The gut microbiota has a strong power: it is able to significantly influence human health. Especially when the gut microbiota is in an equilibrium state, gut bacteria and the human host live in symbiosis and benefit from each other. However, if the equilibrium is disturbed, this can lead to the development or manifestation of unhealthy, dysbiotic states.
Altogether, the results presented in this thesis indicate that chemotherapy treatment and cancer cachexia are associated with perturbations of the gut microbiota equilibrium. To counteract these perturbations and/or to minimize the negative consequences, follow-up studies and collaborative research in this field are urgently required. Hereby, the long-term aim should be to implement gut microbiota analysis and modulation into clinical practice. Ultimately, this will enable us to use the power of the gut microbiota for the benefit of cancer patients.

References

1. Alexander JL, Wilson ID, Teare J, Marchesi JR, Nicholson JK, Kinross JM. Gut microbiota modulation of chemotherapy efficacy and toxicity. Nature reviews Gastroenterology & hepatology. 2017;14(6):356-65.
2. Bhatt AP, Redinbo MR, Bultman SJ. The role of the microbiome in cancer development and therapy. CA Cancer J Clin. 2017;67(4):326-44.
3. LaCourse KD, Zepeda-Rivera M, Kempchinsky AG, Baryiames A, Minot SS, Johnston CD, et al. The cancer chemotherapeutic 5-fluorouracil is a potent Fusobacterium nucleatum inhibitor and its activity is modified by intratumoral microbiota. Cell Rep. 2022;41(7):111625.
4. Daillere R, Vetizou M, Waldschmitt N, Yamazaki T, Isnard C, Poirier-Colame V, et al. Enterococcus hirae and Barnesiella intestinihominis Facilitate Cyclophosphamide-Induced Therapeutic Immunomodulatory Effects. Immunity. 2016;45(4):931-43.
5. An J, Ha EM. Combination Therapy of Lactobacillus plantarum Supernatant and 5-Fluouracil Increases Chemosensitivity in Colorectal Cancer Cells. J Microbiol Biotechnol. 2016;26(8):1490-503.
6. Sougiannis AT, VanderVeen BN, Enos RT, Velazquez KT, Bader JE, Carson M, et al. Impact of 5 fluorouracil chemotherapy on gut inflammation, functional parameters, and gut microbiota. Brain Behav Immun. 2019;80:44-55.
7. Galloway-Pena JR, Smith DP, Sahasrabhojane P, Wadsworth WD, Fellman BM, Ajami NJ, et al. Characterization of oral and gut microbiome temporal variability in hospitalized cancer patients. Genome Med. 2017;9(1):21.
8. Sze MA, Baxter NT, Ruffin MTt, Rogers MAM, Schloss PD. Normalization of the microbiota in patients after treatment for colonic lesions. Microbiome. 2017;5(1):150.
9. Stringer AM, Al-Dasooqi N, Bowen JM, Tan TH, Radzuan M, Logan RM, et al. Biomarkers of chemotherapy-induced diarrhoea: a clinical study of intestinal microbiome alterations, inflammation and circulating matrix metalloproteinases. Support Care Cancer. 2013;21(7):1843-52.
10. Reigner B, Blesch K, Weidekamm E. Clinical pharmacokinetics of capecitabine. Clin Pharmacokinet. 2001;40(2):85-104.
11. Derissen EJ, Jacobs BA, Huitema AD, Rosing H, Schellens JH, Beijnen JH. Exploring the intracellular pharmacokinetics of the 5-fluorouracil nucleotides during capecitabine treatment. Br J Clin Pharmacol. 2016;81(5):949-57.
12. Timmers L, Boons CC, Mangnus D, Van de Ven PM, Van den Berg PH, Beeker A, et al. Adherence and Patients' Experiences with the Use of Capecitabine in Daily Practice. Front Pharmacol. 2016;7:310.
13. Li J, Li J, Lyu N, Ma Y, Liu F, Feng Y, et al. Composition of fecal microbiota in low-set rectal cancer patients treated with FOLFOX. Ther Adv Chronic Dis. 2020;11:2040622320904293.
14. Kawasaki Y, Kakimoto K, Tanaka Y, Shimizu H, Nishida K, Numa K, et al. Relationship between Chemotherapy-Induced Diarrhea and Intestinal Microbiome Composition. Digestion. 2023:1-13.
15. van der Beek CM, Dejong CHC, Troost FJ, Masclee AAM, Lenaerts K. Role of short-chain fatty acids in colonic inflammation, carcinogenesis, and mucosal protection and healing. Nutr Rev. 2017;75(4):286-305.
16. Morrison DJ, Preston T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes. 2016;7(3):189-200.
17. Venema K. The TNO In Vitro Model of the Colon (TIM-2). In: Verhoeckx K, Cotter P, López-Expósito I, Kleiveland C, Lea T, Mackie A, et al., editors. The Impact of Food Bioactives on Health: in vitro and ex vivo models. Cham (CH): Springer

Copyright 2015, The Author(s). 2015. p. 293-304.

18. Spanogiannopoulos P, Kyaw TS, Guthrie BGH, Bradley PH, Lee JV, Melamed J, et al. Host and gut bacteria share metabolic pathways for anti-cancer drug metabolism. Nat Microbiol. 2022;7(10):1605-20.
19. Mancabelli L, Milani C, Lugli GA, Turroni F, Cocconi D, van Sinderen D, et al. Identification of universal gut microbial biomarkers of common human intestinal diseases by meta-analysis. FEMS Microbiol Ecol. 2017;93(12).
20. Tremlett H, Zhu F, Arnold D, Bar-Or A, Bernstein CN, Bonner C, et al. The gut microbiota in pediatric multiple sclerosis and demyelinating syndromes. Ann Clin Transl Neurol. 2021;8(12):2252-69.
21. Zeng MY, Inohara N, Nuñez G. Mechanisms of inflammation-driven bacterial dysbiosis in the gut. Mucosal Immunol. 2017;10(1):18-26.

22. Bindels LB, Neyrinck AM, Loumaye A, Catry E, Walgrave H, Cherbuy C, et al. Increased gut permeability in cancer cachexia: mechanisms and clinical relevance. Oncotarget. 2018;9(26):18224-38.
23. Bindels LB, Neyrinck AM, Claus SP, Le Roy CI, Grangette C, Pot B, et al. Synbiotic approach restores intestinal homeostasis and prolongs survival in leukaemic mice with cachexia. Isme j. 2016;10(6):1456-70.
24. Pötgens SA, Brossel H, Sboarina M, Catry E, Cani PD, Neyrinck AM, et al. Klebsiella oxytoca expands in cancer cachexia and acts as a gut pathobiont contributing to intestinal dysfunction. Sci Rep. 2018;8(1):12321.
25. Zimmer J, Lange B, Frick JS, Sauer H, Zimmermann K, Schwiertz A, et al. A vegan or vegetarian diet substantially alters the human colonic faecal microbiota. Eur J Clin Nutr. 2012;66(1):53-60.
26. Pötgens SA, Thibaut MM, Joudiou N, Sboarina M, Neyrinck AM, Cani PD, et al. Multi-compartment metabolomics and metagenomics reveal major hepatic and intestinal disturbances in cancer cachectic mice. J Cachexia Sarcopenia Muscle. 2021.
27. Terrisse S, Derosa L, Iebba V, Ghiringhelli F, Vaz-Luis I, Kroemer G, et al. Intestinal microbiota influences clinical outcome and side effects of early breast cancer treatment. Cell Death Differ. 2021;28(9):2778-96.
28. Vernaci G, Savarino EV, Patuzzi I, Facchin S, Zingone F, Massa D, et al. Characterization of Gut Microbiome Composition in Patients with Triple-Negative Breast Cancer Treated with Neoadjuvant Chemotherapy. Oncologist. 2023.
29. Argiles JM, Busquets S, Stemmler B, Lopez-Soriano FJ. Cancer cachexia: understanding the molecular basis. Nat Rev Cancer. 2014;14(11):754-62.
30. Fearon KC, Glass DJ, Guttridge DC. Cancer cachexia: mediators, signaling, and metabolic pathways. Cell Metab. 2012;16(2):153-66.
31. Pin F, Barreto R, Couch ME, Bonetto A, O'Connell TM. Cachexia induced by cancer and chemotherapy yield distinct perturbations to energy metabolism. J Cachexia Sarcopenia Muscle. 2019;10(1):140-54.
32. Caillet P, Liuu E, Raynaud Simon A, Bonnefoy M, Guerin O, Berrut G, et al. Association between cachexia, chemotherapy and outcomes in older cancer patients: A systematic review. Clin Nutr. 2017;36(6):1473-82.
33. Olsson LM, Boulund F, Nilsson S, Khan MT, Gummesson A, Fagerberg L, et al. Dynamics of the normal gut microbiota: A longitudinal one-year population study in Sweden. Cell Host Microbe. 2022;30(5):726-39.e3.
34. Clavel T, Horz HP, Segata N, Vehreschild M. Next steps after 15 stimulating years of human gut microbiome research. Microb Biotechnol. 2022;15(1):164-75.
35. Le Bastard Q, Ward T, Sidiropoulos D, Hillmann BM, Chun CL, Sadowsky MJ, et al. Fecal microbiota transplantation reverses antibiotic and chemotherapy-induced gut dysbiosis in mice. Sci Rep. 2018;8(1):6219.
36. Terrisse S, Goubet AG, Ueda K, Thomas AM, Quiniou V, Thelemaque C, et al. Immune system and intestinal microbiota determine efficacy of androgen deprivation therapy against prostate cancer. J Immunother Cancer. 2022;10(3).
37. Hugenholtz F, de Vos WM. Mouse models for human intestinal microbiota research: a critical evaluation. Cell Mol Life Sci. 2018;75(1):149-60.
38. Puschhof J, Pleguezuelos-Manzano C, Clevers H. Organoids and organs-on-chips: Insights into human gut-microbe interactions. Cell Host Microbe. 2021;29(6):867-78.
39. Wensel CR, Pluznick JL, Salzberg SL, Sears CL. Next-generation sequencing: insights to advance clinical investigations of the microbiome. J Clin Invest. 2022;132(7).
40. Haiser HJ, Gootenberg DB, Chatman K, Sirasani G, Balskus EP, Turnbaugh PJ. Predicting and manipulating cardiac drug inactivation by the human gut bacterium Eggerthella lenta. Science. 2013;341(6143):295-8.
41. Schirmer M, Franzosa EA, Lloyd-Price J, McIver LJ, Schwager R, Poon TW, et al. Dynamics of metatranscription in the inflammatory bowel disease gut microbiome. Nat Microbiol. 2018;3(3):337-46.
42. Zhang X, Li L, Butcher J, Stintzi A, Figeys D. Advancing functional and translational microbiome research using meta-omics approaches. Microbiome. 2019;7(1):154.
43. Li L, Ning Z, Zhang X, Mayne J, Cheng K, Stintzi A, et al. RapidAIM: a culture- and metaproteomics-based Rapid Assay of Individual Microbiome responses to drugs. Microbiome. 2020;8(1):33.
44. Hatzenpichler R, Krukenberg V, Spietz RL, Jay ZJ. Next-generation physiology approaches to study microbiome function at single cell level. Nat Rev Microbiol. 2020;18(4):241-56.

