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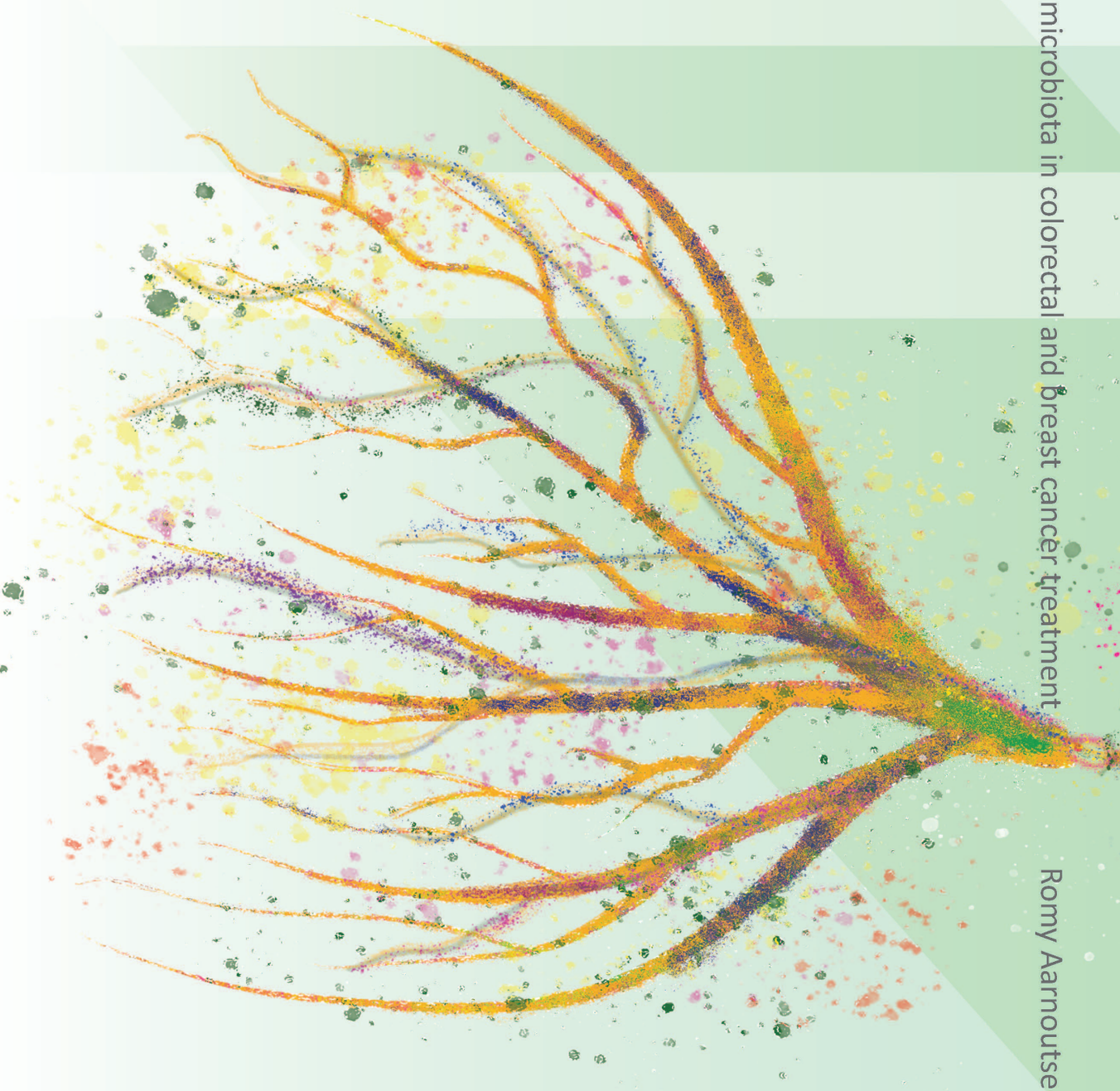
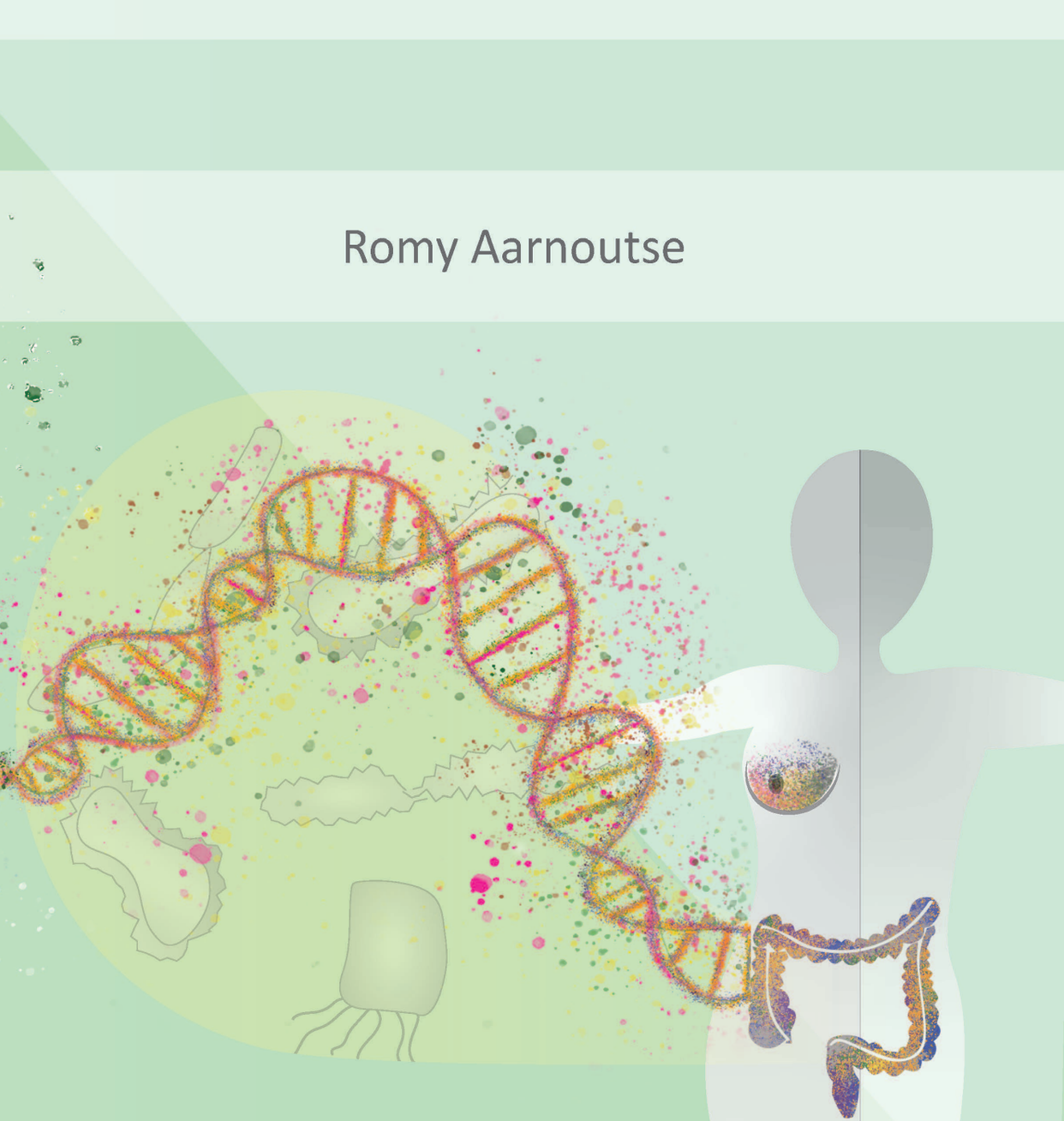
A pathway to personalised medicine?

Romy Aarnoutse





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The role of intestinal microbiota in colorectal and breast cancer treatment

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PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
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in het openbaar te verdedigen
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Contents

CHAPTER 1

General introduction and outline

General introduction

History of intestinal microbiota

For centuries, it has been believed that faecal products reflect health. Hippocrates identified multiple diarrheal syndromes and associated diseases with a disequilibrium of body fluids which resulted from an interaction with the environment[1]. In the early twentieth century, Kendall (1915) published his study observations that not all bacteria living in the human gastro-intestinal tract are harmful, but that they may also be beneficial[2]. During the following decades, Salyers (1977) observed that *Bacteroides* were able to ferment mucin and plant polysaccharides[3].

During the second half of the twentieth century, laboratory techniques improved. Due to this technical evolution, the far-reaching Human Genome Project was initiated in the late eighties. The goal of the Human Genome Project was to identify all the genes of the human genome. The project succeeded after thirteen years. Thanks to the knowledge acquired from the unprecedented Human Genome Project and Moore's law with respect to sequencing costs, it became possible for the National Institute of Health to start the Human Microbiome Project in 2008[4-6]. This project aimed to characterise the human microbiome and understand how the microbiome impacts human health and disease. During recent decades, bacterial identification techniques have improved from cultivation-dependent methods to polymerize chain reaction to modern sequencing techniques targeting the 16S rRNA gene[8]. Finally, these bacterial identification and quantification methods have improved to high-throughput whole metagenomic shotgun sequencing, including the possibility to determine bacterial metabolic capacity[7].

All this research has resulted in the knowledge that trillions of intestinal microorganisms, consisting of bacteria, archaea, fungi, protozoa, and viruses, colonize the human gastrointestinal tract. This ecosystem is often referred to as "intestinal microbiota". The genomes of all the microorganisms within the intestinal microbiota, collectively termed the metagenome, together contain many more genes than our own genome. This implies that humans should actually be considered as holobionts[9]. The metagenome is able to amplify or counteract human functions. The interaction between intestinal microbiota and the human host is essential for the maintenance of metabolism, immune function, and homeostasis[10].

Pre-clinical studies have revealed that human metabolism and a proper immune function depend on host-microbiota interactions[11]. Birth mode, breast-feeding, hygiene, and (dietary) lifestyle are considered as important factors in shaping the intestinal microbiota[12-16]. In healthy conditions, intestinal microbiota live in symbiosis

within a well-balanced host environment. Due to environmental influences, dysbiosis can occur[10,17,18]. In cases of dysbiosis, intestinal microbiota can instigate cancer or affect systemic cancer therapy[19]. The thesis addresses the role of intestinal microbiota in cancer and systemic cancer therapy. Herby focussing on colorectal and breast cancer patients treated with chemotherapy. Figure 1.1 demonstrates the interactions of the human intestinal microbiota with environmental, dietary, and lifestyle determinants.

Intestinal microbiota and carcinogenesis

It has been shown that intestinal microbiota are involved in carcinogenesis[19]. The mechanism, by which intestinal microbiota influence carcinogenesis, has been mostly examined in pre-clinical studies[11]. Despite the availability of pre-clinical evidence, there is only limited clinical evidence on the role of intestinal microbiota in carcinogenesis in cancer patients. Of this clinical evidence, most studies have focussed on gastro-intestinal cancer, for example colorectal cancer. Fortunately, recently more interest has also arisen in the role of the intestinal microbiota in extra-intestinal cancers, for example breast cancer[20,21] (Figure 1.1).

Colorectal cancer carcinogenesis

The role of intestinal microbiota in gastro-intestinal cancer, specifically colorectal cancer, has been studied before[22-24]. Colorectal cancer is the third most common cancer in the world[13]. The incidence of colorectal cancer almost doubled from 1990 to 2016, with 15,000 new patients yearly in the Netherlands. About 20% of colorectal cancer patients already have metastatic disease at diagnosis[25]. Genetic predisposition, obesity, sedentary lifestyle, tobacco, diet, including red meat and alcohol consumption, are considered as important driving factors of colorectal cancer carcinogenesis[13,24].
Despite available knowledge, the mechanism of carcinogenesis is not fully understood. Currently, the interest in the role of intestinal microbiota in colorectal cancer carcinogenesis is rising[22,23]. Several studies have described the role of intestinal microbiota in colorectal carcinogenesis[26-35]. These studies have concluded that members of the genera *Fusobacterium, Porphyromonas, Parvimonas, Peptostreptococcus, Gemella, Prevotella,* and *Solobacterium* are associated with colorectal cancer[27,32,34,35]. In particular, the abundances of *Fusobacterium nucleatum*[28,29,36] and *Enterococcus faecalis* [30,37] were increased in patients with colorectal cancer. Furthermore, Wirbel et al. conducted a meta-analysis and have established microbial signatures specific for colorectal cancer. These signatures form the basis for future clinical diagnostic assays[23].

Breast cancer carcinogenesis

Knowledge is scarce on the role of intestinal microbiota in extra-intestinal cancer, for example breast cancer[38]. Breast cancer is the most common cancer in women worldwide[39]. Approximately one out of seven women (15%) will develop breast cancer during their lifetime[40]. A combination of genetic, epigenetic, and environmental factors is known to contribute to the development of cancer[41]. Factors, such as hormone treatment, heredity, and obesity are known to increase the risk of breast cancer[42,43]. Still, other factors, such as intestinal microbiota, are thought to influence breast cancer carcinogenesis.

Only a limited number of clinical studies have explored the role of intestinal microbiota in breast cancer carcinogenesis[43-45]. These studies showed that specific bacterial species were directly related with clinical stages of breast cancer. Patients with a high histoprognostic grade, according to Scarff-Bloom-Richardson grading, show higher numbers of intestinal *Blautia* species[46]. Only a small number of studies have compared the intestinal microbiota composition in breast cancer patients with a healthy control group. These studies have indicated alterations in the abundance of specific bacterial species[47-51].

Intestinal microbiota and systemic cancer therapy

It has been shown that the intestinal microbiata might influence human metabolism of dietary components and medication, including chemotherapeutic agents[19] (Figure 1.1). Moving to the field of pharmacomicrobiomics. Pharmacomicrobiomics is an emerging field that investigates the interplay between microbiota, medication and the host[52,53]. Consequently, intestinal microbiota are able to extent the metabolic capacity of humans due to their enzymatic activity[53]. The mechanism, by which intestinal microbiota influences response to systemic cancer therapy is mostly studied in a pre-clinical setting. Despite the availability of pre-clinical evidence, there is limited clinical evidence on the role of intestinal microbiota in response to chemotherapy in cancer patients.

During recent years most studies have focussed on gastro-intestinal cancer, for example colorectal cancer. However, recently more interest has also arisen in the role of the intestinal microbiota in extra-intestinal cancers[50,54].

Colorectal cancer treatment with chemotherapy

The role of intestinal microbiota in colorectal cancer treatment with chemotherapy has been examined in pre-clinical studies and in a limited number of clinical studies[55-60].

Despite recent developments in systemic cancer therapy, classical chemotherapeutic agents, such as fluoropyrimidines, for example capecitabine, an oral prodrug of 5-fluorouracil (5-FU), remain the backbone of systemic cancer therapies for colorectal cancer patients[61].

Pre-clinical microbiota studies have indicated 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[57]. Furthermore, *Lactobacillus plantarum* supernatant is able to sensitise colorectal cancer cell lines to 5-FU and stimulate apoptosis in chemo-resistant cells[58]. Another study examined the effect of 5-FU as well as the influence of antibiotics on the gut microbiota and tumour response in colorectal cancer. Administration of an antibiotic combination, composed of vancomycin, ampicillin, neomycin, and metronidazol, reduced antitumour response of 5-FU in mice[59]. After antibiotic administration, opportunistic bacterial pathogens, such as *Escherichia*, *Shigella,* and *Enterobacter* significiantly increased, while other commensals decreased. After 5-FU administration, the relative abundance of *Enterobacter, Lachnospiraceae NK4A136 group, Escherichia, Shigella, Alloprevotella, Bacteoides, Blautia,* and *Mycoplasma* increased, while the relative abundance of *Alistipes, Lactobacillus, Rikenella* and unidentified Lachnospiraceae decreased[59]. Recently, Zimmermann et al. provided the first *in vitro* evidence that capecitabine can be metabolised by several bacterial species[60].

Two studies form the basis of clinical evidence on the role of intestinal microbiota in colorectal cancer treatment originates from two studies. Li at al. studied rectal cancer patients without metastasis who received a combination of 5-FU and oxaliplatin (FOLFOX). Baseline microbiota did not differ between responders and non-responders. However, after FOLFOX treatment microbiota changes were more apparent in the responder group. After treatment, *Lactobacillus fermentum, Pyramidobacter piscolens*, and *Dialister invisus* were increased and *Campylobacter ureolyticus*, *Prevotella timonensis*, and *Solobacterium moorei* nearly disappeared[55]. Another clinical study performed longitudinal microbiota analysis in patients with primary diagnosed colorectal cancer (n=26)[56]. After treatment for colorectal cancer, Sze et al. observed a change in community structure and a shift towards a microbiota comparable to the profile of healthy controls. However, these findings were based on a heterogeneously treated group including surgery, with or without five different types of chemotherapy, with or without radiation[56]. Despite the two available clinical studies on the role of intestinal microbiota in colorectal cancer treatment, no clinical studies in metastatic colorectal cancer setting are available.

Breast cancer treatment with chemotherapy

The role of intestinal microbiota in breast cancer treatment with chemotherapy has been studied in a limited number of pre-clinical studies and has not been studied thoroughly in clinical studies. Despite recent developments in systemic cancer therapy, classical chemotherapeutic agents, such as adriamycin, cyclophosphamide and docetaxel, remain the backbone of (neo)adjuvant chemotherapy regimes in postmenopausal oestrogen receptor positive (ER+) and Human Epidermal growth factor Receptor-2 negative (HER2-) breast cancer patients[62-64].

Pre-clinical microbiota studies have indicated significant interactions between cyclophosphamide and the intestinal microbiota[65-67]. In mice, cyclophosphamide induced 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* promoted the immune response and increased cyclophosphamide efficacy[65-67].

Besides the cyclophosphamide-microbiota interactions, pre-clinical evidence has demonstrated the interaction between intestinal microbiota and adriamycin. Rigby et al. (2016) has concluded that the intestinal microbiota were necessary for adriamycin induced intestinal damage and repair[68]. In contrast to conventionally raised mice (with intestinal microbiota) Rigby et al. (2016) did not observe intestinal damage and repair after adriamycin administration in germ free mice (mice without intestinal microbiota). However, clinical studies need to address the association between intestinal damage and chemotherapy toxicity, including diarrhoea following from mucositis.

Limited evidence exists on the interaction between docetaxel and intestinal microbiota[69]. Flórez et al. (2016) determined the susceptibility profiles of lactic acid and bifidobacteria to multiple chemotherapeutics. The main findings have showed that adriamycin perturbs the intestinal microbiota and that the tested intestinal microbiota was resistant to high doses of cyclophosphamide and docetaxel. However, these *in vitro* tests did not take into account the effect of in vivo transformation of chemotherapeutics to more toxic compounds[70]. Despite the availability of the previously described pre-clinical evidence, no clinical studies have been performed that explored the role of intestinal microbiota in breast cancer treatment with chemotherapy.

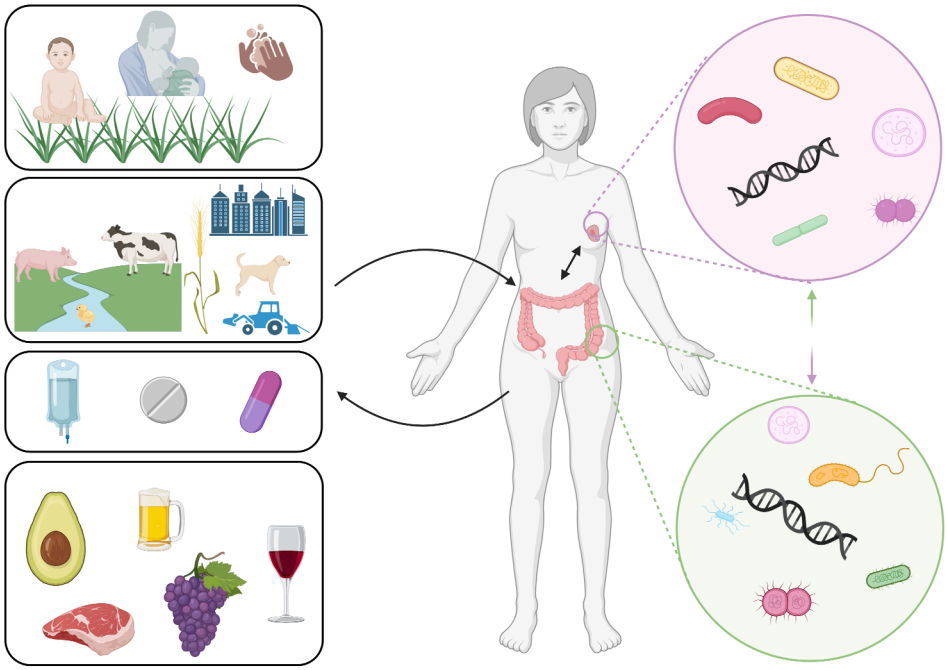

Figure 1.1: Interactions of the human intestinal microbiota with environmental, dietary, and lifestyle determinants. Intestinal microbiota might be involved in carcinogenesis, for example colorectal and breast cancer. Intestinal microbiota are known to influence systemic cancer treatment. In addition, systemic cancer treatment could influence the intestinal microbiota. Furthermore, there may be a link between intestinal microbiota, breast microbiota and breast cancer. Figure created with BioRender.com.

Summary

The mechanism by which intestinal microbiota influence carcinogenesis and response to systemic cancer therapy, has mostly been studied in pre-clinical setting. Firstly, there is limited clinical evidence on the role of intestinal microbiota in carcinogenesis and response to systemic cancer therapy. Secondly, the role of intestinal microbiota in gastro-intestinal cancer has been more widely studied compared to cancers of extra-intestinal origin. Lastly, studies on the role of intestinal microbiota in systemic cancer treatment are scarce compared to studies that explore the role of intestinal microbiota in carcinogenesis.

To elaborate pre-clinical evidence into a clinical setting, additional observational cohort studies are currently needed, focussing on longitudinal faecal sampling. These clinical studies should explore the role of intestinal microbiota in carcinogenesis and cancer

treatment in clinical setting. Clinical knowledge is needed to identify factors, which impact individual susceptibility to cancer development. Furthermore, there needs to be clarification of why cancer patients show variable response and chemotherapy toxicities to cancer therapy and the role of microbiota in this mechanism. Variable response rates could only partially be explained by inter-patient variability of known factors, such as molecular profiles, mutational burden and microsatellite (in)stability. In addition, due to higher cancer survival rates, there is an urgency to reduce cancer treatment morbidity, by reduction of chemotherapy toxicity.

Aims and outline

The overall aim of this thesis was to investigate the role of intestinal microbiota in cancer and systemic cancer therapy exploring new predictive, prognostic, and therapeutic targets. This thesis has focussed on cancer patients treated with systemic cancer therapy and especially on colorectal and breast cancer patients treated with chemotherapy.

Chapter 2 provides an overview of all clinical studies performed until the 22nd of April 2019 that described the clinical association between intestinal microbiota and systemic cancer therapy, including chemotherapy, hormone therapy, and immunotherapy.
Chapter 3 presents our study protocol on the role of intestinal microbiota in colorectal cancer treatment. The study aims to explore whether baseline intestinal microbiota composition is associated with response or chemotherapy toxicity in patients with mCRC treated with capecitabine or trifluridine/tipiracil. Furthermore, the study aims to explore the influence of capecitabine and trifluridine/tipiracil on intestinal microbiota over the course of three cycles of chemotherapy.
Chapter 4 describes the results of the study presented in chapter 3. With longitudinal faecal sampling, the study explored the role of intestinal microbiota in metastatic colorectal cancer patients treated with capecitabine. In total, 33 patients were included and 90 faecal samples were collected. Faecal samples were collected at three timepoints and included 27 paired samples.
Chapter 5 explores with longitudinal faecal sampling the intestinal microbiota in ER+ and HER2- breast cancer patients treated with (neo)adjuvant chemotherapy. In total, 44 patients were included and 153 faecal samples were collected. Faecal samples were collected at four timepoints and included 28 paired samples.
Chapter 6 further reveals the role of intestinal microbiota in breast cancer development in a case-control study, which was conducted in collaboration with the National Dutch Breast Cancer Screening Programme in Maastricht. In total, 81

postmenopausal ER+ and HER2- breast cancer patients and 67 postmenopausal healthy controls were included, resulting in 148 faecal samples.

Chapter 7 presents a literature review on the role of breast microbiota as a biomarker for breast cancer and therapeutic response. The interaction of breast microbiota with the immune system, gut microbiota and anti-cancer therapeutics are discussed. Lastly, possible strategies for targeting microbiota in order to improve breast cancer treatment are evaluated.

Chapter 8 describes the results of this thesis in the context of current literature. The implementations as well as the impact of our findings in clinical practice are discussed, focusing on future clinical microbiota research.

Methods

During this PhD trajectory, four research lines were designed, implemented, and conducted in collaboration with eight Dutch hospitals and the National Dutch Breast Cancer Screening Programme in Maastricht. Participants included in the articles of this thesis originate from five Dutch hospitals and the National Dutch Breast Cancer Screening Programme in Maastricht.

In 2017, the first three clinical research lines were set up to explore the role of intestinal microbiota in metastatic colorectal cancer patients and postmenopausal breast cancer patients treated with chemotherapy or hormone therapy. The first cohort consists of metastatic colorectal cancer patients treated with three cycles of capecitabine or trifluridine/tipiracil. After publishing the study protocol **(chapter 3)**, the study was initiated in seven Dutch hospitals. Patients included in the article described in **chapter 4** originate from four hospitals. The second cohort consists of postmenopausal ER+ and HER2- breast cancer patients treated with (neo)adjuvant adriamycin, cyclophosphamide and docetaxel. The study was initiated in four Dutch hospitals. Patients included in the articles in **chapters 5 and 6** originate from all four hospitals. The third cohort consists of postmenopausal ER+ and HER2- breast cancer patients who were treated with hormone therapy. The study was initiated in four Dutch hospitals. Patients included in **chapter 6** originate from three hospitals that participated in the hormone therapy cohort study and four hospitals that participated in the chemotherapy cohort study. To match postmenopausal ER+ and HER2- breast cancer patients, derived from research lines two and three, with healthy controls, a fourth research line was designed.

In 2018, the fourth cohort study was initiated in collaboration with the National Dutch Breast Cancer Screening Programme in Maastricht. Postmenopausal women without

breast cancer were included and faecal samples and questionnaires were collected. **Chapter 6** presents the results.

Simultaneous to the conducting of the research lines described above, two literature reviews were performed. The results are included in **chapter 2** and **chapter 7**. A systematic literature search was performed for the review presented in **chapter 2**.

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CHAPTER 2

The clinical link between human intestinal microbiota and systemic cancer therapy

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Abstract

Clinical interest in the human intestinal microbiota has increased 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.

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 fulfils 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 found to be increased in colorectal cancer and it is suggested that this bacterial species might be involved in intestinal tumour genesis and modulation of the tumour 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 oestrogen 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 leukaemia 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 2.1). We focused on chemotherapy, immunotherapy, and hormonal therapy. In addition, Table S2.1 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.

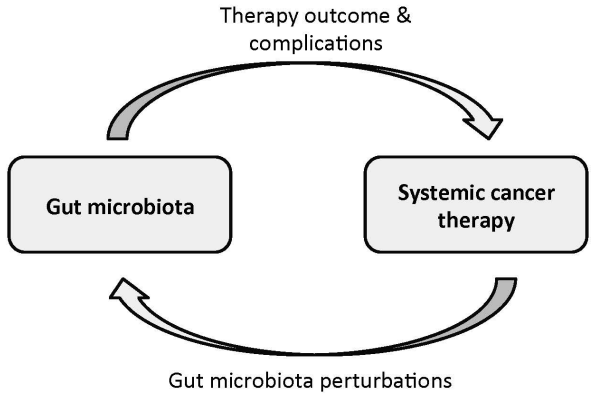

Figure 2.1: Overview of the main questions addressed in this review.

Materials and methods

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

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 on the 22nd of April 2019.

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 analysed 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 fulfil 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.

Study selection

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 11 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 2.2 provides an overview of the article selection process.

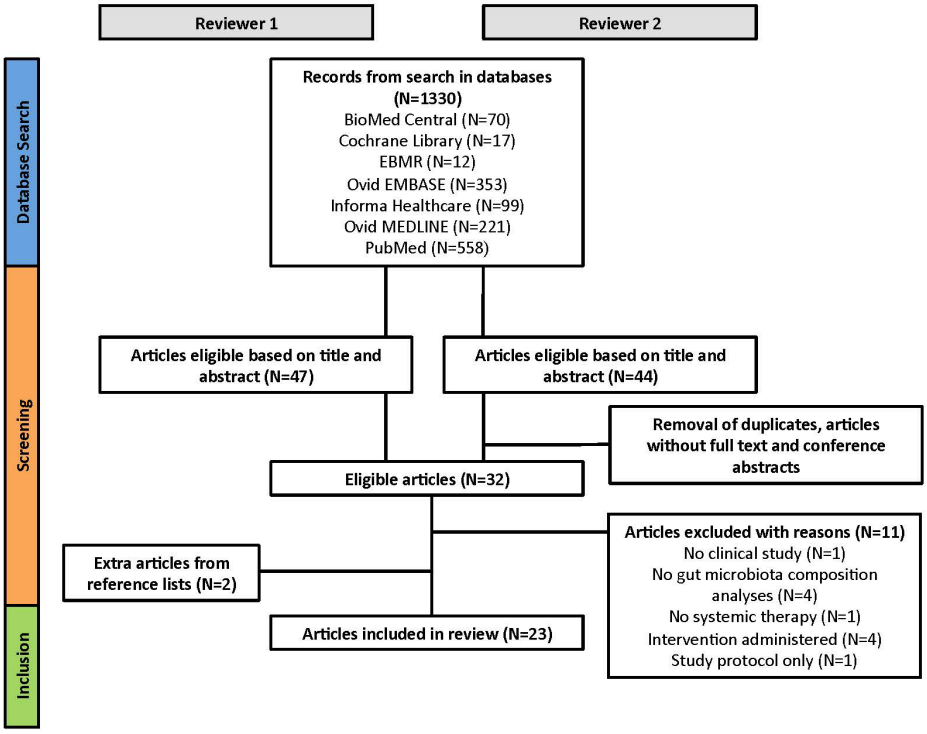

Figure 2.2: Schematic overview of the article selection procedure.

Data collection process

Data extraction was conducted following a data extraction sheet conform Tables 2.1-2.3. 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.

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 [19]. The QUIPS tool consisted 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 (Table S2.2 and Figure S2.1). Two reviewers (R.A. and J.Z.) independently assessed the risk of bias and consensus was reached afterwards.

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

Chemotherapy

Infectious complications are a common side effect of cancer therapy and have a considerable impact on patients' prognosis and quality of life [20]. 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 leukaemia (AML) suffering from infectious complications after induction chemotherapy compared to patients without infections [21]. 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 analysed stool temporal variability as indicator of microbial instability and its association with induction chemotherapy outcome [22]. Baseline samples were collected up to eight days before and 24 h following chemotherapy initiation. It was concluded

that AML patients who developed an infection within 90 days post-neutrophil recovery had significantly higher microbial instability [22]. Moreover, patients developing an infection during induction chemotherapy had a significantly higher relative abundance of *Stenotrophomonas* [22]. 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 [22]. 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) [23] Principal coordinate analysis (PCoA) of faecal samples collected before start of the treatment demonstrated differences between patients with or without subsequent BSI [23]. 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 faecal samples from patients who developed subsequent BSI [23]. Furthermore, abundance of several bacteria was altered in these patients (Table 2.1). 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 [23]. 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 diarrhoea in patients with metastatic renal cell carcinoma (RCC) was not related to differences in α-diversity of the gut microbiota [24]. 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%) [24]. In the low-risk group, the opposite pattern was apparent with 47% *Prevotella* and 13% *Bacteroides* [24]. This suggests that there might be an interaction between intestinal microbiota composition and VEGF-TKI-induced diarrhoea.

Table 2.1: 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),[22]	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), [21]	AML	*n*=34	Induction chemotherapy	16S rRNA gene sequencing	Increased risk for infections	↓ baseline α-diversity (Shannon index)
Pal et al. (2015), [24]	Metastatic RCC	*n*=20	VEGF-TKI	16S rRNA gene sequencing	Increased risk to develop diarrhoea	↑ *Bacteroides* ↓ *Prevotella*
				Immunotherapy		
Matson et al. (2018), [25]	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), [26]	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*

Table 2.1: (continued)

Study Design					Main Findings	
Study	**Cancer Type**	***n***	**Type of Therapy**	**Analysis Method**	**Therapy Outcome**	**Microbial Outcomes Found to be Different**
		n=39	Anti-PD-1	Metagenomic whole-genome shotgun sequencing	Prolonged PFS (*n*=19)	↑ *Faecalibacterium* ↓ Bacteroidales
Routy et al. (2018), [27]	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*

Table 2.1: (continued)

Study Design					Main Findings	
Study	**Cancer Type**	***n***	**Type of Therapy**	**Analysis Method**	**Therapy Outcome**	**Microbial Outcomes Found to be Different**
	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), [28]	Metastatic melanoma	*n*=26	Ipilimumab	16S rRNA gene sequencing	Colitis and good response	↑ Firmicutes ↓ Bacteroidetes
					↑ PFS ↑ OS ↑ % clinical benefit	↑ *Faecalibacterium* ↑ Firmicutes ↑ unclassified Ruminococcaceae ↑ *Clostridium cluster XIVa* ↑ *Blautia*

Table 2.1: (continued)

Study Design					Main Findings	
Study	**Cancer Type**	***n***	**Type of Therapy**	**Analysis Method**	**Therapy Outcome**	**Microbial Outcomes Found to be Different**
Frankel et al. (2017), [29]	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), [30]	Metastatic Melanoma	*n*=34	Ipilimumab	16S rRNA gene sequencing	Colitis free	↑ Bacteroidaceae ↑ Bacteroides ↑ Barnesiellaceae ↑ unclassified Barnesiellaceae ↑ Rikenellaceae ↑ unclassified Rikenellaceae ↑ Bacteroidetes ↑ Bacteroidia ↑ Bacteroidales ↑ *Bacteroidetes*
				Other		
Montassier et al. (2016), [23]	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 leukaemia; 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.

Immunotherapy

Six articles were available describing the association between baseline human intestinal microbiota and immunotherapy outcome [25-30]. 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 analysed faecal microbiota composition with metagenomic shotgun sequencing.[25-27,29].

Matson et al. (2018) compared the baseline microbiota composition of 42 patients with metastatic melanoma that received anti-PD-1 (*n*=8) or anti-CTLA-4 (*n*=4) immunotherapy [25]. 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 [25]. Principal component analysis (PCA) showed a separation of responders and non-responders [25]. 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 2.1). As conclusions did not change when removing the four anti-CTLA-4 treated patients, these patients were retained in the analysis [25]. 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-tumour 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 might predict the most favourable clinical outcome [25].

Gopalakrishnan et al. (2018) compared the microbiota composition of 43 metastatic melanoma patients treated with anti-PD-1 therapy [26]. Baseline stool samples were collected prior to therapy initiation. Median time from initial faecal 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 [26]. 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 [26]. 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) [26]. 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 [26]. Patients with a high α-diversity and abundance of *Ruminococcaceae* and *Faecalibacterium* were found to have an enhanced systemic and antitumour 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 [26].

Routy et al. (2018) analysed 100 patients who received anti-PD-1 therapy for NSCLC (n=60) or RCC (n=40) [27]. Baseline faecal 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 faecal samples was correlated with clinical response at six months, but not at three months after therapy initiation [27]. Response was defined as the absence of progression defined by the Response Evaluation Criteria in Solid Tumours (RECIST) [27]. By means of the RECIST criteria, tumour response can be graded as complete response (CR), partial response (PR), progressive disease (PD), or stable disease (SD) [31]. In addition, Routy et al. (2018) identified that for instance Firmicutes, *Akkermansia* and *Alistipes* were significantly associated with response (PR and SD) [27]. *Akkermansia muciniphila* was most significantly (p=0.004) overrepresented at diagnosis in the faeces 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 [27].

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 2.1. 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 faeces, patients would later benefit from anti-PD-1 therapy [27].

Chaput et al. (2017) analysed the predictive value of baseline faecal microbiota samples of 26 patients with metastatic melanoma receiving ipilimumab [28]. Baseline faecal samples were collected before the first ipilimumab infusion. PCA analysis at genera level (*p*=0.009), species level (*p*=0.005) or OTU level (*p*=0.008) indicated that metastatic melanoma patients could be clustered into groups with long-term versus poor clinical benefit, based on gut microbiota composition at baseline [28]. Main genera, which contributed to this stratification, were *Faecalibacterium, Gemmiger, Bacteroides*, and *Clostridium cluster XIVa* [28]. Before treatment, patients with poor clinical benefit had a high proportion of *Bacteroid.es* (*p*=0.034). The relative abundance of *Faecalibacterium, Clostridium cluster XIVa*, and *Gemminger* was higher in patients with long-term benefit [28]. 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 [28].

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 cluster XIVa*, 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 [28]. 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) [28]. 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 [28].

Frankel et al. (2017) collected baseline faecal 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)) [29]. 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) [29]. In the IN + N group there were 16 responders and eight non-responders [29]. 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 [29].

Interestingly, overall microbial diversity was not significantly different between responders and patients with progressive disease [29]. 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 faecal microbiota composition with subsequent colitis development in 34 patients with metastatic melanoma to be treated with ipilimumab [30]. In general, faecal 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, 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 faecal samples collected before ipilimumab infusion [30]. Based on this, the authors concluded that increased faecal abundance of Bacteroidetes, Bacteroidaceae, Rikenellaceae, and Barnesiellaceae correlated with a reduced risk to develop ipilimumab-induced colitis [30].

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.

Human intestinal microbiota changes during systemic cancer therapy

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 tumours (NET), it was observed that systemic chemotherapy increased the concentration of *Faecalibacterium prausnitzii* in patients with midgut NET [32]. 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 has been described by Zwielehner et al. (2011).[33]

In a subsequent study, Montassier et al. (2015) verified the previously described results concerning microbial diversity and differences at the phylum level [34]. 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) [34]. 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 [34]. Concurrently, signal transduction (p=0.0002), xenobiotics biodegradation (p=0.002), and glycan metabolism (p=0.0002) were enhanced.[34] Furthermore, several other metabolic pathways, amongst others pathways involved in bacterial motility, virulence, and epithelial repair were altered after chemotherapy.[34]
Galloway-Peña et al. (2016) observed similar dramatic changes in the intestinal microbiota composition in AML patients during induction chemotherapy [21]. 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* [21]. On the other hand, chemotherapy caused increased abundance of *Lactobacillus* [21]. 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 bacteraemia (e.g., *Staphylococcus*, *Enterobacter*, *Escherichia*). Before chemotherapy, this was only 20% [21]. 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 [22]. Furthermore, high intra-patient temporal instability was associated with increased abundance of opportunistic pathogenic genera [22]. High CV values were positively correlated with pathogenic genera such as *Staphylococcus* and *Streptococcus* and negatively associated with the non-pathogenic *Akkermansia* [22]. Thus, a high relative abundance of *Akkermansia*, *Subdilogranulum*, and *Pseudobutvrivi.brio* 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 [22].
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 faecal samples of 26 colorectal cancer (CRC) patients treated with different types of chemotherapy [35]. 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) [35]. However, no significant change in α-diversity between pre- and post-treatment samples was identified [35]. The authors concluded that the community structure was affected by the treatment, but the effect of treatment was not consistent across patients [35]. No subgroup analysis was performed for these very heterogeneous small groups receiving chemotherapy or chemo-radiation. 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) [35]. 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 [35]. These results are contradictory to the studies described before, which indicated deterioration of the gut microbiota instead of improvement.

Youssef et al. (2018) collected faecal samples of 20 treated patients with gastrointestinal neoplasms and 13 healthy controls [36]. Gastrointestinal neoplasms included neoplasms of the stomach (n=6), small intestine (n=1), or rectum (n=13). Treatment included chemotherapy and/or radiotherapy [36]. 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* [36].

Similarly, Deng et al. (2018) compared faecal microbiota composition of 14 CRC patients treated with chemotherapy with 33 healthy controls [37]. 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 [37].