45. Corrêa-Oliveira R, Fachi JL, Vieira A, Sato FT, Vinolo MA. Regulation of immune cell function by short-chain fatty acids. Clin Transl Immunology. 2016;5(4):e73.
46. Russell W, Duthie G. Plant secondary metabolites and gut health: the case for phenolic acids. Proc Nutr Soc. 2011;70(3):389-96.
47. Louis P, Hold GL, Flint HJ. The gut microbiota, bacterial metabolites and colorectal cancer. Nat Rev Microbiol. 2014;12(10):661-72.
48. Oliphant K, Allen-Vercoe E. Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. Microbiome. 2019;7(1):91.
49. Kim HJ, An J, Ha EM. Lactobacillus plantarum-derived metabolites sensitize the tumor-suppressive effects of butyrate by regulating the functional expression of SMCT1 in 5-FU-resistant colorectal cancer cells. J Microbiol. 2022;60(1):100-17.
50. Tintelnot J, Xu Y, Lesker TR, Schönlein M, Konczalla L, Giannou AD, et al. Microbiota-derived 3-IAA influences chemotherapy efficacy in pancreatic cancer. Nature. 2023;615(7950):168-74.
51. Teng H, Wang Y, Sui X, Fan J, Li S, Lei X, et al. Gut microbiota-mediated nucleotide synthesis attenuates the response to neoadjuvant chemoradiotherapy in rectal cancer. Cancer Cell. 2023;41(1):124-38.e6.
52. Koppel N, Maini Rekdal V, Balskus EP. Chemical transformation of xenobiotics by the human gut microbiota. Science. 2017;356(6344).
53. Biernat KA, Pellock SJ, Bhatt AP, Bivins MM, Walton WG, Tran BNT, et al. Structure, function, and inhibition of drug reactivating human gut microbial beta-glucuronidases. Sci Rep. 2019;9(1):825.
54. Ervin SM, Li H, Lim L, Roberts LR, Liang X, Mani S, et al. Gut microbial beta-glucuronidases reactivate estrogens as components of the estrobolome that reactivate estrogens. J Biol Chem. 2019;294(49):18586-99.
55. Flores R, Shi J, Fuhrman B, Xu X, Veenstra TD, Gail MH, et al. Fecal microbial determinants of fecal and systemic estrogens and estrogen metabolites: a cross-sectional study. J Transl Med. 2012;10:253.
56. Key T, Appleby P, Barnes I, Reeves G. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst. 2002;94(8):606-16.
57. Kaaks R, Rinaldi S, Key TJ, Berrino F, Peeters PH, Biessy C, et al. Postmenopausal serum androgens, oestrogens and breast cancer risk: the European prospective investigation into cancer and nutrition. Endocr Relat Cancer. 2005;12(4):1071-82.
58. Arnone AA, Cook KL. Gut and Breast Microbiota as Endocrine Regulators of Hormone Receptor-positive Breast Cancer Risk and Therapy Response. Endocrinology. 2022;164(1).
59. Bailly C. Irinotecan: 25 years of cancer treatment. Pharmacol Res. 2019;148:104398.
60. Wallace BD, Wang H, Lane KT, Scott JE, Orans J, Koo JS, et al. Alleviating cancer drug toxicity by inhibiting a bacterial enzyme. Science. 2010;330(6005):831-5.
61. Bhatt AP, Pellock SJ, Biernat KA, Walton WG, Wallace BD, Creekmore BC, et al. Targeted inhibition of gut bacterial beta-glucuronidase activity enhances anticancer drug efficacy. Proc Natl Acad Sci U S A. 2020;117(13):7374-81.
62. Chamseddine AN, Ducreux M, Armand JP, Paoletti X, Satar T, Paci A, et al. Intestinal bacterial beta-glucuronidase as a possible predictive biomarker of irinotecan-induced diarrhea severity. Pharmacol Ther. 2019;199:1-15.
63. Jariwala PB, Pellock SJ, Goldfarb D, Cloer EW, Artola M, Simpson JB, et al. Discovering the Microbial Enzymes Driving Drug Toxicity with Activity-Based Protein Profiling. ACS Chem Biol. 2020;15(1):217-25.
64. Dashnyam P, Mudududdla R, Hsieh TJ, Lin TC, Lin HY, Chen PY, et al. β-Glucuronidases of opportunistic bacteria are the major contributors to xenobiotic-induced toxicity in the gut. Sci Rep. 2018;8(1):16372.
65. Schloss PD. Identifying and Overcoming Threats to Reproducibility, Replicability, Robustness, and Generalizability in Microbiome Research. mBio. 2018;9(3).
66. Swen JJ, van der Wouden CH, Manson LE, Abdullah-Koolmees H, Blagec K, Blagus T, et al. A 12-gene pharmacogenetic panel to prevent adverse drug reactions: an open-label, multicentre, controlled, cluster-randomised crossover implementation study. Lancet. 2023;401(10374):347-56.
67. Hulshof EC, de With M, de Man FM, Creemers GJ, Deiman B, Swen JJ, et al. UGT1A1 genotype-guided dosing of irinotecan: A prospective safety and cost analysis in poor metaboliser patients. Eur J Cancer. 2022;162:148-57.
68. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59-65.

69. Zhernakova A, Kurilshikov A, Bonder MJ, Tigchelaar EF, Schirmer M, Vatanen T, et al. Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. Science. 2016;352(6285):565-9.
70. Maier L, Pruteanu M, Kuhn M, Zeller G, Telzerow A, Anderson EE, et al. Extensive impact of non-antibiotic drugs on human gut bacteria. Nature. 2018;555(7698):623-8.
71. Zimmermann M, Zimmermann-Kogadeeva M, Wegmann R, Goodman AL. Mapping human microbiome drug metabolism by gut bacteria and their genes. Nature. 2019;570(7762):462-7.
72. Doestzada M, Vila AV, Zhernakova A, Koonen DPY, Weersma RK, Touw DJ, et al. Pharmacomicrobiomics: a novel route towards personalized medicine? Protein Cell. 2018;9(5):432-45.
73. Xavier JB, Young VB, Skufca J, Ginty F, Testerman T, Pearson AT, et al. The Cancer Microbiome: Distinguishing Direct and Indirect Effects Requires a Systemic View. Trends Cancer. 2020;6(3):192-204.
74. Zimmermann M, Zimmermann-Kogadeeva M, Wegmann R, Goodman AL. Separating host and microbiome contributions to drug pharmacokinetics and toxicity. Science. 2019;363(6427).
75. Tripathi A, Debelius J, Brenner DA, Karin M, Loomba R, Schnabl B, et al. The gut-liver axis and the intersection with the microbiome. Nat Rev Gastroenterol Hepatol. 2018;15(7):397-411.
76. Algavi YM, Borenstein E. A data-driven approach for predicting the impact of drugs on the human microbiome. Nat Commun. 2023;14(1):3614.
77. Kolodziejczyk AA, Zheng D, Elinav E. Diet-microbiota interactions and personalized nutrition. Nat Rev Microbiol. 2019;17(12):742-53.
78. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, et al. Linking long-term dietary patterns with gut microbial enterotypes. Science. 2011;334(6052):105-8.
79. Procházková N, Falony G, Dragsted LO, Licht TR, Raes J, Roager HM. Advancing human gut microbiota research by considering gut transit time. Gut. 2022.
80. Vandeputte D, Falony G, Vieira-Silva S, Tito RY, Joossens M, Raes J. Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates. Gut. 2016;65(1):57-62.
81. Vandeputte D, De Commer L, Tito RY, Kathagen G, Sabino J, Vermeire S, et al. Temporal variability in quantitative human gut microbiome profiles and implications for clinical research. Nature communications. 2021;12(1):6740.
82. Maier L, Goemans CV, Wirbel J, Kuhn M, Eberl C, Pruteanu M, et al. Unravelling the collateral damage of antibiotics on gut bacteria. Nature. 2021;599(7883):120-4.
83. Sinha R, Abu-Ali G, Vogtmann E, Fodor AA, Ren B, Amir A, et al. Assessment of variation in microbial community amplicon sequencing by the Microbiome Quality Control (MBQC) project consortium. Nat Biotechnol. 2017;35(11):1077-86.

Chapter 10

Summary / Samenvatting

Summary

The role of the gut microbiota in human cancer - The power of an equilibrium

The present thesis investigates how chemotherapy and cancer cachexia might challenge the gut microbial equilibrium. Furthermore, it shows that prebiotics represent a promising strategy to restore or maintain this equilibrium in cancer patients.

As an introduction into the topic, **Chapter 1** familiarizes the reader with some important concepts of gut microbiota research as well as prior research in the field of the gut microbiota in human cancer. Furthermore, prebiotics, probiotics, synbiotics, postbiotics, other dietary interventions, fecal microbiota transplantation and antibiotics are presented as potential strategies to influence gut microbiota composition.

1. The gut microbiota and chemotherapy

Chapter 2 provides a systematic overview of clinical research concerning the interactions between the gut microbiota and systemic anti-cancer therapies, more specifically chemotherapy, immunotherapy and hormonal therapy. This review indicates not only that the administration of systemic treatments affects the gut microbiota, but that gut bacteria could also influence the outcome of these treatments. However, generalizability of these results is limited because of large differences in study design and sampling timepoints, as well as a considerable heterogeneity in patient characteristics.

The following chapters focus on mutual relationships between gut bacteria and the chemotherapeutic compound 5-Fluorouracil (5-FU). In **Chapter 3**, we analyze microbial diversity (α-diversity), community structure (β-diversity), and bacterial abundances during three cycles of capecitabine in patients with metastatic or unresectable colorectal cancer (CRC). In this relatively small cohort of 33 patients, we do not identify consistent 5-FU-induced effects on gut microbiota composition and no associations between gut microbiota parameters and tumor response. Nevertheless, this pilot study provides a framework and insights into potential challenges of gut microbiota research in complex patient cohorts, which will be of significant benefit for future longitudinal studies in similar populations.

In 44 patients derived from the same patient cohort, we also quantify fecal levels of the gut bacteria-derived short-chain fatty acids (SCFA) and branched-chain fatty acids (BCFA) (**Chapter 4**). Particularly SCFA have been previously described to exert beneficial metabolic, anti-inflammatory, as well as anti-carcinogenic effects. In our cohort, fecal levels of the SCFA valerate and caproate decrease significantly during three cycles of capecitabine. Furthermore, baseline levels of the BCFA iso-butyrate are significantly lower in fecal samples from patients with partial response, compared to patients with stable disease or progressive disease, indicating a potential link between BCFA levels and tumor response to 5-FU-based chemotherapy. Nutritional status (MUST score), physical performance (Karnofsky Performance Score), and chemotherapy-induced toxicity (CTCAE criteria) are not significantly associated with SCFA or BCFA in these patients. Surprisingly, baseline SCFA correlate positively with blood neutrophil counts, which is contradictory to the expected anti-inflammatory effects. To combine data on bacterial abundances with metabolite data, different associations between SCFA/BCFA and the abundance of bacterial families are described.

As a follow-up of these two clinical studies, the effects of 5-FU on both gut microbiota composition, as well as SCFA/BCFA levels are investigated using the TNO *in vitro* model of the colon (TIM-2) and a human-derived microbial consortium (**Chapter 5**). The use of this validated, computer-controlled model, that closely mimics the physiological conditions of the colon, allows the close monitoring of molecular gut microbiota-chemotherapy interactions in a more controlled setting. By means of these *in vitro* experiments, we identify 5-FU-induced microbiota shifts, and demonstrate that these shifts could be counteracted by the addition of prebiotics during 5-FU treatment. Consequently, it is hypothesized that prebiotics might represent a promising strategy to prevent manifestation of microbial dysbiosis during 5-FU-based chemotherapy and that this requires further investigation in a clinical setting.

In **Chapter 6** we investigate the gut microbiota in 44 postmenopausal breast cancer patients during combinatorial treatment with Adriamycin, Cyclophosphamide and Docetaxel (AC-D) and identify AC-D-induced gut microbiota changes. More specifically, abundance of *Ruminococcaceae NK4A214 group*, *Christensenellaceae R7 group*, *Ruminococcaceae UCG-005* and *Marvinbryantia* decreases during AC-D, while abundance of Proteobacteria, unclassified Enterobacterales and *Lactobacillus* increases. Furthermore, the occurrence of diarrhea during the Docetaxel cycles seems to be associated with microbial species richness. However, there is no association between the gut microbiota and tumor response in a subgroup of patients who received AC-D in the neoadjuvant setting.

2. The gut microbiota and cancer cachexia

Another aspect of human cancer that is investigated in the context of this thesis, is cancer cachexia, a metabolic wasting syndrome. In **Chapter 7**, we summarize the current knowledge about cachexia-associated gut microbiota profiles, as described in previous (preclinical) research. Furthermore, we describe associations between gut bacteria and different metabolic hallmarks of cancer cachexia, including systemic inflammation, gut permeability, muscle wasting, insulin sensitivity, food intake, as well as body weight regulation.

Subsequently, **Chapter 8** presents one of the first clinical gut microbiota studies in the context of cancer cachexia. For this cross-sectional study, patients suffering from pancreatic-, breast-, lung-, or ovarian cancer are included before treatment initiation and are classified as being either cachectic or non-cachectic, based on weight loss. As a control group, cancer-free partners are included. This study indicates that abundance of the pro-inflammatory Proteobacteria, a genus from the Enterobacteriacea family, as well as *Veillonella* are enriched in cachectic cancer patients, while the genera *Megamonas* and *Peptococcus* are more abundant in non-cachectic cancer patients. In addition, fecal levels of SCFA tend to be reduced in cachectic cancer patients, but this is statistically significant for acetate only. Markers of intestinal inflammation (calprotectin) or systemic inflammation (CRP and leukocyte counts) are not significantly elevated in cachectic cancer patients.

To conclude, **Chapter 9** integrates the results from all previous chapters and provides a framework, as well as practical recommendations for future studies, with the aim to stimulate and facilitate further research in this field.

Samenvatting

De rol van de darmmicrobiota bij humane kanker - De kracht van een evenwicht

Dit proefschrift onderzoekt hoe chemotherapie en cachexie het evenwicht van de darmmicrobiota mogelijk kunnen verstoren. Bovendien toont het aan dat prebiotica een veelbelovende strategie vormen om het microbiële evenwicht in kankerpatiënten te herstellen of te behouden.

In **Hoofdstuk 1** wordt de lezer ingeleid in het onderwerp en worden belangrijke concepten van darmmicrobiota onderzoek besproken, evenals eerdere onderzoeken op het gebied van de darmmicrobiota bij kanker. Verder worden prebiotica, probiotica, synbiotica, postbiotica, andere voedingsinterventies, fecale microbiota-transplantatie en antibiotica gepresenteerd als mogelijke strategieën om de samenstelling van de darmmicrobiota te beïnvloeden.

1. De darmmicrobiota en chemotherapie

Hoofdstuk 2 geeft een systematisch overzicht van klinisch onderzoek naar de interacties tussen de darmmicrobiota en systemische behandeling van kanker, meer specifiek chemotherapie, immunotherapie en hormonale therapie. Uit dit overzicht blijkt niet alleen dat de systemische behandelingen invloed kunnen hebben op de darmmicrobiota, maar ook dat de darmmicrobiota de uitkomst van deze behandelingen kan moduleren. De generaliseerbaarheid van deze resultaten is echter beperkt vanwege grote verschillen in onderzoeksopzet en verzamelingstijdstippen, evenals aanzienlijke heterogeniteit in patiëntkenmerken.

De volgende hoofdstukken richten zich op wederzijdse relaties tussen darmbacteriën en het chemotherapeutische middel 5-Fluorouracil (5-FU). In **Hoofdstuk 3** analyseren we de diversiteit (α-diversiteit) en samenstelling (β-diversiteit) van de darmmicrobiota, evenals de relatieve hoeveelheid van speciefieke bacteriën tijdens drie cycli van capecitabine bij patiënten met gemetastaseerde of niet-resectabele colorectaal carcinoom. In deze groep van 33 patiënten identificeren we geen consistente effecten van 5-FU op de samenstelling van de darmmicrobiota en geen verbanden tussen darmmicrobiota parameters en tumorrespons. Desondanks biedt deze pilotstudie inzichten in potentiële uitdagingen van darmmicrobiota onderzoek bij complexe patiëntenpopulaties. Dit zal aanzienlijke voordelen opleveren voor toekomstige longitudinale studies in vergelijkbare populaties.

Bij 44 patiënten uit dezelfde patiëntenpopulatie hebben we ook de fecale concentraties van korteketenvetzuren (SCFA) en vertakte-ketenvetzuren (BCFA) gemeten (**Hoofdstuk 4**). Deze metabolieten worden geproduceerd door de darmmicrobiota. Met name SCFA werden eerder beschreven als stoffen met gunstige metabole, ontstekingsremmende en anti-carcinogene effecten. In onze patiëntengroep nemen de fecale waarden van de SCFA valeraat en caproaat significant af gedurende drie cycli van capecitabine. Bovendien zijn de basis concentraties van de BCFA iso-butyraat significant lager in fecale monsters van patiënten met een gedeeltelijke respons in vergelijking met patiënten met stabiele ziekte of progressieve ziekte, wat wijst op een mogelijk verband tussen BCFA en de tumorrespons op 5-FU-gebaseerde chemotherapie. Voedingsstatus (MUST-score), fysieke conditie (Karnofsky Performance Score) en toxiciteit (CTCAE-criteria) vertonen geen significante associaties met SCFA of

BCFA bij deze patiënten. Het valt echter op dat de basis waarden van SCFA positief correleren met het aantal neutrofielen in het bloed, wat in tegenspraak is met de verwachte ontstekingsremmende effecten. Daarnaast beschrijven we ook verschillende associaties tussen SCFA/BCFA en bacteriële families.

Als vervolg op deze twee klinische onderzoeken worden de effecten van 5-FU op zowel de samenstelling van de darmmicrobiota als ook op SCFA/BCFA concentraties onderzocht. Hiervoor wordt het TNO *in vitro* model van het colon (TIM-2) en een menselijke microbiota gebruikt (**Hoofdstuk 5**). Het gebruik van dit gevalideerde, computer-gecontroleerde model, dat de fysiologische omstandigheden van het colon simuleert, maakt het mogelijk om moleculaire interacties tussen de darmmicrobiota en chemotherapie in een gecontroleerde omgeving te monitoren. Met behulp van deze *in vitro* experimenten identificeren we 5-FU-geïnduceerde verschuivingen in de microbiota en tonen we aan dat deze verschuivingen kunnen worden tegengegaan door de toevoeging van prebiotica tijdens de behandeling met 5-FU. Hieruit volgt de hypothese dat prebiotica een veelbelovende strategie kunnen vormen om microbiële dysbiose tijdens 5-FU-gebaseerde chemotherapie te voorkomen, en dat dit verder onderzocht moet worden in een klinische setting.

In **Hoofdstuk 6** onderzoeken we de darmmicrobiota bij 44 postmenopauzale borstkankerpatiënten tijdens de combinatietherapie met Adriamycine, Cyclofosfamide en Docetaxel (AC-D). Bij deze patiënten zijn AC-D-geïnduceerde veranderingen in de darmmicrobiota gevonden. De hoeveelheid van *Ruminococcaceae NK4A214-groep*, *Christensenellaceae R7-groep*, *Ruminococcaceae UCG-005* en *Marvinbryantia* neemt af tijdens AC-D, terwijl de hoeveelheid van Proteobacteria, ongeclassificeerde Enterobacterales en *Lactobacillus* toeneemt. Bovendien lijkt het optreden van diarree tijdens Docetaxel geassocieerd te zijn met het aantal verschillende darmbacteriën. Er is echter geen verband gevonden tussen de darmmicrobiota en tumorrespons bij een subgroep van patiënten die AC-D in de neoadjuvante setting kregen.