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 [33]. Likewise, total bacterial abundance declined after chemotherapeutic treatment (p=0.037) and was also restored within a few days [33]. Next to *Clostridium cluster IV*, the bacteria found to be affected most by chemotherapy were *Bacteroides*, *Bifidobacteria*, as well as *Clostridium cluster XIVa* [33].

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 [38]. Increased relative abundance was found for *Escherichia coli* and *Staphylococcus spp*. [38]. 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 10 days [38].

Besides, some studies investigated the effect of chemotherapy on the gut microbiota in paediatric 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 [39].

In addition, another study with paediatric AML patients revealed that there was a considerable decrease in bacterial diversity during chemotherapy treatment, which restored quickly after chemotherapy [40]. Furthermore, the total number of bacteria has found to be significantly reduced in patients during treatment but resembled the bacterial count in healthy samples six weeks after the last chemotherapy cycle [40]. 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 cluster XIVa* and *Faecalibacterium prausnitzii* levels were restored six weeks after treatment [40]. 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 [40]. 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 [40].

On the contrary, Rajagopala et al. (2016) indicated that there was no difference in microbial diversity before and during induction chemotherapy in patients with paediatric 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 [15,40]. 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 cluster IV and XIV*) *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 [41], 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.

Immunotherapy

Six articles of five human clinical studies were identified that described human intestinal microbiota changes during immunotherapy assed by longitudinal sampling [26-29,32,42]. 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 tumours (NET).

Routy et al. (2018) collected longitudinal faecal samples of 32 patients that received two months anti-PD-1 therapy for NSCLC (*n*=15) or RCC (*n*=17) [27]. Faeces 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*, and *Eubacterium eligens* [27].

Chaput et al. (2017) collected longitudinal faecal samples of 26 patients with metastatic melanoma [28]. Patients received four cycles of ipilimumab every three weeks. Faecal 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 [28]. However, it should be noted that the number of faecal samples analysed decreased to four over time [28]. 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, faecal 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 cluster IV*, *Eubacterium*, unclassified Lachnospiraceae, and *Pseudoflavonifracto*) was significantly reduced in metastatic melanoma patients with ipilimumab-induced colitis [28]. They all belong to the Firmicutes phylum. Furthermore, 18 other bacteria, mostly Firmicutes, were significantly reduced (Table 2.2). Ipilimumab-induced colitis was also associated with lower α-diversity [28]. 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 [42]. These patients were later also described in the article of Chaput et al. (2017) in relation to ipilimumab-induced colitis [28]. 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* [42]. During ipilimumab treatment, the proportion of patients in cluster C increased (p=0.05) whereas it decreased in cluster B (p=0.007) [42]. Interestingly, it has been shown that tumours in mice treated with ipilimumab respond better to faecal 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 favourable cluster C [42].

Additional to the studies of Routy, Chaput, and Vetizou, three studies performed longitudinal faecal 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 [26]. 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) [29]. They concluded that specific gut microbiota abundances changed, but that these numbers were too small to draw conclusions [29]. In 2012, Dörffel et al. collected faecal samples in 11 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 [32].

Hormonal therapy

Currently, only two studies are available that investigated human intestinal microbiota changes during hormonal therapy [32,43].

Dörffel et al. (2012) collected faecal samples of 27 patients receiving somatostatin analogues for NET. It was observed that somatostatin analogues had no influence on the abundance of specific bacterial groups in these patients [32].

Sfanos et al. (2018) compared intestinal microbiota of patients with prostate cancer treated with androgen axis-targeted therapies compared to no hormonal medication use [43]. 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 tumours (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 [43]. 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 faecal 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 2.3) [43]. In addition, it was confirmed that *Akkermansia muciniphila* was significantly more prevalent in men taking oral ATT, using quantitative polymerase chain reaction (qPCR) [43]. As indicated in Table 2.3, 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) [43].

Table 2.2: 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), [22]	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), [35]	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), [21]	AML	*n*=34	Induction chemotherapy	Baseline: before therapy; Follow-up: every 96 h until neutrophil recovery	16S rRNA gene sequencing	↑ *Lactobacillus* ↓ α-diversity (Shannon index) ↓ *Blautia*
Rajagopala et al. (2016), [15]	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)

Table 2.2: (continued)

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**
Montassier et al. (2015), [34]	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), [6]	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), [38]	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)

Table 2.2: (continued)

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**
Dörffel et al. (2012), [32]	NET	*n*=13	Chemotherapy	Before and during therapy	FISH	↑ *Faecalibacterium prausnitzii* (midgut NET only)
Zwielehner et al. (2011), [33]	Different types of cancer	*n*=17	Chemotherapy	(1) Before chemotherapy (2) Day 1–4 after chemotherapy (3) 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), [39]	Different types of cancer	*n*=23	Chemotherapy	(1) Before chemotherapy (2) Within 24 h after initiation (3) Once weekly	Bacterial cultures (*n*=3)	↑ Enterobacteriaceae
Van Vliet et al. (2009), [40]	Paediatric 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), [27]	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*

Table 2.2: (continued)

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**
Chaput et al. (2017), [28]	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 cluster 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), [42]	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), [32]	Midgut NET	*n*=11	Interferon alpha-2b	Before and during therapy	FISH	↑ *Faecalibacterium prausnitzii*

↑: Increase; ↓: Decrease; AML: acute myeloid leukaemia; CRC: colorectal cancer; ALL: acute lymphoblastic leukaemia; dHPLC: denaturing high-performance liquid chromatography; NET: neuroendocrine tumour; 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 2.3: 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), [36]	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), [37]	CRC	Oxaliplatin + tegafur	*n*=14	*n*=33	16S rRNA gene sequencing	↑ *Veillonella* ↑ *Veillonella dispar* ↑ *Prevotella copri* ↑ *Bacteroides plebeius*
Stringer et al. (2013), [44]	CRC, breast cancer, laryngeal cancer, oesophageal 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), [40]	Paediatric AML	Chemotherapy	*n*=9	*n*=11	FISH	↑ Enterococci ↓ total number of bacteria ↓ *Bacteroides* ↓ *Clostridium cluster XIVa* ↓ *Faecalibacterium prausnitzii* ↓ *Bifidobacterium* ↓ Streptococci

Table 2.3: (continued)

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**
				Hormonal Therapy		
Sfanos et al. (2018), [43]	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 cluster 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 leukaemia; CRC: colorectal cancer; FISH: fluorescent in situ hybridization; ATT: androgen receptor axis-targeted therapy; GNRH: gonadotropin-releasing hormone.

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.

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 diarrhoea. 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 chemo resistant cells [16]. In line with this, administration of an antibiotic cocktail markedly diminished the antitumour efficacy of 5-FU in mice [45]. Considering the complexity of the interaction, various metabolic pathways might be involved in microbial metabolism of

chemotherapeutic drugs. The field of pharmacomicrobiomics focuses on unravelling these interactions between drugs and the human microbiome [46].

In this context, Alexander et al. (2017) suggested the TIMER mechanistic framework (translocation, immunomodulation, metabolism, enzymatic degradation, reduced diversity) to describe the mechanisms through which the gut microbiota might modulate chemotherapy treatment [47]. According to this concept, bacterial translocation might be facilitated by chemotherapeutic drugs, which damage the intestinal barrier [47]. Subsequently, intestinal bacteria or their products can shape the chemotherapy-induced immune response by immunomodulation [47]. 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 metabolism and enzymatic degradation. Several gut bacteria-derived enzymes metabolize chemotherapeutic drugs and their metabolites, thereby modulating efficacy as well as toxicity [46,47]. Zimmermann et al. (2019) indicated that the orally administered capecitabine, cyclophosphamide, melphalan, and paclitaxel can be metabolized by specific bacterial strains [48]. 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 In contrast, the same thymidine phosphorylase enhanced cytotoxicity of capecitabine, probably by converting the pro-drug into the cytotoxic 5-FU [49.]

Another factor with a considerable impact on cancer therapy outcome is chemotherapy toxicity. High chemotherapy toxicity 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 chemotherapy toxicity. 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 [50]. In support of this, targeted inhibition of bacterial β-glucuronidase has been shown to alleviate gastrointestinal toxicity in mice [51].

Lastly, reduced microbial diversity and ecological variation might also affect the chemotherapy response of the host [47]. 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.

A limited number (*n*=6) of 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* [25], *Faecalibacterium* [26,28,29], and *A. muciniphila* [27] and a reduced number of Bacteroidales [26] and Bacteroides [28]) 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 [26]. An active and well-developed immune system stimulates beneficial T-cell activation and consequently a diverse repertoire of T-cells [52]. 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 tumour response as well as inflammatory side effects like colitis. Consequently, it might be speculated that tumours 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 tumour growth besides tumour 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 faecal microbiota of responder and non-responder patients to germ-free and tumour bearing mice, it was revealed that the clinical response was repeated in the majority of mice [25-27]. Moreover, the immune stimulatory effect of anti-CTLA-4 blockade reactivated T-cells, which resulted in anti-cancer response [28], but also immune-mediated colitis [28,30]. Bacteroidetes seemed to be associated with this clinical presentation [28,30]. Bacteroidetes could stimulate differentiation of regulatory T-cell [53] 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 tumour-suppressing pathways will inhibit tumour 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.

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 faecal 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 [6,21,34,40]. Furthermore, we identified key species that have 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 fulfil a crucial role in colonic health. In particular, butyrate is essential for gut barrier integrity, since it serves as energy source for colonocytes [54]. Moreover, *in vitro* studies showed that SCFA regulate the expression of tight-junction proteins and mucins [54]. In addition, SCFA have been shown to have potent anti-inflammatory as well as direct anti-carcinogenic effects [54]. 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 fulfil crucial physiological roles. In this context, secondary bile acids, branched chain fatty acids, as well as amino acids are repeatedly suggested as key metabolites [55]. The gut microbiota produces secondary bile acids by converting primary bile acids which were produced in the liver [56.] Mikó et al. (2018) showed that the secondary bile acid lithocholic acid inhibited cancer cell proliferation, tumour infiltration, as well as metastasis formation

and improved the anti-tumour immune response [57]. 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) [58]. 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 signalling pathways through GPCR agonism [58]. Furthermore, microbial metabolites might also play a role in the indirect modulation of drug response [46].

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) [33] 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 chemotherapy toxicity 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 [27], but did not affect microbiota diversity and the abundance of Firmicutes and Bacteroides in another [28]. In this study, only patients who developed colitis showed a reduced diversity and a significant difference in bacteria belonging to the Firmicutes phylum [28]. Vetizou et al. (2015) [42], who used the same patient population as described by Chaput et al. (2017) [28], observed microbiota changes during ipilimumab treatment.

Mechanisms by which immunotherapy influences intestinal microbiota composition are sparsely studied and are mainly based on mouse studies [59]. There are indications that anti-PD-1 therapy stimulates T-cell responses against intestinal bacteria and consequently improves cancer cell surveillance and detection [27,60]. 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 [42].

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 analogues or androgen axis-targeted therapies with ATT or GNRH [32,43]. No explanation is available as to why somatostatin analogues 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 [61]. 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 [12,62,63].

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 [64], 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 [64]. Attention and correction for these confounders was very different between the studies (Table S2.2 and Figure S2.1). 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 [65]. Besides, it has been recently demonstrated that antibiotics modulate gut microbiota composition and metabolite production as well as key metabolic processes and tumour growth [66]. 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 [67]. 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.

Future directions

The rapidly growing number of publications concerning microbiota-based 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:

1. The predictive ability of pre-treatment intestinal microbiota concerning development of complications and response to cancer treatment.
2. 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 faeces, 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 faecal 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 [68].

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.

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Supplemental material

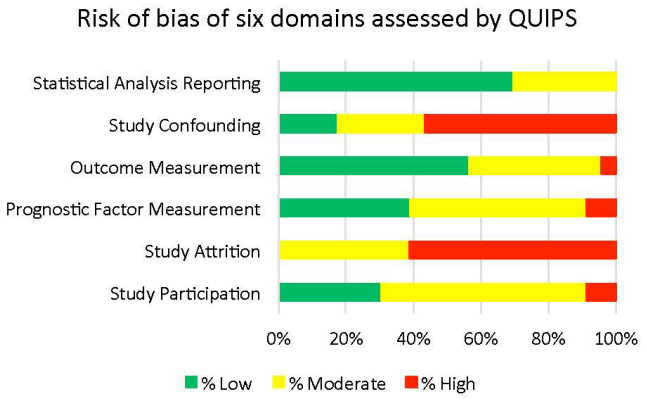
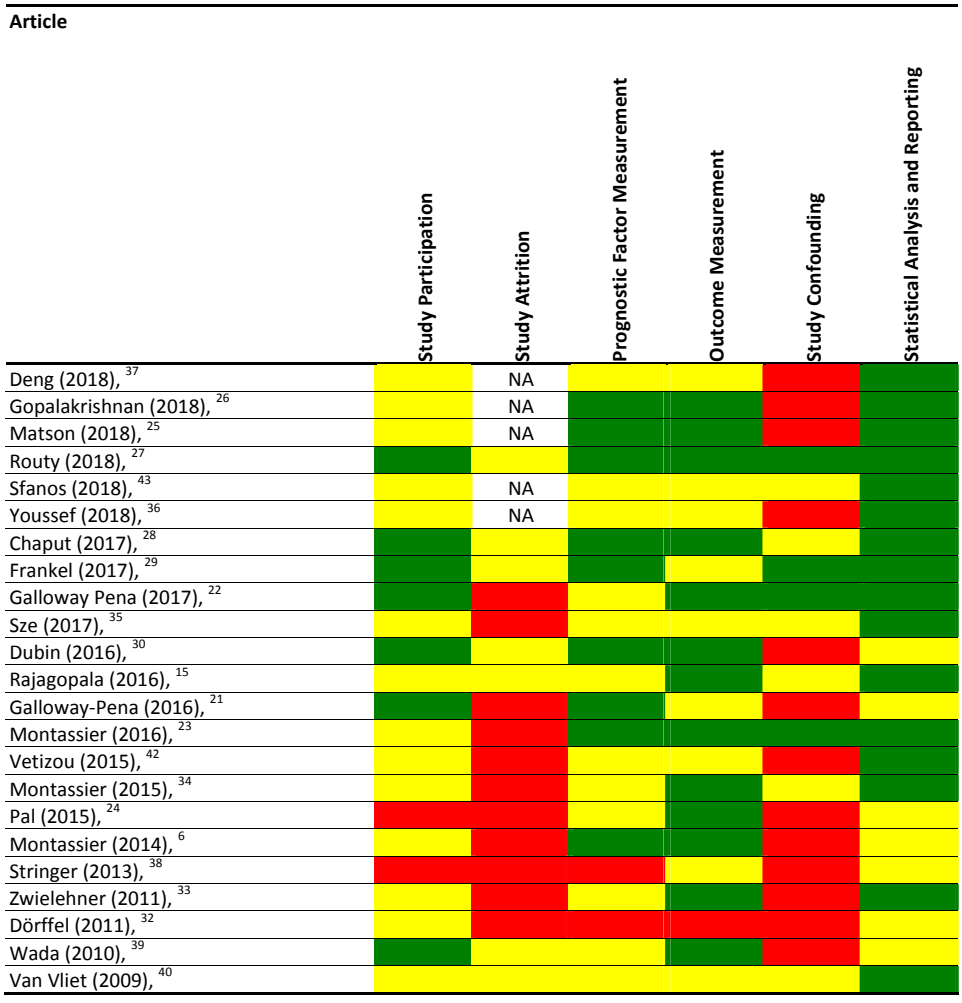


Figure S2.1: Risk of bias of six domains assessed by QUIPS.

Table S2.1: 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

Table S2.2: Risk of bias of included studies assessed by Quality in Prognosis Studies tool (QUIPS).



In red: high risk of bias; in yellow: moderate risk of bias; and in green: low risk of bias. NA: not applicable.

CHAPTER 3

Study protocol on the role of intestinal microbiota in colorectal cancer treatment: a pathway to personalised medicine 2.0

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Abstract

Purpose

Investigate in patients with metastatic and/or unresectable colorectal cancer treated with systemic treatment with capecitabine or trifluridine/tipiracil whether:

1. Intestinal microbiota composition can act as a predictor for response.
2. Intestinal microbiota composition changes during systemic treatment and its relation to chemotherapy toxicity.

Background

Gut microbiota and host determinants evolve in symbiotic and dependent relationships resulting in a personal ecosystem. *In vitro* studies showed prolonged and increased response to 5-fluorouracil, a fluoropyrimidine, in the presence of a favourable microbiota composition. Capecitabine and trifluridine/tipiracil are both fluoropyrimidines used for systemic treatment in colorectal cancer patients.

Methods

An explorative prospective multicentre cohort study in the Maastricht University Medical Centre+ and Zuyderland Medical Centre will be performed in 66 patients. Before, during and after three cycles of systemic treatment with capecitabine or trifluridine/tipiracil, faecal samples and questionnaires (concerning compliance and chemotherapy toxicity) will be collected. Response will be measured by CT/MRI using RECIST-criteria. Faecal microbiota composition will be analysed with 16S rRNA Next Generation Sequencing. Absolute bacterial abundance will be assessed with quantitative polymerase chain reaction. Multivariate analysis will be used for statistical analysis.

Conclusions

We aim to detect a microbiota composition that predicts if patients with metastatic and/or unresectable colorectal cancer will respond to systemic treatment and/or experience zero to limited chemotherapy toxicity. If we are able to identify a favourable microbiota composition, faecal microbiota transplantation might be the low burden alternative to chemotherapy switch in future.

Background

The interest in intestinal microbiota in relation to cancer is rapidly growing. The human gastro-intestinal tract contains over one-kilogram bacteria. The collective genome of all these bacteria is called the microbiome. The intestinal microbiota exerts crucial functions, that humans cannot exert themselves, while human hosts offer a nutrient rich environment. In healthy people a dynamic equilibrium exists. However, environmental factors and host genetic factors all can influence microbiota composition and generate dysbiosis related to carcinogenesis and disturbed metabolism [1-3]. Microbiota differences were reported between healthy people and patients with colorectal and gastric cancer [4,5]. Especially in gastric cancer, the presence of *Helicobacter pylori* is highly associated with cancer development [4]. A recent study showed that the human intestinal microbiota signature is able to predict in vivo treatment response to antibiotics in patients with *Clostridium difficile* infections [6]. Another in vivo study used microbiota composition of the female urogenital tract to predict failure of implementation of an embryo in In Vitro Fertilization (IVF) [7]. The pilot study results of the Erasmus Medical Centre showed that IVF fails with 96% certainty in the presence of an unfavourable microbiota profile [8].

Colorectal cancer (CRC) is the third most common cancer in the world. Standard systemic treatment in CRC in metastatic and/or unresectable setting is mostly based on fluoropyrimidine, such as 5-fluorouracil (5-FU), orally administered capecitabine or trifluridine/tipiracil. Capecitabine is a precursor of 5-FU [9]. Capecitabine is frequently administered in combination with bevacizumab, which is an antibody that binds to vascular endothelial growth factor (VEGF). Bevacizumab inhibits angiogenesis and its related tumour growth. Trifluridine/tipiracil consists of cytotoxin trifluridine and the thymidine phosphorylase inhibitor tipiracil (TPI). Tipiracil prevents rapid metabolism of trifluridine, increasing the bioavailability of trifluridine, which inhibits tumour cell growth [10].

Response to systemic treatment is often defined as complete or partial response, stable or progressive disease. Only 10-15% of the advanced CRC patients actually respond positively to the administration of 5-FU alone. Furthermore, patients treated with the fluoropyrimiden capecitabine frequently experience chemotherapy toxicity with grade 3-4 chemotherapy toxicity diarrhoea of 5%, 2% nausea or vomiting, 4% mucositis, 3% anaemia and 1% hand foot syndrome [9,10]. Response to systemic therapy also depends on patient's dihydropyrimide deficiency (DPD) status [11]. DPD is a molecular determinant of capecitabine efficacy in CRC patients [12]. It is known that patients with DPD experience more chemotoxic effects, since dihydropyrimidine cannot process thymine

and uracil [13]. Therefore, patients DPD status will be standardly determined in an increasing number of hospitals.

There is little evidence in the field of CRC (treatment) and the role of human intestinal microbiota composition. Existing evidence is limited to *in vitro* studies. One *in vitro* study recently indicated a better response of CRC cell lines to 5-FU in the presence of *Lactobacillus plantarum* supernatant [14]. Another *in vitro* study indicated that 5-fluorocytosine (5-FC) (structurally related to 5-FU) samples, incubated with viable *Escherichia coli* showed a higher increase in active 5-FU concentration compared to samples incubated with non-viable *Escherichia coli*. When 5-FC was incubated with human faecal samples a significant degradation reaction of 5-FC was observed when compared to samples that were incubated with human faeces that received an antimicrobial treatment. These *in vitro* studies indicate better responses in the presence of human intestinal bacteria [15,16].

Thus, in the field of CRC treatment, no studies currently bridge the high potential translational gap between previous promising (*in vitro*) studies results and clinic. This study aims to bridge this translational gap in order to explore the importance of the human intestinal microbiota in CRC treatment by initiating an explorative prospective multicentre cohort study in the Maastricht University Medical Centre+ and Zuyderland Medical Centre.

If the human microbiota can predict response to systemic treatment with a non-invasive method, chemotherapy toxicity can be reduced in non-responders and/or systemic therapy regimes can be changed. Further, change of the intestinal microbiota related to response and chemotherapy toxicity can also help to identify with a non-invasive method "the favourable microbiota composition". In case of Inflammatory Bowel Syndrome and *Clostridium difficile* infection faecal microbiota transplantations are already performed. If faecal microbiota transplantations could be applied in order to increase and prolong response to systemic treatment with limited chemotherapy toxicity, wouldn't that be an impressive step forward?

In conclusion, the field of research of the human microbiota is new. By further exploring the intestinal microbiota composition before and after systemic treatment in patients with metastatic and/or unresectable CRC, an area of personalised medicine 2.0 can be created. Resulting in an increased response rate and improved chemotherapy toxicity profiles. In future, new therapeutic pathways could be created by providing faecal microbiota transplantation to replenish, maintain or create an optimal microbiota composition [17].

Methods

Study objectives

Primary objective

The primary object is to investigate whether the microbiota composition can act as a predictor for response and/or chemotherapy toxicity to three cycles of systemic treatment with capecitabine (with or without bevacizumab) or trifluridine/tipiracil in patients with metastatic and/or unresectable CRC.

Secondary objective

The secondary objective of this study is to investigate microbiota composition changes during systemic treatment with capecitabine (with or without bevacizumab) or trifluridine/tipiracil and its relation to response and/or chemotherapy toxicity in patients with metastatic and/or unresectable CRC.

Study design

An explorative multicentre cohort study will be performed in the Maastricht University Medical Centre+ (MUMC+) and Zuyderland Medical Centre.

Population

All patients with metastatic and/or unresectable CRC who will receive three cycles systemic treatment are eligible to participate in this study. Systemic treatment can consist of either oral capecitabine (with or without intravenous bevacizumab) or oral trifluridine/tipiracil. This patient group is selected, since these patients receive only one chemotherapeutic agent, and this will optimize homogeneity. To further optimize homogeneity, patients with Micro Satellite Instability (MSI) will be excluded. Patients visiting the outpatient clinic of the MUMC+ or Zuyderland Medical Centre can be selected for participation.

Inclusion criteria

- Patients diagnosed with metastatic and/or unresectable CRC who will be treated with oral capecitabine (with or without intravenous bevacizumab) or oral trifluridine/tipiracil
- Aged 18 years or older

- Written informed consent

Exclusion criteria

- Micro Satellite instability (MSI)
- Has not received any prior systemic therapy for the treatment of CRC during the previous four weeks prior to start current line of capecitabine or trifluridine/tipiracil
- Patients treated with additional systemic treatments during planned treatment period
- Radiotherapy within two weeks
- Therapeutic antibiotic use within past three months
- Renal function: calculated creatinine clearance (Cockroft – Guilt) < 30 ml/min
- Pregnant or nursing
- Physically or mentally incapable or incompetent

Patient accrual

The medical oncologist will propose the study during a regular hospital visit to each eligible patient. The patient is asked for permission to talk with the investigator directly after their regular appointment. The investigator will then inform him/her about the goal and reason for the study and answer questions. If a patient approves, permission will be asked to use clinical data and collect faecal samples. A patient information letter will be provided. Finally, he or she is asked to sign informed consent. The patient will have minimal three days to decide to participate. Informed consent will be collected (by phone) before or at the start of the first treatment day. Subjects can leave the study at any time for any reason if they wish to do so without any consequences.

Study procedure

Patients follow the regular treatment program initiated by an oncologist of MUMC+ or Zuyderland Medical Centre. Since treatment schedules of capecitabine (with or without bevacizumab) and trifluridine/tipiracil slightly differ, both study procedures will be described in the following section.

Patients that receive capecitabine (with or without bevacizumab)

Before the start of each treatment cycle, the patient visits the hospital for a regular check up and blood samples collection. On day 1-14 capecitabine therapy 1250 mg/m^2 is orally administered twice daily, every three weeks. Each dose capecitabine should be

ingested with water 30 minutes after a light meal. The total daily dose amount is 2500 mg/m^2. Dose reductions are allowed following regular institutional practice. The treating medical oncologist is allowed to give additional treatment of the VEGF-inhibitor bevacizumab 7,5 mg/kg, which will be administered intravenously at the day care clinic on day one of every cycle. During the second week of third treatment cycle, a Computer Tomography (CT) - or Magnetic Resonance Imaging (MRI) - scan will be performed for response monitoring.

In addition to regular treatment the patient is asked to collect a faecal sample and complete a questionnaire before (T1), during (T2) and after (T3) three treatment cycles with systemic treatment. The patient can collect the faecal samples in the hospital or up to 2 days before hospital visit at home. Sample collection can be performed easily and hygienically with the collection device provided in less than 5 minutes. If collected at home, the sample should be stored in the freezer (-20°C) and need to be transported to the hospital with a cool transport container (Sarstedt), that will be distributed to all patients.

During all sample collection moments, patients visit the hospital as outlined in Table 3.1. The second week of the third treatment cycle with capecitabine (with or without bevacizumab) is chosen to collect the faecal sample and questionnaire during treatment (T2), since patients receive one of the last doses of capecitabine during that treatment cycle. It is expected that during these days the highest capecitabine concentration and potential chemotoxic effects will be reached. No additional hospital visit is needed for the collection of the faecal sample of T2, since patients visit the hospital for response evaluation with a CT- or MRI-scan during that week. Figure 3.1 provides a schematic overview of the study procedure.

Table 3.1: Indicates which regular and study related data will be collected during different time points for patients treated with capecitabine (with or without bevacizumab).

	Capecitabine		**Cycle 1**			**Cycle 2**			**Cycle 3**			**Cycle 4**
	Day	<1	1	2 -14	15-21	22	23-35	36-42	43	44-56	57-63	64
Regular treatment	**Hospital visit**	X	X			X			X	X		X
	Bevacizumab		X			X			X			X
	Capecitabine		X	X		X	X		X	X		X
	A + PA	X				X			X			X
	CT/MRI	X								X		
	Laboratory	X				X			X			X
Study related	**Time point**	**T1**								**T2**		**T3**
	Questionnaire	X								X		X
	Faecal sample	X								X		X

A: Anamnesis; PA: Physical Examination; CT: Computer Tomography; MRI: Magnetic Resonance Imaging.

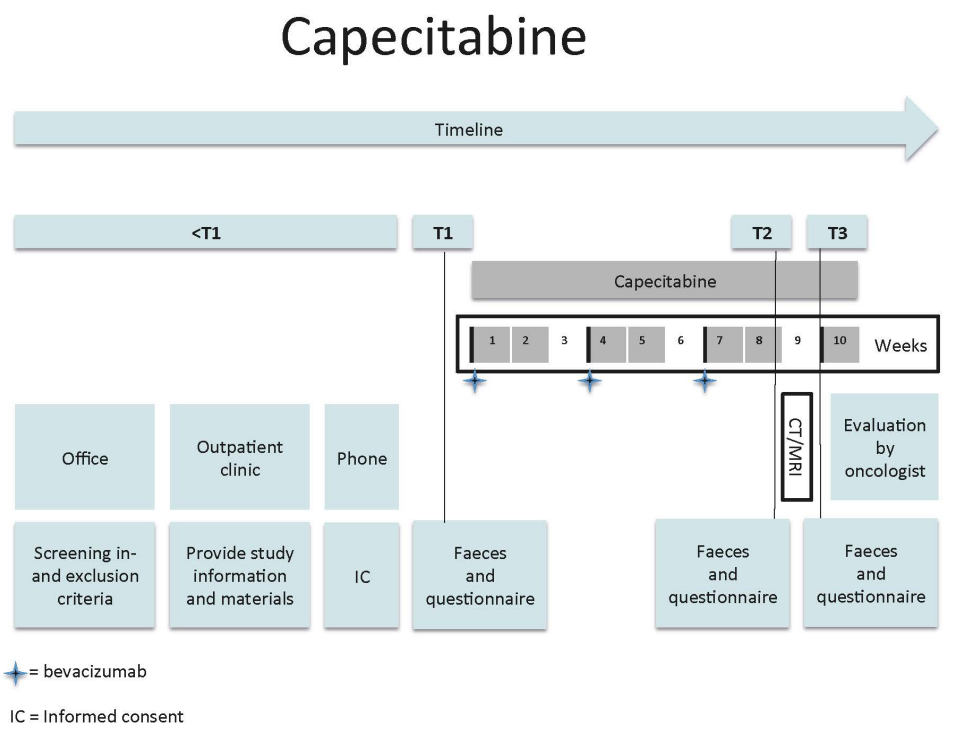

Figure 3.1: Schematic overview of the study procedure for patients treated with capecitabine (with or without bevacizumab).

Patients that receive trifluridine/tipiracil

Before the start of each treatment cycle, the patient visits the hospital for a regular check up and blood samples collection. Trifluridine/tipiracil is orally administered (35 mg/m^2/dose) twice daily on day 1-5 and day 8-12, every four weeks [18]. During the third treatment cycle, the patient visits the hospital to analyse response by a CT- or MRI-scan.

In the same ways as for patients that receive capecitabine, patients will be asked to collect a faecal sample and complete a questionnaire before (T1), during (T2) and after (T3) three treatment cycles with systemic treatment. Sample collection should be performed as described in the capecitabine group.

During sample collection of T1 and T3, patients visit the hospital as outlined in Table 3.2. The second week of the third treatment cycles with trifluridine/tipiracil is chosen to collect faecal sample and questionnaire during treatment (T2), since patients receive one of the last doses of trifluridine/tipiracil during the third treatment cycle. It is

expected that during this days the highest trifluridine/tipiracil concentration and potential chemotoxic effects will be reached. No additional hospital visit is needed since faecal sample of T2 can be collected at home and stored in the freezer for one week. The patients can bring both faecal sample and the questionnaire to the hospital during their next hospital visit, which will be approximately one week later for response evaluation with a CT- or MRI-scan. Figure 3.2 provides a schematic overview of the study procedure.

In general, there will be no risk for the patient, since no intervention or treatment will be initiated. Patients will follow regular treatment program initiated by their oncologist. There will be almost no burden for participating patients in this study. The faecal samples and questionnaires could be collected at home or during hospital visits. Faecal sample collection is very easy with the provided collection device and can be performed hygienically in less than 5 minutes.

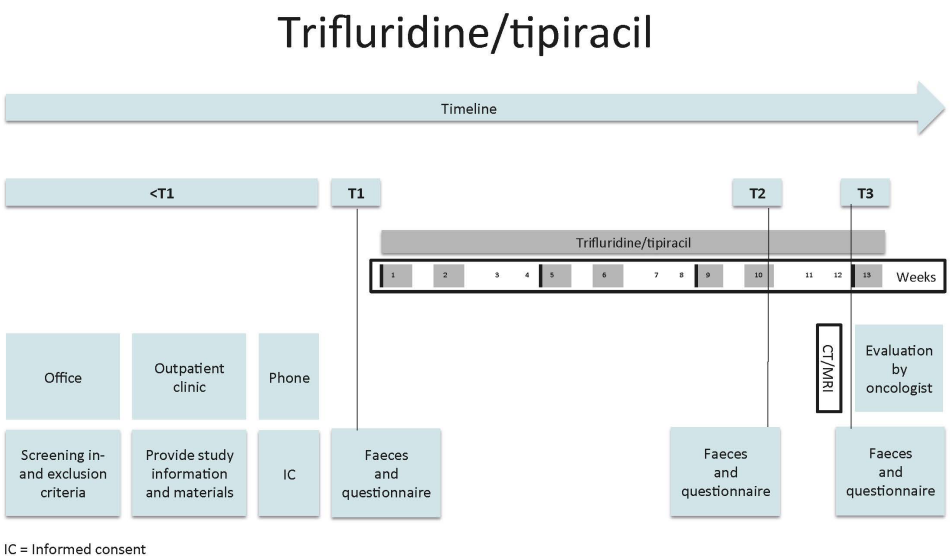

Figure 3.2: Schematic overview of the study procedure for patients treated with trifluridine/tipiracil.

Table 3.2: Indicates which regular and study related data will be collected during different time points for patient treated with trifluridine/tipiracil.

	Trifluridine/tipiracil		**Cycle 1**					**Cycle 2**					**Cycle 3**					**Cycle 4**
	Day	<1	1-5	6-7	8-12	13-14	15-28	29-33	34-35	36-40	41-42	43-56	57-61	62-63	64-68	69-70	71-84	85
Regular treatment	**Hospital visit**	X	X					X					X				X	X
	Trifluridine/tipiracil		X		X			X		X			X		X			X
	A + PA	X						X					X					X
	CT/MRI	X															X	
	Laboratory	X						X					X					X
Study related	**Time point**	**T1**													**T2**			**T3**
	Questionnaire	X													X			X
	Faecal sample	X													X			X

A: Anamnesis; PA: Physical Examination; CT: Computer Tomography; MRI: Magnetic Resonance Imaging.

Data collection and sample handling

After the patient has given informed consent, faecal samples, questionnaires and data from patients medical record, including chemotherapy dose reduction, will be collected. All data, except faecal samples, will be collected in the Case Report Form (CRF). Personal data will be handled with strict care securing the patients privacy. Results will only be used for research purposes, not for clinical purposes. All patients CRF's and their faecal samples receive a study code. All coded data will be entered into a secured database and statistically analysed. The key linking study code to patient data (hospital ID and name) will only be accessible to the investigator. Conform current guidelines, all CRF's, faecal samples and questionnaires will be stored for a maximum of 15 years for any future studies in line with current study.

Patients inclusion number:

ID_MB_CRC_001 to ID_MB_CRC_066

Coding of faecal sample collection collected on T1, T2 and T3:

T1_FS_MB_CRC_001 to T1_FS_MB_CRC_066
T2_FS_MB_CRC_001 to T2_FS_MB_CRC_066
T3_FS_MB_CRC_001 to T3_FS_MB_CRC_066

Once faecal samples arrive in the hospital, samples will be coded and stored at -20°C before being transported in bulk badges to the Biobank of Maastricht for long time storage at -80°C. The intestinal microbiota composition and abundance will be analysed with 16S rRNA Next Generation Sequencing. Subsequent quantitative polymerase chain reaction (qPCR) will be conducted to convert relative abundance to absolute abundance.

Response to systemic treatment will be measured with CT or MRI: Tumour response is classified by Response Evaluation Criteria in Solid Tumours (RECIST) version 1.1 published in January 2009. The following categories are used [19]:

- CR (complete response) = disappearance of all target lesions
- PR (partial response) = 30% decrease in the sum of the longest diameter of target lesions
- PD (progressive disease) = 20% increase in the sum of the longest diameter of target lesions
- SD (stable disease) = small changes that do not meet above criteria

Chemotherapy toxicity will be scored with the Common Terminology Criteria for Adverse events (CTCAE) version 4.03 [20]. The following criteria will be scored: nausea, vomiting, oral mucositis, diarrhoea, constipation, fever, (febrile) neutropenia, peripheral sensory neuropathy, hand-foot syndrome, fatigue, alopecia.
Severity will be graded from 1-5. A Dutch translated version will be used in our study. These data will be obtained from the questionnaires and medical records. Patient compliance to oral chemotherapy will be registered.

Sample size calculation

Since the relation of the microbiota composition with response prediction to systemic treatment in vivo has not been studied before, a power analysis for sample size calculation is not possible. This explorative study will provide much needed data to explore the differences in microbiota composition between patients that will respond and will not respond to systemic treatment with capecitabine (with or without bevacizumab) or trifluridine/tipiracil. Previous studies on microbiota in other fields (obesity and infections) showed that sample size between 12-88 patients is sufficient for this kind of explorative studies [2,6,21]. In this study 60 patients are needed for data analysis. Considering 10% drop out, we need to include 66 patients. In MUMC+ 30 patients will meet our in- and exclusion criteria each year. In Zuyderland Medical Centre this will be 40 patients each year. Considering 50% willing to participate, 66 patients can be included within 2 years.

Data analysis

Microbial analysis of the faecal samples will be achieved by Next Generation Sequencing using the MiSeq platform. Metagenomic DNA from faecal samples will be isolated using a combination of repeated bead-beating and column-based purification in accordance with the recommendations of the International Human Microbiome Standards consortium [22]. The V3-V4 hypervariable regions of the 16S rRNA gene will be amplified using bar-coded fusion primers and sequenced using MiSeq 300 PE sequencing (~25,000 reads/sample). This approach has been proven a powerful tool to provide a complete picture of the diversity and relative abundance of complex microbial communities. Subsequent quantitative polymerase chain reaction (qPCR) will be conducted to convert relative abundance to absolute abundance. Although the current project focuses on the taxonomic microbial profiles, samples are being properly stored to enable future (functional) metagenomic analyses.

Statistics

For bioinformatic analysis of MiSeq-data, the expandable software package QIIME will be used [23]. QIIME integrates many third party tools that have become standard in the field of microbial community analysis (such as tools for chimera checking, denoising, clustering, aligning, classifying, phylogeny reconstruction and calculation of diversity measures). After quality filtering and chimera checking, reads are clustered into Operational Taxonomic Units (OTUs) against the Greengenes reference database [24]. For all subsequent analyses, we will normalize the count-table of OTUs using variant stabilization by the R-package DESeq2 [25] to account for differences in sequencing depth between the samples.

Faecal samples will be analysed by taxonomic composition and alpha- and beta-diversity indices will be calculated. Gut microbiota analysis will include alpha-diversity analysis of OTU richness and evenness within each sample, and beta-diversity analysis between samples. Differential abundance of each OTU between responders and non responders and patient that experience chemotherapy toxicity or not in relation to microbiota changes will be tested using DESeq2. Results will be reported as log2 fold changes and associated adjusted p-values.

For visualization and exploration of these complex data sets, cluster analysis and ordination (e.g. Principal Coordinate Analysis) will be used. To identify the main variables that affect the bacterial communities, constrained analysis such as distance-based Redundancy Analysis (db-RDA) will be applied.

To further test the potential clinical relevance of bacterial communities and clinical factors in CRC, we will carry out a Random Forest analysis combining the OTU abundances and clinical data. Random forests represent a method to correlate metadata with a set of features (OTUSs/ clinical factors) and are an effective approach for analysing and interpreting high-dimensional data. Hereto, we will use the R package randomForestSRC [26] and the Boruta algorithm for feature selection. The bootstrapped feature selection will be repeated 1000 times with differing random seeds.

Final data analysis will compare microbiota composition before, during and after three treatment cycles with systemic therapy between responders and non responders measured with CT/MRI and compare microbiota composition between patients that experience chemotoxic effects and those who have limited chemotoxic effects measured with CTCAE criteria. A prediction model will be developed to predict systemic treatment response and/or chemotherapy toxicity based on patients intestinal microbiota composition. The prediction model will be developed by selecting bacteria and their abundance associated with response and/or chemotherapy therapy toxicity.

Future

In case of metastatic and/or unresectable colorectal cancer, time and quality of life are the most important issues. To limit chemotherapy toxicity and prolong and increase effects of systemic treatment, a great need exists to explore the role of the intestinal microbiota. *In vitro* studies already suggest that intestinal microbiota plays a putative role in colorectal cancer treatment. By exploring microbiota composition and changes in relation to response and chemotherapy toxicity the favourable microbiota composition can be detected. In the future, low burden faecal microbiota transplantation might result in more time and improved quality of life for cancer patients.

Trial status

The national Dutch Research Ethics Committee of Maastricht University Medical Centre+ approved the study protocol in December 2016. Patient inclusion started in March 2017. The total duration of the project will be approximately two years.

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CHAPTER 4

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

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Abstract

Background

Previous pre-clinical research has indicated that the intestinal microbiota can potentiate anti-tumour 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 multicentre cohort study. Patients collected a faecal sample and completed a questionnaire before, during, and after three cycles of capecitabine. Several clinical characteristics, including tumour response, toxicity and antibiotic use were recorded. Intestinal microbiota were analysed 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 faecal 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 effect of capecitabine on the intestinal microbiota and vice versa. Intestinal microbiota composition and diversity before, during, and after three cycles of capecitabine were not associated with response. 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 tumour 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 optimise 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 sensitises CRC cell lines to 5-FU and stimulates apoptosis in chemo-resistant cells [8]. Administration of an antibiotic cocktail (vancomycin, ampicillin, neomycin, and metronidazol) reduced antitumour efficacy of 5-FU in mice [9]. Very recently, Zimmermann et al. provided the first *in vitro* evidence that capecitabine can be metabolised 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 hypothesised 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/m^2 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

Patients collected faecal samples and completed questionnaires at three time points: before the start of the first capecitabine cycle (T1), between days 7 and 14 of the third cycle (T2), and at day 20 or 21 of the third cycle (T3) (Figure S4.1). After collection, samples were immediately stored in the freezer 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

Tumour 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 Tumours) version 1.1 [14]. Response was defined as complete response (CR): disappearance of all target lesions and partial response (PR): ≥30% decrease in the sum of 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 [14].
Toxicity was scored with CTCAE version 4.0 [15]. The following aspects were scored: diarrhea with or without colostomy, peripheral sensory neuropathy, hand-foot syndrome, fatigue, nausea, oral mucositis, vomiting, and constipation.

Faecal microbiota analyses

Metagenomic DNA was isolated using the Ambion MagMax™ 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, amplicons were sequenced on a MiSeq platform, as described previously [16].
Pre-processing of the sequencing data was performed using R. A standardised in-house pipeline using the software package DADA2 (R version 4.0.3) was applied [17]. After pre-processing, 908 taxa remained for downstrean analysis. For further details on DNA isolation, sequencing, and data pre-processing see the supplementary methods.

Statistical analysis of clinical data

Baseline characteristics were analysed 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 [18]. For the calculation of α-diversity indices on Amplicon Sequence Variant (ASV) level (Shannon effective and observed richness) and prior data normalisation, the standard script and settings of the Rhea pipeline were used [19]. 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), generalised UniFrac and Bray-Curtis distances were calculated on ASV level, using Rhea [19] and the R packages GUniFrac [20] and phyloseq [21] 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 [21], vegan [22], microbiome [23], dplyr [24], ggplot2 [25], and microViz [26] were used for ordination and visualisation 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 [27]. 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 4.1 and S4.1). 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 faecal samples were collected. Figure S4.2 provides an overview of all samples available for 16S rRNA gene sequencing.

Baseline characteristics

Median age was 75 years. Mean BMI was 27 kg/m^2. 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 tumour. In total 88% underwent resection of the primary tumour (Table 4.1). 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 tumour, 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 faecal 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 faecal 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 4.1 and S4.1).

Table 4.1: Clinical characteristics.

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

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

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 S4.2). 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 S4.3-S4.5). Toxicity grades of peripheral sensory neuropathy, hand foot syndrome, oral mucositis, and bone marrow toxicity increased significantly over the study period (Figure 4.1, Table S4.6 and S4.7). All other toxicity

measures, including diarrhea, did not change during three cycles of capacitabine (Table S4.6).

Compared to baseline, KPS was significantly lower after three cycles of capecitabine (p=0.002) (Table S4.8). The MUST score was not significantly different before, during or after three cycles of capecitabine (Table S4.8).

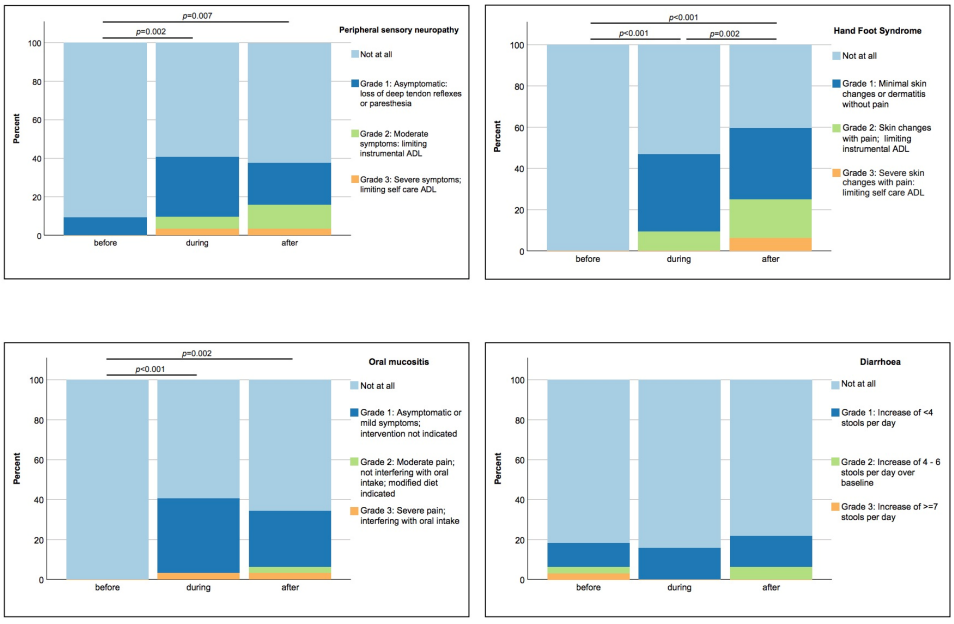

Figure 4.1: Stacked bar charts presenting percentage toxicity grades before, during, and after three cycles of capecitabine. For peripheral sensory neuropathy and oral mucositis significant differences were observed between T1-T2 and T1-T3. For hand foot syndrome significant differences were observed between all time points.

Intestinal microbiota composition and diversity

Similar α-diversity in responders and non-responders

Before (Figure S4.3 and Table S4.9) and during (Figure 4.2A and Table S4.10) 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 4.2B and Table S4.11).

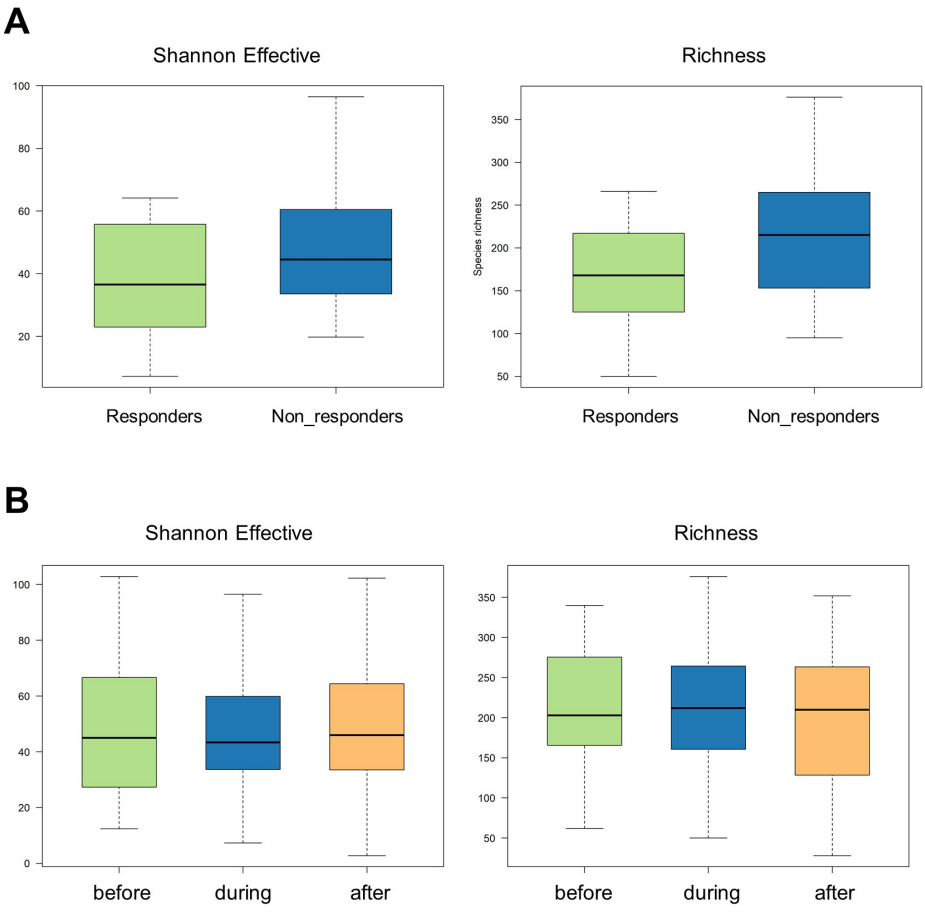

Figure 4.2: α-diversity measures. **A:** Microbial diversity and richness of responders and non-responders at T2, measured in terms of Shannon effective (p=0.301) and observed richness (p=0.145) (Table S4.10). **B:** α-diversity before, during, and after three cycles of capecitabine, measured in terms of Shannon effective (p=0.640) and observed richness (p=0.240) (Table S4.11). 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 4.3). However, on phylum level, responders tended to cluster in the direction of Proteobacteria and

Actinobacteria (Figure 4.3A). 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 4.3A). On genus level, Lachnospiraceae ND3007 group, *Dialister, Veillonella, Anerostipes,* and *Flavonifractor* contributed the most to the variation in the overall microbiota community structure (Figure 4.3B).

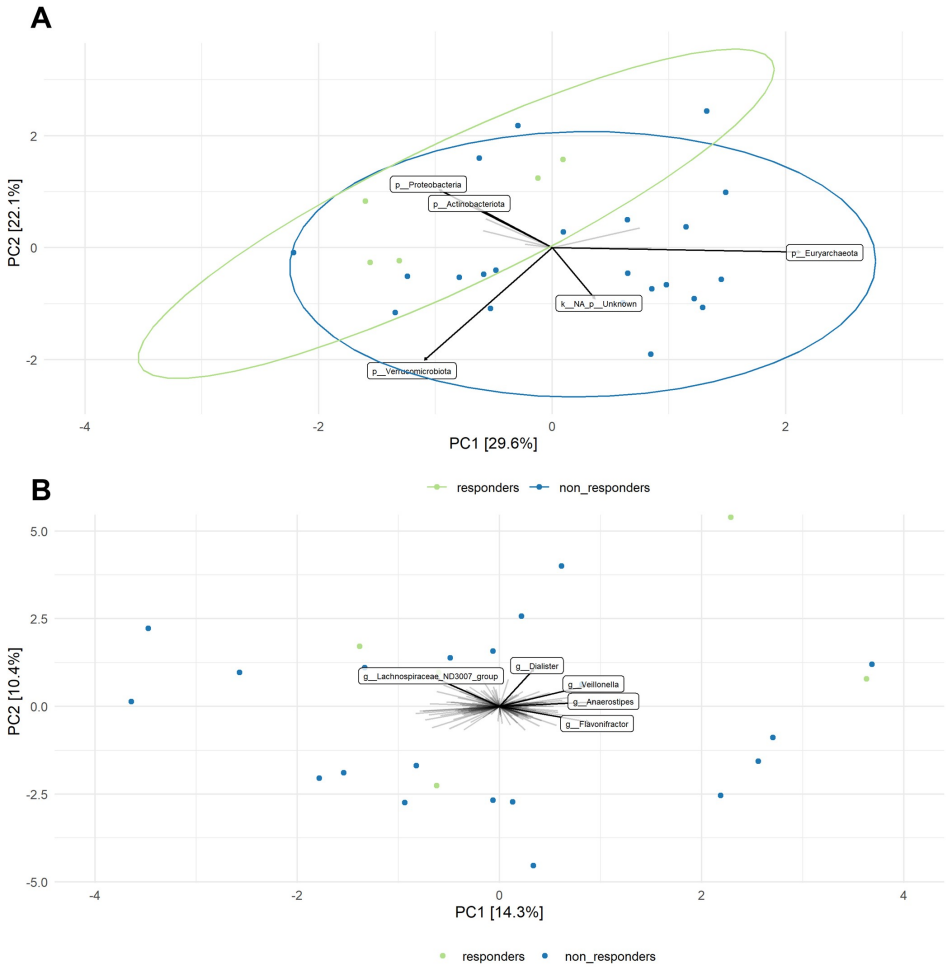

Figure 4.3: Ordination plots derived from unconstrained Principal Components Analysis (PCA), showing overall composition of the microbial community on phylum (A) and genus level (B) at T2. Aitchison distance was used. 10 phyla and 150 genera were included for this analysis. Data were transformed using centre-log-ratio transformation. Names are given for genera which contributed most to overall microbial variation.

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 S4.4). 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 generalised UniFrac as well as Bray-Curtis distances. P08 showed considerably large instability between T2-T3 and T1-T3 (Figure S4.5 and Table S4.12).
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 4.4A). This was confirmed by PERMANOVA, which revealed no association between sampling time point and microbial community structure on phylum (*p*=0.96) level.
Figure 4.4B 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 4.4B), 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 faecal sample and showed relatively high levels of *Bacteroides* and *Streptococcus* in this sample. P01 received oral ciprofloxacin 24 days before the second faecal sample collection, resulting in relatively high levels of *Streptococcus.*

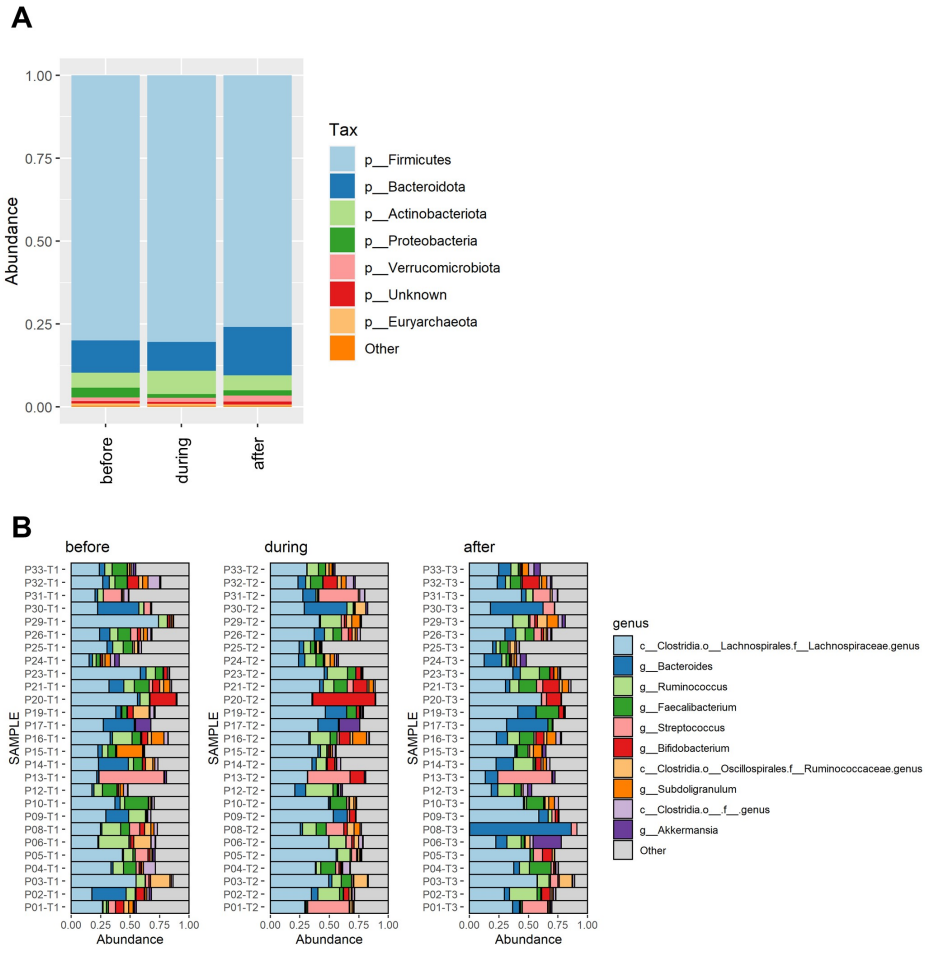

Figure 4.4: **A:** Composition plot at phylum level, before, during, and after three cycles of capecitabine, indicating relative abundance of the most common phyla **B:** Changes in relative abundance of the most common genera before, during, and after three cycles of capecitabine indicate a large inter-individual 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 effect of capecitabine (without surgery, radiation or chemotherapy or combinations thereof) on the intestinal microbiota composition and

diversity and vice versa. Intestinal microbiota composition and diversity before, during or after three cycles of capecitabine were not associated with treatment response. 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.

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 more detail, 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 hospitalisation and lifestyle changes (desirable and undesirable) [28]. 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 faecal 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. [29] 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 [29]. 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) [30]. Recently, Zimmermann et al. provided *in vitro* evidence that capecitabine can be metabolised 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 [31]. These individual changes indicate that the impact of antibiotics is substantial compared to the impact of the relatively mild chemotherapeutic capecitabine [2-4]. The faecal sample of P20 contained a relatively high relative abundance of Bifidobacteria. Surprisingly, this patient also showed the highest tumour 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 [32]. These observations of individual patients 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.

In general, our study was limited by an unequal group size 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 [28,33]. Furthermore, it is known that capecitabine is converted in tumour tissue to its cytotoxic moiety 5-FU. The lack of a direct cytotoxic effect of capecitabine in the intestines might have contributed to the lack of association between capecitabine treatment and microbiota modulation [34].

Before proceeding to clinical interventions studies with pre- and/or probiotics or even faecal microbiota transplantation (FMT) in this population 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. 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. High inter- and intra-individual microbiota variations were observed during capecitabine treatment. This is most likely due to an extensive medical history in this patient group. This highly variable microbiota composition and diversity is a great challenge for the application of personalised medicine and microbiota-based therapies. 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 faecal microbiota transplantations.

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Supplemental material and methods

Faecal microbiota analyses

In order to extract metagenomic DNA, 250 mg of the frozen faecal samples were homogenised 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 MagMax™ Total Nucleic Acid Isolation Kit *(Thermo Fisher Scientific)* and mixed with 175 µl supernatant of faeces 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 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 [1]. 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) [2].

The pre-processing of sequencing data, using an in-house pipeline based upon DADA2 (R version 4.0.3) [3], consisted of the following steps: reads filtering, identification of sequencing errors, dereplication, and removal of chimeric sequences. In order to assign taxonomy, DECIPHER [4] 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 indentification of contamination in marke-gene and metagenomics data [5]. 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 [6].

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Supplemental figures

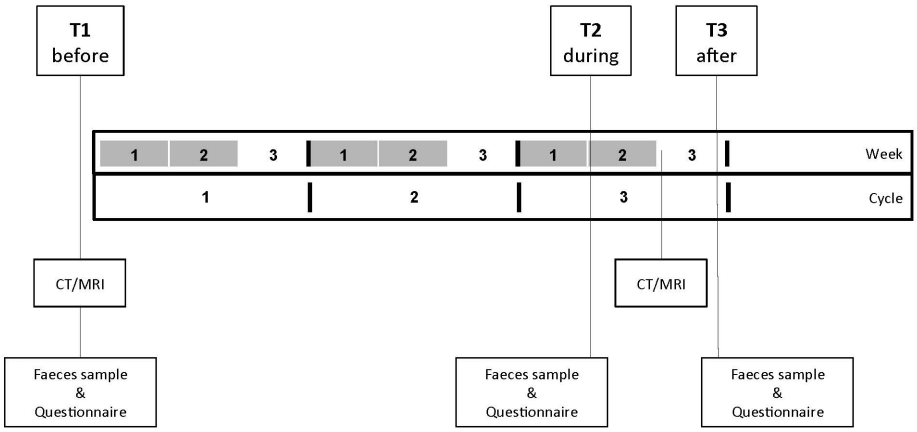

Figure S4.1: Study design.
Patients collected faecal samples and completed questionnaires at three time points. **T1** is collected before the start of the first capecitabine cycle, **T2** is collected between day 7-14 of the third cycle, and **T3** is collected at day 20 or 21 of the third cycle. Tumour 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.

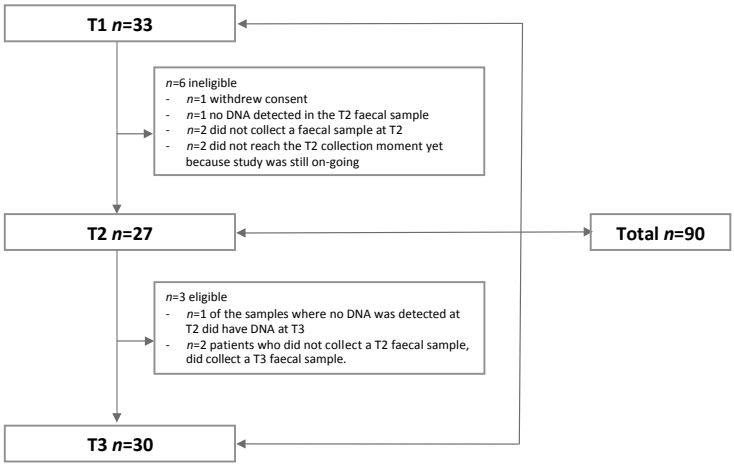

Figure S4.2: Overview of faecal samples used for 16S rRNA gene sequencing of the V4 hypervariable region.

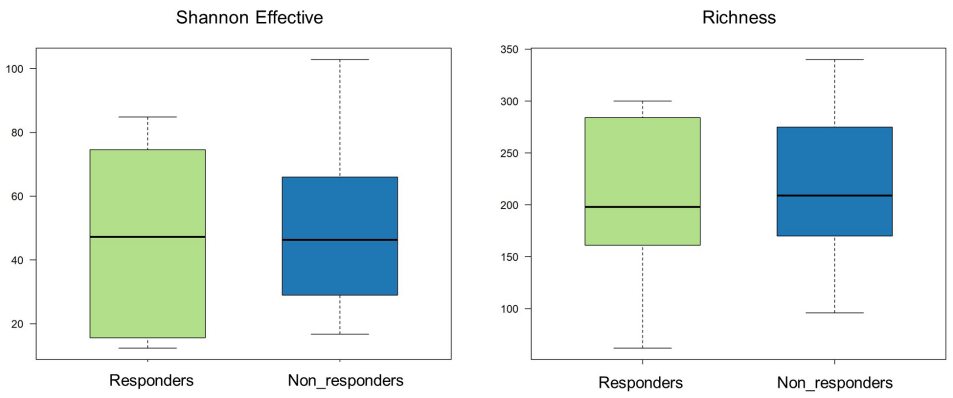

Figure S4.3: α-diversity measures.
Microbial diversity and richness of responders and non-responders at T1, measured in terms of Shannon effective (p=0.786) and observed richness (p=0.528) (Table S4.9). Numbers presented in median (IQR).

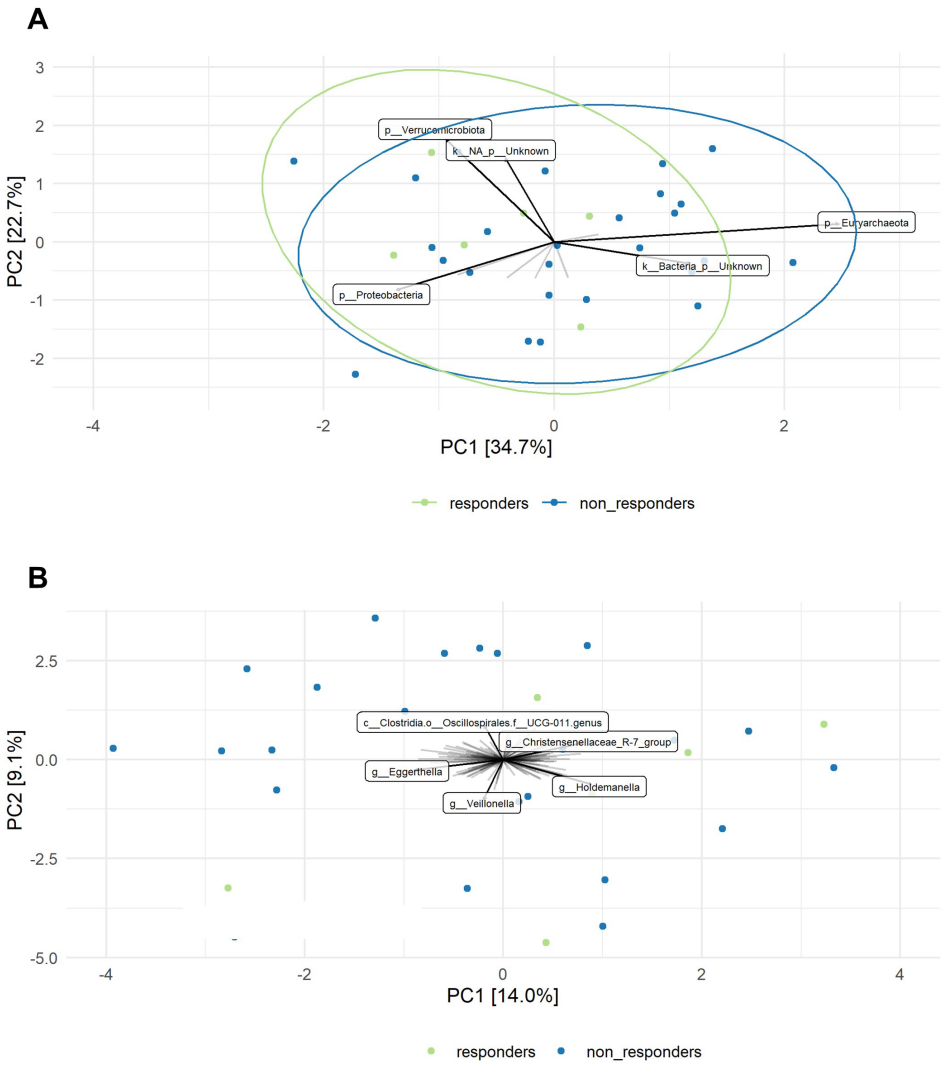

Figure S4.4: Ordination plots derived from unconstrained Principal Components Analysis (PCA), showing overall composition of the microbial community on phylum (A) and genus level (B) at T1. Aitchison distance was used. 10 phyla and 156 genera were included for this analysis. Data were transformed using centre-log-ratio transformation. Names are given for genera which contributed most to overall microbial variation.

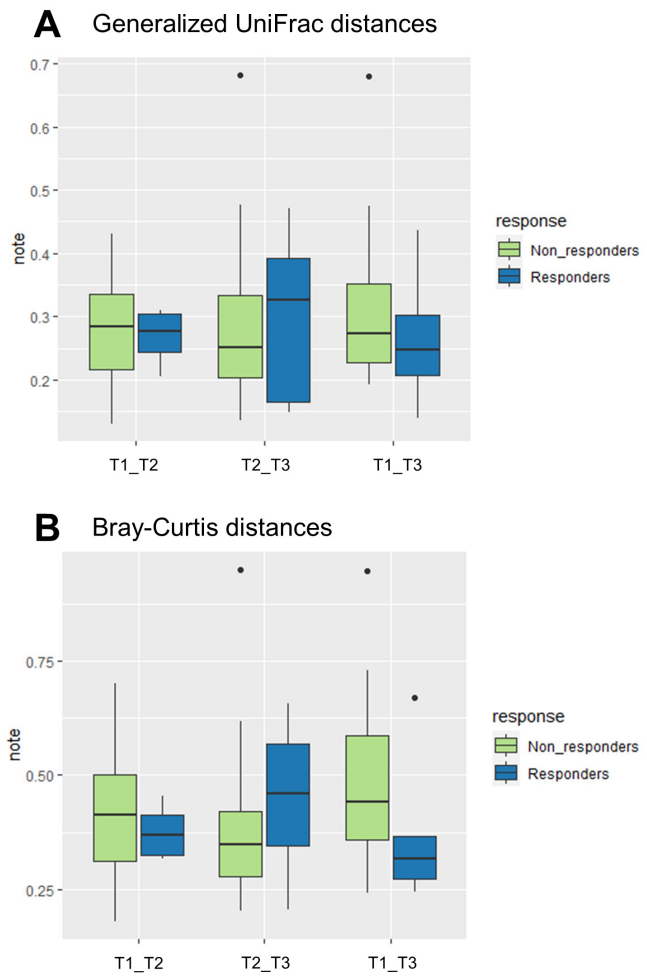

Figure S4.5: Temporal (in)stability in microbial communicty structure (β-diversity). Changes of generalized UniFrac distances (A) and Bray-Curtis distances (B) between T1 versus T2, T2 versus T3 and T1 versus T3. At all time points, distances were not significantly different between responders and non-responders.

Supplemental tables

Table S4.1: Clinical characteristics.

Baseline characteristics	Total	Responders	Non-responders	*p*-value
Total no. of weeks previous systemic treatment				0.436
Median (IQR)	0 (24)	13 (31)	0 (24)	
25-75%	0-24	0-31	0-24	
Time between collection faecal sample and last systemic treatment – days				0.446
Median (IQR)	686 (1000)	425 (1452)	705 (869)	
25-75%	261-1261	140-1592	392-1261	
Therapeutic antibiotic use last year – No. (%)	8 (24)	0 (0)	8 (31)	0.296
Time between collection faecal sample and last therapeutic antibiotic treatment – days		NA		NA
Mean (SD)	197 (±101)		197 (±101)	
Range	93-394		93-394	
Prophylactic antibiotics use last year – No. (%)	10 (30)	2 (33)	7 (27)	1.000
Time between collection faecal sample and last prophylactic antibiotic treatment – days				0.083
Mean (SD)	96 (±92)	23 (±24)	91 (±74)	
Range	0-276	6-40	0-171	
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				0.559
Mean (SD)	32 (±17)	26 (±22)	32 (±17)	
Range	2-68	7-50	2-68	
Type of colorectal surgery – No. (%)*				0.316
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)	
Tumour mutation status – No. (%)				0.865
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)	

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

Table S4.2: 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		NA		NA
Median (IQR)	7 (27)		7 (27)	
% Capecitabine administered – *Median (IQR)*				
Cycle 1	94 (19)	86 (23)	95 (17)	0.356
Cycle 2	95 (16)	84 (22)	96 (14)	0.131
Cycle 3	95 (16)	84 (21)	96 (15)	0.119
Compliant at T2	24 (89)	4 (100)	20 (87)	1.000
% Tumour change				**<0.001**
Mean (SD)	-13 (17)	-34 (14)	-8 (13)	
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 S4.3: CTCAE at T1.

Toxicity grade	Total	Responders	Non-responders	*p*-value
Diarrhoea without colostomy – No. (%)				1.000
0	19 (86)	4 (100)	14 (82)	
1	3 (14)	0 (0)	3 (18)	
Diarrhoea with colostomy – No (%)*				0.186
0	7 (70)	1 (50)	6 (75)	
1	1 (10)	0 (0)	1 (13)	
2	1 (10)	0 (0)	1 (13)	
3	1 (10)	1 (50)	0 (0)	
Diarrhoea with or without colostomy – No (%)				0.384
0	27 (82)	5 (83)	21 (81)	
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 (%)				0.476
0	30 (91)	5 (83)	24 (92)	
1	3 (9)	1 (17)	2 (8)	
Hand Foot Syndrome – No. (%)				NA
0	32 (100)	6 (100)	25 (100)	
Fatigue – No (%)*				0.753
0	14 (42)	3 (50)	10 (39)	
1	14 (42)	2 (33)	12 (46)	
2	5 (15)	1 (17)	4 (15)	
Nausea – No (%)				0.226
0	27 (82)	6 (100)	20 (77)	
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 (%)				0.773
0	27 (82)	6 (100)	21 (81)	
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 S4.4: CTCAE at T2.

Toxicity grade	Total	Responders	Non-responders	*p*-value
Diarrhoea without colostomy – No. (%)				1.000
0	17 (94)	3 (100)	14 (93)	
1	1 (6)	0 (0)	1 (7)	
Diarrhoea with colostomy – No (%)				0.133
0	6 (60)	0 (0)	6 (75)	
1	4 (40)	2 (100)	2 (25)	
Diarrhoea 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 (%)*				0.193
0	19 (59)	5 (83)	14 (54)	
1	10 (31)	1 (17)	9 (35)	
2	2 (6)	0 (0)	2 (8)	
3	1 (3)	0 (0)	1 (4)	
Hand Foot Syndrome – No. (%)*				0.800
0	17 (53)	3 (50)	14 (54)	
1	12 (38)	3 (50)	9 (35)	
2	3 (9)	0 (0)	3 (12)	
Fatigue – No (%)*				0.067
0	7 (22)	4 (67)	3 (12)	
1	20 (63)	1 (17)	19 (73)	
2	4 (13)	1 (17)	3 (12)	
3	1 (3)	0 (0)	1 (4)	
Nausea – No (%)				0.637
0	22 (69)	5 (83)	17 (65)	
1	10 (31)	1 (17)	9 (35)	
Oral mucositis – No (%)				0.221
0	19 (59)	5 (83)	14 (54)	
1	12 (38)	1 (17)	11 (42)	
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 (%)				0.773
0	26 (81)	5 (83)	21(81)	
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 S4.5: CTCAE at T3.

Toxicity grade	Total	Responders	Non-responders	*p*-value
Diarrhoea without colostomy – No. (%)				0.442
0	19 (86)	4 (100)	15 (83)	
1	2 (9)	0 (0)	2 (11)	
2	1 (5)	0 (0)	1 (6)	
Diarrhoea with colostomy – No (%)*				1.000
0	6 (60)	1 (50)	5 (63)	
1	3 (30)	1 (50)	2 (25)	
2	1 (10)	0 (0)	1 (13)	
Diarrhoea with or without colostomy – No (%)				0.592
0	25 (78)	5 (83)	20 (77)	
1	5 (16)	1 (17)	4 (15)	
2	2 (6)	0 (0)	2 (8)	
Peripheral Sensory Neuropathy – No (%)*				0.458
0	20 (63)	4 (67)	16 (62)	
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. (%)*				0.784
0	13 (41)	2 (33)	11 (42)	
1	11 (34)	2 (33)	9 (35)	
2	6 (19)	2 (33)	4 (15)	
3	2 (6)	0 (0)	2 (8)	
Fatigue – No (%)*				**0.026**
0	5 (16)	3 (50)	2 (8)	
1	22 (69)	3 (50)	19 (73)	
2	4 (13)	0 (0)	4 (15)	
3	1 (3)	0 (0)	1 (4)	
Nausea – No (%)*				0.271
0	21 (66)	5 (83)	16 (62)	
1	8 (25)	1 (17)	7 (27)	
2	3 (9)	0 (0)	3 (12)	
Oral mucositis – No (%)				0.692
0	21 (66)	4 (67)	17 (65)	
1	9 (28)	2 (33)	7 (27)	
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 (%)				0.716
0	26 (81)	5 (83)	21 (81)	
1	5 (16)	1 (17)	4 (15)	
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 S4.6: Longitudinal CTCAE.

Toxicity grade	T1	T2	T3	*p*-value
Diarrhoea without colostomy – No. (%)*				0.449
0	19 (86)	17 (94)	19 (86)	
1	4 (13)	1 (6)	2 (9)	
2	0 (0)	0 (0)	1 (5)	
Diarrhoea with colostomy – No (%)				0.819
0	7 (70)	6 (60)	6 (60)	
1	1 (10)	4 (40)	3 (30)	
2	1 (10)	0 (0)	1 (10)	
3	1 (10)	0 (0)	0 (0)	
Diarrhoea with or without colostomy – No (%)				0.407
0	27 (82)	27 (84)	25 (78)	
1	4 (12)	5 (16)	5 (16)	
2	1 (3)	0 (0)	2 (6)	
3	1 (3)	0 (0)	0 (0)	
Peripheral Sensory Neuropathy – No (%)*				**0.002**[1]
0	30 (91)	19 (59)	20 (63)	
1	3 (9)	10 (31)	7 (22)	
2	0 (0)	2 (6)	4 (13)	
3	0 (0)	1 (3)	1 (3)	
Hand Foot Syndrome – No. (%)				**<0.001**[2]
0	32 (100)	17 (53)	13 (41)	
1	0 (0)	12 (38)	11 (34)	
2	0 (0)	3 (9)	6 (19)	
3	0 (0)	0 (0)	2 (6)	
Fatigue – No (%)*				0.154
0	14 (42)	7 (22)	5 (16)	
1	14 (42)	20 (63)	22 (69)	
2	5 (15)	4 (13)	4 (13)	
3	0 (0)	1 (3)	1 (3)	
Nausea – No (%)				0.132
0	27 (82)	22 (69)	21 (66)	
1	5 (15)	10 (31)	8 (25)	
2	1 (3)	0 (0)	3 (9)	
Oral mucositis – No (%)				**<0.001**[3]
0	33 (100)	19 (59)	21 (66)	
1	0 (0)	12 (38)	9 (28)	
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 (%)				0.761
0	27 (82)	26 (81)	26 (81)	
1	6 (18)	5 (16)	5 (16)	
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 S4.7: Bone marrow toxicity.

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

Table S4.8: Longitudinal data.

	T1	T2	T3	*p*-value
MUST score – No (%)*				0.554
Low risk	26 (79)	25 (83)	29 (94)	
Medium risk	4 (12)	4 (13)	0 (0)	
High risk	3 (9)	1 (3)	2 (7)	
Karnofsky Performance Score – No (%)*				**0.013****
Median (IQR)	90 (20)	80 (20)	80 (23)	
50	1 (3)	1 (3)	1 (3)	
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 S4.9: 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 S4.10: 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 S4.11: 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 S4.12: 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.

CHAPTER 5

Changes in intestinal microbiota in oestrogen receptor positive breast cancer patients treated with (neo)adjuvant chemotherapy

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Abstract

Background

Previous pre-clinical research has indicated that the intestinal microbiota can potentiate anti-tumour efficacy of chemotherapy and that chemotherapy can affect intestinal microbiota. Using a longitudinal design, this clinical study explored the associations between the intestinal microbiota, chemotherapy toxicity, and treatment response in breast cancer patients.

Patients and methods

Oestrogen receptor positive postmenopausal breast cancer patients treated with 4 cycles of (neo)adjuvant adriamycin, cyclophosphamide (AC) followed by 4 cycles of docetaxel (D) were prospectively enrolled in a multicentre cohort study. Patients collected a faecal sample and completed a questionnaire before, during AC, during D, and after completing AC-D. Chemotherapy toxicity and tumour response were determined. Intestinal microbiota was analysed by amplicon sequencing of the 16S rRNA V4 gene-region.

Results

In total, 44 patients were included and 153 faecal samples were collected before AC-D (*n*=44), during AC (*n*=43), during D (*n*=29), and after AC-D treatment (*n*=37), resulting in 28 paired samples. Observed species richness significantly 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 diarrhoea during docetaxel treatment had a significantly lower observed species richness compared to patients without diarrhoea. Pathologic response in neoadjuvant treated patients was not related to baseline intestinal microbiota richness, diversity and composition.

Conclusions

While the baseline microbiota is not predictive for pathological response in neoadjuvant treated patients, AC-D treatment did impact subsequent shifts in microbial richness as well as the abundance of specific bacterial taxa.

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 oestrogen receptor positive breast cancer patients. Besides reducing tumour load in the neoadjuvant setting and improving disease free- and overall survival, AC-D treatment may induce toxicity, which impacts quality of life and may require dose reductions. Most common non-haematological 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 diarrhoea and peripheral sensory neuropathy [2-4].

In order to reduce toxicity and optimize treatment outcome, factors need to be identified which impact 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 harbours trillions of intestinal microorganisms, consisting of bacteria, archaea, fungi, protozoa, and viruses. It is well-established that a crosstalk exists 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 breast cancer models, general *in vitro* and mouse studies indicate that significant interactions between the intestinal microbiota and cyclophosphamide, adriamycin, and docetaxel occur [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 increased 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. 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 the potential effect of in vivo transformation to more toxic compounds into account [10].