2. De darmmicrobiota en cachexie

Een ander aspect van kanker, dat in het kader van dit proefschrift wordt onderzocht, is cachexie, een metabool wasting syndroom. In **Hoofdstuk 7** wordt de huidige kennis over cachexie-geassocieerde darmmicrobiota profielen samengevat. Deze kennis komt vooral voort uit eerdere (preklinische) onderzoeken. Bovendien beschrijven we de verbanden tussen de darmmicrobiota en verschillende metabole kenmerken van cachexie, waaronder systemische ontsteking, integriteit van de darmbarrière, spierverlies, insulinegevoeligheid, voedselinname en gewichtsregulatie.

Vervolgens presenteert **Hoofdstuk 8** een van de eerste klinische studies naar de darmmicrobiota in het kader van cachexie. Voor deze cross-sectionele studie worden patiënten met alvleesklier-, borst-, long- of eierstokcarcinoom geïncludeerd vóór aanvang van de behandeling en geclassificeerd als cachectisch of niet-cachectisch op basis van gewichtsverlies. Als controlegroep worden partners van patiënten zonder carcinomen opgenomen. Deze studie geeft aan dat de pro-inflammatoire Proteobacteria, een genus uit de Enterobacteriaceae-familie, en *Veillonella* verhoogd zijn bij cachectische kankerpatiënten, terwijl *Megamonas* en *Peptococcus* meer aanwezig zijn bij niet-cachectische kankerpatiënten.

Bovendien neigen de fecale concentraties van SCFA verlaagd te zijn bij cachectische patiënten, maar dit is alleen voor acetaat statistisch significant. Markers van darmontsteking (calprotectine) of systemische ontsteking (CRP en leukocytenaantallen) zijn niet significant verhoogd door cachexie.

Tot slot integreert **Hoofdstuk 9** de resultaten van alle voorgaande hoofdstukken en biedt een raamwerk en praktische aanbevelingen voor toekomstige studies, met als doel verdere onderzoeken in dit vakgebied te stimuleren en te faciliteren.

Chapter 11

Impact

Preamble

The present thesis aimed to investigate how **chemotherapy** and **cancer cachexia** interact with the **gut microbiota**, which comprises the bacterial communities residing in the digestive tract. Interactions between the gut microbiota and the two different chemotherapies, 5-Fluorouracil (5-FU)-based chemotherapy, as well as Adriamycin, Cyclophosphamide, Docetaxel (AC-D)), were investigated among patients with colorectal cancer (CRC) and breast cancer.

Although we did not detect consistent effects of capecitabine (a 5-FU-based chemotherapy) on the gut microbiota in CRC patients, this treatment seemed to interact with metabolites produced by gut bacteria (the so-called short-chain fatty acids (SCFA) and branched-chain fatty acids (BCFA)). Furthermore, we observed 5-FU-induced gut microbiota shifts in an experimental model and explored that these shifts could be counteracted by supplementing the gut microbiota with prebiotics, thereby stimulating the growth of potentially beneficial bacteria.
In addition, it was shown that chemotherapy with AC-D in breast cancer patients had a major impact on various gut bacteria.
The role of the gut microbiota in cancer cachexia was examined across four different cancer types (pancreatic cancer, breast cancer, lung cancer, ovarian cancer). Here, we found that the abundance of specific gut bacteria and fecal SCFA levels were different in cachectic cancer patients, as compared to non-cachectic cancer patients and cancer-free controls. The results from our own studies were complemented by two reviews, summarizing the current literature concerning the gut microbiota and chemotherapy or cancer cachexia respectively.

These data strongly suggest that the gut microbiota should not be neglected when treating human cancer. Since this is a relatively new concept which has not yet been implemented in clinical practice, these results are expected to have a considerable impact in different domains.

1. Scientific impact: from *"too high risk"* to *"promising"*

For the scientific community, the work presented in this thesis contains a highly important message: it encourages further research in this field.

We showed not only that clinical gut microbiota research with longitudinal fecal sampling is feasible in cancer patients, but also provided first indications that the gut microbiota and its metabolites are associated with chemotherapy treatment and the development and manifestation of cancer cachexia. Furthermore, this thesis provided insights into lessons learned from our studies and practical recommendations for future research.

These first results are essential for the progress of gut microbiota research in the cancer setting since they provide a scientific basis to justify further research. In the first years after starting our research line, our research proposals were regularly rated as *"too high risk"* by most funding agencies and evaluating commissions. This means that the risk of not finding a significant relationship was estimated as too high to fund the research. Nevertheless, we believed in our ideas, and could proceed due to the help of some early supporters. The results of these early explorative studies are presented in this thesis and will hopefully not only simplify the acquisition of future research funding but will also inspire other researchers to investigate the gut microbiota in the context of human cancer.

Two important milestones of our research team, which were considerably facilitated by the outcomes and knowledge obtained in the context of this thesis, were the provision of funding from the *Dutch Research Council* (NWO) as well as from the *Top Consortium for Knowledge and Innovation* (TKI) *Agri&Food*.
The recently initiated NWO-funded **OPTIMA study** (NCT05655780) aims to explore different biomarkers (amongst others gut microbiota parameters) during irinotecan-based chemotherapy for metastatic CRC. The experiences, results, and collaborations that we already gathered in the context of 5-FU-based chemotherapies formed the basis for the design of this new study, which also has the ultimate aim to implement targeted gut microbiota modulation. This closely corresponds, with the TKI-funded **Oncobiotics study**, investigating the effect and feasibility of a prebiotic intervention during 5-FU-based chemotherapy, as a direct follow-up of the results presented in this thesis. Together, these two studies will form the scientific basis for the implementation of gut microbiota-modulating interventions in patients with metastatic CRC.

These two follow-up studies illustrate that the results presented in this thesis have contributed significantly to a paradigm shift: currently, gut microbiota modulation in cancer patients is no longer considered to be of ***"too high risk"***, but to be ***"promising"***. And this paradigm shift opens new opportunities to develop from standardized anti-cancer therapies to gut microbiota-based personalized medicine.

However, gut microbiota research in the cancer setting is still in its infancy. Consequently, the connection of different research groups working on this same topic is of high relevance, to stimulate the exchange of different perspectives and complementary techniques. Therefore, we established external collaborations with (amongst others) *Wageningen University* (the Netherlands), the *University of North Carolina at Chapel Hill* (United States), as well as the *University of California, San Francisco* (United States) and aim to further expand our collaborative network in the near future. The publication of the current results in peer-reviewed journals will support this process, by attracting the attention of other researchers for this work.

2. Societal impact: from bed to bench and back

In 2022, almost 50,000 people in the Netherlands suffered from CRC and approximately 67,000 patients from breast cancer (1, 2). Many have been or will be treated with chemotherapy and will potentially be confronted with a suboptimal tumor response and/or chemotherapy toxicity. For instance, for 5-FU-based chemotherapies, a pooled analysis of 16 trials reported that only 34.2% of patients with advanced CRC showed response to capecitabine and 34.6% to 5-FU (3). Furthermore, it is well known that a proportion of patients experiences toxicity during 5-FU-based treatment, for instance in the form of the hand-foot syndrome (swollen and red hand/feet), diarrhea, nausea, or fatigue (4).
In addition, the presence of cancer cachexia can also negatively affect chemotherapy efficacy and toxicity (5). Altogether, this has a strong negative impact on the patients' quality of life and prognosis.

These clinical challenges from the ***"bedside"*** were the underlying motivation to conduct the studies described in this thesis (***"the bench"***).
Currently, cancer patients mainly receive standardized chemotherapy regimens. However, each patient enters the therapy with an individual gut microbiota profile, comparable to a fingerprint. Therefore, it is not surprising that tumor response and the experience of toxicity varies significantly between individuals. By using new knowledge on interactions between gut bacteria and chemotherapies, it would be possible to optimize the current treatment through implementation of microbiota-based personalized medicine.
For example, if a chemotherapy would work better in the presence or absence of specific gut bacteria, these bacteria could be stimulated or inhibited before and during the treatment by means of targeted microbiota-modulating interventions. This would considerably improve the patient's quality of life since patients would not be exposed to a toxic therapy without considerable therapeutic effect. Simultaneously, gut bacteria with anti-inflammatory properties or their metabolites could help to reduce chemotherapy- or cachexia-induced inflammation in cancer patients, with a potential positive effect on toxicity.

All patients who participate in our studies, do this without any personal benefit but with the purpose to help future patients in the same situation. Therefore, one of our main points of attention is the rapid **translation of research results back to clinical practice**, so that patients can benefit from scientific results as soon as possible. To achieve this, we collaborate with *Danone Nutricia Research*, a manufacturer of medical nutrition including prebiotics. This collaboration enables us to choose the best suitable prebiotic mixture for gut microbiota modulation in our target populations and to incorporate it into a consumable product. On the other hand, our research results also help them to develop new markets.
Of course, the collaboration between academic and private partners is not without controversies and requires critical reflection to ensure that research results are not influenced by economic interests. According to our experiences this can best be tackled by adhering to the principles of scientific integrity and by being aware of and communicate openly about potential competing interests. In this way, the partnership with industrial partners can help tremendously to make sure that the society can benefit from scientific advances and that evidence-based products become available on the market.

3. Key players involved and ways to target them

To make sure that relevant scientific results are not lost somewhere between bench and bedside, science communication is of high relevance. The results presented in this thesis are of potential interest for different target groups. To ensure that all of them are approached, different communication tools are used.

Scientific community: Our results have been presented on scientific conferences, including the *International Human Microbiome Consortium* (IHMC) Congress as well as on several editions of the Scientific Spring Meeting of the *Nederlandse Vereniging voor Medische Microbiologie* (NVMM) & *Koninklijke Nederlandse Vereniging voor Microbiologie* (KNVM). In addition, the results were published in peer-reviewed scientific journals and communicated on several internal meetings and research symposia. We also used *LinkedIn*® to share important research-related news with our professional network.

Clinicians: Clinicians represent another highly relevant target group of our research, since they are the connection between "bench" and "bedside". Therefore, we intentionally also published our work in journals with a clinical scope (e.g. *Clinical & Experimental Medicine* or *Clinical Colorectal Cancer*). Furthermore, the results were also presented on clinical symposia, such as the *Mammacongres Harderwijk* and a symposium on Nutrition & Cancer at the *Catalan Institute of Oncology* in Barcelona. In addition, an open and regular communication with all participating centers is actively maintained and centers are informed about the study progress by means of newsletters.

Students: As discussed in detail in Chapter 9, the training and education of next-generation scientists and clinicians is of great importance to further advance the research field and guarantee a continuity of high-quality research. Therefore, a total of thirteen students (medical as well as biomedical) was supervised and trained in our team. Hereby, critical scientific thinking, independent and careful laboratory work, as well as a broad interest in microbiota-related questions was stimulated. Furthermore, I was involved in tutoring, the supervision of laboratory practicals, as well as the revision of a tutor instruction. In the future, I aim to continue and further expand my educational tasks, for instance by participating in planning groups or giving lectures about gut microbiota research.

Patients and the society: To fulfil our ambition to give the results "back" to the patients, several communication tools to reach the broader public were applied. First of all, our articles were published *Open Access*, so that interested patients or family members have the opportunity to inform themselves about the study results. Furthermore, we recently implemented the possibility to subscribe to a patient newsletter concerning recent advances in our research line. We also involved patient organizations and patient representatives in the design of new studies, for instance the already mentioned OPTIMA study. For the future, a website containing information about our research activities is already planned. Finally, I will give a workshop on the gut microbiota at a public educational institution for adults (*Volkshochschule Aachen*) in March 2024.

References

1. IKNL. Prevalentie per jaar, 5-jaarsprevalentie. Borstkanker 2023 [Available from: https://nkr-cijfers.iknl.nl/#/viewer/634a725a-4333-418d-b7d3-bb4939cec741.
2. IKNL. Prevalentie per jaar, 5-jaarsprevalentie. Dikkedarm- en endeldarmkanker 2023 [Available from: https://nkr-cijfers.iknl.nl/#/viewer/d03267a6-025d-485e-a4ac-23c0e9cfe870.
3. Petrelli F, Cabiddu M, Barni S. 5-Fluorouracil or capecitabine in the treatment of advanced colorectal cancer: a pooled-analysis of randomized trials. Med Oncol. 2012;29(2):1020-9.
4. Timmers L, Boons CC, Mangnus D, Van de Ven PM, Van den Berg PH, Beeker A, et al. Adherence and Patients' Experiences with the Use of Capecitabine in Daily Practice. Front Pharmacol. 2016;7:310.
5. Schmidt SF, Rohm M, Herzig S, Berriel Diaz M. Cancer Cachexia: More Than Skeletal Muscle Wasting. Trends Cancer. 2018;4(12):849-60.

Addendum

Dankwoord

Nu is het dan eindelijk zo ver: na 5 jaar poep scheppen, talrijke brainstorm sessies, nieuwe onderzoeksideeën en samenwerkingen, lange lab dagen en intense schrijf periodes staat alles op papier en mag ik beginnen met het schrijven van mijn dankwoord. Als ik terug kijk, ben ik enorm dankbaar voor de afgelopen 5 jaar en heb ik – ondanks het feit dat vanwege de COVID-19 pandemie veel dingen anders gingen dan normaal – een enorm leuke en leerzame tijd als PhD kandidaat gehad. Mijn PhD tijd werd vooral heel leuk door de mensen die ik mocht ontmoeten en die aanzienlijk hebben bijgedragen aan de totstandkoming van dit proefschrift. Daarnaast ben ik heel trots dat het nog is gelukt om het op tijd af te maken (met op tijd bedoel ik de avond voor de geboorte van mijn dochtertje), maar dit was zonder de support van mensen uit mijn omgeving zeker niet mogelijk geweest! En daarom wil ik nu aan het einde van dit proefschrift een aantal bijzondere mensen bedanken.

Allereerst wil ik mijn promotor **Prof. Dr. Marjolein Smidt** van harte danken. Beste Marjolein, bedankt voor jouw groot vertrouwen in mij. Een van mijn stellingen is van Michelle Obama en zegt dat *"People who are truly strong lift others up. People who are truly powerful bring others together."* Ik heb dit citaat gekozen omdat ik het als leidraad voor mijn toekomstige carrière mee wil nemen maar ik vind het ook heel goed passen bij jou. Jij geloofde in mijn vaardigheden vanaf de eerste dag. Dit heeft mij niet alleen enorm gemotiveerd maar heeft er ook aan bijgedragen dat ik kon groeien naar de onderzoeker die ik vandaag ben. Ik ben heel blij dat we ook in de toekomst samen zullen werken en kijk er al naar uit om samen met jou onze research line verder uit te bouwen. Ik wist al langer dat ik mijn plek had gevonden, maar jouw positieve en ondersteunende reactie op mijn zwangerschap heeft nog een keer bevestigd dat dit de goede keuze is. Bedankt dat jij het mogelijk maakt om wetenschap en familie te combineren en dat je me hebt geholpen om met mijn schuldgevoelens om te gaan toen ik opeens niet meer kon werken. Ik waardeer jouw leiderschaps stijl enorm en heb ook op dit gebied veel van jou geleerd.
Achter iedere sterke vrouw staat ook een sterk gezin: bedankt ook aan Ivo, Linde, Nienke, Gijs en Pip voor de gezellige etentjes bij jullie thuis!

Prof. Dr. Venema, beste Koen. Ik herinner me nog precies hoe ik het eerste mailtje naar jou stuurde (toen nog voor mijn eerste NUTRIM GP aanvraag) en hoe je direct een enthousiast antwoord terug stuurde. Sindsdien is er veel gebeurd en ik ben er heel trots op dat we de experimenten die we toen hebben bedacht inmiddels ook heel succesvol hebben uitgevoerd. Bedankt voor het hartelijk welkom in jouw team in Venlo, ik heb bij jullie echt een heel waardevolle en leerzame tijd gehad (ondanks het feit dat ik echt heel vroeg moest opstaan wat echt niet mijn sterke punt is). Jij was voor mij altijd een belangrijk aanspreekpunt. Je hebt alle vragen geduldig beantwoord en samen met mij naar oplossingen gezocht. Hartelijk dank daarvoor! Ik waardeer jouw enorme kennis over TIM-2 en de gut microbiota en meetings met jou waren altijd heel motiverend en inspirerend voor mij. Dankjewel Koen voor alle support en begeleiding!

Dr. Penders, beste John. Jij bent DE microbiota expert in het MUMC+ en wat ben ik blij dat jij ook deel uitmaakte van mijn promotie team! Jij was altijd bereikbaar voor advies en kon de meest ingewikkelde dingen in een paar zinnen helder uitleggen. Hoe vaak dacht ik al dat ik

niet verder kwam en was er na overleg met jou toch weer een oplossing. Jouw indrukwekkende expertise heeft aanzienlijk bijgedragen aan de kwaliteit van mijn projecten en ik heb van jou echt heel veel over het microbioom geleerd. Vooral ook jouw kritische en analytische blik heeft me enorm geholpen mijn experimenten en artikelen te verbeteren. Door de integratie in het MMI Department heb je me in contact gebracht met andere onderzoekers in hetzelfde onderzoeksgebied. Dit was voor mijn werk maar ook voor mij persoonlijk heel erg waardevol. Hartelijk dank voor jouw inzet en begeleiding en ik kijk uit naar gezamenlijke toekomstige projecten!

Dear **Prof. Dr. Daniel Keszthelyi, Prof. Dr. Frans Ramaekers, Prof. Dr. Matthew Redinbo and Dr. Dieuwertje Kok**, members of the assessment committee. Thank you very much for taking time to assess and evaluate my dissertation as well as defense. I also thank the other members of the **promotion committee** for attending and evaluating my defense.

Mijn paranimfen: **Lars** en **Heike.** Ik ben heel blij dat jullie gedurende mijn verdediging naast me zullen staan! Jullie zijn allebei heel bijzondere mensen voor mij, op zowel professioneel als ook persoonlijk gebied.
Beste **Lars**, we zijn in 2018 samen begonnen bij de chirurgie, ik als PhD kandidaat en jij als student. Vanaf dag 1 waren we een super goed team, heel complementair, maar toch altijd op een lijn. Het is een groot genoegen met jou samen te werken, samen oplossingen te vinden, te brainstormen en tussendoor gewoon even urenlang te filosoferen (bijvoorbeeld tijdens de lange rit naar Venlo, waardoor het contract voor mijn telefoon opeens aanzienlijk duurder werd...). Ik kon altijd op je rekenen en 100% vertrouwen en dit waardeer ik enorm! Toen ik zwanger werd, heb je geen seconde geaarzeld, mijn experimenten overgenomen en ze meer dan succesvol afgerond. Dit zal ik nooit vergeten, dankjewel Lars! We hebben samen veel bereikt: de succesvolle TIM-2 experimenten, het uitbreiden van de onderzoekslijn, de nieuwe samenwerkingen en analyses. Ik zeg het altijd aan het eind van mijn praatjes: onderzoek is team-work en ik ben echt heel dankbaar voor het team-work met jou, want zonder jou waren we nooit zo ver gekomen! Van harte bedankt Lars – voor alles! Je bent een heel gewardeerde collega, een ambiteuze wetenschapper en een fantastische arts en ik wens je voor de toekomst het allerbeste, dat heb je verdiend.
Beste **Heike**, in de afgelopen jaren is er bij ons allebei veel gebeurd: bruiloft, kind, huis en promotie... daarom ben ik heel blij dat we dit samen mochten beleven en dat ik met jou zowel de correcte samenstelling van het TIM-2 dialysaat als ook corona maatregelen op de bruiloft kon bediscussiëren. Je bent een heel secure, zorgvuldige en slimme wetenschapper en het uitwisselen van gedachtes met jou heeft me altijd verder geholpen bij het maken van de juiste keuzes. Ik geniet enorm van onze *"Scientific Spaziergänge"* en kijk al uit naar tijd samen in de speeltuin met onze meisjes, koffie en diepgaande discussies. En misschien nemen we ook eten mee, zoals de mensen in Kopenhagen...
Gefeliciteerd ook met jouw eigen promotie en met jouw geweldige gezin, Mia en Christian. Hopelijk kom je ooit weer terug in de wetenschap, want we hebben jouw diepgaande kennis en kritische blik meer dan nodig!

Het onderzoek gepresenteerd in dit proefschrift was nooit tot stand gekomen zonder de bereidheid van **patiënten en gezonde vrijwilligers** om te participeren in onze studies. Daarom wil ik alle patienten en proefpersonen, evenals hun naasten, van harte danken dat jullie deel hebben genomen – ondanks het feit dat jullie er zelf geen voordeel aan hebben. Ik

heb grote bewondering voor jullie, dat jullie ondanks het hebben van een dodelijke ziekte extra moeite nemen om toekomstige patienten te helpen. Ik ben ervan overtuigd dat wetenschappelijk onderzoek de sleutel is om de impact van deze vreselijke ziekte steeds meer te beperken, maar zonder jullie zou dit belangrijke onderzoek niet mogelijk zijn. Ik zal daarom ook steeds streven om zo veel mogelijk informatie uit jullie samples te halen, want alleen meer kennis kan ons helpen kanker succesvol te bestrijden.