Despite the availability of the previously described pre-clinical evidence, no clinical studies with longitudinal microbiota sampling are available that explored the interaction between AC-D and the intestinal microbiota regarding chemotherapy toxicity and tumour response in breast cancer patients [17]. We hypothesize that intestinal microbiota changed during AC-D treatment, as well as that intestinal microbiota are associated with chemotherapy toxicity and tumour response in patients with breast cancer.

Patient, materials and 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 oestrogen receptor positive (ER+) and human epidermal growth factor receptor-2 negative (HER2-) breast cancer 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 is registered in the Dutch Trial Register (NTR-6296) and 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 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.

Faecal sample and data collection

Patients collected a faecal 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) (Figure S5.1). Samples were immediately stored in the freezer and transported to the hospital in a cooled container (Sarstedt). 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 therapeutic antibiotic treatment up to one year until three months prior to T0 faecal sample collection. Prophylactic cefazolin administration at the start of the operation and prophylactic amoxicillin/clavulanic acid administration after the operation were summarized as perioperative prophylactic antibiotic use. Nutritional status was assessed with the Malnutrition Universal Screening Tool (MUST).

Response and chemotherapy toxicity measurement

In neoadjuvant patients, pathologic tumour 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 [18]. Toxicity was scored with Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 [19] (Supplementary methods).

Faecal microbiota analyses

Metagenomic DNA was isolated using the Ambion MagMax™ Total Nucleic Acid Isolation Kit (*Thermo Fisher Scientific*) and consisted of a manual pre-processing procedure 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 previously described [20].

Bioinformatic analysis of the sequencing data was performed using R studio. For the pre-processing, a standardized in-house pipeline using the software package DADA2 (R version 4.0.3) was applied [21]. Contaminated Amplicon Sequence Variants (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%. After filtering, 816 taxa

remained in the analysis. For further details on DNA isolation, sequencing and data pre-processing, see supplementary methods.

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 analysed 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 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

Bioinformatic analysis of the sequencing data was performed using R Studio (R version 4.0.0) [22]. 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 [23]. 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 [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 all analyses. Unconstrained ordination was performed using Principal Component Analysis (PCA) based on Aitchison distances at genus and phylum level [28]. Permutational multivariate analysis of variance

(PERMANOVA) was used to analyse longitudinal changes in overall microbiota composition (based on Aitchison distances) and to assess the association between diarrhoea, nausea, oral mucositis, hand-foot-syndrome, and peripheral sensory neuropathy with overall microbiota composition. Within the neoadjuvant subgroup, PERMANOVA was used to analyse the association between treatment response and overall microbiota composition [28]. 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 which accounts for the underlying compositional structure and sparseness of microbiota data [29]. We set $\alpha<0.05$ at 70% (W) of comparisons as threshold for significance. For the purpose of visualisation, bacterial relative abundance were transformed into $\log^{10}(1+x)$ abundance by means of the microbiome package [25].

Results

In total, 44 patients were included (Figure 5.1). 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.

In the year prior to inclusion, 27% of the patients used therapeutic antibiotics with a median use of 7 days. None of the patients used therapeutic antibiotics within three months prior to inclusion. The mean time between the last therapeutic antibiotic dosage and baseline faecal 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 faecal 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 (Table 5.1 and Table S5.1).

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 ($p=0.338$) (Table S5.2).

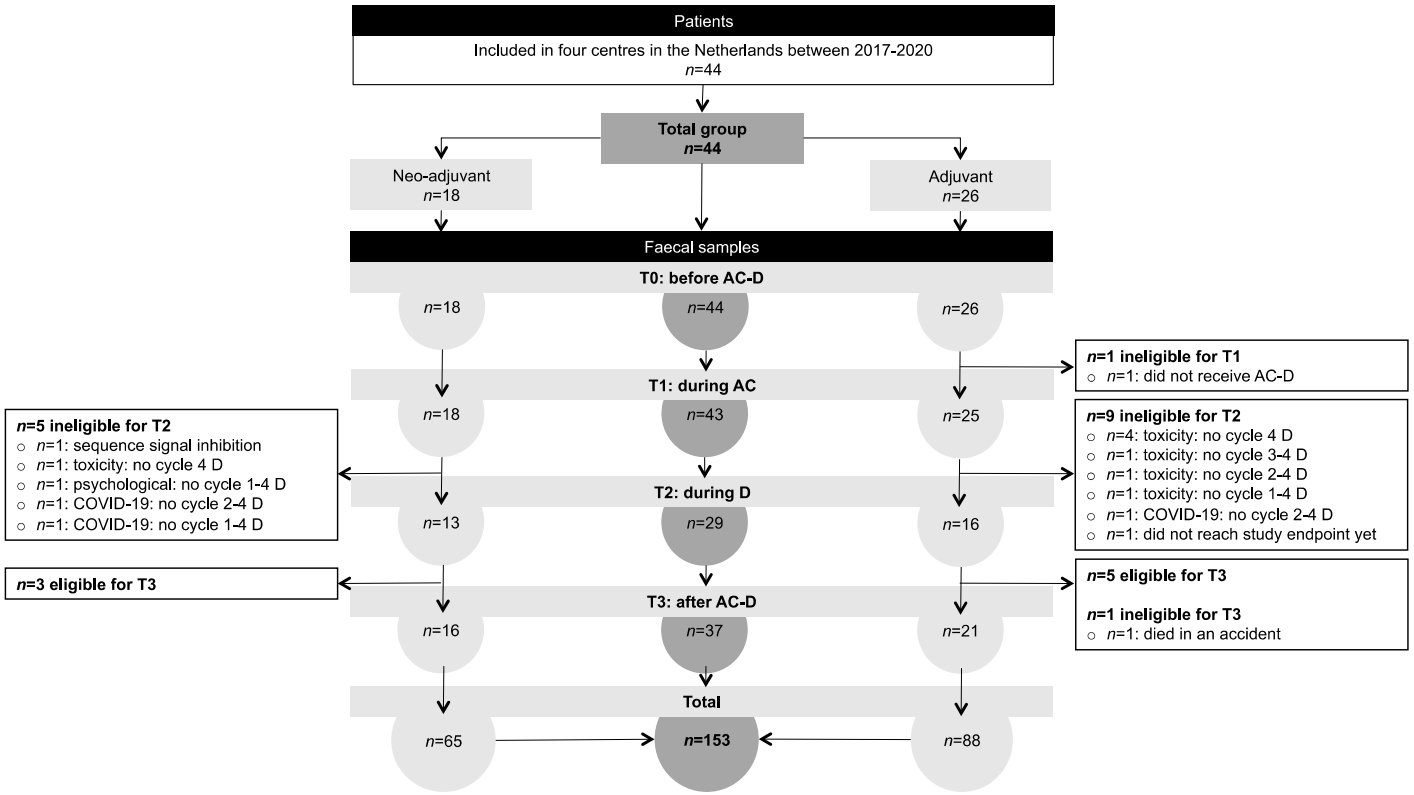

Figure 5.1: The flow chart presents the number of patients included and the number of faecal samples collected by those patients during the study period. Multiple patients who did not collect a faecal sample at T2 were able to collect a faecal sample at T3. In total, 44 patients collected 153 faecal samples at four time points, resulting in 28 paired samples. The total group is presented in the middle. On the left and right side, the total group is subdivided in a neoadjuvant and adjuvant group.

Table 5.1: 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				0.478
Mean (SD)	59 (6)	59 (6)	58 (5)	
BMI - kg/m^2				0.943
Median (IQR)	26 (5)	26 (4)	26 (7)	
Weight loss past 3-6 months - in kg				1.000
<5%	40 (91)	24 (92)	16 (89)	
5-10%	4 (9)	2 (8)	2 (11)	
Clinical tumour stage - No (%)*				0.001
Stage I	17 (40)	15 (58)	2 (12)	
Stage II	23 (54)	11 (42)	12 (71)	
Stage III	3 (7)	0 (0)	3 (18)	
Tumour-type - No (%)				0.089
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)	
Therapeutic antibiotic use last year - No. (%)				0.733
	12 (27)	8 (31)	4 (22)	
Weeks between collection T0 faecal sample and last therapeutic antibiotic treatment				0.713
Mean (SD)	31 (13)	29 (12)	33 (15)	
Karnofsky Performance Score - No (%)*				**0.006**
70-80	9 (21)	7 (27)	2 (11)	
90-100	35 (79)	19 (73)	16 (89)	
MUST-score - No (%)				0.688
Low risk	38 (86)	22 (85)	16 (89)	
Medium risk	6 (14)	4 (15)	2 (11)	
High risk	0 (0)	0 (0)	0 (0)	
Oral contraception use past	34 (77)	19 (73)	15 (83)	0.489

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

Between T0-T1, 21% of the patients used antibiotics; 38% between T1 and T2 and 5% between T2 and T3. Most commonly 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 faecal sample collection, antibiotic administration during AC-D treatment was not different between adjuvant and neoadjuvant treated patients (Table S5.3).

Dose intensity was high, with a median of 94% of the chemotherapy dosage administered during AC-D treatment (Table S5.4).

Intestinal microbiota composition of the total study population

In total, 153 faecal samples were collected. Faecal samples were collected before AC-D (*n*=44), during AC (*n*=43), during D (*n*=29), and after AC-D treatment (*n*=37), resulting in 28 paired samples (Figure 5.1).

In the total study population, Firmicutes was the most abundant phylum, followed by Bacteroidetes and Actinobacteria (Figure 5.2A). Figure 5.2B indicates the changes in relative abundance of the most common genera.

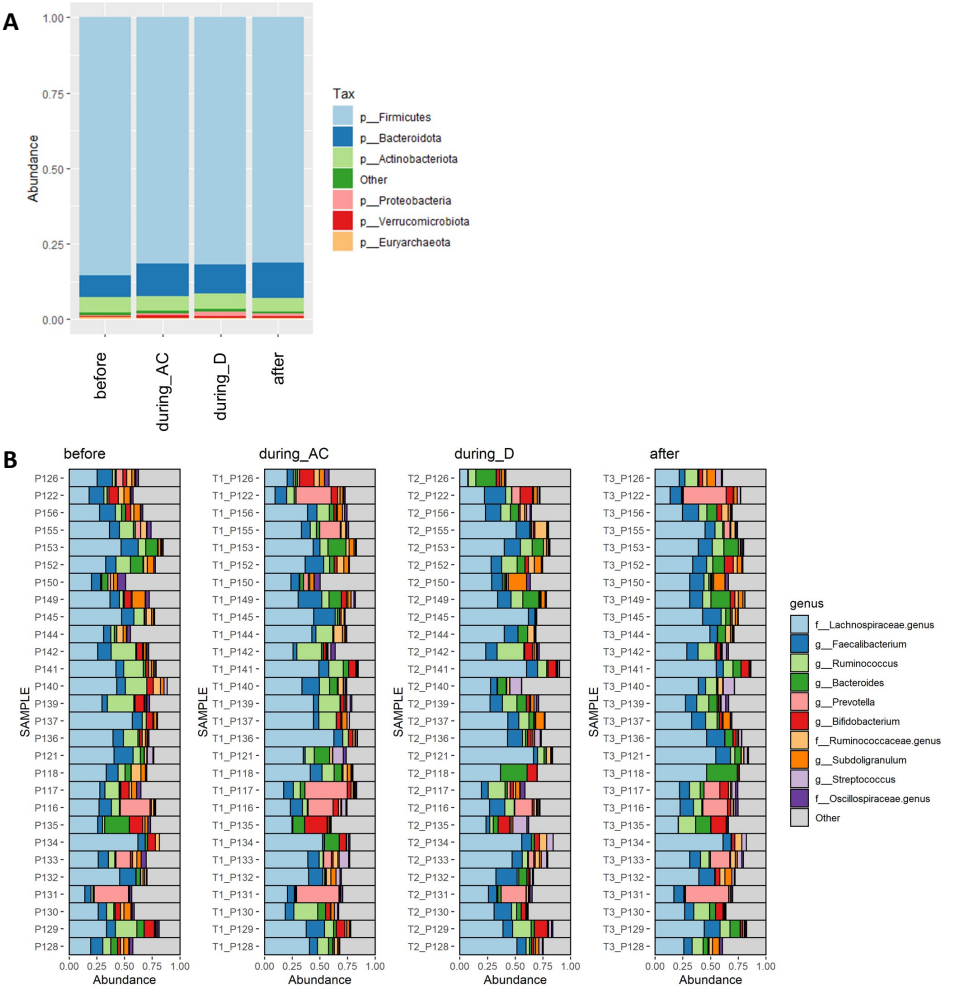

Figure 5.2: **A:** Relative abundances of different phyla before AC-D (*n*=44), during AC (*n*=43), during D (*n*=29), and after AC-D treatment (*n*=37). **B:** Composition plot of individual samples (paired samples only, *n*=28) indicating changes in relative abundance of most common genera over the course of AC-D treatment.

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 5.3A and Table S5.5). Pairwise comparison of all samples revealed a significant decrease in observed species richness between T0-T3 (p=0.003; n=37) (Figure 5.3B and Table S5.6).

Additional analyses were performed to assess the influence of antibiotic exposure 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 (Table S5.7). In addition, baseline α-diversity measures were not influenced by perioperative antibiotic administration in adjuvant treated patients.

Antibiotic administration between T0 and T1 was negatively correlated with observed species richness (p=0.002) and Shannon index (p=0.003) at T1 (Table S5.8). Cumulative therapeutic and prophylactic antibiotic use from the year prior to baseline faecal sample collection until the index sample, was not correlated with lower α-diversity at T1, T2 or T3 (Table S5.9).

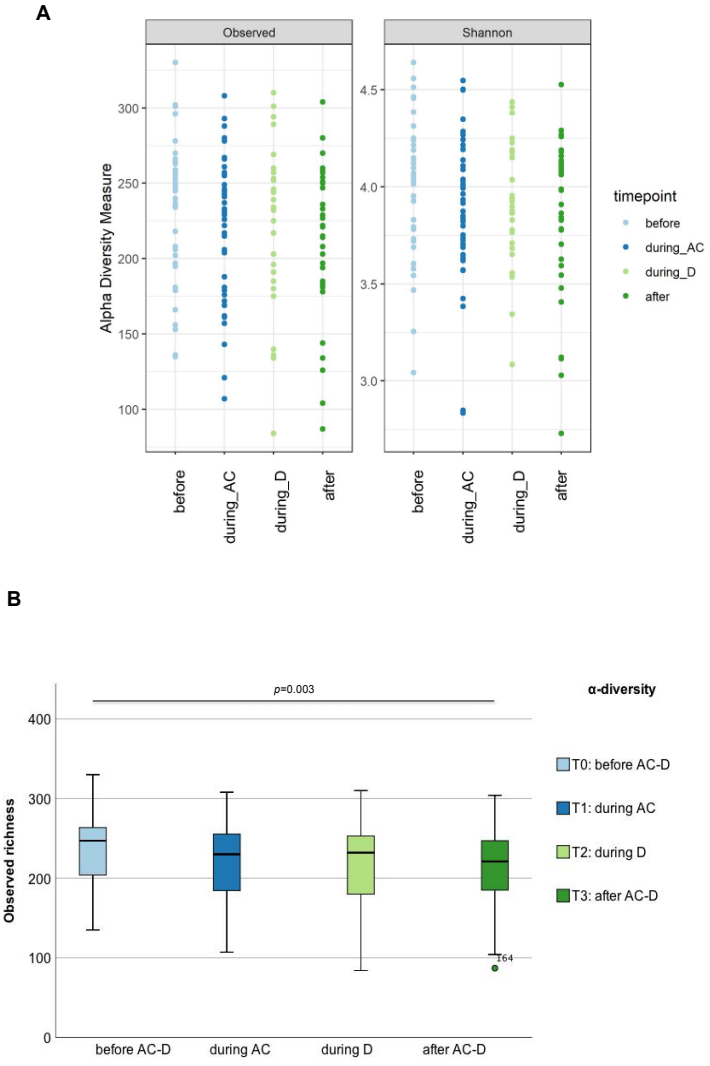

Figure 5.3: **A:** Changes in α-diversity measures before AC-D, during AC, during D, and after AC-D treatment, measured in terms of observed species richness (p=0.042) and Shannon index (p=0.206; n=28) (Table S5.5). **B:** Pairwise comparison (Wilcoxon signed-rank sum test) of all samples revealed significant differences in observed species richness between T0-T3 (p=0.003; n=37) (Table S5.6).

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=0.0864) and genus (p=0.1024) level and the different sampling time points (Figure 5.4).

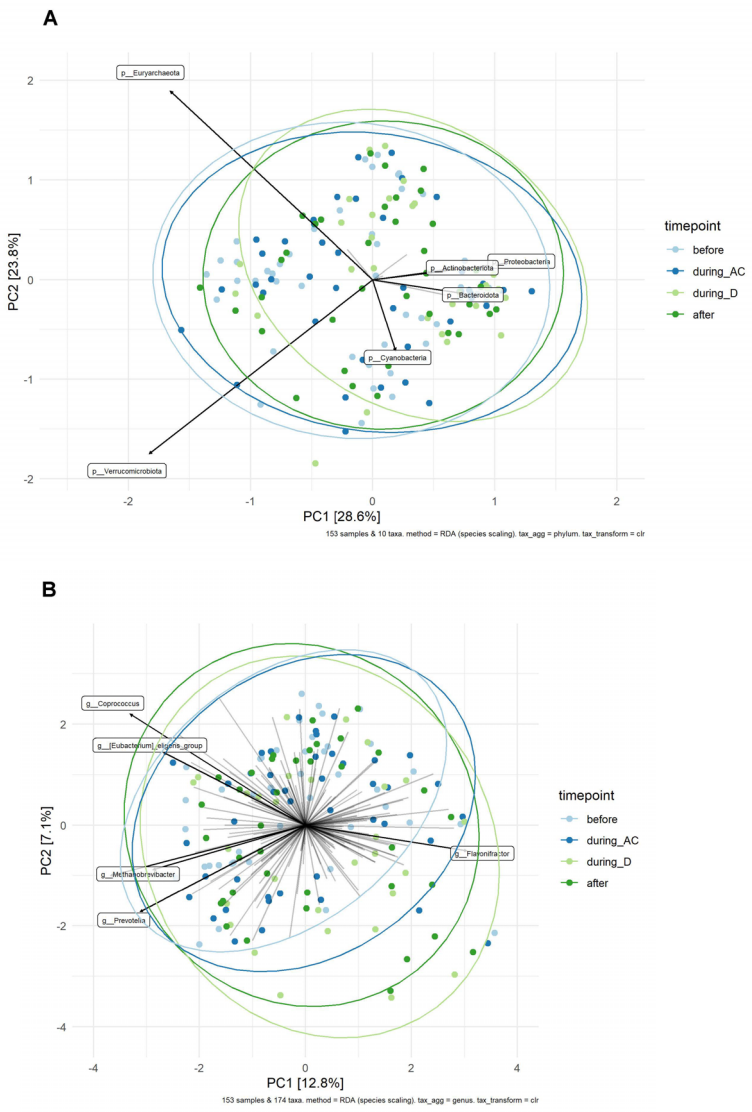

Figure 5.4: Ordination plots derived from unconstrained Principal Components Analysis (PCA) based on the Aitchison distance, showing overall composition of the microbial community at phylum **(A)** and genus level **(B)** before AC-D, during AC, during D, and after AC-D treatment. Taxa that were present 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.

At phylum level, ANCOM-II analysis identified that Proteobacteria were differently abundant during the course of AC-D (Figure 5.5A). 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, eight genera were differently abundant during the course of AC-D (Figure 5.5B). Except for *Turicibacter* and *Intestinibacter*, Friedman's ANOVA confirmed these results and indicated significant changes in the abundance of 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).

The abudance of unclassified Enterobacterales and *Lactobacillus* increased during AC-D treatment. After AC-D treatment, the abundance of 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 5.5 and Table S5.10).

To rule out the effect of perioperative prophylactic antibiotic exposure in the adjuvant group, differential abundant taxa during the course of AC-D were separately analysed in the neoadjuvant group. In the neoadjuvant group, Friedman's ANOVA confirmed similar significant changes in abundance of the taxa that were differential abundant in the total group (Figure 5.5), except for *Christensenellaceae R7 group*. In the neoadjuvant subgroup, *Christensenellaceae R7 group* showed the same decreasing trend compared to the total group, except between T2 and T3, where a slight decrease was observed compared to an increasing trend in the total group.

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 abundance of *Marvinbryantia* at T2 (p=0.028).

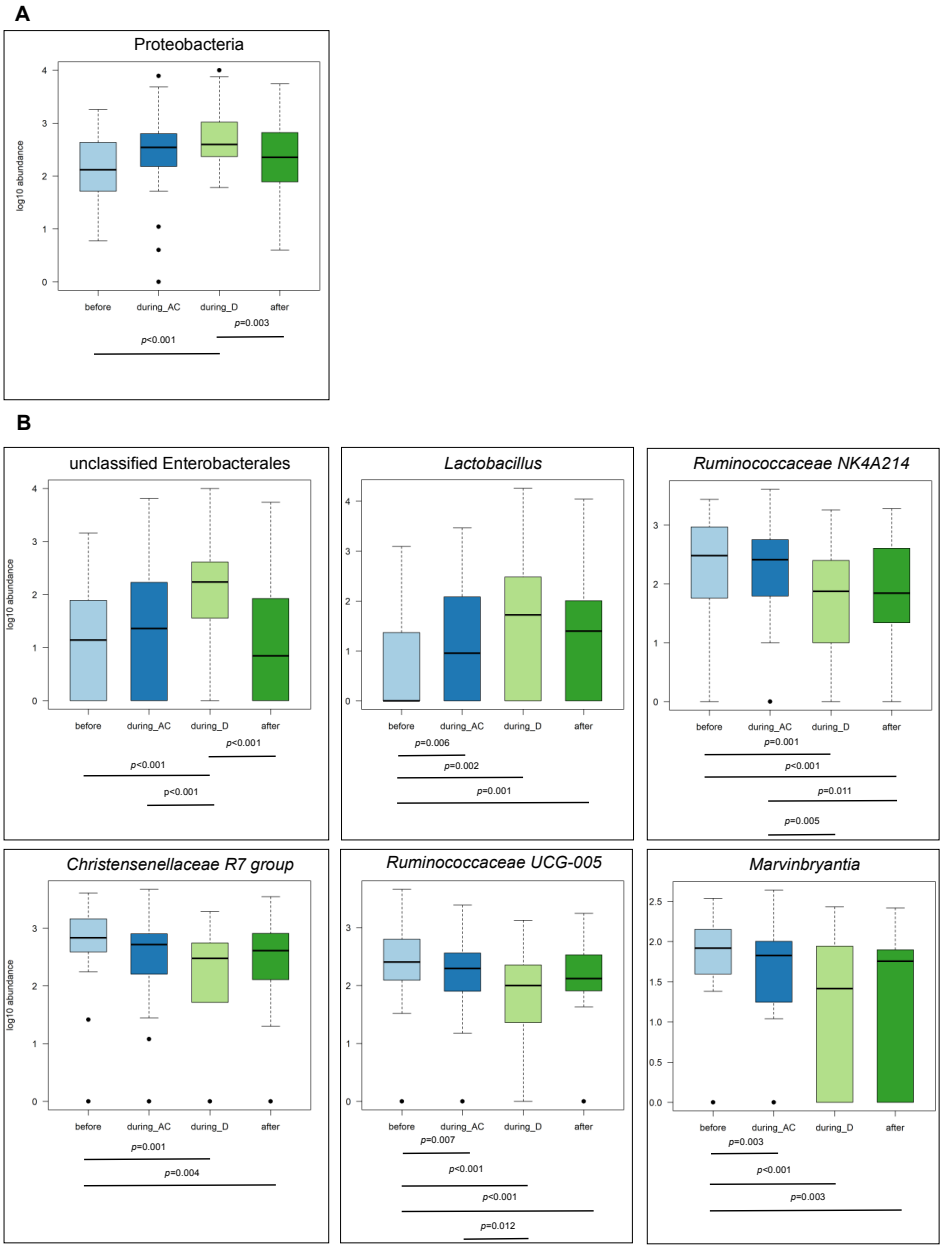

Figure 5.5: Log^{10} abundance of taxa with significant differential abundance during the course of AC-D treatment. *P*-values below boxplots indicate significant differential abundances analysed with a pairwise Wilcoxon signed-rank sum test. **A**: Phylum level. **B**: Genus level.

Associations of microbiota richness, diversity and composition with chemotherapy toxicity

Most common CTCAE toxicities are reported in Figure S5.2 and Table S5.11 and S5.12. During docetaxel (T2), 19% experienced grade 1 diarrhoea and 19% grade 2. Observed species richness and Shannon index at T2 as well as T3 were negatively correlated with diarrhoea at T2. Patients with any grade diarrhoea during D (T2) had a significantly lower level of observed species richness at T2 compared to patients without diarrhoea (p=0.039). Patients with any grade diarrhoea after AC-D treatment (T3) had a lower Shannon index at T3 compared to patients without diarrhoea (p=0.006). Diarrhoea at T3 was negatively correlated with the abundance 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 diarrhoea, nausea, oral mucositis, hand-foot syndrome or peripheral sensory neuropathy (Table S5.13).

Diarrhoea was not correlated to previous therapeutic antibiotics, perioperative prophylactic antibiotic administration, or antibiotic exposure during the course of AC-D treatment.

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 neoadjuvant subgroup is comparable to the total study population over the course of AC-D treatment regarding clinical characteristics (Table S5.14), toxicity (Table S5.15), antibiotic use, and therapy adjustments (Table S5.16). Except that, neoadjuvant patients did not have an increased risk of malnutrition during the course of AC-D treatment compared to the total group (Table S5.14).

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, six patients (35%) presented with <10% remaining tumour cells, four patients (24%) with 10-50% remaining tumour cells, and six patients (35%) with >50% remaining tumour cells. Accordingly, ten patients were classified as low-responders and seven as high-responders (Table S5.17). Baseline

characteristics were not different between low and high-responders (Table S5.18). No differences in clinical characteristics were observed between low and high responders after AC-D (Table S5.19). Before AC-D, during AC, during D, and after AC-D, both α-diversity measures were not significantly different between low and high-responders (Table S5.20). PERMANOVA revealed that there was no statistically significant association between baseline microbial community structure and response after AC-D at phylum (p=0.0733) and genus (p=0.1301) level. There were no differences in bacterial abundances at baseline between low response and high responders.

Discussion

This is the first longitudinal study that examined the impact of adriamycin, cyclophosphamide and docetaxel (AC-D) on intestinal microbiota as well as the impact of the intestinal microbiota on chemotherapy toxicity and tumour 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, diarrhoea was associated with lower α-diversity. Furthermore, pathologic response in neoadjuvant treated patients was not related to baseline microbiota richness, diversity, and composition.

Concerning the observed changes in microbiota richness, diversity, and composition in breast cancer patients, no comparable longitudinal clinical studies during AC-D treatment are available. According to our knowledge, only Yulzari et al. (2020) [30] collected faecal 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 analyse longitudinal microbiota changes. 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). Despite the lack of longitudinal comparable clinical studies, our results will be compared to pre-clinical studies or studies in patients with different types of cancer.

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) [31] and Galloway-Peña et al. (2017) [32], in patients with acute myeloid leukaemia 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 [33]. The consequences of reduced α-diversity warrants further investigation in this study population, for example studying microbial functions and long-term clinical effects.

Beside the reduction of α-diversity, the abundance of specific microbial taxa changed during the course of AC-D treatment. In the present population, a general trend was observed where the abundance of 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 the abundance of Proteobacteria and unclassified Enterobacterales significantly decreased to levels comparable to baseline.
Our results suggest that the *Ruminococcaceae NK4A214 group*, *Christensenellaceae R7 group*, *Ruminococcaceae UCG-005*, and *Marvinbryantia* are 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 [34,35]. Therefore, reduction of these bacteria during chemotherapy might contribute to manifestation of intestinal inflammation and dysregulated homeostasis.
In contrast to the genera that decrease during AC-D, the abundance of 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 attenuate the intestinal barrier, which resulted in the translocation of bacteria via the intestinal wall causing systemic inflammation in mice [13,14,36,37]. In addition, the Enterobacterales order includes amongst others 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 related phylum of Proteobacteria. Subsequently, the bloom of Proteobacteria/Enterobacterales at the expense of genera from the Ruminococcaceae family might promote further intestinal inflammation during AC-D.

Concerning chemotherapy toxicity, we detected that patients with any grade diarrhoea during docetaxel treatment had significantly lower observed species richness compared to patients without diarrhoea. Furthermore, diarrhoea 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 diarrhoea. 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 diarrhoea in patients undergoing chemotherapy [38]. It might be speculated that patients with lower microbial richness have a higher risk to develop diarrhoea or vice versa. However, the exact mechanism by which AC-D induced diarrhoea occurs should be further revealed, for example using the TIMER (translocation, immunomodulation, metabolism, enzymatic degradation, and reduced diversity) model that was recently proposed by Alexander et al. (2017) [39].

Pathologic response in neoadjuvantly treated patients was not related to intestinal microbiota richness, diversity and composition. Due to the lack of clinical studies in breast cancer patients, our observations were compared to studies with other types of cancer and response-related outcomes. To our knowledge, only 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 tumour response [11]. In our population, species level differences could not be observed. In addition, the effects of adriamycin and docetaxel were not taken into account in the study described by Goubet et al. (2018). Furthermore, our results were based on a relative small sample size of 18 patients. Therefore, these observations should be interpreted carefully and warrants further investigation in a larger study population.

Since it is widely described that antibiotic exposure interferes with the intestinal microbiota and diversity, 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 patients who received perioperative prophylactic antibiotics. To rule out the effect of perioperative prophylactic antibiotic exposure on the microbiota composition, the impact of AC-D was studied again in the neoadjuvant treated patients. Compared to the total group, similar significant changes in the abundance of differential abundant taxa were observed during the course of AC-D treatment in the neoadjuvant group, except for the *Christensenellaceae R7 group*. In addition, 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 likely to be attributable to differences in antibiotic exposure or microbiota modulating agents.

There are several limitations and strengths of this study. First of all, no analysis of bacterial metabolites (e.g. SCFA), has been performed. Insights in the levels of SCFA will provide more knowledge on the intestinal bacterial activities involved in the regulation of host's immune system and metabolism, as well as their associations with cancer treatment [40]. Another limitation is that it was only possible to conduct chemotherapy response measures in the subgroup of neoadjuvant treated patients. As a consequence, the group size reduced from 44 to 18 patients. In addition, response measurement based on residual tumour will never be possible in adjuvant treated patients, since adjuvant patients will be subjected to tumour resection first. To circumvent this, increased samples sizes and other response measurement should be used, for instance disease free survival or progression free survival [41].
Unique of this study is its homogenous study population of postmenopausal ER+ and HER2- breast cancer patients as well as the longitudinal design including collection of faecal 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. In conclusion, this is the first clinical study with longitudinal faecal sampling in breast cancer patients that explored the effect of adriamycin, cyclophosphamide, and docetaxel on the intestinal microbiota as well as the impact of the intestinal microbiota on chemotherapy toxicity and tumour response in ER+ and HER2- postmenopausal breast cancer patients. We reported shifts of intestinal microbiota richness and composition during AC-D treatment. Our findings provide important first insights into the effect of chemotherapy on intestinal microbiota in 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.

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Supplementary material and methods

Response measurement

The scoring system according to EUSOMA was applied to determine pathologic response after neoadjuvant AC-D. 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. Below the complete definition of the EUSOMA scoring system is presented [1].

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 tumour cells or tumour cells located in small groups).
- **(ii):** evidence of response to therapy but with 10-50% of tumour remaining.
- **(iii):** >50% of tumour 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 [2] (Supplementary methods). The following aspects were scored: diarrhoea, 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.

Faecal microbiota analyses

In order to extract metagenomic DNA, 250 mg of the frozen faecal samples were homogenised 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 MagMax™ Total Nucleic Acid Isolation Kit *(Thermo Fisher Scientific)* and mixed with 175 µl supernatant of faeces 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 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 [3]. 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) [4].

The pre-processing of sequencing data, using an in-house pipeline based upon DADA2 (R version 4.0.3) [5], consisted of the following steps: reads filtering, identification of sequencing errors, dereplication, and removal of chimeric sequences. In order to assign taxonomy, DECIPHER [6] 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 marke-gene and metagenomics data [7]. 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 816 ASVs were maintained for downstream analysis. The final file was saved in the phyloseq format [8].

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Supplementary figures

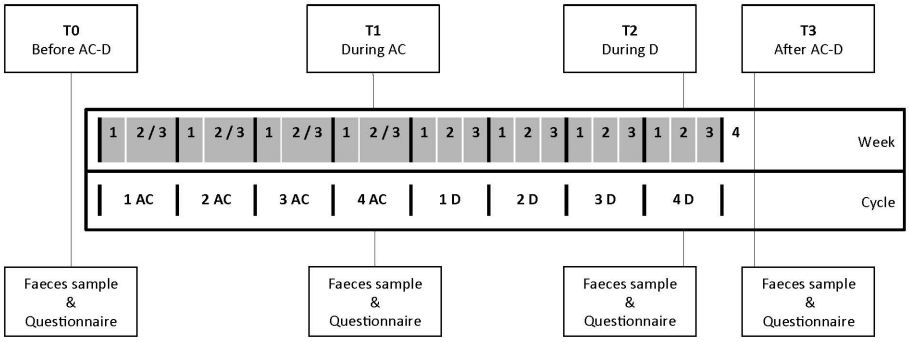

Figure S5.1: Study design.
Patients collected a faecal sample and completed a questionnaire at four time points: **T0** was collected before the start of the AC-D, **T1** during week 2 of cycle 4 AC, **T2** during week 2 of cycle 4 D, and **T3** one month after the dose D. During the study period, patients received four cycles adriamycin (A), 60 mg/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 was followed by four cycles of docetaxel (D), 100 mg/m^2 i.v. on day 1, in a three-weekly cycle.

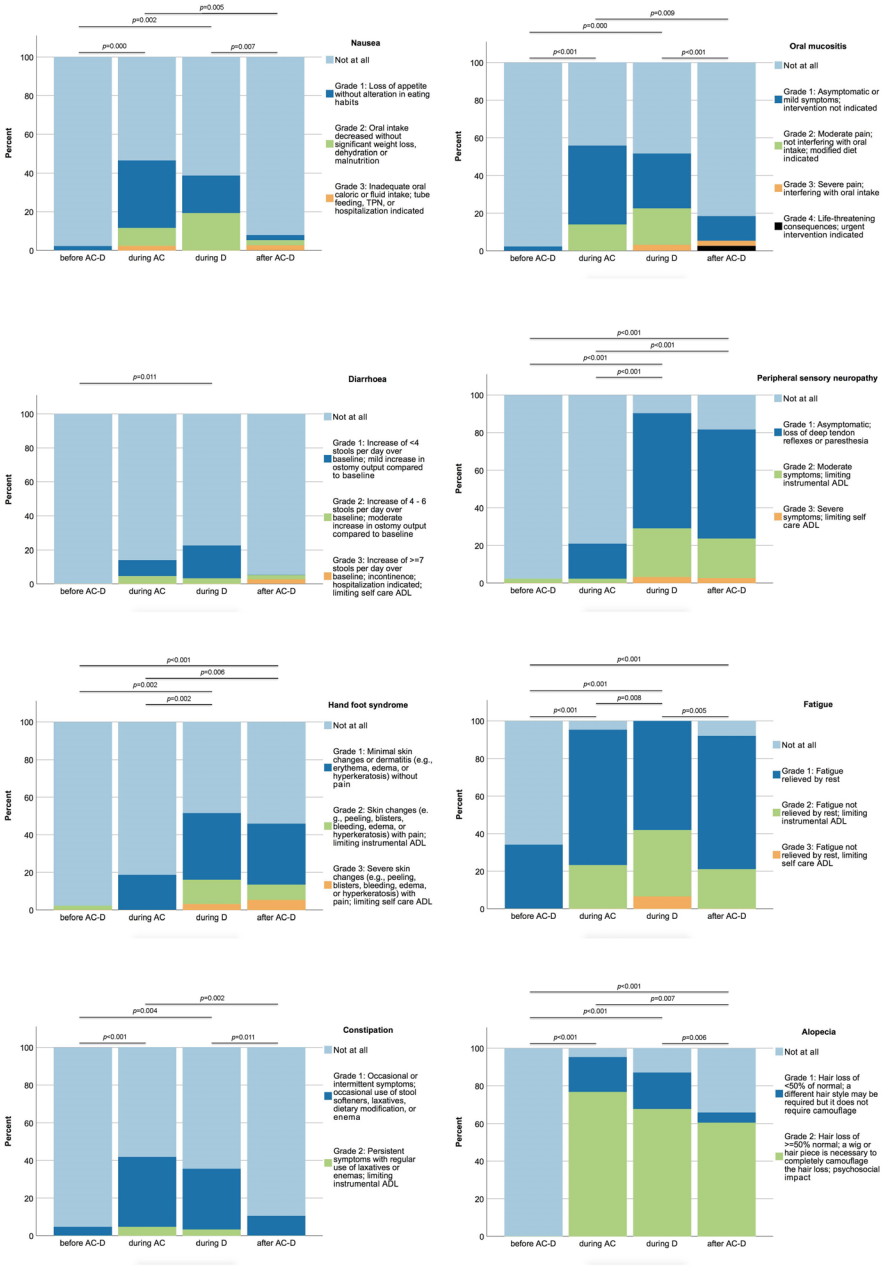

Figure S5.2: Stacked bar charts presenting percentage toxicity grades before AC-D, during AC, during D, and after AC-D. Friedman tests indicated that nausea, oral mucositis, diarrhoea, peripheral sensory neuropathy, hand foot syndrome, fatigue, constipation and alopecia changed significantly over time. Presented *p*-values indicate significant differences between the different time points revealed by the Wilcoxon test with Bonferoni correction (Table S5.11).

Table S5.1: Clinical characteristics of the total study population (*N*=44) at baseline including the comparison between adjuvant and neoadjuvant treated patients.

Baseline characteristics	Total	Adjuvant	Neoadjuvant	*p*-value
Focality - No. (%)				0.714
Unifocal tumour	33 (75)	19 (73)	14 (78)	
Multifocal tumour	10 (23)	7 (27)	3 (17)	
Unknown	1 (2)	0 (0)	1 (6)	
cT stage - No. (%)*				**0.003**
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)	
cG grade - No. (%)*				0.638
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)	
cN stage - No. (%)*				**0.016**
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)	
cT size - in mm				**0.012**
Median (IQR)	22 (16)	19 (12)	28 (16)	
(y)pT size -				0.257
Median (IQR)	20 (11)	21 (14)	15 (13)	
25%-75%	15-26	15-29	13-25	
MIB1%				0.492
Median (IQR)	15 (25)	20 (20)	10 (24)	
25%-75%	5-30	10-30	5-29	
Ki-67%				0.850
Mean (SD)	26 (20)	27 (17)	25 (25)	
Range	2-75	5-60	2-75	

Table S5.1: (continued)

Baseline characteristics	Total	Adjuvant	Neoadjuvant	*p*-value
OK-type - No (%)				0.954
Lumpectomy	20 (46)	12 (46)	8 (44)	
Mastectomy	23 (52)	14 (54)	9 (50)	
Unknown	1 (2)	0 (0)	1 (6)	
ER- No (%)				-
Negative	0 (0)	0 (0)	0 (0)	
Positive	44 (100)	26 (100)	18 (100)	
ER %				0.263
Median (IQR)	100 (0)	100 (0)	100 (5)	
25%-75%	100-100	100-100	95-100	
PR- No (%)				0.307
Negative	18 (41)	9 (35)	9 (50)	
Positive	26 (59)	17 (65)	9 (50)	
PR %				0.745
Median (IQR)	25 (79)	30 (75)	12 (91)	
25%-75%	1-80	5-80	0-92	
DM-type II - No. (%)*				1.000
No	39 (89)	23 (89)	16 (89)	
Yes	5 (11)	3 (12)	2 (11)	
Prior systemic treatment - No. (%)				-
No	44 (100)	26 (100)	18 (100)	
Yes	0 (0)	0 (0)	0 (0)	
Days therapeutic antibiotic use last year				0.286
Median (IQR)	7 (4)	6 (3)	9 (-)	
25-75%	5-9	5-8	7- -	
Days from operation				-
Mean (SD)	50 (23)	50 (23)	-	
Range	18-93	18-93		
Prophylactic antibiotic use during operation - No. (%)				**<0.001**
No	14 (54)	14 (54)	18 (100)	
Yes	12 (46)	12 (46)	0 (0)	
Oral contraception use - years				0.104
Median (IQR)	12 (13)	10 (15)	15 (19)	
25%-75%	8-21	5-20	10-29	

Table S5.1: (continued)

Baseline characteristics	Total	Adjuvant	Neoadjuvant	*p*-value
Oral contraception use - years				0.104
Median (IQR)	12 (13)	10 (15)	15 (19)	
25%-75%	8-21	5-20	10-29	
Years between T0 faecal sample and last oral contraception use				0.274
Mean (SD)	20 (13)	22 (14)	17 (12)	
Range	0.1-49.3	0.2-49	0.1-39	
Years between T0 faecal sample and last hormone IUD use				0.229
Mean (SD)	7 (5)	5 (5)	9 (5)	
Range	1-15	1-12	5-15	

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

Table S5.2: Longitudinal clinical characteristics of the total study population (*N*=44).