Verder wil ik ook mijn microbiota collegas bedanken. Allereerst natuurlijk **Romy**. Jij hebt de researchlijn opgestart en hebt samenwerkingen met diverse ziekenhuizen geïnitieerd. Daarnaast heb jij ervoor gezorgd dat ik in de onderzoeksgroep van Marjolein terecht kwam, om samen met jou het microbiota onderzoek verder uit te breiden. Jij als arts-onderzoeker met een grote passie voor de kliniek en ik als meer fundamentele wetenschapper met lab skills – een goede combinatie! Hartelijk dank voor de leuke jaren samen en voor al je harde werk dat de basis heeft gevormd voor alle follow-up studies. Uiteraard wil ik je ook bedanken voor al je waardevolle tips met betrekking tot de promotie en het afronden van mijn boekje.
Pien, bedankt voor jouw werk in de opstart-periode van de OPTIMA studie en bij het verwerken van de eerste samples. Ik wens je het allerbeste voor de toekomst en veel succes bij je nieuwe baan. **Eva**, wat ontzettend leuk dat jij ook in ons team terecht bent gekomen! Jouw ervaring met klinisch onderzoek en jouw oplossingsgericht denken zijn echt van grote toegevoegde waarde voor de OPTIMA studie. Bovendien ben je een heel leuk en positief persoon met een groot hart. Ik ben blij om met jou samen te mogen werken. **Milou & Emma,** de *"next-generation"* microbiota onderzoekers in ons team. Bedankt voor jullie harde werk tijdens jullie afstudeerstages, het was leuk om jullie te mogen begeleiden. En nog leuker vind ik het dat jullie het zien zitten om verder door te gaan met het *"poep-onderzoek"* en dat we dus de komende jaren nog verder samen zullen werken. Ik kijk al uit naar gezamenlijke brainstorm sessies, creatieve plannen en het implementeren van onze klinische (interventie) studies. Ik ben heel trots en blij dat mijn proefschrift niet het eind is, maar heeft bijgedragen aan de ontwikkeling van nieuwe studies op dit gebied.

Daarnaast mocht ik tijdens mijn PhD tijd ook nog andere studenten begeleiden: **Anne (Heuft), Anne (Vievermans), Aurelia, Bob, Coco, Elvira, Janneke, Jasper, Lisa (Coolen), Lisa (Dohmen), Sabine (Dieleman)** en **Saskia**. Ik vond het heel leuk om jullie te introduceren in de wereld van het microbioom onderzoek en jullie de basics te leren. Iedereen van jullie is enorm gegroeid tijdens de stage en ik ben enorm trots op jullie allemaal. Bedankt voor jullie enthousiasme voor ons onderzoek en de praktische ondersteuning van ons team. Het was mij een groot genoegen om jullie begeleider te zijn en de ervaringen met jullie hebben mij duidelijk gemaakt dat ik dit ook in de toekomst wil doen: onderwijs geven en jonge wetenschappers begeleiden. Ik wens jullie allemaal heel veel succes met jullie toekomstige carrière!

Ik ben altijd heel graag naar mijn werk gegaan en dit ook mede door onze super leuke en gezellige onderzoeksgroep.
Mijn *"Roomies"* **Evie, Sabine (de Wild), Roxanne en Veerle** wil ik in het bijzonder bedanken voor de gezellige tijd in ons kantoortje, voor het *"even samen nadenken"* en de gezamenlijke koffie- of theepauzes. Bij de Ups en Downs van een PhD-leven is het soms gewoon heel fijn om naar een vriendelijk gezicht te kijken, samen te lachen en te kletsen. Ik ben heel dankbaar dat ik mijn PhD tijd met jullie samen heb mogen beleven! Hartelijk dank voor alle mentale en

praktische ondersteuning en de leuke momenten samen. En sorry voor de ZOOM meetings met allemaal ingewikkelde bacterie namen waar jullie soms naar moesten luisteren...
Dank ook aan **Sanaz, Renée, Kees, Sabine (Dieleman), Lidewij, Melissa** en **Florien**. We hebben samen een ontzettend mooie tijd gehad, die ik nooit zal vergeten. Naast het werk gingen we ook regelmatig samen uit eten of mochten we genieten van de culinaire kookkunsten van Kees – bedankt voor deze gezelligheid en leuke herinneringen! Een deel van jullie was ook op onze bruiloft, super leuk dat jullie speciaal daarvoor naar Aken zijn gekomen, dat waardeer ik enorm. Daarnaast wil ik jullie ook bedanken voor de mentale steun en de attenties toen ik aan het eind van mijn zwangerschap thuis in bed moest blijven. Dat jullie aan mij hebben gedacht, heeft me echt enorm geholpen tijdens deze moeilijke tijd!
Renée, jou wil ik nog in het bijzonder bedanken voor de ondersteuning bij het leren van de *"R language"* en jouw oneindig geduld bij het beantwoorden van mijn vragen. Zonder jou had ik het nooit zo snel kunnen leren – hartelijk dank!
Ik wens jullie allemaal van harte heel veel succes en geluk voor de toekomst en (voor diegenen die niet meer in Maastricht zijn) hopelijk zien we elkaar snel weer!
Janine & Thiemo, de post-docs van ons team. Bedankt voor goede gesprekken en ideeën. Ik bewonder jullie diepgaande expertise en professionele uitstraling en ik hoop dat we ook als post-docs nog lang samen zullen werken (ondanks het feit dat dit echt enorm verwarrend is dat Janine en ik niet alleen dezelfde voornaam, maar ook bijna dezelfde achternaam hebben).
Loes, de patholoog in onze groep. Bedankt voor de leuke samenwerking op het gebied van tumor microbiota en jouw expertise over alles wat met weefsel te maken heeft!

Verder wil ik bij deze ook alle andere huidige en voormalige **collega's van de chirurgie** bedanken voor de gezellige en leerzame tijd op de afdeling.
In het bijzonder wil ik **Mo, Bas, Annemarie, Hans, Chantal** en **Cathy** bedanken voor de ondersteuning in het lab en het meedenken over allerlei praktische vragen. Jullie ervaring met diverse lab analyses en deskundigheid op dit gebied heeft me vaak verder geholpen. Dank aan **Prof. Dr. Steven Olde-Damink** en **Dr. Sander Rensen** voor de samenwerking binnen de cachexie studie. Beste Sander, door mijn master stage kwam ik bij de chirurgie terecht. Ik herinner me nog ons eerste gesprek, midden tussen de verhuisdozen, omdat de hele afdeling naar de huidige gang ging verhuizen. Bedankt voor de goede begeleiding tijdens mijn stage en de prettige samenwerking daarna, die twee artikelen in dit proefschrift heeft opgeleverd. Ik heb veel wetenschappelijke vaardigheden van jou geleerd en waardeer jouw kritische blik en wetenschappelijke inschatting. Daarnaast ben jij ook een van de personen die altijd in mijn talent geloofde en mij gemotiveerd heeft door te gaan – ook na meerdere niet succesvolle grant aanvragen. Hartelijk dank voor jouw vertrouwen, support en motiverende woorden!
Beste **Jorne**, jou wil ik bedanken voor de leuke en leerzame tijd tijdens mijn stage, die aanzienlijk heeft bijgedragen tot mijn besluit om een PhD traject te gaan volgen. Samen hebben we de microbiota in cachexie studie tot een succesvol eind gebracht, waar ik ook heel trots op ben. Dank voor alle gezellige koffie momenten en goede gesprekken, evenals voor jouw overtuiging dat *"alles wel goed komt",* die me verder heeft geholpen als ik het zelf even niet meer zag zitten.
Tessa, ik had het groot geluk om met jou samen te mogen werken bij zowel de heelkunde als bij de MMI. Al tijdens onze afstudeerstages hadden we een leuke tijd samen en was jij mijn nr.1 aanspreekpunt voor allerlei lab vragen (en vooral berekeningen van concentraties en hoeveelheden). Bedankt voor jouw hulp en het meedenken bij problemen. Ik was altijd onder de indruk hoe secuur en efficiënt jij in het lab kon werken. Daarnaast ben je ook een uitermate

vriendelijk, vrolijk en eerlijk mens en wil ik je bedanken voor de leuke tijd samen en de gezamenlijke koffie pauzes, die me altijd weer nieuwe energie gaven (en dit niet alleen door de cafeïne).
Ook mijn collega's van het MMI Department (MMI staat trouwens voor *Medische Microbiologie Infectieziekten en Infectiepreventie*) wil ik graag bedanken voor al jullie kennis, kritische reflectie en ondersteuning van mijn onderzoek. **Niels**, bedankt voor de gezellige koffiepauzes met (semi)-post-doc-geklets. Jij bent een erg enthousiaste en getalenteerde wetenschapper en ik kijk uit naar veel toekomstige brainstorm sessies, wetenschappelijke discussies en gezamenlijke projecten. Ik besef me nu trouwens dat ik echt heel veel mensen voor koffie pauzes bedank, dus blijkbaar heb ik best veel koffie gedronken tijdens mijn PhD...
David (Barnett), first of all, thank you for inventing microViz, which is such a useful package for R Dummies like me! Furthermore, thank you so much for all your support and patience! I highly appreciate your immense knowledge and that you helped me to find my way in the complex world of R analysis. You are an extraordinary scientist as well as a pleasant colleague and I am very happy that you will also stay as a post-doc! Looking forward to our future collaborations! **Giang**, thank you very much for your help with the preprocessing of the sequencing data and the very useful analysis pipeline – I highly appreciate your skills and support!
Beste **Christel**, jou wil ik van harte bedanken voor alle hulp en praktische ondersteuning in het lab, evenals voor het uitvoeren van onze sequencing runs. Ik kon altijd met alle vragen bij jou terecht en dankzij jou heb ik heel veel nieuwe lab vaardigheden opgedaan of verfijnd. Jij bent echt mijn grote voorbeeld wat betreft nauwkeurigheid en zorgvuldigheid in het lab en ik bewonder jouw kennis en ervaring. Daarnaast vind ik het ook altijd leuk om even bij jullie binnen te lopen om jouw mening te vragen of om gewoon even bij te kletsen. Jij bent een zeer gewaardeerde collega voor mij, dankjewel voor alles!
Beste **Erik**, evenals Christel, sta jij ook altijd klaar om te helpen. Je hebt een enorme expertise met betrekking tot lab analyses, maar ook een groot hart. Hartelijk dank voor al je hulp met de β-glucuronidase assay en de bactron, maar ook voor alle vriendelijke en motiverende woorden. Zonder jou waren we nooit zo ver gekomen met onze analyses, bedankt voor jouw support!
Melissa, jij hebt me geleerd hoe ik DNA uit feces kan isoleren. Bedankt voor jouw geduld en al jouw hulp wanneer het niet lukte, of als ik onzeker werd omdat iets anders was dan verwacht. En samen met jou waren de wacht- en incubatiestappen ook veel minder saai... Ik wens je van harte veel succes met jouw nieuwe baan en het allerbeste voor de toekomst.