Clinical characteristics	T0 before AC-D	T1 during AC	T2 during D	T3 after AC-D	*p*-value	Pairwise comparison
Karnofsky Performance Score - No (%)*					**<0.001**	**T0 vs T1 < 0.001**
20-30	0 (0)	0 (0)	1 (3)	0 (0)		**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 (%)					**0.023**	T0 vs T1 = 0.117
Low risk	38 (86)	34 (79)	22 (67)	32 (84)		T0 vs T2 = 0.022
Medium risk	6 (14)	5 (12)	6 (18)	6 (16)		T0 vs T3 = 0.739
High risk	0 (0)	4 (9)	5 (15)	0 (0)		**T1 vs T2 < 0.001**
						T1 vs T3 = 0.531
						T2 vs T3 = 0.005
BMI - kg/m^2					0.338	T0 vs T1 = 0.024
Median (IQR)	26 (5)	26 (5)	27 (5)	26 (4)		T0 vs T2 = 0.265
25%-75%	24-29	23-28	24-28	24-28		T0 vs T3 = 0.777
						T1 vs T2 = 0.470
						T1 vs T3 = 0.330
						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.

Table S5.3: 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 (%)				0.060
No	33 (79)	17 (68)	16 (94)	
Yes	9 (21)	8 (32)	1 (6)	
Between T1-T2				
Antibiotic use - No (%)				1.000
No	18 (62)	10 (63)	8 (62)	
Yes	11 (38)	6 (38)	5 (39)	
Between T2-T3				
Antibiotic use - No (%)				1.000
No	35 (95)	20 (95)	15 (94)	
Yes	2 (5)	1 (5)	1 (6)	

Table S5.4: Therapy adjustments of the total study population (*N*=43) 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.

Table S5.5: Longitudinal α-diversity measures of paired samples of the total study population (*n*=28).

α-diversity measures	**T0 before AC-D**	**T1 during AC**	**T2 during D**	**T3 after AC-D**	**ANOVA *p*-value**
Observed richness					**0.042**
Mean (SD)	240 (48)	229 (40)	218 (57)	217 (47)	
Range	136-330	157-308	84-310	87-304	
Shannon index					0.206
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	

Repeated measure ANOVA was performed to test differences in α-diversity measures over time.

Table S5.6: Longitudinal α-diversity measures of all samples of the total population (*N*=44).

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

Table S5.7: α-diversity measures in patients with or without therapeutic antibiotics 1 year prior to T0 (*N*=44).

α-diversity measures	Total	- Antibiotics	+ Antibiotics	*p*-value
T0 Observed richness				0.388
Mean (SD)	234 (45)	230 (48)	244 (39)	
Range	135-330	135-330	166-301	
T1 Observed richness				0.802
Mean (SD)	222 (47)	221 (46)	225 (52)	
Range	107-308	121-293	107-308	
T2 Observed richness				0.789
Mean (SD)	217 (57)	216 (48)	222 (78)	
Range	84-310	134-294	84-310	
T3 Observed richness				0.606
Mean (SD)	213 (48)	215 (41)	206 (66)	
Range	87-304	104-280	87-304	
T0 Shannon index				0.856
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	
T1 Shannon index				0.996
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	
T2 Shannon index				0.413
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	
T3 Shannon index				0.671
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	

Table S5.8: Correlation between antibiotic administration during AC-D treatment and α-diversity.

α-diversity measures	Antibiotics during T0-T1		Antibiotics during T1-T2		Antibiotics during T2-T3	
	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.

Table S5.9: Correlation between cumulative antibiotic administration and α-diversity.

α-diversity measures	Cumulative antibiotics until T1		Cumulative antibiotics until T2		Cumulative antibiotics until T3	
	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 faecal sample collection until the index sample.

Table S5.10: Longitudinal differential abundant taxa of the total study population.

Taxa	T0 before AC-D	T1 during AC	T2 during D	T3 after AC-D	*p*-value	Pairwise comparison
Proteobacteria					**0.006**	T0 vs T1 = 0.023
Median (IQR)	2.12 (0.94)	2.54 (0.69)	2.60 (0.67)	2.36 (0.98)		**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					**<0.001**	T0 vs T1 = 0.122
Median (IQR)	1.15 (1.91)	1.36 (2.28)	2.24 (1.24)	0.85 (1.95)		**T0 vs T2 < 0.001**
25%-75%	0.00-1.91	0.00-2.28	1.49-2.74	0.00-1.95		T0 vs T3 = 0.877
						T1 vs T2 < 0.001
						T1 vs T3 = 0.117
						T2 vs T3 < 0.001
Lactobacillus					**0.004**	**T0 vs T1 = 0.006**
Median (IQR)	0.00 (1.39)	0.95 (2.10)	1.72 (2.57)	1.40 (2.04)		**T0 vs T2 = 0.002**
25%-75%	0.00-1.39	0.00-2.10	0.00-2.57	0.00-2.04		**T0 vs T3 = 0.001**
						T1 vs T2 = 0.024
						T1 vs T3 = 0.245
						T2 vs T3 = 0.174
Ruminococcaceae NK4A214 group					**<0.001**	T0 vs T1 = 0.132
Median (IQR)	2.48 (1.25)	2.41 (0.97)	1.88 (1.91)	1.85 (1.36)		**T0 vs T2 = 0.001**
25%-75%	1.72-2.97	1.79-2.76	0.50-2.41	1.30-2.66		**T0 vs T3 < 0.001**
						T1 vs T2 = 0.005
						T1 vs T3 = 0.011
						T2 vs T3 = 0.927
Intestinibacter					0.347	T0 vs T1 = 0.013
Median (IQR)	2.00 (1.44)	1.54 (2.47)	2.23 (1.90)	2.46 (1.26)		T0 vs T2 = 0.200
25%-75%	1.21-2.65	0.00-2.47	1.17-3.07	1.65-2.90		T0 vs T3 = 0.219
						T1 vs T2 = 0.041
						T1 vs T3 = 0.002
						T2 vs T3 = 0.716

Table S5.10: (continued)

Taxa	T0 before AC-D	T1 during AC	T2 during D	T3 after AC-D	*p*-value	Pairwise comparison
Marvinbryantia					**0.020**	**T0 vs T1 = 0.003**
Median (IQR)	1.92 (0.56)	1.83 (0.91)	1.41 (1.95)	1.76 (1.91)		**T0 vs T2 < 0.001**
25%-75%	1.59-2.15	1.11-2.02	0.00-1.95	0.00-1.91		**T0 vs T3 = 0.003**
						T1 vs T2 = 0.041
						T1 vs T3 = 0.140
						T2 vs T3 = 0.685
Christensenellaceae R7 group					**0.008**	T0 vs T1 = 0.013
Median (IQR)	2.83 (0.60)	2.72 (0.74)	2.48 (1.93)	2.61 (1.24)		**T0 vs T2 = 0.001**
25%-75%	2.57-3.17	2.17-2.91	0.86-2.79	1.71-2.95		**T0 vs T3 = 0.004**
						T1 vs T2 = 0.387
						T1 vs T3 = 0.489
						T2 vs T3 = 0.548
Ruminococcaceae UCG-005					**<0.001**	**T0 vs T1 = 0.007**
Median (IQR)	2.41 (0.73)	2.30 (0.73)	2.00 (1.31)	2.12 (0.65)		**T0 vs T2 < 0.001**
25%-75%	2.09-2.81	1.87-2.60	1.16-2.47	1.90-2.54		**T0 vs T3 < 0.001**
						T1 vs T2 = 0.012
						T1 vs T3 = 0.105
						T2 vs T3 = 0.317
Turicibacter					0.069	T0 vs T1 = 0.098
Median (IQR)	1.42 (2.27)	0.95 (1.79)	1.89 (1.68)	1.45 (2.26)		T0 vs T2 = 0.062
25%-75%	0.00-2.27	0.00-1.79	0.85-2.52	0.00-2.26		T0 vs T3 = 0.962
						T1 vs T2 = 0.015
						T1 vs T3 = 0.066
						T2 vs T3 = 0.067

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.

Table S5.11: Longitudinal CTCAE in grade of the total study population (*N*=44).

Toxicity grade	T0 before AC-D	T1 during AC	T2 during D	T3 after AC-D	*p*-value	Pairwise comparison
Nausea - No. (%)*					**<0.001**	**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)		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
0	44 (100)	42 (98)	30 (97)	37 (97)		T0 vs T2 = 0.317
1	0 (0)	1 (2)	1 (3)	1 (3)		T0 vs T3 = 0.317
						T1 vs T2 = 1.000
						T1 vs T3 = 1.000
						T2 vs T3 = 1.000
Oral mucositis - No (%)*					**<0.001**	**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)		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**
Diarrhoea - No (%)*					**0.005**	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)		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. (%)*					**<0.001**	**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)		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

Table S5.11: (continued)

Toxicity grade	T0 before AC-D	T1 during AC	T2 during D	T3 after AC-D	*p*-value	Pairwise comparison
Fever - No (%)*					0.072	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)		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 (%)					**<0.001**	T0 vs T1 = 0.021
0	43 (98)	34 (79)	3 (10)	7 (18)		**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 - No. (%)*					**<0.001**	T0 vs T1 = 0.083
0	43 (98)	35 (81)	15 (48)	20 (54)		**T0 vs T2 = 0.002**
1	0 (0)	8 (19)	11 (36)	12 (32)		**T0 vs T3 < 0.001**
2	1 (2)	0 (0)	4 (13)	3 (8)		**T1 vs T2 = 0.002**
3	0 (0)	0 (0)	1 (3)	2 (5)		**T1 vs T3 = 0.006**
						T2 vs T3 = 0.323
Fatigue - No (%)*					**<0.001**	**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)		**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 (%)*					**<0.001**	**T0 vs T1 < 0.001**
0	44 (100)	2 (5)	4 (13)	13 (34)		**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.

Table S5.12: Longitudinal clinical characteristics of the total study population (*N*=44) - bone marrow toxicity.

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

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.

Table S5.13: Microbial community structures and chemotherapy toxicity of the total study population.

Any grade CTCAE	T1 during AC		T2 during D	
	Genus *p*-value	Phylum *p*-value	Genus *p*-value	Phylum *p*-value
Diarrhoea	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.

Table S5.14: Longitudinal clinical characteristics of neoadjuvant treated patients (n=18).

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

* Percentages do not add up to 100% due to rounding. Wilcoxon signed-rank sum test with Bonferroni correction was used for pairwise comparison.

Table S5.15: 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. (%)					**0.001**	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)		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 (%)*					**0.002**	**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)		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
Diarrhoea - No (%)*					**0.019**	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)		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. (%)					**0.019**	**T0 vs T1 = 0.008**
0	18 (100)	11 (61)	10 (71)	16 (100)		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

Table S5.15: (continued)

Toxicity grade	T0 before AC-D	T1 during AC	T2 during D	T3 after AC-D	*p*-value	Pairwise comparison
Fever - No (%)*					0.194	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)		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 (%)*					**<0.001**	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)		**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 - No. (%)*					**0.014**	T0 vs T1 = 0.046
0	18 (100)	14 (78)	8 (57)	11 (69)		T0 vs T2 = 0.024
1	0 (0)	4 (22)	3 (21)	3 (19)		T0 vs T3 = 0.039
2	0 (0)	0 (0)	3 (21)	1 (6)		T1 vs T2 = 0.063
3	0 (0)	0 (0)	0 (0)	1 (6)		T1 vs T3 = 0.336
						T2 vs T3 = 0.157
Fatigue - No (%)*					**<0.001**	**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)		**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 (%)*					**<0.001**	**T0 vs T1 < 0.001**
0	18 (100)	2 (11)	0 (0)	7 (44)		**T0 vs T2 = 0.001**
1	0 (0)	5 (28)	5 (36)	2 (13)		T0 vs T3 = 0.005
2	0 (0)	11 (61)	9 (64)	7 (44)		T1 vs T2 = 0.705
						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.

Table S5.16: Longitudinal clinical data - therapy adjustments of neoadjuvant treated patients.

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

Table S5.17: 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)	7
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.

Table S5.18: 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				0.199
Mean (SD)	58 (5)	60 (5)	57 (5)	
Range	49-71	55-71	49-65	
BMI - kg/m^2				0.429
Mean (SD)	28 (6)	27 (4)	29 (6)	
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. (%)*				1.000
Unifocal tumour	14 (78)	6 (86)	8 (80)	
Multifocal tumour	3 (17)	1 (14)	2 (20)	
Unknown	1 (6)	0 (0)	0 (0)	
cT stage - No. (%)*				0.325
1	3 (17)	2 (29)	1 (10)	
2	10 (56)	2 (29)	8 (80)	
3	2 (11)	1 (14)	1 (10)	
4	2 (11)	2 (29)	0 (0)	
Unknown	1 (6)	0 (0)	0 (0)	
cG grade - No. (%)*				0.621
1	2 (11)	1 (14)	1 (10)	
2	12 (67)	4 (57)	7 (70)	
3	3 (17)	2 (29)	1 (10)	
Unknown	1 (6)	0 (0)	1 (0)	
cN stage - No. (%)*				0.665
0	11 (61)	4 (57)	7 (70)	
1	4 (22)	2 (29)	2 (20)	
2	1 (6)	0 (0)	0 (0)	
3	2 (11)	1 (14)	1 (10)	
cT size - in mm				0.812
Median (IQR)	28 (16)	24 (20)	29 (16)	
25%-75%	22-38	20-40	22-38	
MIB1%				0.190
Median (IQR)	10 (24)	50 (-)	10 (17)	
25%-75%	5-29	25- -	4-20	
Ki-67%				0.190
Median (IQR)	25 (25)	50 (-)	10 (24)	
25%-75%	5-30	25- -	4-28	
Tumour-type - No (%)				0.485
Invasive carcinoma of no special type (NST)	16 (89)	7 (100)	8 (80)	
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)	

Table S5.18: (continued)

Baseline characteristics	Total *N*=18	High responders *n*=7	Low responders *n*=10	*p*-value
ER %				0.962
Median (IQR)	100 (5)	100 (5)	100 (6)	
25%-75%	95-100	95-100	94-100	
PR- No (%)*				0.637
Negative	9 (50)	4 (57)	4 (40)	
Positive	9 (50)	3 (43)	6 (60)	
PR %				0.417
Median (IQR)	12 (91)	8 (80)	33 (95)	
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 (%)				0.232
Low risk	16 (89)	6 (86)	10 (100)	
Medium risk	2 (11)	1 (14)	0 (0)	
High risk	0 (0)	0 (0)	0 (0)	
DM-type II - No. (%)*				1.000
No	16 (89)	6 (86)	9 (90)	
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.

Table S5.19: 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 (%)*				1.000
Lumpectomy	8 (44)	3 (43)	5 (50)	
Mastectomy	9 (50)	4 (57)	5 (50)	
Unknown	1 (6)	0 (0)	0 (0)	
pG grade - No. (%)*				0.251
1	4 (22)	1 (14)	3 (30)	
2	5 (28)	2 (29)	3 (30)	
3	1 (6)	1 (14)	0 (0)	
Unknown	8 (44)	3 (43)	4 (40)	
pN stage - No. (%)*				0.690
0	9 (50)	4 (57)	5 (50)	
1	6 (33)	3 (43)	3 (30)	
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				0.638
Mean (SD)	21 (14)	19 (15)	22 (13)	
Range	0-48	0-47	6-48	
pT stage - No. (%)*				0.232
0	1 (6)	1 (14)	0 (0)	
1	9 (50)	4 (57)	5 (50)	
2	7 (39)	2 (29)	5 (50)	
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 %				0.682
Mean (SD)	36 (29)	39 (39)	33 (21)	
Range	-20-100	-20-100	-5-73	

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

Table S5.20: 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				0.708
Mean (SD)	238 (52)	232 (70)	242 (41)	
Range	136-330	136-330	153-302	
T1 Observed richness				0.907
Mean (SD)	233 (34)	232 (45)	234 (29)	
Range	169-293	172-293	169-266	
T2 Observed richness				0.674
Mean (SD)	218 (48)	211 (31)	225 (57)	
Range	136-294	175-239	136-294	
T3 Observed richness				0.931
Mean (SD)	218 (48)	212 (36)	210 (48)	
Range	136-294	178-257	126-260	
T0 Shannon index				0.238
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	
T1 Shannon index				0.669
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	
T2 Shannon index				0.999
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	
T3 Shannon index				0.456
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	

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

CHAPTER 6

Intestinal microbiota in postmenopausal breast cancer patients and healthy controls

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2021; under revision

Abstract

Background

Previous pre-clinical and clinical research has investigated the role of the intestinal microbiota in carcinogenesis. Growing evidence exists that the intestinal microbiota can influence breast cancer carcinogenesis. However, the role of the intestinal microbiota in breast cancer needs to be further investigated. This study aimed to identify microbiota differences between postmenopausal breast cancer patients and healthy controls.

Patients and methods

This prospective cohort study compared the intestinal microbiota richness, diversity, and composition in postmenopausal histologically proven ER+/HER2- breast cancer patients and postmenopausal healthy controls. Patients scheduled for (neo)adjuvant adriamycin, cyclophosphamide (AC) and docetaxel (D) followed by endocrine therapy, or endocrine therapy (tamoxifen) were prospectively enrolled in a multicentre cohort study in the Netherlands. Patients collected a faecal sample and completed a questionnaire before starting systemic cancer treatment. Healthy controls, enrolled from the National Dutch Breast Cancer Screening Programme, also collected a faecal sample, and completed a questionnaire. Intestinal microbiota was analysed by amplicon sequencing of the 16S rRNA V4 gene-region.

Results

In total, 81 postmenopausal ER+/HER2- breast cancer patients and 67 postmenopausal healthy controls were included, resulting in 148 faecal samples. Observed species richness, Shannon index, and overall microbial community structure were not significantly different between breast cancer patients and healthy controls. There was a significant difference in overall microbial community structure between patients who received recent breast cancer surgery, patient without recent breast cancer surgery, and healthy controls on phylum (p=0.042) and genus level (p=0.015). *Dialister* (p=0.001) and its corresponding family Veillonellaceae (p=0.001) were higher in patients who received recent breast cancer surgery, compared to patient without recent breast cancer surgery, but a higher abundance was not associated with intravenous prophylactic antibiotic administration with cefazolin.

Conclusions

Intestinal microbiota richness, diversity and composition are not associated with postmenopausal breast cancer. This study identified that *Dialister* and Veillonellaceae were increased in patients who had received recent breast cancer surgery, independent of intravenous prophylactic cefazolin administration during recent breast cancer surgery, suggesting potential antibiotic-independent microbiota-modulating effects of breast cancer surgery.

Introduction

Breast cancer is the most common cancer in women worldwide [1]. Approximately one out of seven women (15%) will develop breast cancer during their lifetime [2]. A combination of genetic, epigenetic, and environmental factors are known to contribute to the development of cancer [3]. Factors such as hormonal treatment, heredity, and obesity are known to increase the risk of breast cancer [4,5]. Still, other factors, such as intestinal microbiota, are thought to influence breast cancer carcinogenesis [4,6,7].

During the last decade, there has been a growing interest in the role of the human intestinal microbiota and the development of cancer. The intestinal microbiota is a collective term for all micro-organisms that colonize the gastrointestinal tract, like bacteria, yeasts, and fungi. The intestinal microbiota plays an important role in health and disease and carries out crucial functions in the immune system and metabolism of humans [3]. In healthy people, an important equilibrium in the composition of the intestinal microbiota exists, resulting in a personal ecosystem which is essential to maintain homeostasis [8,9]. However, environmental factors and host genetic factors both influence microbiota diversity and composition and can generate dysbiosis leading to a disturbed metabolism and even carcinogenesis [10,11].

Several pre-clinical and clinical studies already investigated the link between dysbiosis of the intestinal microbiota and development of different types of cancer [12-14]. However, there are few studies which have specifically investigated the relationship between the intestinal microbiota and breast cancer [4,6,7]. A clinical study in breast cancer patients showed that increased relative abundance of *Blautia* sp. and *Faecalibacterium prausnitzii* was directly related with higher clinical breast cancer stages. In addition, a higher abundance of *Blautia* sp. was associated with higher histoprognostic grades according to according to Scarff-Bloom-Richardson [15]. Only a small number of studies have compared the intestinal microbiota composition in breast cancer patients with a healthy control group. The outcome of these studies has indicated differences in microbiota composition [16-20], of which only one study indicated alterations in the relative abundance of 45 bacterial species between breast cancer patients and healthy controls [19]. However, these studies were limited by their relatively small sample sizes of which the largest included 48 patients and 48 controls [16]. Since there is a direct link between breast cancer risk and high levels of circulating oestrogens, especially in postmenopausal women, it is important to study homogeneous groups concerning pre- and postmenopausal state [21,22].

In summary, growing evidence exists for a link between postmenopausal breast cancer and the intestinal microbiota composition. Current clinical information is limited; therefore, further investigation on the role of the intestinal microbiota in

postmenopausal breast cancer patients is required. We hypothesized that the intestinal microbiota richness, diversity, and composition of postmenopausal breast cancer patients differs from postmenopausal women without breast cancer. In this paper, we explored the intestinal microbiota richness, diversity, and composition in postmenopausal women with histologically proven oestrogen receptor positive breast cancer and postmenopausal healthy control women.

Materials and methods

Participants

Between November 2017 and February 2020, breast cancer patients were prospectively enrolled in four Dutch hospitals. Eligible patients were postmenopausal women with histologically proven oestrogen receptor positive (ER+) and human epidermal growth factor receptor-2 negative (HER2-) breast cancer scheduled for (neo)adjuvant chemotherapy with, adriamycin (doxorubicin), cyclophosphamide, and taxane docetaxel (AC-D) followed by endocrine therapy, or adjuvant endocrine therapy (tamoxifen). Exclusion criteria included distant metastasis, previous chemotherapy and therapeutic antibiotics use within three months prior to faecal sampling.
Between September and November 2018, healthy postmenopausal women with a negative mammography during the National Dutch Breast Cancer Screening Programme in Maastricht, were enrolled as a healthy control group. Exclusion criteria for the healthy control group included any type of cancer in history, inflammatory bowel disease, mammography older than 8 weeks, and therapeutic antibiotics use within three months prior to faecal sampling.
The studies were registered in the Dutch Trial Register (NTR-6296 and NTR-7478) and at ToetsingOnline (NL61646.068.17). All studies are approved by the Medical Ethics Committee azM/UM. The studies were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. Each participant provided written informed consent.

Sample and data collection

Faecal samples and a questionnaire were collected from all participants. For the breast cancer group, this was done before start of systemic cancer treatment. After collection, samples were immediately stored in the freezer and transported to the hospital in a cooled transport container (Sarstedt). In the hospital, the samples were stored at -20°C first and at -80°C for long-term storage. Baseline characteristics were registered

including Karnofsky performance score (KPS), nutritional status assessed with the Malnutrition Universal Screening Tool (MUST), prophylactic or therapeutic antibiotic administration, prebiotic/probiotic use, exogenous oestrogen use, and the use of nutritional supportive drinks.

Faecal microbiota analyses

Metagenomic DNA was isolated using the Ambion MagMax™ Total Nucleic Acid Isolation Kit (*Thermo Fisher Scientific*) and consisted of a manual pre-processing procedure 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 previously described [23].

Bioinformatic analysis of the sequencing data was performed using R studio. For the pre-processing, a standardized in-house pipeline using the software package DADA2 (R version 4.0.3) was applied [24]. Contaminated Amplicon Sequence Variants (ASVs) identified by decontam [25] were filtered out together with ASVs presented in less than 5% of all samples and a total abundance of less than 0.001%. After filtering, 816 taxa remained in the analysis. For further details on DNA isolation, sequencing, and data pre-processing, see supplementary methods.

Statistical analysis of clinical data

Baseline characteristics were analysed in IBM SPSS version 27. For continuous data, normality was tested using the Kolmogorov-Smirnov 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. Two-tailed tests were used and *p*-values <0.05 were considered statistically significant. Correlations between differentially abundant taxa and clinical characteristics were measured by means of Kendall's Tau correlation coefficients (τ).

Statistical analysis of intestinal microbiota data

Bioinformatic analysis of the sequencing data was performed using R Studio (R version 4.0.0) [26]. Observed species richness and the Shannon index, reflecting microbial diversity, were calculated on ASV level, using the phyloseq package [27]. Testing the

assumptions of normality and homogeneity of variance as well as subsequent statistical testing was performed as described for clinical data.
The R packages, phyloseq [27], microbiome [28], dplyr [29], ggplot2 [30] and microViz [31] were used for ordination and visualization of taxonomic composition. Again, taxa present in less than 5% of 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 [31]. Permutational multivariate analysis of variance (PERMANOVA) was used to analyse differences in overall microbiota composition between groups [31]. Homogeneity of multivariate dispersions was assessed using the microViz package [31] and revealed no significant differences in dispersion. The workflow of ANCOM v2.1, which accounts for the underlying compositional structure and sparseness of microbiota data, was used to identify taxa with differential abundance between healthy controls and breast cancer patients or healthy controls and breast cancer patients treated with adjuvant or neoadjuvant systemic therapy, respectively [32]. We set $\alpha<0.05$ at 70% (W) of comparisons as threshold for significance. Afterwards, bacterial counts were transformed into $\log^{10}(1+x)$ abundance by means of the microbiome package [28]. Significant differences identified with the ANCOM v2.1 workflow were confirmed by the Kruskal-Wallis test using IBM SPSS version 27. Subsequently pairwise comparison with Bonferroni correction for multiple testing was performed. Boxplots were made by means of GraphPad Prism 5 version 5.02.

Results

In total, 81 postmenopausal ER+ and HER2- breast cancer patients and 67 postmenopausal healthy controls were included. From the breast cancer group, 18 patients were scheduled for neoadjuvant chemotherapy and 63 patients for adjuvant chemotherapy or tamoxifen (Figure 6.1).

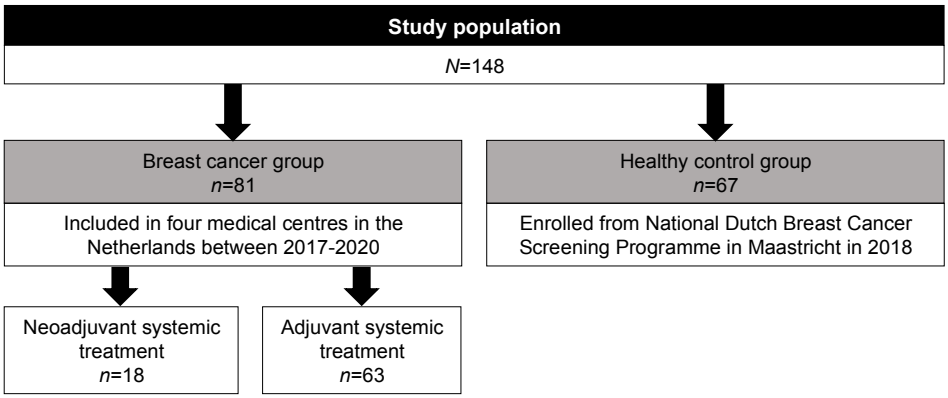

Figure 6.1: Flowchart study population. The flow chart presents the number of participants included and the number of faecal samples collected during the study period. Patients scheduled for neoadjuvant systemic treatment were eligible to receive adriamycin (doxorubicin), cyclophosphamide and docetaxel (AC-D) followed by endocrine therapy. Patients scheduled for adjuvant systemic treatment were eligible to receive AC-D (*n*=26) or tamoxifen (*n*=37). All faecal samples were collected before systemic cancer treatment with chemotherapy or tamoxifen and analysed by amplicon sequencing of the 16S rRNA V4 gene-region.

Baseline characteristics

Median age (p=0.929) and BMI (p=0.450) were similar in breast cancer patients and healthy controls. In the year prior to inclusion, 25% of the breast cancer patients compared to 16% of the healthy controls had used therapeutic antibiotics (p=0.236), with a median of 25 weeks since the last antibiotic use for the total group. One patient used prebiotics, and two patients and nine healthy controls used probiotics in the year prior to inclusion. None of the participants used nutritional supportive drinks in the year prior to inclusion. There was no significant difference in past oral contraceptives use between breast cancer patients (73%) and healthy controls (72%), with a median use of 15 years in the total group (p=0.288). All other assessed baseline characteristics also did not differ between breast cancer patients and healthy controls (Table 6.1 and Table S6.1).

Table 6.1: Clinical characteristics of the study population.

Baseline characteristics	**Total** *n*=148	**Breast cancer** *n*=81	**Healthy controls** *n*=67	***p*-value**
Age - Years				0.929
Median (IQR)	62 (11)	62 (12)	62 (10)	
BMI - kg/m^2				0.450
Median (IQR)	25.3 (5)	25.3 (5)	25.2 (6)	
Karnofsky Performance Score - No. (%)*				0.452
60	5 (3)	3 (4)	2 (3)	
70	6 (4)	5 (6)	1 (2)	
80	29 (20)	17 (21)	12 (18)	
90	61 (41)	30 (37)	31 (46)	
100	46 (31)	26 (32)	20 (30)	
MUST score - No. (%)*				1.000
Low risk	136 (91)	74 (91)	62 (93)	
Medium risk	10 (7)	6 (7)	4 (6)	
High risk	0 (0)	0 (0)	0 (0)	
Unknown	2 (1)	1 (1)	1 (1)	
Diabetes Type 2 - No. (%)	11 (7)	7 (9)	4 (6)	0.755
Therapeutic antibiotics use last year - No. (%)	31 (21)	20 (25)	11 (16)	0.236
Duration of antibiotic use last year - Days				0.154
Mean (SD)	6 (3)	7 (3)	5 (3)	
Time since last antibiotic use - Weeks				0.337
Mean (SD)	25 (14)	22 (15)	28 (14)	
Oral contraceptives use past - No (%)	107 (72)	59 (73)	48 (72)	0.889
Oral contraceptives use - Years				0.288
Median (IQR)	15 (16)	12 (14)	17 (15)	
Time from last oral contraceptives use - Years				0.582
Mean (SD)	24 (14)	23 (15)	24 (13)	
Time hormonal IUD used - Years				0.123
Mean (SD)	9 (7)	7 (5)	13 (9)	

* Percentages do not add up to 100% due to rounding. IUD: intrauterine device.

More in detail, the breast cancer group consisted of 18 patients scheduled for neoadjuvant chemotherapy and 63 patients scheduled for adjuvant chemotherapy or tamoxifen after breast cancer surgery. Most tumours consisted of a ductal type (70%), followed by a lobular (21%) and mucinous (7%) type. Patients scheduled for neoadjuvant systemic treatment had a significantly larger clinical tumour size (p<0.001) and more advanced clinical breast cancer stage (p<0.001). All tumours were oestrogen receptor positive, according to the inclusion criteria. All other baseline characteristics were similar in patients scheduled for neoadjuvant chemotherapy or patients scheduled for adjuvant chemotherapy or tamoxifen (Table 6.2 and Table S6.2).

Table 6.2: Clinical characteristics of the breast cancer group.

Clinical characteristics	**Breast cancer** ***n*=81**	**Neoadjuvant** ***n*=18**	**Adjuvant** ***n*=63**	***p*-value**
Breast cancer stage – No. (%)*				**<0.001**
Stage I	42 (52)	2 (11)	40 (64)	
Stage II	35 (43)	12 (67)	23 (37)	
Stage III	3 (4)	3 (17)	0 (0)	
Unknown	1 (1)	1 (6)	0 (0)	
Clinical tumour size (cT) - mm				**<0.001**
Median (IQR)	20 (13)	28 (16)	18 (10)	
Clinical tumour grading - No. (%)*				0.202
Grade 1	21 (26)	2 (11)	19 (30)	
Grade 2	42 (52)	12 (67)	30 (48)	
Grade 3	12 (15)	3 (17)	9 (14)	
Unknown	6 (7)	1 (6)	5 (8)	
Tumour focality - No. (%)*				1.000
Unifocal tumour	64 (79)	14 (78)	50 (79)	
Multifocal tumour	16 (20)	3 (17)	13 (21)	
Unknown	1 (1)	1 (6)	0 (0)	
Tumour type - No. (%)*				0.051
Ductal	57 (70)	16 (89)	41 (65)	
Lobular	17 (21)	2 (11)	15 (24)	
Mucinous	6 (7)	0 (0)	6 (10)	
Unknown	1 (1)	0 (0)	1 (2)	
PR status - %				0.218
Median (IQR)	50 (85)	11.5 (91)	55 (80)	

* Percentages do not add up to 100% due to rounding. PR: Progesterone Receptor.

Intestinal microbiota in postmenopausal breast cancer patients and healthy controls

Microbial richness and diversity

In total, 148 faecal samples were collected. Faecal samples from breast cancer patients (*n*=81) were collected before starting neoadjuvant chemotherapy (*n*=18) or before starting adjuvant chemotherapy or tamoxifen (*n*=63) (Figure 6.1). Observed species richness (*p*=0.561) and Shannon index (*p*=0.207) were not different between breast cancer patients and the healthy controls (Figure 6.2).

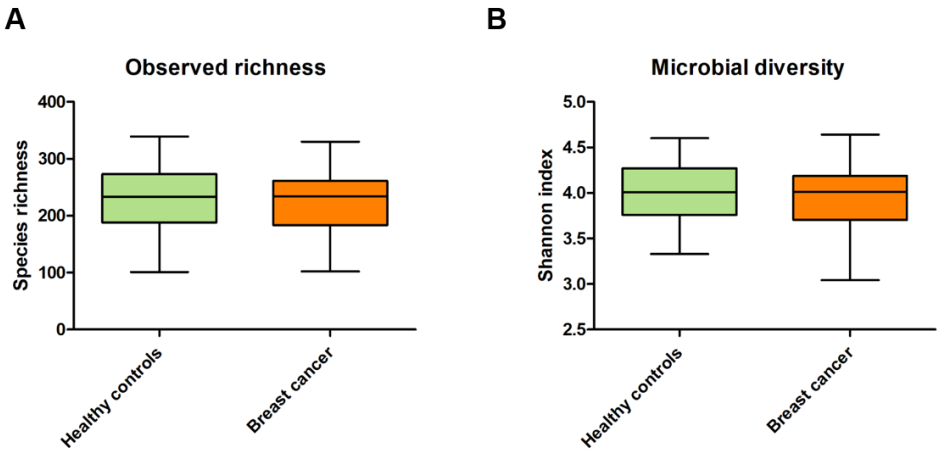

Figure 6.2: Microbial richness and diversity measures between breast cancer patients and the healthy controls. Observed species richness was analysed with a Mann-Whitney U test (**A**) and Shannon index with an unpaired t-test (**B**). For observed species richness the median and IQR are presented and for the Shannon index the mean and SD are presented (Table S6.3). Observed species richness (p=0.561) and Shannon index (p=0.207) were not different between breast cancer patients and healthy controls.

Microbial composition and community structure

In the total study population, Firmicutes were the most abundant phylum, followed by Bacteroidota and Actinobacteriota (Figure 6.3A). At family level, Lachnospiraceae and Ruminococcaceae were most abundant (Figure 6.3B).

While abundance of microbial genera varied per individual (Figure 6.3C), unconstrained ordination by means of Principal Components Analysis (PCA) indicated no clustering of samples from the breast cancer group or healthy control group. Similarly, PERMANOVA showed no statistically significant differences in overall microbial community structure at phylum (p=0.514) and genus level (p=0.292) between breast cancer patients and healthy controls (Figure 6.4). In addition, we did not found taxa that were differential abundant between breast cancer patients and healthy controls at phylum, family, or genus level.

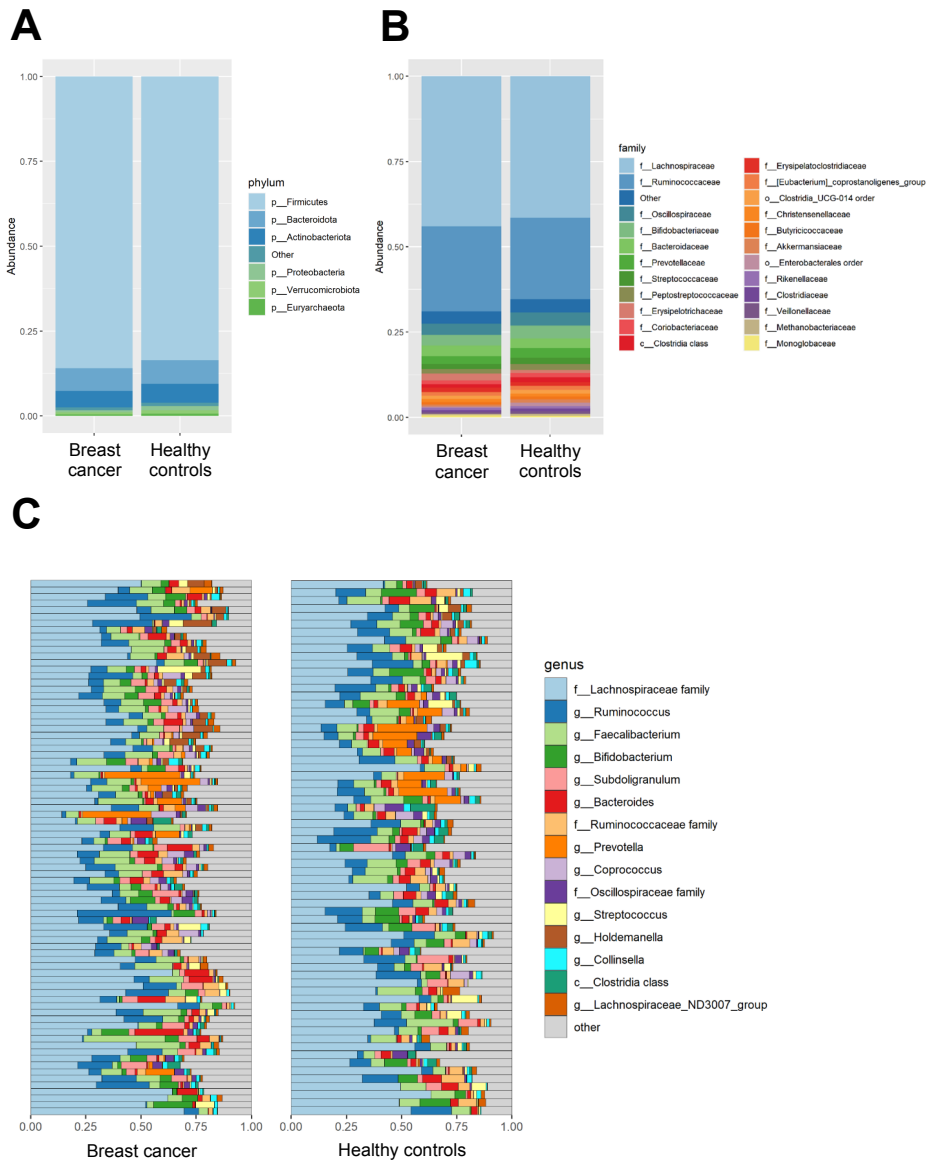

Figure 6.3: Relative abundances of most common bacterial taxa in breast cancer patients (*n*=81) and healthy controls (*n*=67). **A:** Relative abundance of bacterial phyla with prevalence >10% on group level. **B:** Relative abundance of bacterial families with prevalence >10% on group level. **C**: Relative abundance of the 15 most common genera in individual patients and healthy controls.

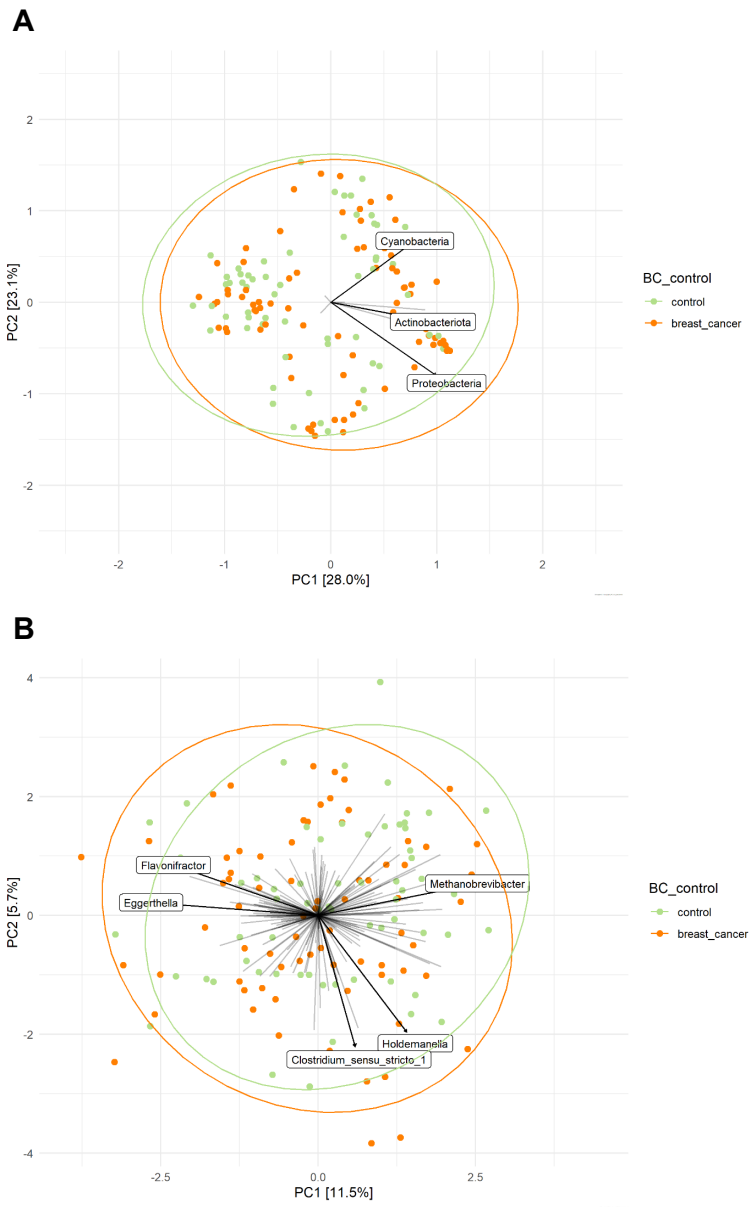

Figure 6.4: Ordination plots derived from unconstrained Principal Components Analysis (PCA) based on the Aitchison distance, showing composition of the microbial community at phylum (**A**) and genus (**B**) level for breast cancer patients and healthy controls. Taxa present in <5% of the samples were excluded for this analysis. Data were transformed using centre-log-ratio transformation. Names are given for taxa, which contributed most to overall microbial variation.

Intestinal microbiota in breast cancer patients scheduled for neoadjuvant systemic treatment or adjuvant systemic treatment compared to healthy controls

We defined two groups with a different treatment schedule within the group of breast cancer patients. Patients scheduled for adjuvant systemic therapy (*n*=63) underwent recent breast cancer surgery before inclusion. Patients scheduled for neoadjuvant systemic treatment (*n*=18) did not receive breast cancer surgery yet and had a larger clinical tumour size and a more advanced clinical breast cancer stage, as outlined above. To identify the potential influence of these factors on the intestinal microbiota, additional analyses were performed between these groups and healthy controls.

Microbial richness and diversity

Observed species richness (*p*=0.288) and the Shannon index (*p*=0.057) were not statistically significant different between patients scheduled for neoadjuvant systemic treatment, adjuvant systemic treatment, and healthy controls (Figure 6.5 and Table S6.4).

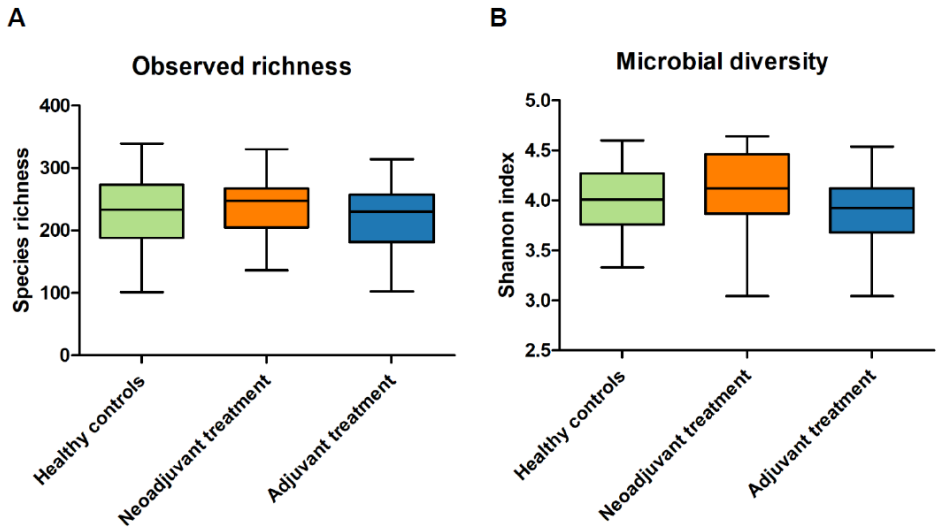

Figure 6.5: Microbial richness and diversity measures, in terms of observed richness (*p*=0.288) and Shannon index (*p*=0.057), of patients scheduled for neoadjuvant systemic treatment, adjuvant systemic treatment, and healthy controls analysed with the Kruskal-Wallis test (Table S6.4).

Microbial composition and community structure

PERMANOVA revealed a significant difference in overall microbial community structure between the three groups on phylum (p=0.042) and genus level (p=0.015) (Figure 6.6A). To identify which taxa contributed to the differences in overall microbial community structure, differential abundance analyses were performed. At phylum level ANCOM-II analysis did not identify differently abundant taxa between the three groups.

At family level, Veillonellaceae was found to be significantly different in abundance between the three groups, which was confirmed by a Kruskal-Wallis test (p=0.004) (Figure 6.6B$_1$; Table S6.5). Pairwise comparison with Bonferroni correction for multiple testing identified a higher abundance in patients scheduled for adjuvant systemic treatment compared to patients scheduled for neoadjuvant systemic treatment (p=0.004; Table S6.6). No differences in the abundance of Veillonellaceae were found in between patients scheduled for adjuvant systemic treatment or neoadjuvant systemic treatment compared to the healthy controls.

Additionally, the abundance of the genus *Dialister* was found to be significantly different between the three groups (Figure 6.6B$_2$; Table S6.5). The significance was confirmed with a Kruskal-Wallis test (p=0.003). Pairwise comparison with Bonferroni correction for multiple testing identified a higher abundance in patients scheduled for adjuvant systemic treatment compared to patients scheduled for neoadjuvant systemic treatment (p=0.003; Table S6.6). No differences in the abundance of *Dialister* were found in between patients scheduled for adjuvant systemic treatment or neoadjuvant systemic treatment compared to the healthy controls.

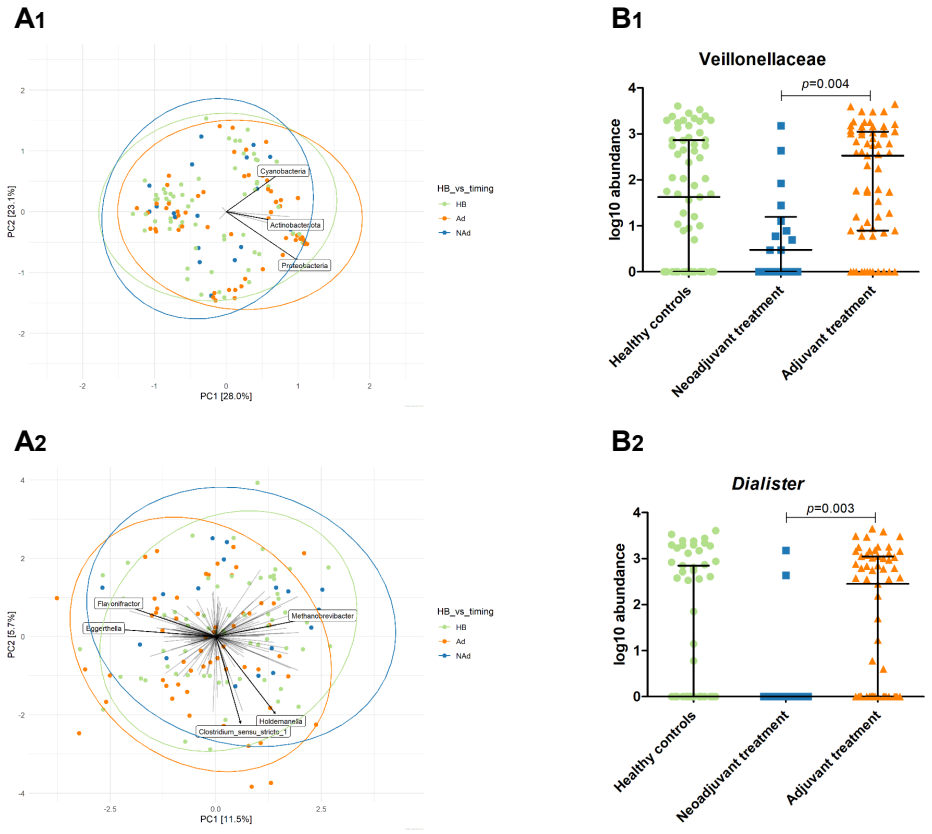

Figure 6.6: **A**: Ordination plots derived from unconstrained Principal Components Analysis (PCA) based on the Aitchison distance, showing composition of the microbial community at phylum (**A_1**) and genus (**A_2**) level for the neoadjuvant systemic treatment group, adjuvant systemic treatment group, and the healthy control group. Taxa that were present in <5% of the 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. **B**: Scatterplots showing the log^{10} abundance of taxa with significant differential abundance identified with ANCOM-II analyses between patients scheduled for neoadjuvant systemic treatment, adjuvant systemic treatment, and healthy controls. Kruskal-Wallis analyses confirmed significant differences between the three groups identified by ANCOM-II for Veillonellaceae (p=0.004) and *Dialister* (p=0.003). Adjusted p-values in the figures indicate significant differences in log^{10} abundance analysed with pairwise Mann-Whitney U test (Table S6.5 & S6.6).

Correlations between differentially abundant taxa and clinical characteristics

In the whole group, breast cancer stage was negatively correlated to the abundance of Veillonellaceae (*p*=0.003) and *Dialister* (*p*=0.007). In line with this, increasing clinical tumour size was associated with a lower abundance of Veillonellaceae (*p*=0.010). Other clinical characteristics showed no significant correlations with differentially abundant taxa (Table 6.3).

Table 6.3: Correlations by means of Kendall's Tau correlation coefficients (τ) between differentially abundant taxa and baseline characteristics.

Baseline characteristics	***Dialister***		**Veillonellaceae**	
	Correlation coefficient	***p*-value**	**Correlation coefficient**	***p*-value**
Clinical breast cancer stage	-0.264**	0.007	-0.272**	**0.003**
Clinical tumour grade	-0.072	0.465	-0.104	0.270
Clinical tumour size in mm	-0.156	0.063	-0.204*	**0.010**
BMI in kg/m^2	0.055	0.373	0.050	0.390
Intravenous prophylactic antibiotic use	0.051	0.606	0.102	0.278

** Correlation is significant at the 0.01 level (2-tailed), * Correlation is significant at the 0.05 level (2-tailed).

Influence of intravenous prophylactic antibiotic administration during operation on the intestinal microbiota in breast cancer patients scheduled for adjuvant systemic treatment

Within the patients who were scheduled for adjuvant systemic treatment, 51% (*n*=32) received intravenous prophylactic antibiotics during the operation; this included mostly patients who received breast reconstruction surgery. To rule out antibiotic administration as factor that may have influenced the microbiota composition, patients who were scheduled for adjuvant systemic treatment were divided into two groups: patients with prophylactic antibiotic administration and without prophylactic antibiotic administration during breast cancer surgery. Microbial richness and diversity measures in terms of observed species richness and Shannon index were not different between patients with or without prophylactic antibiotic administration during the operation. Moreover, no significant differences in Veillonellaceae and *Dialister* were found between these two groups (Figure 6.7 and Table S6.7 en S6.8).

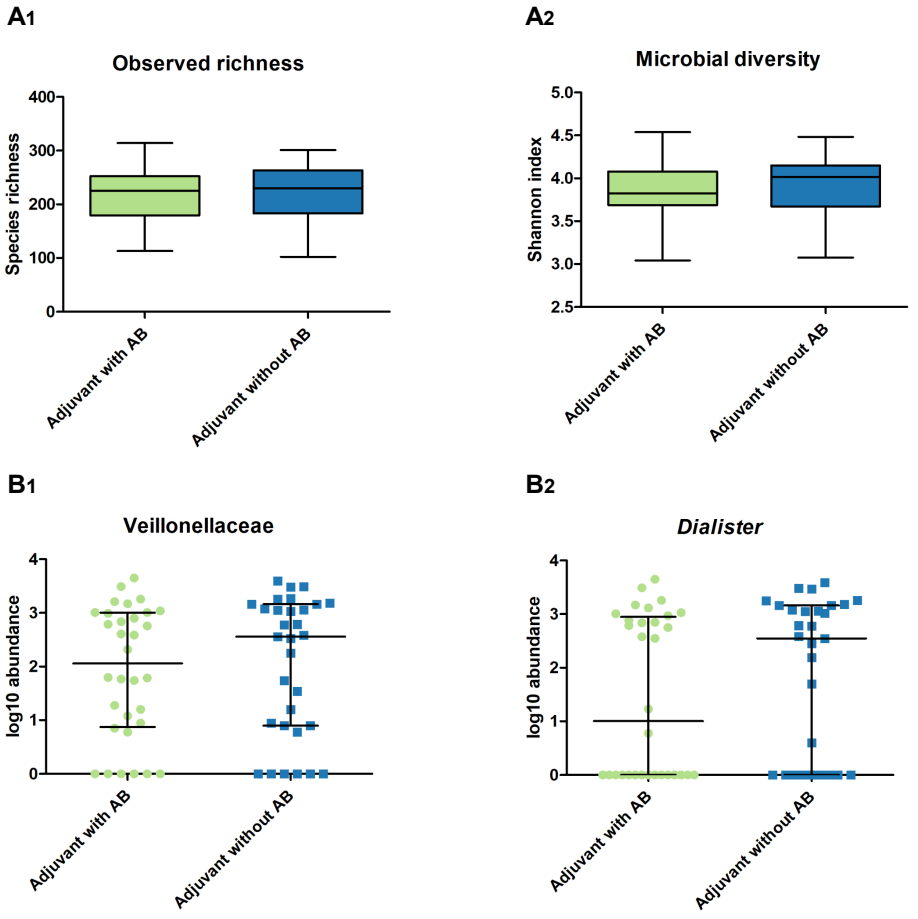

Figure 6.7: **A:** Microbial richness and diversity measures of patients scheduled for adjuvant systemic treatment with or without prophylactic antibiotic administration during breast cancer surgery. Microbial richness and diversity measures were analysed with a unpaired t-test, means and SD are presented. Observed species richness (p=0.947) and Shannon index (p=0.526) were not different between the two groups (Table S6.7). **B:** Scatterplots showing the $\log^{10}$ abundance of Veillonellaceae and *Dialister*. These were analysed with a Mann-Whitney U test, medians and IQR are presented. Veillonellaceae (p=0.629), and *Dialister* (p=0.315) were not different between the two groups (Table S6.8).

Discussion

This prospective cohort study investigated the intestinal microbiota richness, diversity, and composition in postmenopausal women with histologically proven ER+/HER2- breast cancer and postmenopausal healthy controls. Our study showed that microbial richness and diversity in terms of observed species richness and Shannon index and the abundance of specific microbial taxa did not significantly differ between breast cancer patients and healthy controls. Additional analysis of patients scheduled for neoadjuvant systemic treatment, and patients scheduled for adjuvant systemic treatment, and healthy controls also showed no significant differences in microbial richness and diversity. However, at phylum and genus level, faecal microbial community structures differentiated the three groups. Significant differences were found in the abundance of *Dialister* and its corresponding family Veillonellaceae between patients scheduled for neoadjuvant systemic treatment and adjuvant systemic treatment. This suggests that microbiota-modulating effects of recent breast cancer surgery may exist. Notably, antibiotic administrations during recent breast cancer surgery in patients scheduled for adjuvant systemic treatment did not significantly impact microbial richness and diversity or explain the increased abundance of Veillonellaceae and *Dialister*.

Regarding the microbiota richness, diversity and composition in breast cancer patients compared to healthy controls, the availability of clinical studies is limited. Our results showed that there were no significant differences in microbial richness and diversity in terms of observed species richness and Shannon index between breast cancer patients and healthy controls. These results contrast with two other clinical studies that investigated the association between the intestinal microbiota and pre-treatment postmenopausal breast cancer patients [19,20]. Goedert et al. observed a significantly lower microbial richness and diversity, in terms of observed species richness and Chao1 index, in breast cancer patients compared to healthy controls [20]. The opposite results were found in a study by Zhu et al., who showed that breast cancer patients had higher observed species richness and Chao1 index [19]. In the studies by Goedert et al. and Zhu et al., no distinction was made between different types of breast cancer. This makes the breast cancer group less homogenous than our breast cancer group, which only included ER+/HER2- breast cancer patients.
When examining differences in microbial composition, Zhu et al. found differences on species level [19], where Goedert et al. [20] did not find any differences in microbial composition after adjustment for multiple comparisons. Concerning microbiota composition, our results are more in line with the results of Goedert et al. and suggest that the intestinal microbiota composition is not associated with postmenopausal

breast cancer. Even though no differences were found in microbial composition based on 16S rRNA gene sequencing, no conclusion could be drawn concerning the functional potential and activity of the bacteria present. Because the presence of bacteria does not necessarily indicate that they also perform their presumed function. Previous studies have indicated that members of the intestinal microbiota could belong to the so-called estrobolome, which is defined as the aggregate of intestinal bacterial genes capable of metabolising oestrogens [11]. For instance, the bacterial enzyme β-glucuronidase has been shown to increase intestinal oestrogen reabsorption into the circulation [6]. A relationship between intestinal microbiota-related oestrogen metabolism and systemic oestrogen levels has already been demonstrated in small groups [11,33,34]. Specifically in breast cancer, high levels of circulating oestrogens are related to the development of oestrogen receptor positive breast cancer [11]. To further study microbiota-host interactions, future research should also include functional assessments of intestinal microbiota activity. For example, bacterial β-glucuronidase activity in postmenopausal ER+/HER2- breast cancer patients could be explored by conducting β-glucuronidase activity assays, as described by Biernat et al [35]. In addition, it will be highly relevant to combine β-glucuronidase activity assay outcomes with high-throughput whole metagenomic shotgun sequencing to determine bacterial metabolic capacity [4].

Remarkably, an increased abundance of *Dialister* and its corresponding family Veillonellaceae was observed in patients scheduled for adjuvant systemic treatment compared to patients scheduled for neoadjuvant systemic treatment. In addition, breast cancer stage and clinical tumour size were negatively correlated with the abundance of Veillonellaceae and *Dialister* in the whole breast cancer group, which might be caused by the fact that patients scheduled for neoadjuvant systemic treatment had significant higher clinical breast cancer stages and increased tumour sizes compared to patients scheduled for adjuvant systemic treatment. Since patients scheduled for adjuvant systemic treatment received breast cancer surgery before inclusion, while patients scheduled for neoadjuvant systemic treatment not, recent breast cancer surgery might have influenced the differential abundance of *Dialister* and Veillonellaceae.

Increased abundance of *Dialister* potentially resulted from reduced abundance of other bacteria, more susceptible to microbiota-modulating effects of breast cancer surgery, creating an environment where *Dialister* can bloom [36]. Possible microbiota-modulating effects of breast cancer surgery may include antibiotic administration or tumour resection. As antibiotic administration is a well-known microbiota-modulating factor, prophylactic antibiotic administration with cefazolin, which was administered in 51% of

the patients, might be one of the microbiota-modulating effects of breast cancer surgery associated with the bloom of *Dialister* [37].

Surprisingly, in patients scheduled for adjuvant treatment, the abundance of *Dialister* was not different between patients with or without intravenous prophylactic antibiotic cefazolin administration during breast cancer surgery. This means that prophylactic cefazolin administration was not associated with the bloom of Dialister in patients scheduled for adjuvant systemic treatment. Cefazolin has been shown to be antimicrobial active against gram-positive bacteria and only a few specific gram-negative bacteria, like *Escherichia coli* and *Proteus mirabilis*. It can therefore be assumed that cefazolin has no effect on the *Dialister* and Veillonellaceae, which are gram-negative stains [38,39]. Consequently, other non-antibiotic related perioperative microbiota-modulating factors may be responsible for the bloom of *Dialister* and its corresponding family Veillonellaceae in patients scheduled for adjuvant systemic treatment.

To the best of our knowledge, no studies are available that have investigated the effect of breast cancer surgery or extra-gastrointestinal surgery on intestinal microbiota and specifically described the effect of breast cancer surgery on *Dialister* and its corresponding family Veillonellaceae [40]. Similar to the increased Veillonelaceae in our study, increased levels of the pathogenic *Veillonella* of the family Veillonellaceae have been observed in colorectal cancer patients after colorectal cancer surgery [41]. Liang et al. (2019) [42] have demonstrated an increased abundance of *Dialister* after gastrectomy in patients with gastric cancer. In addition, the abundances of *Veillonella* and *Dialister* have been shown to be increased after bariatric surgery [43,44]. This may indicate that not specifically breast cancer surgery, but surgery in general might modulate the postoperative intestinal microbiota composition. However, specific surgery-related factors that might contribute to the bloom of *Dialister* and Veilonellaceae e.g. weight reduction after bariatric surgery were not discussed in these prior studies. In addition to the surgery itself, it might be speculated that also the hospital environment, surgery-related medication, stress, or short-term fasting might contribute to the bloom of *Dialister* and Veillonellaceae in patients scheduled for adjuvant systemic treatment. To further explore the long-term effects and impact on quality of life of microbiota-modulating effects of recent (breast) cancer surgery, longitudinal faecal sampling of breast cancer patients without adjuvant systemic cancer treatment is recommended.

There are several strengths and limitations of this study. Unique to this study is its homogenous study population of postmenopausal ER+ and HER2- breast cancer patients. In addition, in-depth analyses of antibiotic administration have been performed to reveal potentially confounding effects of antibiotic administration.

Limitations of the present study can be found in the fact that even if bacteria are present, this does not necessarily mean that they also perform their presumed function. Therefore, we highly recommend the further study of functional microbiota analyses, for instance β-glucuronidase activity assays and whole metagenomic shotgun sequencing. This will provide more insight into the capacity of the intestinal microbiota to metabolise oestrogen [6,11]. Another limitation is that, although none of the patients had received previous systemic cancer treatment, 63 of the 81 breast cancer patients recently received breast cancer surgery. To omit the microbiota-modulating effects of breast cancer surgery, additional analyses were performed. As a result, the breast cancer group size that had not received breast cancer surgery yet (patients scheduled for neoadjuvant systemic treatment) was reduced to 18 patients. Nonetheless, the potential microbiota-modulating effects of breast cancer surgery should not be neglected. An increased sample size of treatment-naive breast cancer patients who are scheduled for neoadjuvant systemic treatment is warranted to confirm our observations. Alternatively, faecal sample collection prior to surgery in patients scheduled for adjuvant systemic treatment is highly recommended. Ideally future studies should perform longitudinal faecal sampling to assess intestinal microbiota changes over time and related this to clinical characteristics.

In conclusion, intestinal microbiota richness, diversity and composition were not associated with postmenopausal breast cancer. Surprisingly, this study identified that *Dialister* and Veillonellaceae were increased in patients who received recent breast cancer surgery. This finding was independent of intravenous prophylactic antibiotic administration with cefazolin during surgery. The potential antibiotic-independent microbiota-modulating effect of breast cancer surgery requires further investigation with longitudinal faecal sampling.

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Supplementary material and methods

Faecal microbiota analyses

In order to extract metagenomic DNA, 250 mg of the frozen faecal samples were homogenised 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 MagMax™ Total Nucleic Acid Isolation Kit *(Thermo Fisher Scientific)* and mixed with 175 µl supernatant of faeces 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 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 [1]. Pooled amplicons from the triplicate reactions were purified using AMPure XP purification beads (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*). 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) [2].

The pre-processing of sequencing data, using an in-house pipeline based upon DADA2 (R version 4.0.3) [3], consisted of the following steps: reads filtering, identification of sequencing errors, dereplication, and removal of chimeric sequences. In order to assign taxonomy, DECIPHER[4] 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 [5]. 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 816 ASVs were maintained for downstream analysis. The final file was saved in the phyloseq format [6].

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Supplementary tables

Table S6.1: Clinical characteristics of the study population.

Baseline characteristics	Total *N*=148	Breast cancer *n*=81	Healthy breast *n*=67	*p*-value
Age - Years				0.929
Median (IQR)	62 (11)	62 (12)	62 (10)	
25-75%	57-68	57-69	58-68	
BMI - kg/m^2				0.450
Median (IQR)	25.3 (5)	25.3 (5)	25.2 (6)	
25-75%	23.7-28.4	23.9-28.9	22.9-28.5	
Karnofsky Performance Score				0.582
Median (IQR)	90 (20)	90 (20)	90 (10)	
25-75%	80-100	80-100	90-100	
Karnofsky Performance Score - No. (%)				0.452
60	5 (3)	3 (4)	2 (3)	
70	6 (4)	5 (6)	1 (2)	
80	29 (20)	17 (21)	12 (18)	
90	61 (41)	30 (37)	31 (46)	
100	46 (31)	26 (32)	20 (30)	
Unknown	1 (1)	0 (0)	1 (1)	
MUST score - No. (%)*				1.000
Low risk	136 (91)	74 (91)	62 (93)	
Medium risk	10 (7)	6 (7)	4 (6)	
High risk	0 (0)	0 (0)	0 (0)	
Unknown	2 (1)	1 (1)	1 (1)	
Diabetes Type 2 - No. (%)*				0.755
No	136 (91)	74 (91)	62 (93)	
Yes	11 (7)	7 (9)	4 (6)	
Unknown	1 (1)	0 (0)	1 (1)	
Antibiotic use last year - No. (%)*				0.236
No	116 (78)	61 (75)	55 (82)	
Yes	31 (21)	20 (25)	11 (16)	
Unknown	1 (1)	0 (0)	1 (1)	
Duration of antibiotic use last year – Days				0.154
Mean (SD)	6 (3)	7 (3)	5 (3)	
Min - Max	1-14	1-14	1-10	
Time since last antibiotic use – Weeks				0.370
Mean (SD)	25 (14)	22 (15)	28 (14)	
Min - Max	2-52	2-52	13-48	
Oral contraceptives use past - No (%)*				0.889
No	39 (26)	21 (26)	18 (27)	
Yes	107 (72)	59 (73)	48 (72)	
Unknown	2 (1)	1 (1)	1 (1)	
Oral contraceptives use - Years				0.288
Median (IQR)	15 (16)	12 (14)	17 (15)	
25-75%	8-24	8-22	10-25	
Time from oral contraceptives use - Years				0.582
Mean (SD)	24 (14)	23 (15)	24 (13)	
Min - Max	12-35	0-49	4-49	
Time hormone IUD used - Years				0.123
Mean (SD)	9 (7)	7 (5)	13 (9)	
Min - Max	1-28	1-15	5-28	

* Percentages do not add up to 100% due to rounding. IUD = intrauterine device.

Table S6.2: Clinical characteristics of the study population – Breast cancer group.

Baseline characteristics	**Breast cancer** ***n*=81**	**Neoadjuvant** ***n*=18**	**Adjuvant** ***n*=63**	***p*-value**
Breast cancer stage – No. (%)*				**<0.001**
Stage I	42 (52)	2 (11)	40 (64)	
Stage II	35 (43)	12 (67)	23 (37)	
Stage III	3 (4)	3 (17)	0 (0)	
Unknown	1 (1)	1 (6)	0 (0)	
Clinical tumour size (cT) - mm				**<0.001**
Median (IQR)	20 (13)	28 (16)	18 (10)	
25-75%	15-28	22-38	14-24	
Clinical tumour grading – No. (%)*				0.202
Grade 1	21 (26)	2 (11)	19 (30)	
Grade 2	42 (52)	12 (67)	30 (48)	
Grade 3	12 (15)	3 (17)	9 (14)	
Unknown	6 (7)	1 (6)	5 (8)	
Tumour focality - No. (%)*				1.000
Unifocal	64 (79)	14 (78)	50 (79)	
Multifocal	16 (20)	3 (17)	13 (21)	
Unknown	1 (1)	1 (6)	0 (0)	
Tumour type - No. (%)*				0.051
Ductal	57 (70)	16 (89)	41 (65)	
Lobular	17 (21)	2 (11)	15 (24)	
Mucinous	6 (7)	0 (0)	6 (10)	
	1 (1)	0 (0)	1 (2)	
Prophylactic antibiotic use - No. (%)				**<0.001**
No	49 (60)	18 (100)	31 (49)	
Yes	32 (40)	0 (0)	32 (51)	
Chemotherapy past - No. (%)				0.345
No	78 (96)	18 (100)	60 (95)	
Yes	3 (4)	0 (0)	3 (5)	
Time since last chemo – Weeks		-		
Mean (SD)	196 (278)		196 (278)	
Min-Max	22-517		22-517	
Radiotherapy received - No. (%)				**<0.001**
No	16 (20)	3 (17)	13 (21)	
Yes	65 (80)	15 (83)	50 (79)	
Timing radiotherapy - No. (%)*				**<0.001**
Before therapy	18 (22)	0 (0)	18 (29)	
During therapy	19 (24)	0 (0)	19 (30)	
After therapy	28 (35)	15 (83)	13 (21)	
Unknown	16 (20)	3 (17)	13 (21)	

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

Table S6.3: Microbial richness and diversity measures.

Microbial richness and diversity	Total *N*=148	Breast cancer *n*=81	Healthy controls *n*=67	*p*-value
Observed richness				0.561
Median (IQR)	234 (79)	234 (78)	233 (85)	
25-75%	185-264	184-261	188-273	
Shannon index				0.207
Mean (SD)	3.95 (0.36)	3.92 (0.38)	4.00 (0.33)	
Min - Max	3.04-4.64	3.04-4.64	3.33-4.60	

Table S6.4: Microbial richness and diversity measures.

Microbial richness and diversity	Healthy Controls *n*=67	Neoadjuvant *n*=18	Adjuvant *n*=63	*p*-value
Observed richness				0.288
Median (IQR)	233 (85)	248 (62)	230 (76)	
25-75%	188-273	205-267	181-257	
Shannon index				0.057
Mean (SD)	4.00 (0.33)	4.07 (0.44)	3.88 (0.36)	
Min - Max	3.33-4.60	3.04-4.64	3.04-4.54	

Table S6.5: Differential abundant taxa on family and genus level of patients scheduled for neoadjuvant treatment (NA), adjuvant treatment (A), and healthy controls (HC).

Taxa	Healthy Controls *n*=67	Neoadjuvant *n*=18	Adjuvant *n*=63	*p*-value
Veillonellaceae				**0.004**
Median (IQR)	1.63 (2.87)	0.48 (1.2)	2.53 (2.15)	
25%-75%	0.00-2.87	0.00-1.20	0.90-3.05	
Dialister				**0.003**
Median (IQR)	0.00 (2.85)	0.00 (0.00)	2.45 (3.05)	
25%-75%	0.00-2.85	0.00-0.00	0.00-3.05	

$\log^{10}$ abundance of taxa are presented.

Table S6.6: Adjusted *P*-values of pairwise comparison of the differential abundant taxa of patients scheduled for neoadjuvant treatment (NA), adjuvant treatment (A), and healthy controls (HC).

Taxa	HC vs. NA	HC vs. A	NA vs. A
Veillonellaceae	0.116	0.226	**0.004**
Dialister	0.089	0.234	**0.003**

P-values have been adjusted by the Bonferroni correction for multiple testing. $\log^{10}$ abundance of taxa are presented.

Table S6.7: Microbial richness and diversity measures of breast cancer patients scheduled for adjuvant treatment with or without prophylactic antibiotic administration during the operation.

Microbial richness and diversity	**Adjuvant with AB** ***n*=32**	**Adjuvant without AB** ***n*=31**	***p*-value**
Observed richness			0.947
Mean (SD)	218 (48)	219 (50)	
Min - Max	113-314	102-301	
Shannon index			0.526
Mean (SD)	3.85 (0.33)	3.91 (0.38)	
Min - Max	3.04-4.54	3.08-4.48	

Table S6.8: Differential abundant taxa on family and genus level of breast cancer patients scheduled for adjuvant treatment with or without prophylactic antibiotic administration during the operation.

Taxa	**Adjuvant with AB** ***n*=32**	**Adjuvant without AB** ***n*=31**	***p*-value**
Veillonellaceae			0.629
Median (IQR)	2.06 (2.13)	2.56 (2.26)	
25%-75%	0.87-3.00	0.90-3.16	
Dialister			0.315
Median (IQR)	1.00 (2.95)	2.55 (3.16)	
25%-75%	0.00-2.95	0.00-3.16	

log^{10} abundance of taxa are presented.

CHAPTER 7

Exploring the potential of breast microbiota as biomarker for breast cancer and therapeutic response

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Abstract

Breast cancer tissue contains its own unique microbiota. Emerging preclinical data implicates that breast microbiota dysbiosis contributes to breast cancer initiation and progression. Furthermore, the breast microbiota may be a promising biomarker for treatment selection and prognosis. Differences in breast microbiota composition have been found between breast cancer subtypes and disease severities that may contribute to immunosuppression, enabling tumour cells to evade immune destruction. Interactions between breast microbiota, gut microbiota, and immune system are proposed, all forming potential targets to increase therapeutic efficacy. In addition, since the gut microbiota affects the host immune system and systemic availability of oestrogen and bile acids, known to influence tumour biology, gut microbiota modulation could be used to manipulate breast microbiota composition. Identifying breast and gut microbial compositions that respond positively to certain anti-cancer therapeutics, could significantly reduce cancer burden. Additional research is needed to unravel the complexity of breast microbiota functioning and its interactions with the gut and the immune system. In this review, developments in the understanding of breast microbiota and its interaction with the immune system and the gut microbiota are discussed. Furthermore, the biomarker potential of breast microbiota is evaluated in conjunction with possible strategies to target microbiota in order to improve breast cancer treatment.

Introduction

Breast cancer is a heterogeneous malignancy, accounting for 30% of all female cancers [1,2]. Molecular subtypes of breast cancer have been characterized, including luminal A, luminal B, human epidermal growth factor receptor-2 (*HER2)* enriched, and triple negative breast cancer (TNBC) [3]. Luminal A subtype is enriched with the oestrogen-receptor (ER), and has the best clinical prognosis among all intrinsic subtypes, which is mostly due to the favourable response to endocrine therapy. Compared to luminal A cancers, luminal B cancers show lower expression of *ER*-related genes, but have higher expression of proliferation-related genes and variable expression of *HER2*-related genes [4]. The HER2 subtype comprises approximately 15% of all invasive breast cancers and is more aggressive in nature than luminal-like cancers [4]. This subtype is associated with the absence of ER and PR expression, and overexpression of *HER2/HER2* signalling-associated genes and genes located in *HER2* amplicon on chromosome *17q12*. TNBC lacks expression of ER, progesterone receptor (PR), and HER2.

Segregating breast tumours into subtypes is valuable for predicting prognosis and guiding clinical decision making, in which an array of anti-cancer therapeutics can be selected, including chemotherapy, anti-oestrogen therapy, and anti-HER2-targeted agents [5]. Nevertheless, limitations in traditional subtyping exist, since it does not take interactions with the tumour microenvironment (TME) into account. Healthy tissue stroma normally acts as a physiological barrier against tumour formation [6]. During development of neoplastic cells, the adjacent tissue microenvironment is transformed by autocrine and paracrine mechanisms of cancer cells to maintain optimal conditions for tumour survival and progression. These alterations result in tumour-associated stroma, known as the TME. The TME consists of a heterogeneous collection of fibroblasts, myofibroblasts, neuroendocrine cells, adipocytes, extracellular matrix (ECM), blood and lymphatic vascular networks, and is enriched with many immune and inflammatory cells [6].

Recent findings demonstrate the existence of microbiota, both in healthy stroma and in the TME of extra-intestinal organs, including the lung, pancreas, and breast [7-9]. It is suggested that organ-specific microbiota play a role in tumour development and therapeutic resistance. In this review, microbial compositions in tumorous and healthy breast tissue are discussed. Moreover, microbiota interactions with the immune system, the gut microbiota and anti-cancer therapeutics are illustrated. Lastly, the biomarker potential of breast microbiota is evaluated in conjunction with possible strategies to target microbiota in order to improve breast cancer treatment.

Breast microbiota in health and disease

Many extra-intestinal tissues were traditionally considered sterile until the advancement of culture-independent, DNA sequencing techniques, using next-generation sequencing (NGS) technology [10]. The discovered link between colon cancer and certain gut bacteria, led to novel investigations of organ-specific microbiota in the development of cancers in multiple tissues [11]. The existence of microbes in breast tissue was firstly demonstrated in 2014 by Xuan et al. [8]. There is increasing evidence of the existence of a unique microbiota in breast tissue that is distinct from the overlying breast skin, and is unrelated to mastitis [12]. The breast microbiota is dominated by the phyla Proteobacteria and Firmicutes, which likely can be attributed to the fatty acid rich environment in the breast. The discovery of microbiota in breast tissue has brought the attention to its potential role in the pathophysiological process of breast carcinogenesis.

Difference between healthy and tumorous breast tissue

Several studies have examined normal breast tissue adjacent to the breast tumour, commonly approximately 5 cm away from the tumour margin. One study found a higher bacterial load and bacterial richness in breast tumour tissue, compared to adjacent normal breast tissue [13]. At the phylum level, highest presence of Proteobacteria was found in breast cancer tissue, compared to highest presence of Actinobacteria in adjacent normal breast tissue [14]. Families *Pseudomonadaceae, Sphingomonadaceae, Alcaligenaceae Ruminococcaceae* and *Clostridia* seemed to be decreased in adjacent breast tissue compared to breast cancer tissue [8,15,16]. At the class level, the absolute abundance in breast tumour tissue was highest for the classes Clostridia and Bacteroidia [15], while at the genera level *Ralstonia*, followed by *Methylobacterium* and *Sphingomonas* had the highest abundance [15,17]. In normal adjacent breast tissue, the family *Enterobacteriaceae*, of which *Escherichia coli (E. coli)* is a member, was increased compared to healthy controls [18]. *E. coli* isolates, cultured from normal adjacent tissue of breast cancer patients, induced DNA double-stranded breaks *in vitro* in HeLa cells [18]. Similarly, pks-positive *E. coli* bacteria induced DNA damage in human intestinal organoids by the production of the genotoxin colibactin [19]. This finding provides an explanation for a possible pathway by which bacteria, present in the breast, may contribute to breast carcinogenesis.