Alle andere huidige en voormalige **MMI collega's** bedank ik van harte voor de interessante wetenschappelijke discussies, jullie input en kritische blik tijdens de research meetings, evenals voor de gezelligheid tijdens borrels of barbecues!

Een van de grote uitdagingen, maar tegelijkertijd ook een van de meest leuke dingen tijdens mijn PhD was dat ik in drie verschillende laboratoria mocht werken. Daarom bedank ik ook **Jessica en Sanne** uit de onderzoeksgroep van Koen op de UM Campus in Venlo van harte voor de voortvarende hulp bij de TIM-2 experimenten. Heel indrukwekkend hoe jullie deze ingewikkelde machine met al die slangetjes en knopjes onder controle hebben en echt precies weten welk slangetje en knopje waar hoort! Bedankt voor jullie geduld bij het beantwoorden van vragen en het tussendoor redden van onze experimenten. De tijd bij jullie in Venlo was behoorlijk druk en intens, maar het was ook een van de leukste ervaringen die ik heb mogen

maken! En iedere ochtend wanneer ik binnen liep en zag dat TIM-2 nog aan het pruttelen was, was ik gewoon echt heel erg blij!

Ik heb in mijn impact paragraaf ook aandacht besteed aan de samenwerking tussen de academie en de industrie. Op het gebied van microbiota modulatie werken we samen met Danone Nutricia Research. In deze context bedank ik in eerste instantie **Dr. Ardy van Helvoort**. Beste Ardy, jij was ook een van de mensen die altijd in de relevantie mijn onderzoek geloofde en dit in een tijd toen er ook nog veel sceptici waren. Hartelijk dank voor jouw vertrouwen en de goede en succesvolle samenwerking door de jaren heen. Ook **Dr. Guus Roeselers** en **Lotte Dopheide** wil ik bedanken voor hun medewerking binnen het TKI project. Ik kijk uit naar alle plannen die we nog samen hebben!

Uiteraard was het uitvoeren van de klinische studies alleen mogelijk door de hulp van clinici. Daarom wil ik ook alle artsen en verpleegkundigen uit alle deelnemende ziekenhuizen bedanken. Allereerst natuurlijk de oncologen **Dr. Judith de Vos-Geelen** en **Dr. Liselot Valkenburg-van Iersel**. Van harte bedankt voor jullie inzet, betrokkenheid en enthousiasme. Jullie zijn voor mij altijd het eerste aanspreekpunt bij klinische oncologie-gerelateerde vragen en jullie klinisch perspectief is van onschatbare waarde voor onze onderzoeken. Een kort gesprek met jullie heeft al vaak aanzienlijk bijgedragen aan het verbeteren van de kwaliteit van zowel onderzoeksdesigns als ook resultaten en ik ben heel blij dat jullie ook bij onze nieuwe projecten weer aan boord zijn!
Een grote dank ook aan alle andere **oncologen** uit het MUMC+, evenals aan de verpleegkundig specialisten **Lieke Baade-Corpelijn**, **Kim Puts-van der Burgt**, **Nicol Pepels-Aarts**, en **Maud Widdershoven** voor het ondersteunen van de patienteninclusies. Dank ook aan **Elly de Jong-Vrancken**, **Conny Starren-Goessens**, **Janine Lipsch-Crijns**, en **Christel Meers-Haekens**.
Daarnaast wil ik **Dr. Robin van Geel** bedanken voor de hulp bij het berekenen van de juiste concentraties en het begrijpen van de ingewikkelde farmacokinetiek van 5-FU.

Naast alle wetenschappelijke en klinische collega's, werken op een universiteit ook veel mensen die ons ondersteunen zodat we ons op het inhoudelijke werk kunnen focussen. Dank aan **Miriam Habex-Froidmont**, **Sabeth Walpot**, en **Nicole Hermans**. Ook het team van GROW, **Prof. Dr. Manon van Engeland**, **Judith Doomen-Dormans**, **Maarten Vandermeulen**, **Lina Nelissen** en **Brigitte Custers** wil ik bedanken voor de dagelijkse ondersteuning van PhD kandidaten en de organisatie van research days, PhD retreats en symposia. Dit zijn allemaal heel leuke en leerzame events, die ook een belangrijk deel van mijn opleiding uitmaakten. Ook dank aan **Suzanne ten Hoeve** en **Helena Bossini Castillo** voor de hulp bij juridische vragen.

Ook in de andere ziekenhuizen, die in onze studies participeren, mogen we samen werken met enthousiaste artsen en verpleegkundigen. Hartelijk dank aan de research teams uit het Catharina Ziekenhuis, Ziekenhuis Gelderse Vallei, St. Anna Ziekenhuis, Van Weel Bethesda Ziekenhuis, VieCuri Medisch Centrum, Zuyderland Medisch Centrum en het Elkerliek Ziekenhuis voor de participatie in onze onderzoeken! Bijzondere dank aan **Dr. Geert-Jan Creemers**, **drs. Yvonne van Riet, Dr. Irene van Hellemond**, **Dr. Birgit Vriens, Ramon Bax, Marjan Laven** en **Maaike van Dam** uit het Catharina Ziekenhuis, **Dr. Arnold Baars, Alina van de Vendel, Janneke van den Brink** uit het Ziekenhuis Gelderse Vallei, **Dr. Hanneke**

Vestjens, **Dr. Yes van de Wouw, Ilona van Rooij** en **Wendy Heuts** uit het Viecuri Medisch Centrum en **Jeroen Vincent, Monique Vercoulen** en **Sandra Silvis** uit het Elkerliek Ziekenhuis.

Furthermore, I would like to thank our international collaborators **Prof. Dr. Matthew Redinbo** from the University of North Carolina at Chapel Hill as well as **Prof. Dr. Peter Turnbaugh** from the University of California, San Francisco for the pleasant and fruitful collaborations. Matt, thanks a lot for supporting our research group with the conduction of the β-glucuronidase assay and thank you for sharing your unique expertise with us. Peter, thank you very much for your enthousiasm and interest in our research, I am looking forward to our future collaboration!

Ook alle leden van het **OPTIMA consortium** en onze project manager **Joëlle Vos** wil ik bedanken. Ik heb met veel plezier aan het grant proposal en het interview meegewerkt en kijk ernaar uit om samen met jullie de komende jaren aan de OPTIMA studie te werken!

Maar naast het werk heb ik ook het grote geluk dat ik goede vrienden en een fantastische familie om me heen heb. Ik ben niet alleen wetenschapper, maar vooral ook Janine. Daarom wil ik ook mijn familie en vrienden van harte bedanken. En daardoor wordt dit dankwoord net zoals mijn leven drietalig 😊

Liebe **Anne**, du bist meine beste Freundin und ich bin so froh dich an meiner Seite zu wissen! Als ich letztes Jahr zu einem Vortrag in Barcelona war, haben wir relativ spontan noch ein paar gemeinsame Tage in Barcelona verbracht. Das war wirklich toll! Mit dir kann ich über alles reden und einfach völlig ungefiltert ich selbst sein. Danke für diese wertvolle Freundschaft, es ist schön dass es dich gibt!
Liebe **Regina**, **Sarah**, **Kris** und „die Jungs“ **Yannick**, **Philipp**, **Mark**, **Levin** und **Thilo**. Euch alle kenne ich jetzt schon eine ganze Weile und ihr wart auch während meines PhDs wichtige Wegbegleiter für mich. Ich danke euch für eure Freundschaft, eure Unterstützung und die Freude die ihr in unser Leben bringt.
Danke auch an alle **Aachener Zontians**, sowie **Susanne**, **Irene, Christin, Norina** und **Maja**. Der gemeinsame Einsatz für Zonta und die Rechte von Frauen sowie die Gespräche und Erlebnisse mit euch sind ein wichtiger Teil meines Lebens und haben mich in den letzten Jahren entscheidend geprägt. Vor allem auch die gemeinsamen Besuche von Zonta Konferenzen, sind immer wieder eine wichtige Energiequelle für mich (und sonst wäre ich auch niemals auf die Idee gekommen jemals nach Tartu zu fahren…).

Ein herzliches Dankeschön auch an meine wunderbare Familie. Ihr alle habt mich mein Leben lang geprägt und mich zu der Frau gemacht, die ich heute bin. Ein besonderes Dankeschön an meine **Großeltern**, die immer Anteil an meinem Leben nehmen und gespannt den Neuigkeiten aus Maastricht lauschen. Danke für eure Unterstützung und dass ihr an mich glaubt! Danke an meine Paten **Iris** und **Lothar**, sowie deren Ehepartner **Stefan** und **Stefanie**, die bis heute immer eine besondere Rolle in meinem Leben spielen. Danke an **Thomas & Anne** und **Silke & Friedrich**, sowie die ganze Schar Cousins und Cousinen: **Johan, Leonard, Lorenz, Florian, Verena** und **Johanna** und ganz besonders natürlich an mein Patenkind **Julius**!