The microbiota composition of breast tissue adjacent to the tumour has also been compared with healthy breast tissue of women without breast cancer. Compared to healthy breast tissue, adjacent normal breast tissue contained higher relative

abundance of bacteria belonging to the phylum Bacteroidetes, the family *Comamondaceae*, and the genera *Bacillus* and *Staphylococcus* [18], which could also suggest a gradual change in microbiota from healthy to cancerous states. However, not all studies identified differences between breast tumour tissue microbiota and adjacent normal breast tissue microbiota [16-18]. Similar microbiota in paired normal tissue and tumour tissue could suggest a predisposition of the entire breast tissue to carcinogenesis, and thereby potentially predict breast cancer risk.

Microbiota composition in malignant versus benign breast diseases

The breast microbiota composition of malignant disease, mainly invasive ductal carcinoma, can clearly be distinguished from that of benign breast disease, including fibro adenoma, intraductal papilloma and atypical hyperplasia [20]. Phylum Proteobacteria, families *Micrococcaceae, Caulobacteraceae, Rhodobacteraceae, Nocordioidaceae* and *Methylobacteriaceae,* and genus *Propionicimonas*, were significantly more present in malignant breast cancer than in benign breast disease [20]. In another study, investigating breast tissue of women with ER+ breast cancer, increased abundance of specific genera, including *Fusobacterium, Atopobium, Hydrogenophaga, Gluconacetobacter* and *Lactobaccilus*, was demonstrated in invasive breast cancer compared to benign disease [12].

Different stages of breast cancer also coincide with a specific microbial profile. Higher stages were associated with reduced bacterial load [8]. In stage 1 breast cancer, Proteobacteria, *Ruminococcaceae* and *Hyphomicrobium* were most abundant, whereas stage 2 breast cancer showed highest abundance in Euryarchaeota, Firmicutes, Spirochaetes, and the genus *Sporosarcina* [15]. Stage 3 and stage 4 breast cancer exhibited elevation in Thermi, Gemmatimonadetes, and Tenericutes, and higher abundance of *Bosea*. The genus *Agrococcus* was progressively enriched with increased malignancy [20].

Breast microbiome in breast cancer subtypes

Alongside, the breast tumour subtypes luminal A, luminal B, TNBC and HER2+ were found to exhibit unique subtype-associated microbiota [13,15,21,22]. Luminal A tumour tissue exhibited highest abundance in order Xanthomonadales and luminal B tumour tissue showed highest abundance of the genus *Clostridium* [15]. In most hormone positive breast cancer tissues, the genus *Methylobacterium* had a decreased presence compared to healthy breast tissue [16]. In HER2 tumours, *Akkermansia* was most abundantly found [15]. TNBC tissue was shown to harbour *Streptococcaceae* and *Ruminococcus* at the highest level in one study [15]. In another study, using PathoChip

array, a higher percentage of *Prevotella, Brevundimonas, Arcanobacterium, Escherichia, Sphingobacterium, Actinomyces,* and *Rothia,* was found in triple negative breast cancer tissue compared to healthy breast tissue [21].

All these studies show considerable heterogeneity in microbiota composition of healthy breast, normal adjacent and tumorous breast microbiota. It seems clear that a breast microbiome exists and that it may change in the course of breast cancer development. However, a clear breast cancer microbiota profile has not been defined yet. This could be due to several reasons: (1) the breast organ contains a low microbial biomass, which increases the risk of contamination influencing the results, (2) small sample sizes, (3) and the variety of extraction and sequencing methods used. Understanding microbial differences and their role in carcinogenesis will be important to estimate breast cancer risk from a microbiome perspective. In Table 7.1, studies evaluating breast microbiota composition are chronologically illustrated in detail. In Table 7.2, the breast microbiota compositions of various breast cancer subtypes can be found.

Immune involvement in breast carcinogenesis

The immune system plays a significant role in the initiation, progression and control of cancer. This is illustrated by the process of cancer immuno-editing, which describes the evolving interactions between host immunity and cancer cells, consisting of three distinct phases: elimination, equilibrium and escape [23]. In the elimination phase, tumour cells are successfully recognized and eliminated by immune cells. During equilibrium, transformed cells escape elimination, and are able to proliferate. However, this proliferation is still controlled by the immune system, in contrast to the escape phase, which is defined by uncontrolled proliferation. In a healthy situation, a balance exists between pro-inflammatory and anti-inflammatory signals, which is partly regulated by co-stimulation and co-inhibition of T cells [24]. This enables sufficient clearance of foreign antigens, but concurrently prevents uncontrolled inflammation. Tumours are able to avoid immune destruction by several mechanisms, including loss of antigenicity and recruitment of immunosuppressive leukocytes [25].

Table 7.1: Overview of studies analysing breast microbiota composition.

Study	Sample size	Sample source	Breast cancer subtype	Microbiota composition of breast cancer tissue, adjacent breast tissue and healthy breast tissue	Microbiota composition of breast cancer subtypes, grades and stages
Xuan et al [8]	Analysis 1: 20 breast cancer patients; breast cancer tissue and adjacent breast tissue obtained from same patient Analysis 2: 39 breast cancer tissue samples, subdivided in breast cancer stages: Stage I: *n*=18 Stage II: *n*=9 Stage III: *n*=12	FFPE* & Fresh frozen	ER+	Comparing adjacent breast tissue with breast cancer tissue, higher absolute abundance of *Sphingomonas yanoikuyae* was found in adjacent tissue. Comparing breast cancer tissue with adjacent breast tissue, higher relative abundance of *Methylobacterium radiotolerans* was found in breast cancer tissue.	Decreased bacterial load was found in breast cancer tissue with more severe breast cancer stages.
Urbaniak et al [18]	58 adjacent breast tissue samples 13 benign breast tumours 45 malignant breast cancers 23 healthy breast tissue samples	Fresh tissue	†	Comparing healthy breast tissue with adjacent breast tissue, increased relative abundance of *Prevotella, Lactococcus*, *Streptococcus, Corynebacterium,* and *Micrococcus* was found in healthy breast tissue. Comparing adjacent breast tissue with healthy breast tissue, increased relative abundance of genera *Bacillus* and *Staphylococcus*, families *Enterobacteriaceae* and *Comamondaceae*, and phylum Bacteroidetes was found in adjacent breast tissue. Comparing adjacent breast tissue from patients with benign tumours with adjacent breast tissue from patients with malignant tumours and healthy tissue, microbial profiles of benign tumours were more similar to microbial profiles of malignant tumours than to microbial profiles of healthy breast tissue.	Comparing microbiota composition of adjacent breast tissue of women with different cancer stages, no differences were found.

Table 7.1: (continued)

Study	Sample size	Sample source	Breast cancer subtype	Microbiota composition of breast cancer tissue, adjacent breast tissue and healthy breast tissue	Microbiota composition of breast cancer subtypes, grades and stages
Banerjee et al [21]	100 triple-negative breast cancer tissue samples 17 matched (adjacent breast tissue) and 20 non-matched controls (healthy breast tissue)	FFPE	TNBC‡	Comparing TNBC tissue with healthy breast tissue, higher percentage of probes of the genera *Prevotella, Brevundimonas, Arcanobacterium, Escherichia, Sphingobacterium, Actinomyces,* and *Rothia* was found in TNBC tissue. Comparing TNBC tissue with healthy breast tissue, higher percentage of probes of the viruses MMTV, Hepatitis C1, EBV1, BPSV, HCMV, KSHV, PCPV, HPV2, HTLV-2, HPV6B, MCPV, HTLV1, HPV18, Hepatitis B, SV40, HPV16, HHV1, Okra Mosaic Virus, FSV, Hepatitis GB, Viroids, and Orf Virus was found in TNBC tissue. Comparing TNBC tissue with healthy breast tissue, higher percentage of probes of the fungi *Pleistophora* and *Paecilomyces* was found in TNBC tissue.	In TNBC, highest prevalence of probes detecting *Arcanobacterium* (in 75% of samples) was found, followed by *Brevundimonas, Sphingobacteria, Providencia, Prevotella, Brucela, Escherichia, Actinomyces, Mobiluncus, Propiniobacteria, Geobacillus, Rothia, Peptinophilus,* and *Capnocytophaga.* The highest hybridization signal in TNBC was found for the bacterial probe of *Prevotella,* for the viral probe of Herpesvirus, for the fungal probe of *Piedra,* and for the parasitic probe of *Trichuris.*
Hieken et al [12]	28 adjacent breast tissue samples 13 benign breast tumours 15 malignant breast cancer (67% stage I, 33% stage II, 13% positive lymph node)	Fresh frozen	All ER/PR+, 29% HER2+	Comparing malignant breast cancer tissue with benign breast tumours, higher relative abundance of *Fusobacterium, Atopobium, Hydrogenophaga, Gluconacetobacter,* and *Lactobaccilus* was found in malignant breast tissue.	†
Wang et al [16]	57 breast cancer tissue samples 21 healthy breast tissue samples Breast cancer stages: Stage I: *n*=22 Stage II: *n*=30 Stage III: *n*=5	Fresh frozen	ER/PR+ (*n*=50) HER2+ (*n*=9)	Comparing breast cancer tissue with healthy breast tissue, decreased relative abundance of *Methylobacterium* and increased relative abundance of Alcaligenaceae was found in breast cancer tissue. Comparing breast cancer tissue with adjacent breast tissue, no significant differences were found.	Comparing HR+ breast cancer tissue with HR- breast cancer tissue, increased Shannon diversity and decreased relative abundance of *Methylobacterium* was found in HR+ breast cancer.

Table 7.1: (continued)

Study	Sample size	Sample source	Breast cancer subtype	Microbiota composition of breast cancer tissue, adjacent breast tissue and healthy breast tissue	Microbiota composition of breast cancer subtypes, grades and stages
Thompson et al [14]	The Cancer Genome Atlas (TCGA) 668 breast cancer tissue samples 72 adjacent breast tissue samples	Fresh frozen	ER+, HER2+, TNBC (distribution is not given)	In breast cancer tissue, phylum Proteobacteria was most abundant, followed by Actinobacteria and Firmicutes. In adjacent breast tissue, highest presence of phylum Actinobacteria was observed.	†
Banerjee et al [22]	148 breast cancer tissue samples 20 healthy breast tissue samples	FFPE	ER/PR+ (*n*=50) HER2+ (*n*=34) ER/PR+, HER2+ (*n*=24) TNBC (*n*=40)	For all breast cancer subtypes, significant hybridization signals were found for *Actinomyces, Bartonella, Brevundimonas, Coxiella, Mobiluncus, Mycobacterium, Rickettsia,* and *Sphingomonas.* No bacterial signatures in healthy breast tissue were found of the following bacteria that were detected in breast cancer tissue: *Actinomyces, Aerococcus, Arcanobacterium, Bifidobacterium, Bordetella, Cardiobacterium, Corynebacterium, Eikenella, Fusobacterium, Geobacillus, Helicobacter, Kingella, Orientia, Pasteurella, Peptinophilus, Prevotella, Rothia, Salmonella,* and *Treponema.*	In ER/PR+ breast cancer, highest hybridization signals were found for probes of viruses Anelloviridae and Flaviviridae, and for fungal probes of *Filobasidiella, Mucor,* and *Trichophyton.* Comparing ER/PR+ breast cancer tissue with healthy breast tissue, signals for *Arcanobacterium, Bifidobacterium, Cardiobacterium, Citrobacter*, and *Escherichia* were associated with ER/PR+ breast cancer tissue. In HER2+ breast cancer, highest hybridization signals were found for probes of the virus Togaviridae, and the fungi *Epidermophyton, Fonsecaea, Pseudallescheria.* The virus Nodoviridae was only detected in HER2+ breast cancer. In ER/PR+, HER2+ breast cancer, highest hybridization signals were found for probes of the virus Polyomaviridae and the fungus *Penicillum.*

Table 7.1: (continued)

Study	Sample size	Sample source	Breast cancer subtype	Microbiota composition of breast cancer tissue, adjacent breast tissue and healthy breast tissue	Microbiota composition of breast cancer subtypes, grades and stages
					Comparing ER/PR+, HER2+ breast cancer tissue with healthy breast tissue, signals for *Bordetella, Campylobacter, Chlamydia, Chlamydophila, Legionella,* and *Pasteurella* were associated with ER/PR+, HER2+ breast cancer. In TNBC, highest hybridization signals were found for probes of viruses Picornaviridae and Anelloviridae, and fungi *Alternaria, Malassezia, Piedraia,* and *Rhizomucor*. Comparing TNBC tissue with healthy breast tissue, signals for *Aerococcus, Arcobacter, Geobacillus, Orientia* and *Rothia* were associated with TNBC tissue.
Meng et al [20]	94 breast tumour tissue samples 22 benign breast tumours 72 malignant breast cancer (grade I: *n*=7, grade II: *n*=36, grade III: *n*=13, NO-grade: *n*=16)	Fresh frozen	ER+ (*n*=47) ER- (*n*=25)	Comparing malignant breast cancer tissue with benign breast tumour tissue, increased relative abundance of Proteobacteria, *Micrococcaceae, Caulobacteraceae, Rhodobacteraceae, Nocordioidaceae, Methylobacteriaceae, and Propionicimonas* was found in malignant breast cancer tissue.	Comparing grade III breast cancer tissue with grade I and II breast cancer tissue, higher alpha diversity was found in grade III breast cancer. With increasing grade, decreased relative abundance of *Bacteroidaceae* and increased relative abundance of *Agrococcus* was found.
Smith et al [15]	53 breast cancer tissue samples 11 adjacent breast tissue samples 8 healthy breast tissue samples	Fresh frozen	Luminal A (*n*=22) Luminal B (*n*=14) HER2+ (*n*=6) TNBC (*n*=15) Missing (*n*=7)	Comparing healthy breast tissue with breast cancer tissue, higher alpha diversity, decreased relative abundance of *Pseudomonadaceae, Sphingomonadaceae,* and *Ruminococcaceae*, and increased relative abundance of *Actinomycetaceae* was found in healthy breast tissue.	In luminal A breast cancer, order Xanthomonadales was most abundant. In luminal B breast cancer, genus *Clostridium* was most abundant.

Table 7.1: (continued)

Study	Sample size	Sample source	Breast cancer subtype	Microbiota composition of breast cancer tissue, adjacent breast tissue and healthy breast tissue	Microbiota composition of breast cancer subtypes, grades and stages
	Breast cancer stage: Stage I: *n*=13 Stage II: *n*=24 Stage III and IV: *n*=19 Missing: *n*=8 Non-hispanic whites (NHW): *n*=51 Non-hispanic blacks (NHB): *n*=12			Comparing adjacent breast tissue to breast cancer tissue, lower relative abundance of *Ruminococcaceae* and *Clostridia* were found in adjacent breast tissue. Comparing adjacent breast tissue with healthy breast tissue, higher relative abundance of *Pseudomonadaceae* was found in adjacent breast tissue. In breast cancer tissue, class Clostridia, Bacteroidia, and family *Ruminococcaceae* was found to be most abundant. Family *Xanthomonadaceae* was most abundant in breast cancer tissue of non-hispanic white women. Genus *Ralstonia* was most abundant in breast cancer tissue of non-hispanic black women. Families *Pseudomonadaceae, Sphingomonadaceae,* and *Caulobacteraceae* were most abundant in adjacent breast tissue.	In the luminal subtypes, phyla Tenericutes, Proteobacteria, and Planctomycetes were most abundant. In HER2+ breast cancer, genus *Akkermansia* and phyla Thermia and Verrucomicrobia were most abundant. In TNBC, genera *Streptococcaceae* and *Ruminococcus*, and phyla Euryarchaeota, Cyanobacteria, and Firmicutes were most abundant. In stage I breast cancer, family *Ruminococcaceae,* and genus *Hyphomicrobium* were most abundant. In stage II breast cancer, genus *Sporosarcina* was most abundant. In stage III and IV breast cancer, genus *Bosea* was most abundant.
Costantini et al [17]	16 breast cancer tissue samples 16 adjacent breast tissue samples	Fresh tissue	ER/PR+ (*n*=14) ER/PR- (*n*=2) HER2+ (*n*=1) HER2- (*n*=15) TNBC (*n*=1)	In breast cancer tissue, phylum *Proteobacteria* was most abundant, followed by *Firmicutes, Actinobacteria,* and *Bacteroidetes*. 50-75% of relative abundances belonged to genera *Ralstonia, Methylobacterium,* and *Sphingomonas.* 25-50% of relative abundances belonged to genera *Staphylococcus* and *Pseudomonas,* and to families *Bradyrhizobiaceae* and *Rhodocyclaceae*. Comparing breast cancer tissue with adjacent breast tissue, no significant differences were found.	†

Table 7.1: (continued)

Study	Sample size	Sample source	Breast cancer subtype	Microbiota composition of breast cancer tissue, adjacent breast tissue and healthy breast tissue	Microbiota composition of breast cancer subtypes, grades and stages
Nejman et al [13]	355 breast cancer tissue samples 173 adjacent breast tissue samples 54 healthy breast tissue samples	Fresh frozen and FFPE (majority of samples)	ER+, PR+, HER2+ (distribution is not given)	In breast cancer tissue, 9190 bacterial species were detected in total. Breast tumours had a richer and more diverse microbiome than other tumours (melanoma, lung, ovary, bone, and glioblastoma multiforme tumours). Comparing breast cancer tissue with adjacent breast tissue and healthy breast tissue, higher bacterial load and richness were found in breast cancer tissue.	Differences in microbiota composition were found between breast cancer subtypes, based on ER, PR and HER2 expression. Most enriched pathways in bacteria within ER+ breast cancer tissue were arsenate detoxification and mycothiol biosynthesis.

*FFPE = Formalin-Fixed Paraffin-Embedded; †not described or investigated in the study; ‡ TNBC = Triple-negative breast cancer.

Table 7.2: Microbiota composition of breast cancer subtypes.

Study	Breast cancer subtype definition	Microbiota composition of breast cancer subtype
Xuan et al [8], Smith et al [15], Banerjee et al [22]	HR+	Comparing HR+ breast cancer tissue to adjacent breast tissue, higher relative abundance of *Methylobacterium radiotolerans* was found in HR+ breast cancer tissue. Comparing HR+ breast cancer tissue with healthy breast tissue, signals for *Arcanobacterium, Bifidobacterium, Cardiobacterium, Citrobacter*, and *Escherichia* were associated with HR+ breast cancer tissue. Absolute abundance of order Xanthomonadales and phyla Tenericutes, Proteobacteria and Planctomycetes were found highest in luminal A breast cancer. Absolute abundance of genus *Clostridium* and phyla Tenericutes, Proteobacteria and Planctomycetes were found highest in luminal B breast cancer.
Banerjee et al [22]	HER2+	Comparing HR+/HER2+ breast cancer with healthy breast tissue, signals for *Bordetella, Campylobacter, Chlamydia, Chlamydophila, Legionella,* and *Pasteurella* were associated with HR+/HER2+ breast cancer. Absolute abundance of genus *Akkermansia* and phyla Thermia and Verrucomicrobia were highest in HER2+ breast cancer.
Smith et al [15], Banerjee et al [21], Banerjee et al [22]	TNBC	Comparing TNBC tissue with healthy breast tissue, higher signals for *Prevotella, Brevundimonas, Arcanobacterium, Escherichia, Sphingobacterium, Actinomyces, Aerococcus, Arcobacter, Geobacillus, Orientia* and *Rothia* were found in TNBC tissue. Absolute abundance of genera *Streptococcaceae* and *Ruminococcus*, and phyla Euryarchaeota, Cyanobacteria, and Firmicutes were highest in TNBC.

Immunosuppression in breast tumour tissue

Breast cancer is characterized by infiltrated immune cells in the tumour tissue, where immunosuppressive cells are dominant over pro-inflammatory cells. High magnitude of tumour-infiltrating lymphocytes (TILs) in the breast is associated with better prognosis and therapeutic response in certain breast cancer subtypes [26]. Lymphocytes exhibiting anti-tumour activity include CD8+ cytotoxic T lymphocytes (CTLs), which eliminate cancer cells, and CD4+ T helper 1 (Th1) lymphocytes, which activate CTLs [27]. In ER+ HER2+ tumours, presence of tumour-infiltrating cytotoxic (CD8+) T cells was associated with a 27% reduction in the hazard of dying from breast cancer [28].

It is hypothesized that tumours recruit immunosuppressive cells in order to evade immune destruction [28]. Repressors of T cells, such as myeloid-derived suppressor cells (MDSCs) and T regulatory cells (Tregs), are found in higher numbers in patients with breast cancer compared to healthy controls, and increase with tumour stage [29,30]. Tumour-associated macrophages (TAMs), which resemble the anti-inflammatory M2-

polarized macrophages, promote tumour growth by secretion of anti-inflammatory cytokines, such as IL-10 and transforming growth factor (TGF)-β [31,32]. High infiltration of TAMs in breast tissue is associated with malignancy, negative hormone receptor status, and poor disease-free and overall survival [33].

Communication between the immune system and microbiota

The innate immune system is trained in recognizing microbes via pattern recognition receptors (PRRs) that bind to bacterial components, known as pathogen-associated molecular patterns (PAMPs) [34]. Examples of PRRs include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and C-type lectin receptors (CLRs), which are expressed by cells such as macrophages, dendritic cells (DCs) and natural killer (NK) cells [34]. TLRs induce an inflammatory response in reaction to binding with microbial structures such as lipopolysaccharide (LPS), peptidoglycan, flagella, or microbial DNA or RNA [35]. Activation of TLRs can either have tumour promoting or inhibiting effects, depending on the TLR subset, cancer type and involved immune cells in the tumour [36]. In breast carcinomas of mice xenografts, TLR5 was found to be highly expressed [37]. In vivo administration of *Salmonella typhimurium* flagellin, a ligand of TLR5, stimulated secretion of pro-inflammatory cytokines and chemokines, mediating anti-tumour activity [37]. In another study, LPS/TLR4 signalling resulted in a pro-tumourigenic effect by up-regulating production of IL-6 and IL-10 [38]. These observations might explain the importance of breast microbiota in breast cancer, since certain microbial components recognized by PRRs in the breast can induce a tumour-inhibiting inflammatory response, contributing to recruitment of tumour-killing cells.

Most of our understanding regarding immune cell and tissue-specific microbiota interactions, originates from studies investigating pancreatic and lung microbiota. In a study exploring microbiota in pancreatic cancer, microbiome diversity correlated with CD8+ T cell infiltration [39]. Additionally, CD8+ immune infiltration was associated with three genera that were most abundant in long-term survivors, including *Saccharopolyspora, Pseudoxanthomonas,* and *Streptomyces*. Interestingly, in another pancreatic cancer study, TLR2 and TLR5 ligation was found to promote pancreatic cancer and induce immunosuppression [40]. Moreover, these immune-suppressive effects were absent when macrophages were deficient in TLR signalling, which suggests that immunosuppression is dependent upon TLR ligation between the tumour microbiome and immune cells. In mice, lung microbiota manipulation was demonstrated to reduce the local immunosuppressive environment. Treatment with antibiotic or probiotic aerosol, 2 weeks before melanoma cell injection, resulted in decreased bacterial load in

the lung accompanied by enhanced activation of NK and T effector cells, promoting tumour immunity against lung metastases [41].
In summary, correlations exist between intratumoural microbiota composition and immune cell infiltration, which indicates that an unfavourable microbiota composition may contribute to tumour immune evasion.

Therapeutic efficacy and cytotoxicity governed by intratumoural microbiota?

Therapeutic resistance, which can be intrinsic or required, remains to be the limiting factor in achieving successful breast cancer treatment. Responsible determinants of drug resistance include alteration in expression or mutation of a drug target, tumour heterogeneity, reduced blood flow to the tumour, and prevention of immune evasion by the tumour microenvironment [42]. Biomarkers to predict therapeutic sensitivity of breast tumour cells are urgently required, so that therapy and dose can be adjusted accordingly. In this context, gut microorganisms have been shown to mediate toxicity and related side effects of anti-cancer agents. For example, the inactive SN-38G form of the pro-drug irinotecan (CPT-11) is reactivated by intestinal bacterial β-glucuronidases into the active and toxic SN-38 [43]. It is proposed that breast microbiota composition influences local availability and cytotoxicity of anti-cancer therapeutics as well, and thus could be used as a marker to determine drug efficacy and toxicity.
Via endogenous enzymes, bacteria have the ability to transform organic compounds [44]. Administration of bacteria found in breast cancer tissue, namely Gram-negative *E. coli* and Gram-positive *Listeria welshimeri* (*L. welshimeri)*, are able to either enhance or reduce efficacy and cytotoxicity of different chemotherapeutics through biotransformation in both *in vitro* and *in vivo* cancer models [44]. In this study, *E. coli* increased *in vitro* cytotoxicity of tegafur, fludarabine de phosphate, 5-fluorcytosine, 6-Mercaptopurine-2'-deoxyriboside, AQ4N, and CB1954, and decreased cytotoxicity of cladribine, vidarabine, gemicitabine, doxorubicin, daunorubicin, etoposide phosphate, mitoxantrone, B-Lapachone, and menadione. *In vitro* findings were confirmed in a CT26 murine colon carcinoma model. Intratumoural growth of *E. coli,* together with gemicitabine administration, resulted in increased tumour volume and reduced survival compared to a control group with only gemicitabine administered [44].
To confirm that bacteria determine effectiveness of anti-cancer therapy, a pancreatic cancer study demonstrated reduction in gemcitabine concentration due to the presence of intratumoural Gammaproteobacteria, and increased chemotherapeutic sensitivity when treated with antibiotics [45]. Proteobacteria are abundant in pancreatic

cancer tissue, and when transferred to a colon cancer mouse model, these bacteria were shown to mediate gemcitabine resistance by metabolizing and inactivating the drug via the long form of bacterial enzyme cytidine deaminase (CDD_L) [45]. Chemotherapeutics can also induce changes in intratumoural microbiota composition, since neoadjuvant chemotherapy reduced bacterial diversity in breast tumour tissue [46].
Current immunotherapeutic strategies aim to block co-inhibitory molecules in the tumour microenvironment, in order to reduce immunosuppression found in cancer. Immune checkpoint inhibitors (ICIs), such as programmed cell death 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4) blocking antibodies, function by preventing T-cell inhibition [47]. However, the clinical benefit of ICIs in breast cancer is not as effective as in other cancer types [48]. To enhance immunotherapeutic efficacy, it is needed to better understand the mechanisms underlying immunotherapy resistance. The tumour microenvironment is a promising target to improve responsiveness to immunotherapy, which can be done by modulating pro-tumour inflammation [25]. The influence of gut microbiota on immunotherapeutic efficacy has been described previously [48-50]. However, it remains to be established if intratumoural breast microbiota influence the functioning of immunotherapeutics as well. This is a reasonable assumption, since activation of TLRs by bacterial products stimulates maturation and priming of immune cells.

Gut-breast microbiota axis

Other than the direct effects of organ-specific microbiota on local tissue, the gut microbiota possibly affect breast cancer development through several mechanisms [11]. Disruption of gut microbiota homeostasis, characterized by low gut microbial diversity and less beneficial bacteria, is associated with breast cancer [51]. In addition, antibiotic treatment, which is known to disrupt gut microbiota homeostasis, is associated with increased breast cancer risk [52,53]. Furthermore, established risk factors for breast cancer, such as obesity and alcohol consumption, are associated with dysbiosis of the intestinal microbiota [54,55].
The breast parenchyma can be influenced by the gut microbiota through different mechanisms, including via enterohepatic recycling of oestrogens and bile acids, and microbial interaction with the innate and adaptive immune system.

Oestrogen and bile acids

Oestrogens are normally conjugated in the liver and delivered into the gut via bile excretion [56]. In the gut reside bacteria with beta-glucuronidase enzymatic activity, capable of de-conjugating conjugated oestrogen. De-conjugated oestrogen is reabsorbed into the circulation, resulting in systemically increased oestrogen exposure and thereby increasing breast cancer risk [57].

A main metabolic feature of intestinal microbes is bile acid conversion. Bile acids are soluble amphipathic molecules derived from cholesterol in the liver [58,59]. Microbiota that reside in the intestinal lumen have the capacity to convert primary bile acids into secondary bile acids by the process of deconjugatation and 7α-dehydroxylation [58]. Certain specific bile acids are functionally similar to hormones, due to their capacity to alter metabolic pathways in distant organ tissues by activating several receptors, including farnesoid X receptor (FXR), pregnane X receptor (PXR), vitamin D receptor (VDR) and Takeda G-protein-coupled receptor 5 (TGR5) [58]. The farnesoid X receptor has been detected in invasive breast carcinoma [60]. Bile acids were found to be accumulated in breast tumours, and were associated with anti-proliferative effects and improved patient prognosis [61]. Another study evaluated the effects in breast cancer of a separate bile acid. Lithocholic acid (LCA) decreased breast cancer cell proliferation by oxidative stress induction and improved anti-tumour immunity by increasing TIL count in the breast [62].

Shaping systemic immunity

The intestinal microbiota has been recognized to shape the immune system. It is already established that gut dysbiosis modulates immune functioning, which might contribute to carcinogenesis [63,64]. Various experimental mouse models exist, demonstrating carcinogenic regulation by gut microbes via interaction with the immune system. In mice, commensal dysbiosis of the gut, induced by oral antibiotics, significantly increased the number of myeloid cells present in normal adjacent breast gland tissue at both early and advanced stages of breast tumour progression [65]. Moreover, these infiltrated myeloid cells exhibited high expressions of inflammatory mediators arginase-1 and IL-6. Myeloid recruitment into breast tissue was explained by corresponding increases in myeloid chemoattractants, namely CXCL10 and CCL2, which were upregulated in dysbiotic mice compared to non-dysbiotic mice [65].

Neutrophils, members of the innate immune system, have been identified to mediate between the gut and extra-intestinal organs. A specific bacterium, *Helicobacter hepaticus (H. hepaticus)*, residing in the gut was shown to affect distant neoplastic progressions in breast tissue, in which neutrophils played a tumourigenic role [66]. Mice

with a predisposition for breast cancer underwent accelerated cancer progression when injected with *H. hepaticus*, compared to non-infected mice. However, mice systemically depleted of neutrophils showed only pre-neoplastic and early neoplastic lesions in the breast tissue, thus inhibited carcinogenesis.

These findings indicate that the gut microbiota has the capacity to affect immune cell expression in breast tissue as well. In breast cancer patients, higher gut microbiota diversity was associated with increased TIL expression in breast tissue [67]. A summary of breast microbiota interactions is illustrated in Figure 7.1.

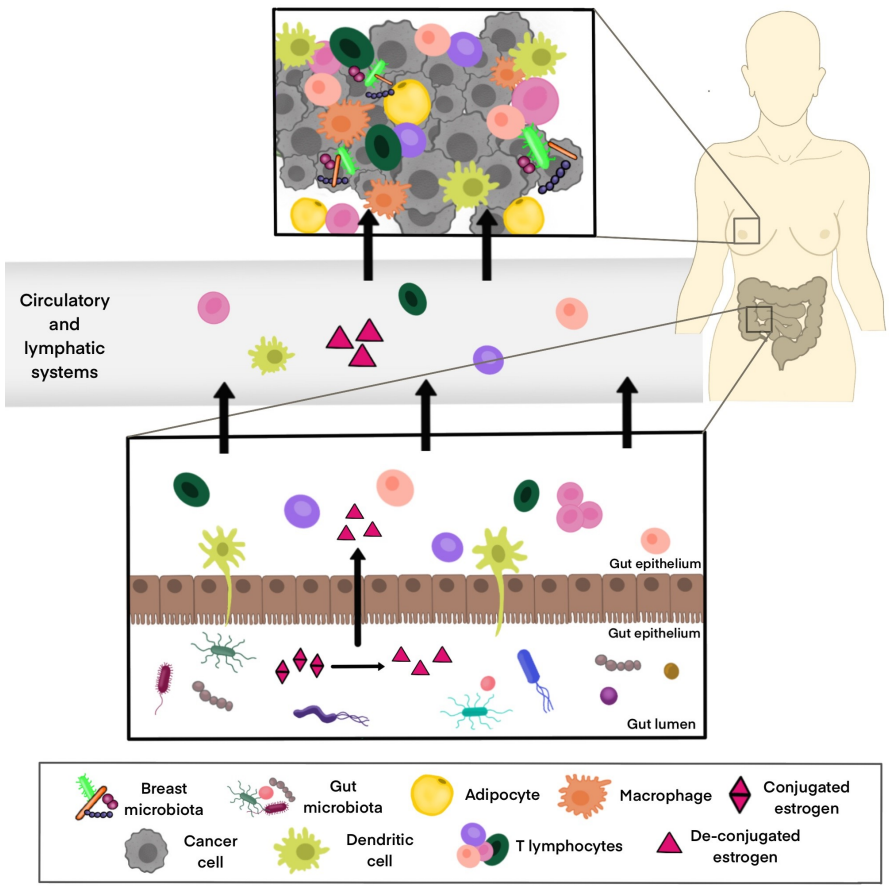

Figure 7.1: Interaction mechanisms between the gut and the breast. A connection between the gut and the breast has been demonstrated in human and mouse studies. The gut microbiota composition interacts with the host immune system and subsequently influences systemic immunity and the local immune environment in the breast. Furthermore, gut microbiota has the capacity to influence systemic availability of oestrogen through enterohepatic recycling of oestrogen, since certain bacteria contain the enzyme beta-glucuronidase.

Translational gaps; the future of using microbiota to fight cancer

The microbiota is a promising field of research, in which the breast microbiota could be used as biomarker to establish disease characteristics and used as a therapeutic target. Accumulated evidence confirms presence of a unique microbial community in the breast. However, small sample sizes, interindividual heterogeneity of the microbiota, variances in methodological approaches (DNA extraction kit, target hypervariable region selection for sequencing, tissue extraction and storage) and patient characteristics (demography, dietary habits, menopausal status, breast cancer subtypes), limit comparability of results. Furthermore, the observational design of most studies only provides associative but not causal evidence for the relationship between microbial dysbiosis and breast carcinogenesis. Moreover, since tumour samples contain a low bacterial biomass, contamination could have contributed to inaccurate findings. A major challenge in this field will be to make the shift from descriptive analysis to functional metagenomics and metabolomics, in order to outline the functional roles of bacteria present in the breast and its interaction with the internal environment. Furthermore, rather than holding a single pathogen responsible for cancer progression, the accumulative effects of an entire microbial community are more likely to determine disease processes.

The breast microbiota as a biomarker for cancer

The breast microbiota could be used as a biomarker in several ways; (1) pre-treatment, to determine the molecular characteristics of the cancer, so that response or resistance to therapeutics can be predicted (2) during treatment, enabling the adjustment of the therapy when unresponsive (3) and during tumour progression, to elucidate the achieved resistance to therapeutics [68]. Using microbiota as a pre-treatment biomarker would provide most value to oncologists, since microbiota analysis would offer additional information on tumour aggressiveness and tumour sensitivity to anti-cancer therapeutics, after which treatment type and regimen could be adjusted accordingly. Moreover, regarding current protocols in breast cancer diagnosis, tissue specimens have already been obtained prior to treatment, placing no additional burden on patients.

To bring a biomarker to the clinic, five phases have been outlined to provide structure in this development [69]. These include preclinical studies, clinical assay development, retrospective studies, prospective studies, and control studies. Currently, research regarding the breast microbiota is still in the preclinical, exploratory phase, since most

studies have focused on identifying characteristics that are unique to breast tumour tissue, by comparing the microbiota of breast tumour tissue to healthy tissue.
Even though the breast microbiome exhibits great potential as a prognostic and predictive biomarker, further investigations are still required to consider the breast microbiome as a biomarker for prognosis or therapeutic response. First, more participants should be included in future studies, taking clinicopathological variability into account. Furthermore, by performing prospective studies, the prognostic value of pre-treatment breast microbiota composition can be evaluated. Second, confounding factors should be taken into consideration, including menopausal status, age, ethnicity, BMI, and lifestyle-related factors, such as alcohol consumption and dietary habits. Lastly, microbiota compositions should be identified which are able to predict efficacy and resistance to anti-cancer therapeutics, by comparing microbial composition of patients with differences in tumour progression and between responders and non-responders. In order to obtain in-depth mechanistic insight into the relationship between breast cancer microbiota and anti-cancer agents, the use of animal models will be required.

Modulating breast microbiota

No studies have been conducted yet on how the breast microbiota composition could be manipulated. However, since the gut microbiota is able to affect the breast through several mechanisms, discussed above, the gut microbiota could be targeted. Beta-glucuronidase activity and gut-derived metabolites can be modulated by diet [56]. Diets high in fat were found to increase beta-glucuronidase activity in gut bacteria and diets high in fibre reduced this activity. Other factors that are known to elevate oestrogen levels in the circulation are alcohol ingestion and adiposity [70]. A more rigorous approach to manipulate gut microbiota composition, is by the method of faecal microbiota transplantation (FMT) [71]. Another strategy encompasses the intake of pro- or prebiotics [72]. However, more research is needed to understand how gut microbiota interacts with microbiota residing in the breast, and other breast microbial targeting possibilities need to be explored. In Figure 7.2, the proposed strategies to influence the gut-breast axis are summarized.

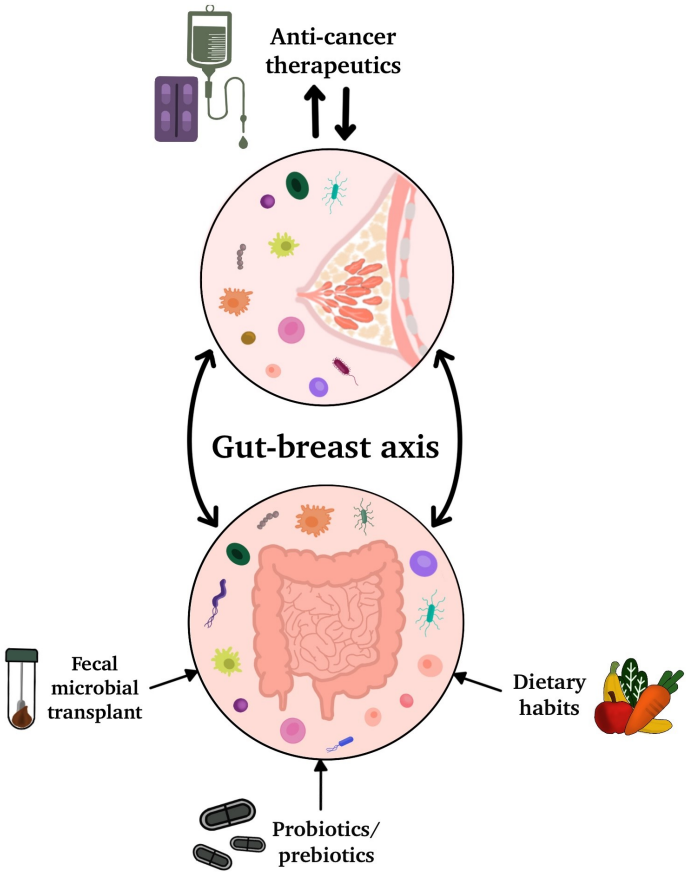

Figure 7.2: Proposed strategies to influence the gut-breast axis. Breast cancer is characterized by a unique microbiota and immune composition. Though little is known about manipulating these compositions in the breast, alteration of the gut microbiota composition might influence the breast tumour microenvironment as well. Strategies to positively alter gut microbiota comprise healthy dietary habits, pre- or probiotics, or more rigorously, faecal microbiota transplantation. Moreover, certain bacterial species potentially have the capacity to modulate anti-cancer agents by bacterial enzymatic activity.

Microbiota investigation techniques

In the future, it remains to be investigated which intratumoural bacteria in the breast modulate anti-cancer agents, in order to identify possible targets for breast cancer therapeutics. Alongside, its interactions with the immune system deserve attention as

well. This is best done by combining metagenomics and metabolomics sequencing with preclinical models, such as gnotobiotic mice and patient-derived tumour xenografts (PDTXs) [73]. Gnotobiotic mice refers to animals with known microbiota composition, either germ-free mice or ex-germ free animals [74]. PDTXs exist of immunodeficient mice to which patient-derived material is transferred [75]. Another possible preclinical model that can be combined with sequencing, is the *in vitro* 3D model termed organoid [75]. Transferring breast cancer microbiota to these models will contribute to the understanding of patient specific tumourigenic and therapeutic modulatory potential of intratumoural bacteria. Identifying which microbial composition favours a positive response to anticancer therapy is important to increase therapeutic effectiveness [73].