Durch unsere Hochzeit wurde unsere eh schon große Familie noch einmal erweitert: ich danke auch allen Mitgliedern der **Familien Prick und Reiß**, dass ihr mich so liebevoll bei euch aufgenommen habt!
Auch wenn sie nicht lesen können, will ich sie der Vollständigkeit halber doch nennen: danke an unsere Haustiere **Fiete & Finn**, sowie **Coffee & Cookie** – ihr habt mir die einsame Corona-Zeit sehr viel erträglicher gemacht! Danke, dass ihr mich immer daran erinnert habt, dass man beim Home Office zwischendurch auch mal (Streichel) Pausen machen muss!
Ein ganz ganz großes Dankeschön auch an meine Schwiegereltern **Petra & Guido**, sowie an **Alina & Niklas**. Danke für eure unerschütterliche Liebe und Unterstützung. Danke, dass ihr in der finalen Phase der Schwangerschaft und Dissertation für mich gesorgt habt, sodass ich mich aufs Schreiben fokussieren konnte. Danke für eure tatkräftige Unterstützung in allen Lebensbereichen, wir sind unendlich dankbar, dass wir immer auf euch zählen können!
Danke an meinem Bruder **Marvin** und seine Frau **Sophia**. Marvin, auch wenn wir so unterschiedlich sind (oder vielleicht gerade deswegen?) bist du mir nah wie kaum ein anderer Mensch. Sophia, ich bin so froh dass mein Bruder dich gefunden hat und du jetzt zu unserer Familie gehörst! Danke, dass ihr immer an meiner Seite seid und wir alle Höhen und Tiefen gemeinsam erleben. Danke für alles, was ihr für uns tut, ich bin sehr dankbar dass ihr Teil meines Lebens seid und habe euch sehr lieb. Danke auch für alle gemeinsamen Erlebnisse (mit oder ohne Tanzeinlagen), die immer wieder sehr viel Freude in mein Leben bringen!
Liebe **Mama**, lieber **Papa**. Dass ihr jetzt diese Dissertation in den Händen haltet, ist zu einem großen Teil euer Verdienst! Danke, dass ihr mir immer den Freiraum gebt meinen eigenen Weg zu finden, und mir gleichzeitig Sicherheit und ein Zuhause gebt. Danke für eure Liebe, und für euer Vertrauen in mich, danke dass ihr immer für uns da seid und mich bedingungslos unterstützt. Papa, wer hätte damals bei unserem 3. Open Dag (mit Fladen!) gedacht, dass ich so lange an der Universität in Maastricht bleiben würde?! Dank eurer liebevollen Unterstützung bei allen Höhen und Tiefen des Studiums und der PhD Zeit vergingen die Jahre wie im Flug und habe ich es jetzt tatsächlich geschafft. Ich danke euch für alles, ohne euch wäre ich nicht die, die ich jetzt bin.
Jarla, auch wenn du noch so klein bist, bist du doch das Größte für mich! Ich hoffe, dass ich dir immer eine gute Mutter und ein Vorbild bin und ich hoffe dass du vielleicht auch ein bisschen stolz bist, wenn du diese Dissertation in ein paar Jahren in deinen Händen hälst. Ich liebe dich sehr mein kleiner Schatz und verspreche dir, dass ich immer mein Bestes für dich geben werde.
Lieber **David**, so wie ich bei unserer Hochzeit gesagt habe: *„du bist mein bester Freund und die Liebe meines Lebens“*. Danke für deinen Rückhalt die ganzen letzten Jahre, du gibst mir die Liebe und Sicherheit die ich brauche. Danke, dass du dir zahllose Präsentationen über das Mikrobiom angehört hast, ohne richtig zu verstehen, worum es eigentlich geht. Danke, dass du bis spätabends am Layout für mein *„boekje“* gesessen hast. Danke, dass du dir meine Zweifel und Probleme angehört hast und mir geholfen hast nach Lösungen zu suchen. Danke, dass du mir zuhörst und ich mit dir über alles sprechen kann. Danke, dass du mit Jarla Abendspaziergänge machst, damit ich an wichtigen Veranstaltungen teilnehmen kann. Danke für alles, was du für mich und unsere Familie tust! Ich bin fest davon überzeugt, dass wir gemeinsam alles schaffen können und ich freue mich auf unser gemeinsames Leben als Familie.

List of publications

This thesis

Ziemons, J., Hillege, L.E., Aarnoutse, R., de Vos-Geelen, J., Valkenburg-van Iersel, L., Mastenbroek, J., van Geel, R., Barnett, D.J.M., Rensen, S.S., van Helvoort, A., Dopheide, L.H.J., Roeselers, G., Penders, J., Smidt, M.L.[#] & Venema, K.[#]. Prebiotic fiber mixtures counteract the manifestation of gut microbial dysbiosis induced by the chemotherapeutic 5 - Fluorouracil (5-FU) in a validated in vitro model of the colon. *Submitted.*

Ziemons, J., Aarnoutse, R., Heuft, A., Hillege, L., Waelen, J., de Vos-Geelen, J., Valkenburg-van Iersel, L., van Hellemond, I.E.G., Creemers, G.J.M., Baars, A., Vestjens, J.H.M.J., Penders, J., Venema, K. & Smidt M.L. (2023). Fecal levels of SCFA and BCFA during capecitabine in patients with metastatic or unresectable colorectal cancer. *Clinical and Experimental Medicine*. 2023 Nov;23(7):3919-3933. doi: 10.1007/s10238-023-01048-7

Aarnoutse, R., **Ziemons, J.**, Hillege, L.E., de Vos-Geelen, J., de Boer, M., Bisschop, S.M.P., (…) Smidt, M.L. (2022). Changes in intestinal microbiota in postmenopausal oestrogen receptor-positive breast cancer patients treated with (neo)adjuvant chemotherapy. *NPJ Breast* Cancer. 2022;8(1):89. doi: 10.1038/s41523-022-00455-5

Aarnoutse, R.*, **Ziemons, J**.*, de Vos-Geelen, J., Valkenburg-van Iersel, L., Wildeboer, A. C. L., Vievermans, A., (...) Smidt, M. L. (2021). The Role of Intestinal Microbiota in Metastatic Colorectal Cancer Patients Treated With Capecitabine. *Clinical Colorectal Cancer*. 2022 Jun;21(2):e87-e97.doi: 10.1016/j.clcc.2021.10.004

Ubachs, J.*, **Ziemons, J.***, Soons, Z., Aarnoutse, R., van Dijk, D. P. J., Penders, J., (…) Rensen, S. S. (2021). Gut microbiota and short-chain fatty acid alterations in cachectic cancer patients. *Journal of Cachexia, Sarcopenia and Muscle. 12*(6), 2007-2021. doi:10.1002/jcsm.12804

Ziemons, J., Smidt, M.L., Damink, S.O., & Rensen, S.S. (2021). Gut microbiota and metabolic aspects of cancer cachexia. *Best practice & research. Clinical endocrinology & metabolism.* 2021 May;35(3):101508. doi: 10.1016/j.beem.2021.101508

Aarnoutse, R.*, **Ziemons, J.***, Penders, J., Rensen, S.S., de Vos-Geelen, J., & Smidt, M.L. (2019). The Clinical Link between Human Intestinal Microbiota and Systemic Cancer Therapy. *International journal of molecular sciences.* 2019 Aug 25;20(17):4145. doi: 10.3390/ijms20174145.

*shared first authorship
[#]shared last authorship

Other

Aarnoutse, R., Hillege, L.E., **Ziemons, J**., De Vos-Geelen, J., de Boer, M., Aerts, E., (...) Smidt, M. L. (2021). Intestinal Microbiota in Postmenopausal Breast Cancer Patients and Controls. *Cancers (Basel).* 2021 Dec 9;13(24):6200. doi: 10.3390/cancers13246200.

Dieleman, S., Aarnoutse, R., **Ziemons, J.**, Kooreman, L., Boleij, A., & Smidt, M. (2021). Exploring the Potential of Breast Microbiota as Biomarker for Breast Cancer and Therapeutic Response. *The American journal of pathology, 191*(6), 968-982. doi:10.1016/j.ajpath.2021.02.020

Terrisse, S., Derosa, L., Iebba, V., Ghiringhelli, F., Vaz-Luis, I., Kroemer, G., (...) **Ziemons, J.**, (...) Zitvogel, L. (2021). Intestinal microbiota influences clinical outcome and side effects of early breast cancer treatment. *Cell death and differentiation, 28*(9), 2778-2796. doi:10.1038/s41418-021-00784-1

Ubachs, J., **Ziemons, J.**, Minis-Rutten, I.J.G., Kruitwagen, R., Kleijnen, J., Lambrechts, S., (...) Van Gorp, T. (2019). Sarcopenia and ovarian cancer survival: a systematic review and meta-analysis. *Journal of Cachexia, Sarcopenia and Muscle. 10*(6), 1165-1174. doi:10.1002/jcsm.12468

Kuepper, M.K., Bütow, M., Herrmann, O., **Ziemons, J.**, Chatain, N., Maurer, A., (…) Schemionek, M. (2019). Stem cell persistence in CML is mediated by extrinsically activated JAK1-STAT3 signaling. *Leukemia, 33*(8), 1964-1977. doi:10.1038/s41375-019-0427-7

About the author

Janine Ziemons was born on September 21st 1993 in Aachen, Germany.

After the completion of secondary school, she moved to Maastricht, the Netherlands, to follow the Bachelor of Science in Health Sciences at *Maastricht University*. Within the bachelor, Janine chose a specialization in Biology & Health and completed the bachelor programme in 2016 with distinction (cum laude). For her bachelor thesis, Janine performed an internship at the *Clinic for Oncology, Hematology, and Stem Cell Transplantation* at the *University Hospital Aachen* and received the student prize of the *"Stichting Wetenschapsbeoefening UM"* for extraordinary academic results and the best bachelor thesis within the bachelor Health Sciences.

Janine continued her education with the Master of Science in Biomedical Sciences at *Maastricht University/Transnationale Universiteit Limburg*, which she completed in 2018 with a specialisation in Nutrition & Metabolism. For her master thesis, Janine conducted an internship at the *Department of Surgery, Maastricht University*. In 2017, Janine also attended a summer course on global health challenges at the *University of Copenhagen*.

Next to her studies at Maastricht University, Janine was also involved in several extracurricular activities, such as the FHML Honours Programme, the language buddy programme for Syrian refugees, and the organizing committee of the MOSA Conference 2018.

During her curricular research internships, Janine discovered her fascination for oncology as well as the gut microbiota. In 2018, she combined these two interests and started her PhD project at the *Department of Surgery (Maastricht University)*, focussing on the role of the gut microbiota in human cancer and supervised by Prof. dr. Marjolein Smidt, Prof. dr. Koen Venema and Dr. John Penders. During her PhD trajectory, Janine presented her work at national and international conferences and was invited as a guest speaker to a symposium on nutrition and cancer at the *Catalan Institute of Oncology* in Barcelona.

Janine lives in Aachen with her family and will continue her academic career as postdoctoral scientist at the *Department of Surgery* at *Maastricht University.*