Summary

Breast microbiota holds many possibilities to reveal more insight into the pathophysiology of breast cancer. It is promising to use the breast microbiome as a marker to predict breast cancer prognosis and therapeutic response. Targeting microbiota could be used to improve therapeutic efficiency and reduce related toxicity. Before breast microbiota analysis can be implemented, major knowledge gaps need to be considered. There is limited understanding of the contribution of breast microbiota to cancer development and treatment response. Since indications exist that breast microbiota components interact with the gut microbiota and the immune system, these need to be explored in parallel.

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CHAPTER 8

General discussion and future perspectives

General discussion

The thesis provides insights into the role of intestinal microbiota in cancer and systemic cancer therapy and explores new predictive, prognostic, and therapeutic targets. It focuses on cancer patients treated with systemic cancer therapy and especially on colorectal and breast cancer patients treated with chemotherapy. In more detail, this thesis starts with the description of clinical studies that explored the associations between intestinal microbiota and systemic cancer therapy. Thereafter, the association between colorectal cancer treatment with chemotherapy and intestinal microbiota was assessed. Furthermore, this thesis examines the role of intestinal microbiota in breast cancer patients and during breast cancer treatment with chemotherapy, as well as the role of breast microbiota in breast cancer.

The role of intestinal microbiota in systemic cancer therapy

While there are strong indications that the intestinal microbiota has a role in carcinogenesis, evidence concerning the influence of the intestinal microbiota on tumour response and toxicity in patients treated with systemic cancer therapy is scarce. Most of the results concerning interactions between intestinal microbiota and systemic cancer therapy originate from *in vitro* studies [1-3]. A comprehensive overview of clinical studies in this field of research was lacking. Therefore, we conducted a systematic literature search to provide an overview of all clinical studies investigating the influence of the intestinal microbiota on systemic cancer therapy as well as the influence of systemic cancer therapy on intestinal microbiota **(chapter 2)**. We focused on chemotherapy, immunotherapy, and hormonal therapy. We identified several strengths and limitations of current microbiota research exploring the role of intestinal microbiota in systemic cancer therapy.

A strength of particularly more recent microbiota studies was the use of 16S rRNA gene sequencing or even whole metagenome sequencing. These techniques are more comprehensive and provide a higher taxonomic resolution as compared to older molecular microbiota profiling techniques, e.g. quantitative polymerase chain reaction (qPCR) or fluorescent in situ hybridization (FISH), which target specific microbial taxa. Moreover, whole metagenome sequencing offers the possibility to determine the functional capacity of the intestinal microbiota [4,5]. In the observational cohort studies presented in this thesis we used amplicon sequencing of the 16S rRNA V4 gene-region.

Despite these promising technical developments, it is known that the intestinal microbiota composition is influenced by several external factors. Consequently, the risk of confounding is generally high in microbiota research [6]. Due to the inclusion of patients with different cancer types and/or different (systemic) cancer therapies, there was a high level of heterogeneity between, but also within the studies included in the review, which limited the comparability. To increase the within study comparability, strict in- and exclusion criteria should lead to more homogeneous study groups for future studies. Depending on the research question, it should be considered if, and to what extend patients with previous (abdominal) operation, systemic cancer therapy, and antibiotic treatment should be included. Moreover, for longitudinal studies, planned operations, perioperative antibiotic use, and planned systemic cancer therapy regimens should be adequately registered. Attention, and if needed, correction for these confounders is advised. Particularly, the frequent assessment of, and correction for prior antibiotic use is relevant, since it has been recently demonstrated that antibiotics do not only modulate intestinal microbiota composition and metabolite production but also tumour growth and key metabolic processes such as protein and short-chain fatty acid (SCFA) metabolism [7,8]. To increase the between study comparability of future reviews, more clinical studies are needed to be able to compare e.g., multiple studies with one type of cancer or one type of systemic cancer therapy. The conduction of studies that compare multiple cancer types and systemic cancer therapies should be discouraged [9]. The use of stricter in- or exclusion criteria in our review aiming to focus on a more homogenous population e.g., one specific cancer type and systemic cancer therapy, would not have been meaningful. This would have resulted in only one or two articles eligible for inclusion.

Another limitation was the relatively small sample size of the majority of the studies included in our review. Sample sizes varied between seven and 43 patients with one outlier of 60 patients. Depending on the influence of the previously described confounding factors, larger sample sizes in future studies are warranted. The problem of small sample sizes increases in longitudinal studies due to loss to follow-up, resulting in even smaller groups to draw conclusions on. Sample size calculation in microbiota research is challenging, because the influence of microbiota is still a relatively unknown and unpredictable area. Calculation of the sample size depends on the exact research question and endpoints, the presence of confounding factors, and the expected attrition rates. Due to the complex microbiota-host interactions, it is recommended to involve a statistician experienced in microbiota data to calculate the sample size when studies evolve from explorative pilot studies to causal designs.

Overall, we can conclude that this review has provided pivotal information on current gaps of knowledge and will facilitate the evidence-based design of future studies in the field of intestinal microbiota and systemic cancer therapy. Additional longitudinal clinical research is needed to confirm or oppose the results of the limited studies that were available. Studies with increased sample sizes are warranted to further explore these highly relevant research questions and overcome the influence of potential confounding factors. If associations can be confirmed, it is essential to further identify prognostic and therapeutic microbial targets and move forward to microbiota-based personalised medicine.

The role of intestinal microbiota in colorectal cancer treatment with chemotherapy

In vitro studies showed prolonged and increased response to chemotherapy, in the presence of specific intestinal microbiota [1,10-12]. Clinical evidence on the role of intestinal microbiota in tumour response and chemotherapy toxicity in metastatic colorectal cancer (mCRC) patients was not available [13]. To bridge the translational gap between these *in vitro* studies and clinical practice, we initiated an observational cohort study **(chapter 3)**. The study aimed to explore whether baseline intestinal microbiota composition is associated with tumour response or chemotherapy toxicity in patients with mCRC treated with capecitabine or trifluridine/tipiracil. Furthermore, the study was designed to investigate the associations between the intestinal microbiota and chemotherapy toxicity and to explore the influence of capecitabine and trifluridine/tipiracil on intestinal microbiota over the course of chemotherapy. To rule out the confounding effect of multiple systemic therapies, only patients who received either capecitabine (± bevacizumab) or trifluridine/tipiracil were eligible to participate. After three cycles of chemotherapy, standardised response measurement was assessed with CT or MRI. In July 2021, 61 patients treated with capecitabine (± bevacizumab) and 41 patients treated with trifluridine/tipiracil were included. These relative high inclusion numbers confirm that patients are willing to participate in studies exploring the intestinal microbiota. After a pilot analysis of the first 33 mCRC patients receiving capecitabine **(chapter 4)**, we concluded that intestinal microbiota composition and diversity before, during, and after three cycles of capecitabine were not associated with response. In addition, capecitabine did not induce significant changes in the intestinal microbiota composition and diversity during the treatment period and induced only mild chemotherapy toxicity symptoms. Individual specific microbial shifts as a result of antibiotic therapy during capecitabine treatment were observed.

It is not possible to compare our results to other studies, since this is the first clinical study with longitudinal intestinal microbiota sampling in mCRC patients that explored the effect of capecitabine (without surgery, radiation or additional chemotherapy or combinations thereof) on the intestinal microbiota composition and diversity and vice versa. Closest are the results of a study in 31 rectal cancer patients without metastasis who were treated with 5-fluoropyrimidine and oxaliplatin (FOLFOX). In contrast to our results, they did observe a decrease in α-diversity in terms of Chao1 after FOLFOX treatment [14]. Due to the administration of the combination of the two chemotherapeutics 5-fluoropyrimidine and oxaliplatin, it is not possible to distinguish the effect of each individual chemotherapeutic. In line with this, Sze et al. observed in 26 patients with primary diagnosed CRC changes in community structure when comparing pre- and post treatment samples, as well as a shift towards a microbiota profile comparable to healthy controls after treatment. However, they did not observe differences in observed richness, Shannon evenness or Shannon diversity between pre- and post treatment. Again, these findings were based on a heterogeneous treated group. These patients were treated with surgery (*n*=12), surgery and chemotherapy (*n*=9) or surgery, chemotherapy, and radiation (*n*=5). Hence, patients in the study of Sze et al. were treated with mono- or combination therapy of capecitabine or 5-fluoropyrimidine and oxaliplatin [15]. As previously described, this heterogeneous treatment highlights the limitations to compare microbiota research and underlines the importance of additional microbiota research with homogenous treatment groups.

In our opinion, there may be two explanations why capecitabine did not induce significant alteration in the intestinal microbiota composition in our mCRC study population. First of all, compared to the combination chemotherapy treatments described in the former studies, capecitabine is a relatively mild chemotherapeutic concerning chemotherapy toxicity. As a result, the effect of only three cycles of capecitabine on the microbiota could also be relatively mild. This potential mild effect of capecitabine is emphasized by the observation of individual antibiotic induced microbiota shifts in our study population. In addition, it is hypothesized that the relative complex medical history of the mCRC may have contributed to an already perturbed intestinal microbiota at baseline. In general, most of the mCRC patients were previously submitted to abdominal surgery, prior chemotherapy treatment and/or antibiotic administration. In our mCRC population, almost half of the patients received prior systemic treatment. Hence, if prior systemic cancer therapy reduced the diversity as a whole, then the impact of the relatively mild chemotherapeutic capecitabine is not significant, because the reduction of low diversity to a lower diversity is in general not significant compared to the reduction of a normal diversity to a low diversity. After

completing the inclusion of 66 patients, it is worthwhile to compare baseline microbiota composition and diversity between patients with or without prior systemic treatment.

Another important limitation of our study is the unequal group size distribution between responders (*n*=6) and non-responders (*n*=26). The unequal group size in combination with large inter and intra-individual microbiota composition and diversity further complicated the detection of differences on group level. An increased sample size is needed to overcome or at least diminish these limitations. After all, we have to deal with current clinical practice of prior (systemic) therapies and differences in tumour response. As a result, the dimension and challenges of the work that has to be done is quite high, nonetheless we have to continue and start upcoming longitudinal microbiota research in mCRC patients, in order to enhance microbiota-based personalised medicine.

The role of intestinal microbiota in breast cancer and during breast cancer treatment with chemotherapy

Molecular interactions between chemotherapy consisting of adriamycin, cyclophosphamide, and docetaxel (AC-D) and microbiota have not been studied in breast cancer patients before we designed our cohort study, general *in vitro* and mouse studies have indicated that significant interactions between the intestinal microbiota and AC-D occur [2, 3, 16-21]. By using a longitudinal design, we explored the associations between the intestinal microbiota, chemotherapy toxicity, and treatment response in 44 postmenopausal oestrogen receptor positive breast cancer patients **(chapter 5)**. Our study showed that observed species richness reduced significantly during (neo)adjuvant AC-D treatment. The abundance of Proteobacteria, unclassified Enterobacterales, and *Lactobacillus* significantly increased during AC-D treatment. After AC-D treatment, the abundance of Proteobacteria and unclassified Enterobacterales significantly decreased to levels comparable to baseline. Contrary, the abundance of *Ruminococcaceae NK4A214 group*, *Marvinbryantia*, *Christensenellaceae R7 group*, and *Ruminococcaceae UCG-005* significantly decreased during (neo)adjuvant AC-D treatment. Patients with any grade diarrhoea during docetaxel had a significantly lower observed species richness compared to patients without diarrhoea. In addition, diarrhoea was not correlated to antibiotic administration.

Very 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 analysed with metagenomic shotgun sequencing [22]. 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.* However, we were not able to identify these microbiota shifts on species level, the observed shifts on species level of the French study do not correspond with our findings on genus level, where *Ruminococcaceae UCG-005 and Ruminococcaceae NK4A214* decreased after chemotherapy. These differences could be due to some limitations of the French study, which limit comparability. First of all, in the French study patients were not homogenous concerning tumour subtype and systemic cancer therapy scheme. In the French study, up to 24% of the breast cancer patients had triple negative breast cancer, where in our study only ER+ patients were included. 60% of the French study patients received additional endocrine therapy before the last faecal sample collection, and 31% of the patients received HER2-directed therapy. In addition, faecal samples were only analysed before and after chemotherapy treatment and not during chemotherapy treatment in the French study. With respect to the potential interaction between the oestrogen metabolism and the intestinal microbiota via microbial β-glucuronidase, no distinction was made between pre- or postmenopausal women.

Another interesting observation in our study was the reduction of Ruminococcaceae during chemotherapy treatment. Many bacteria within the family of Ruminococcaceae are able to produce SCFA by degrading polysaccharides. SCFA positively influence intestinal homeostasis and are known to be involved in immunological and metabolic functions and have protective effects on the intestinal barrier [23, 24]. Therefore, reduction of these bacteria during chemotherapy might contribute to manifestation of intestinal inflammation and dysregulated homeostasis.

There are several strengths and limitations of our study. As partially emphasised above, our study is unique due to its homogenous study population of postmenopausal ER+ and HER2- breast cancer patients, as well as the longitudinal design including the collection of faecal 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. A limitation of our study was that is was only possible to conduct chemotherapy response measures in the subgroup of neoadjuvant

treated patients. As a consequence, the group size reduced from 44 to 18 patients. In addition, response measurement based on residual tumour will never be possible in adjuvant treated patients, since adjuvant patients undergo tumour resection first. To circumvent this, increased samples sizes in the neoadjuvant setting are required and/or other outcome measurements should be used, for instance measuring disease free survival or progression free survival [25].

To recapitulate, we emphasize that more attention should be paid to chemotherapy-induced microbiota changes and potential metabolic and immunologic consequences in postmenopausal breast cancer patients. The identified changes in taxa abundance and their functions should be further evaluated in additional longitudinal studies. Longitudinal designs are able to identify the (in)stability of the microbiota within individuals over time as well as differences between patients.

During the last decade, there has also been a growing interest in the role of the human intestinal microbiota and the development of cancer [26-28]. Though, only a small number of pre-clinical [29,30] and clinical studies [31-35] investigated the association between intestinal microbiota and breast cancer. Clinical studies have indicated alterations in the abundance of specific bacterial species in breast cancer patients compared to healthy controls [31-34]. However, these studies were limited by their relatively small sample sizes or had included a heterogeneous group with multiple breast cancer subtypes [35]. The largest accounted for only 48 patients. To provide more insight into the role of intestinal microbiota in breast cancer diagnosis, we set up a prospective cohort study in collaboration with the National Dutch Screening for Breast Cancer **(chapter 6)**. After analysis of 81 postmenopausal ER+/HER2- breast cancer patients and 67 postmenopausal healthy controls, we concluded that intestinal microbiota richness, diversity, and composition were not associated with postmenopausal breast cancer. When comparing patients who received recent breast cancer surgery, patient without recent breast cancer surgery, and healthy controls, this study identified that *Dialister* and Veillonellaceae were increased in patients who had received recent breast cancer surgery. This effect was independent of intravenous prophylactic cefazolin administration during recent breast cancer surgery, suggesting potential antibiotic-independent microbiota-modulating effects of breast cancer surgery.

The lack of differences in microbiota richness, diversity, and composition between breast cancer patients and healthy controls contrasts with two other clinical studies that investigated the association between the intestinal microbiota and pre-treatment postmenopausal breast cancer patients [34,36]. Goedert et al. observed a significantly

lower microbial richness and diversity, in terms of observed species richness and Chao1 index, in breast cancer patients compared to healthy controls [36]. The opposite results were found in a study by Zhu et al., who showed that breast cancer patients had higher observed species richness and Chao1 index [34]. In the studies by Goedert et al. and Zhu et al., no distinction was made between different types of breast cancer. This makes the breast cancer group less homogenous than our breast cancer group, with only ER+ and HER2- breast cancer. When examining differences in microbial composition, Zhu et al. found differences on species level [34], where Goedert et al. [36] did not find any differences in microbial composition. Concerning microbiota composition, our results are more in line with the results of Goedert et al. and suggest that the intestinal microbiota composition is not associated with postmenopausal breast cancer.

Even though no differences were found in microbial composition between breast cancer patients and healthy controls based on 16S rRNA gene sequencing, no conclusion could be drawn concerning the functional potential and activity of these bacteria. Because, as described previously, the fact that even if bacteria are present, this does not necessarily mean that they also perform their presumed function. Therefore, we highly recommend investigating functional microbiota analyses, for instance β-glucuronidase activity assays and whole metagenomic shotgun sequencing. The bacterial enzyme β-glucuronidase has been shown to increase intestinal oestrogen reabsorption into the circulation [29] and a relationship between intestinal microbiota-related oestrogen metabolism and systemic oestrogen levels has already been demonstrated in small groups [37-39]. In addition to the potential microbiota-modulating effects of breast cancer surgery itself, it might be speculated that also the hospital environment, surgery-related medication, stress, or short-term fasting might contribute to the bloom of *Dialister* and Veillonellaceae in patients scheduled for adjuvant systemic treatment. Therefore, the potential microbiota-modulating effects of the peri-operative phase in microbiota studies should not be neglected in clinical microbiota research.

Overall, in our opinion, the intestinal microbiota could not be used as a marker for postmenopausal breast cancer at this moment. On the other hand, the potential antibiotic-independent microbiota-modulating effects of breast cancer surgery might be an interesting lead to further follow-up with longitudinal microbiota sampling in breast cancer patients without adjuvant systemic cancer treatment in order to explore effects on quality of life.

Finally, this thesis reviewed the potential interaction between intestinal microbiota and breast tissue microbiota. Previous studies have suggested that besides intestinal microbiota, organ-specific microbiota play a role in tumour development and therapeutic resistance [40-42]. However, a comprehensive overview of available knowledge on the role of breast tissue microbiota in breast cancer and breast cancer treatment was not available. Therefore, we conduced a review to summarize the developments in the understanding of breast microbiota and its interaction with the immune system and the intestinal microbiota. Furthermore, the biomarker potential of breast microbiota was evaluated in conjunction with possible strategies for targeting microbiota in order to improve breast cancer treatment **(chapter 7)**. Identifying breast and intestinal microbial compositions that respond positively to certain anti-cancer therapeutics, could significantly reduce cancer burden. However, before breast microbiota analysis can be implemented as predictive target for breast cancer diagnosis, additional clinical research is needed to unravel the complexity of breast microbiota functioning and its interactions with the intestines and the immune system.

Main findings

- Only limited and heterogeneous clinical studies were available for our review that summarized knowledge of the role of intestinal microbiota on systemic cancer therapy.

- The review indicated that immunotherapy and chemotherapy induced changes in intestinal microbiota composition with possible consequences for therapy efficacy. Furthermore, there is an association between baseline intestinal microbiota composition and tumour response and toxicity in patients treated with immunotherapy or chemotherapy. Consequently, additional longitudinal as well as larger studies are required to investigate the predictive ability of baseline intestinal microbiota for systemic cancer therapy outcome and complications.

- Patients are willing to participate in observational studies exploring the intestinal microbiota.

- Intestinal microbiota composition and diversity before, during, and after capecitabine treatment were not associated with response in mCRC patients. Capecitabine did not induce significant changes in microbiota composition and diversity. However, individual effects of antibiotic treatment during capecitabine treatment were observed.

- Baseline intestinal microbiota richness, diversity and composition were not associated with pathological response in neoadjuvant treated postmenopausal ER+ and HER2- breast cancer patients, (neo)adjuvant chemotherapy treatment with adriamycin, cyclophosphamide and docetaxel did induce subsequent shifts in microbial richness as well as the abundance of specific bacterial taxa. Furthermore, diarrhoea was associated with lower α-diversity.

- Intestinal microbiota richness, diversity, and composition were not associated with postmenopausal breast cancer. When comparing patients who received recent breast cancer surgery, patient without recent breast cancer surgery, and healthy controls, this study identified that *Dialister* and Veillonellaceae were increased in patients who had received recent breast cancer surgery. This effect was independent of intravenous prophylactic cefazolin administration during recent breast cancer surgery, suggesting potential antibiotic-independent microbiota-modulating effects of breast cancer surgery.

- Before breast microbiota analysis can be implemented as a predictive, prognostic, or therapeutic target for breast cancer diagnosis and treatment, major knowledge gaps concerning the interaction with the intestinal microbiota and immune system needs to be explored.

Future perspectives

Additional longitudinal clinical studies are warranted to increase the general knowledge on the role of microbiota in cancer and systemic cancer therapy. The aforementioned challenges should be tackled to improve the design of future studies. First, studies should be homogenous concerning cancer type, tumour response measurement outcome, systemic cancer therapy, and follow-up period. In addition, upcoming studies should adequately register and analyse potential confounding factors such as antibiotic administration, prebiotic or probiotic use, dietary habits (e.g. veganism), the use of nutritional supportive drinks and surgery. Furthermore, research questions concerning the influence of the intestinal microbiota on tumour response and toxicity in patients treated with systemic cancer therapy are far too important to draw conclusions based on studies with small sample sizes. Lastly, the use of 16S rRNA gene sequencing or even whole metagenomic sequencing should be highly encouraged in future studies. It is highly recommended to combine sequencing techniques with functional microbiota analysis and link these outcomes to the immunological consequences.

To apply the lessons learnt from this thesis in a broader view of intestinal microbiota research in cancer diagnosis and treatment, I would first recommend establishing the importance in high incidence cancers, for example, colorectal and breast cancer. This would create the opportunity to include larger patient numbers in a shorter period of time. Concerning cancer stage, I would advise upcoming researchers to study newly diagnosed and treatment-naive patients. Compared to metastatic cancer patients who will receive a second or third line systemic cancer therapy, newly diagnosed patients mostly have a relatively confined medical history with minor possible influences on the intestinal microbiota. The knowledge and potential new therapeutic targets, mainly obtained from responder patients gathered by these studies, can be ideally tested in metastatic cancer patients. Quality of life is most important here and as a consequence, microbiota-modulating strategies will be ethically more accepted in patients with metastatic disease without any treatment options. Of course, it should be taken into account that second or third line chemotherapy treatment will induce generally less chemotherapy toxicity compared to first line treatment.

In a more profound view of this thesis, we can conclude that the results provide the basis for exploring future intestinal microbiota research in oncologic patients. The clinical studies in mCRC and breast cancer patients, and healthy controls have provided new leads for future steps.

As shown in **chapters 3 and 4**, we designed the first homogenous longitudinal study with faecal microbiota sampling in mCRC patients treated with capecitabine or trifluridine/tipiracil. Results of the 66 patients treated with trifluridine/tipiracil will follow. Results of the 33 mCRC patients treated with capecitabine did not indicate an association between intestinal microbiota composition and tumour response, chemotherapy toxicity or capecitabine-induced changes in intestinal microbiota composition and diversity. Although our study is a strong pilot study, definitive conclusions should be based on the results of the 66 patients with hopefully a more equal distribution between responders and non-responders or at least an increased number of responder patients. Furthermore, this study had a limited follow-up period. Although we are dealing with mCRC, there were many patients with stable disease. These patients should be followed up for a longer period to study the differences between these stable patients from patients with progressive disease. During the extended follow-up period, it is advised to plan faecal sample collections before and during each cycle or, if limited financial support is available, at least during each CT or MRI evaluation. By following patients until progression or other reasons to discontinue capecitabine treatment, more insight can be obtained into intestinal microbiota

alterations associated with tumour progression or chemotherapy toxicity leading to discontinuation of capecitabine therapy. Despite these limitations, which can be resolved by using a longer follow-up period, our preliminary results provide input for the design of future research.

Future research should also include functional microbiota analysis. This brings us to the field of pharmacomicrobiomics. Pharmacomicrobiomics is an emerging field that investigates the interplay between microbiota, medication and the host [43-45]. The intestinal microbiota are able to extend the metabolic capacity of humans due to their enzymatic activity. Consequently, analysis of enzymatic activity and microbial metabolites is an important following step, because the presence of bacteria does not necessarily mean that they also perform their presumed function. Important microbial metabolites are SCFA. SCFA are known to have potent anti-inflammatory and immune-modulatory effects [46]. Furthermore, SCFA can positively influence intestinal homeostasis and are known to be involved in metabolic functions [23,24]. Particularly butyrate is crucial for intestinal barrier integrity, by serving as fuel for colonocytes and regulating tight-junction proteins and the production of mucins [46].

In addition, the interaction between capecitabine and intestinal microbiota and metabolite production can be further explored using *in vitro* models mimicking the colon, such as the TNO *in vitro* model of the colon (TIM-II) [47]. In this model, confounding factors of the human external environment can be easily omitted. Intestinal bacteria derived from healthy participants can be incubated and treated with capecitabine in the TIM-II model. In contrast to the availability of only one faecal sample in the human setting, the TIM-II model is able to elucidate the whole process of capecitabine metabolism, because it is possible to sample at multiple time points. If capecitabine-induced alterations are observed, potential therapeutic strategies to modulate intestinal microbiota composition and reverse capecitabine-induced changes can be investigated. Prebiotics might be promising compounds in this context [48]. A prebiotic is a substrate which is selectively utilized by host microorganisms conferring a health benefit to the host [48]. Prebiotic substrates are fermented by the intestinal bacteria, leading to the formation of SCFA [46]. Prebiotic intervention experiments in the TIM-II can identify suitable prebiotic compounds as well as providing insight into optimal timing to counteract potential capecitabine-induced microbial alterations. Lastly, the TIM-II model can be used to determine if a prebiotic intervention is able to modulate the microbiota composition and function of mCRC patients treated with prior chemotherapeutics and shift the microbiota composition towards a more beneficial profile. All these experiments will provide important knowledge for the design of an

intervention study with prebiotics in the future to further study microbiota-host interactions.

In **chapter 5** we conclude that the abundance of the *Ruminococcaceae NK4A214 group*, *Christensenellaceae R7 group*, *Ruminococcaceae UCG-005, and Marvinbryantia* decreased in postmenopausal breast cancer patients during treatment with AC-D. It is known that Ruminococcaceae can produce SCFA by degrading polysaccharides. As described above, SCFA positively influence intestinal homeostasis and are known to be involved in immunological and metabolic functions [23,24 49]. Therefore, reduction in the abundance of these bacteria during chemotherapy might contribute to manifestation of intestinal inflammation and dysregulated homeostasis. Quantitative assessment of microbial metabolites is an essential step to further reveal host-microbiota interactions. Furthermore, 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 [4]. Ideally metagenomic shotgun sequencing could be combined with the determination of microbial metabolites including SCFA to confirm our hypothesis [50].

Another option to further confirm our observations is to validate our results in an independent breast cancer cohort. If comparable conclusions could be drawn, decreased numbers of bacteria could be supplemented or manipulated with microbial interventions. For example, this can include a prebiotic or probiotic intervention or even faecal microbiota transplantation [51]. Finally, chemotherapy treatment combined with microbiota-modulating strategies could be the next step to personalised medicine in breast cancer patients treated with chemotherapy.

To overcome the influence of a small sample size on tumour response measurement in the neoadjuvant treated group, larger breast cancer cohorts should be created, or other outcome measurements are needed. For example, disease free survival or progression free survival or follow breast cancer patients until the presence of distant metastasis which was used in the study of Chiba et al. [52].

In **chapter 6** we suggest to further study the β-glucuronidase activity in postmenopausal breast cancer patients and healthy controls. This will provide more insight into the capacity of the intestinal microbiota to metabolise oestrogen [29,39]. Specifically in breast cancer, high levels of circulating oestrogens are related to the development of oestrogen receptor positive breast cancer [39]. Though normally, there is

a decrease in circulating oestrogens after the menopause. Yet oestrogen receptor positive breast cancer is the most common subtype within postmenopausal women. This might be caused by specific bacteria in the intestinal microbiota which are capable of metabolizing oestrogens. These bacteria belong to the estrobolome, which includes all bacterial genes involved in oestrogen metabolism. Activity of the bacterial enzyme β-glucuronidase increases intestinal oestrogen reabsorption into the circulation [29]. A relationship between intestinal microbiota-related oestrogen metabolism and systemic oestrogen levels has already been demonstrated in small groups [37-39]. Previously, our research team designed a cohort study to explore the role of bacterial β-glucuronidase activity in postmenopausal oestrogen receptor positive breast cancer patients. On top of that, as we concluded in chapter 2, evidence on an interaction between intestinal microbiota and hormonal therapy is very limited [53]. Our observational cohort study in postmenopausal oestrogen receptor positive breast cancer patients is also able to diminish this existing knowledge gap by comparing microbiota composition and ß-glucuronidase activity before and during endocrine therapy with tamoxifen.

To further explore the long-term effects and impact on quality of life of microbiota-modulating effects of recent (breast) cancer surgery, longitudinal faecal sampling of breast cancer patients without adjuvant systemic cancer treatment is recommended. Depending on the available budget, I will advise to perform faecal sampling each 2 or 4 months until one year after surgery. At the same sampling time points, questionnaires concerning quality of life, medication use, and dietary habits including prebiotic and probiotic use should be collected to assess other microbiota-modulating factors and if these potential surgery-induced microbiota changes are clinically relevant.

As shown in the review of **chapter 7**, current evidence on the link between breast microbiota and intestinal microbiota in breast cancer is limited. To further establish the link between breast microbiota, intestinal microbiota, the immune system, as well as breast tissue microenvironment, future studies should integrate longitudinal faecal microbiota sampling with breast tissue sampling, and the measurement of systemic oestrogen levels, as well as systemic inflammatory parameters. Ideally, this data should associate microbiota with bacterial β-glucuronidase activity, circulating oestrogens, and tumour infiltrating lymphocytes in breast cancer tissue to obtain the bigger picture.

In conclusion, this thesis has provided insights into the influence of intestinal microbiota on systemic cancer treatment as well as the influence of systemic cancer treatment on the intestinal microbiota. For this purpose it focussed especially on

colorectal and breast cancer. Although our studies are not perfect, more suitable alternative studies are not yet available to answer our research questions, which is mainly due to the lack of homogenous groups with reasonable sample sizes and challenges associated with longitudinal faecal sampling in a clinical setting. Our research has provided new leads to further explore the functional role of the intestinal microbiota and their impact on systemic cancer therapy. It will be highly relevant to unravel the functional microbial activity and its influence on systemic cancer therapy metabolism (pharmacomicrobiomics) in combination with the analysis of extensive longitudinal patients cohorts. During recent years, important collaborations have been established between microbiologist, oncologists, pathologist, and surgeons. These collaborations form the basis to explore future intestinal microbiota research in oncologic patients. Increased knowledge on the role of the intestinal microbiota in systemic cancer treatment is essential to improve personalised medicine in cancer patients.

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ADDENDUM

Summary

Summary

The overall aim of this thesis was to investigate the role of intestinal microbiota in cancer and systemic cancer therapy exploring new predictive, prognostic, and therapeutic targets. This thesis has focussed on cancer patients treated with systemic cancer therapy and especially on colorectal and breast cancer patients treated with chemotherapy.

The role of intestinal microbiota in systemic cancer therapy

Chapter 2 provides an overview of all relevant clinical studies performed until April 22nd 2019 that described the clinical association between baseline intestinal microbiota and systemic cancer therapy outcome as well as therapy-related changes in intestinal microbiota composition. The systematic literature search identified 23 articles. There were strong indications for a close association between the intestinal microbiota and immunotherapy related tumour response, survival, and toxicity. Furthermore, the development of chemotherapy-induced infectious complications seemed to be associated with the baseline intestinal microbiota. Both chemotherapy and immunotherapy induced drastic changes in intestinal microbiota composition with possible consequences for treatment efficacy in cancer patients. Evidence of an interaction between intestinal microbiota and hormonal therapy was very limited. Large heterogeneity concerning study design, study population, and faecal microbiota analysis techniques limited comparability and generalizability of results. Despite some studies identified associations between intestinal microbiota and tumour response or therapy induced changes, this is only based on a limited number of studies. Additional longitudinal clinical research is essential to confirm previous results and further identify prognostic and therapeutic microbial targets.

The role of intestinal microbiota in colorectal cancer treatment with chemotherapy

Chapter 3 presents our study protocol of a prospective multicentre cohort study on the role of intestinal microbiota in colorectal cancer treatment. The study aimed to explore whether baseline intestinal microbiota composition is associated with response or chemotherapy toxicity in patients with metastatic colorectal cancer (mCRC) treated with capecitabine or trifluridine/tipiracil. Moreover, the study aimed to explore the influence of capecitabine and trifluridine/tipiracil on intestinal microbiota over the course of chemotherapy. Before, during, and after three cycles of systemic treatment with capecitabine or triflurdine/tipiracil, faecal samples and questionnaires concerning

compliance, chemotherapy toxicity and quality of life were collected. Response was measured by CT or MRI using RECIST-criteria. Intestinal microbiota was analysed by amplicon sequencing of the 16S rRNA V4 hypervariable gene-region.

Chapter 4 described the results of the study presented in chapter 3. With longitudinal faecal sampling, we explored the role of intestinal microbiota in 33 mCRC patients treated with capecitabine. In total, 90 faecal samples were collected. Intestinal microbiota analyses with amplicon sequencing of the 16S rRNA V4 hypervariable gene-region showed that intestinal microbiota composition and diversity before, during, and after three cycles of capecitabine were not associated with response. In addition, capecitabine did not induce significant changes in the intestinal microbiota composition and diversity during the treatment period. Individual specific microbial shifts as a result of antibiotic therapy during capecitabine treatment were observed. Since this is the first clinical study with longitudinal intestinal microbiota sampling in mCRC patients that explored the interaction between capecitabine and the intestinal microbiota, additional longitudinal studies using larger cohorts, longer follow-up, and performing functional microbiota analyses will be highly relevant to further investigate intestinal microbiota-therapy interactions in mCRC patients.

The role of intestinal microbiota in breast cancer and during breast cancer treatment with chemotherapy

In **chapter 5** we investigated with longitudinal faecal sampling the role of intestinal microbiota in postmenopausal oestrogen receptor positive (ER+) and human epidermal growth factor receptor-2 negative (HER2-) breast cancer patients treated with (neo)adjuvant chemotherapy with adriamycin (A), cyclophosphamide (C) and docetaxel (D). In total, 44 patients were included and 153 faecal samples were collected. Patients collected a faecal sample and completed a questionnaire before, during AC, during D and after completing chemotherapy, resulting in 28 paired samples. Pathologic tumour response after neoadjuvant AC-D treatment was assessed using the scoring system according to European Society of Breast Cancer Specialists (EUSOMA). Intestinal microbiota analyses with amplicon sequencing of the 16S rRNA V4 hypervariable gene-region showed that baseline intestinal microbiota was not associated with pathological response in neoadjuvant treated patients. However, our study showed that observed species richness reduced significantly during the course of AC-D in (neo)adjuvant treated patients. The abundance of Proteobacteria, unclassified Enterobacterales, and *Lactobacillus* significantly increased during AC-D treatment. After AC-D treatment, the abundance of Proteobacteria and unclassified Enterobacterales significantly decreased

to levels comparable to baseline. Contrary, the abundance of *Ruminococcaceae NK4A214 group*, *Marvinbryantia*, *Christensenellaceae R7 group*, and *Ruminococcaceae UCG-005* significantly decreased during the course of AC-D treatment. Patients with any grade diarrhoea during docetaxel had a significantly lower observed species richness compared to patients without diarrhoea. Diarrhoea was not correlated to antibiotic administration. We concluded that results from this first longitudinal clinical study in postmenopausal breast cancer patients indicate that AC-D treatment reduced observed species richness and induced subsequent shifts of specific bacterial taxa. Furthermore, diarrhoea was associated with lower microbial diversity.

In **chapter 6** we aimed to investigate if the intestinal microbiota of postmenopausal breast cancer patients differs from postmenopausal women without breast cancer. We compared the intestinal microbiota richness, diversity, and composition of 81 postmenopausal histologically confirmed ER+ and HER2- breast cancer patients and 67 postmenopausal healthy controls enrolled from the National Dutch Breast Cancer Screening Programme. Patients scheduled for (neo)adjuvant AC-D followed by endocrine therapy, or endocrine therapy (tamoxifen) were prospectively enrolled in a multicentre cohort study in the Netherlands. Patients collected a faecal sample and completed a questionnaire before starting systemic cancer treatment. In total, 148 faecal samples were collected. Intestinal microbiota was analysed by amplicon sequencing of the 16S rRNA V4 gene-region. Observed species richness, Shannon index, and overall microbial community structure were not significantly different between breast cancer patients and healthy controls. There was a significant difference in overall microbial community structure between patients who received recent breast cancer surgery, patient without recent breast cancer surgery, and healthy controls on phylum (p=0.042) and genus level (p=0.015). *Dialister* (p=0.001) and its corresponding family Veillonellaceae (p=0.001) were higher in patients who received recent breast cancer surgery, compared to patients without recent breast cancer surgery. Within the recent breast cancer surgery group, this higher abundance of *Dialister* and Veillonellaceae was not associated with intravenous prophylactic antibiotic administration with cefazolin. We concluded that intestinal microbiota richness, diversity and composition are not associated with postmenopausal breast cancer. This study identified that *Dialister* and Veillonellaceae were increased in patients who had received recent breast cancer surgery, independent of intravenous prophylactic cefazolin administration during recent breast cancer surgery, suggesting potential antibiotic-independent microbiota-modulating effects of breast cancer surgery.

Chapter 7 presents a literature review on the role of breast microbiota as a biomarker for breast cancer and therapeutic response. Breast cancer tissue contains its own unique microbiota. Emerging preclinical data implicates that breast microbiota dysbiosis contributes to breast cancer initiation and progression. Furthermore, the breast microbiota may be a promising biomarker for treatment selection and prognosis. Differences in breast microbiota composition have been found between breast cancer subtypes and disease severities that may contribute to immunosuppression, enabling tumour cells to evade immune destruction. Interactions between breast microbiota, intestinal microbiota, and the immune system are proposed, which all form potential targets to increase therapeutic efficacy. Identifying breast and gut microbial compositions that acquire a positive outcome to certain systemic cancer therapies could significantly reduce cancer burden.

Addendum

ADDENDUM

Samenvatting

Samenvatting

Dit proefschrift onderzocht de rol van de darmmicrobiota bij kanker en de systemische behandeling daarvan. Hierbij werd gekeken of de darmmicrobiota een rol kan spelen als diagnostische, prognostische of therapeutische marker. Dit proefschrift richt zich met name op kankerpatiënten die behandeld worden met systemische kankertherapie zoals immunotherapie, chemotherapie of hormonale therapie en in het bijzonder op darm- en borstkankerpatiënten die behandeld worden met chemotherapie.

De rol van de darmmicrobiota bij de behandeling van kanker met systemische kankertherapie.

Hoofdstuk 2 geeft een overzicht van alle relevante klinische studies die onderzochten of er een associatie was tussen de darmmicrobiota voorafgaand aan de therapie, de tumorrespons en bijwerkingen alsmede de invloed van de systemische kankertherapie op de darmmicrobiota. Door middel van een systematisch literatuuronderzoek werden 23 artikelen geïdentificeerd. Er waren sterke aanwijzingen voor een associatie tussen de darmmicrobiota en immunotherapie geïnduceerde tumorrespons, overleving en bijwerkingen. Chemotherapie geïnduceerde infectieuze bijwerkingen waren geassocieerd met een specifieke samenstelling van de darmmicrobiota voorafgaand aan behandeling. Zowel chemotherapie als immunotherapie zorgde voor een verandering in de samenstelling van de darmmicrobiota, met mogelijk gevolgen voor de effectiviteit van die kankerbehandeling. Er was erg weinig bewijs beschikbaar over een mogelijke interactie tussen darmmicrobiota en hormoontherapie. Verder werd geconcludeerd dat er veel heterogeniteit in de opzet, populatie en microbiota analysetechnieken tussen de verschillende studies aanwezig was. Hierdoor werd de vergelijkbaarheid van de studies sterk beperkt. Ondanks dat sommige studies een associatie vonden tussen darmmicrobiota en tumorrespons of bijwerkingen, zijn deze bevindingen gebaseerd op een beperkt aantal beschikbare studies. Aanvullend klinisch onderzoek is daarom noodzakelijk om bovenstaande resultaten te bevestigen en om de rol van de darmmicrobiota als prognostische en therapeutische marker verder te onderzoeken.

De rol van de darmmicrobiota bij de behandeling van darmkanker met chemotherapie

In **hoofdstuk 3** wordt het studieprotocol van een exploratieve observationele cohortstudie gepresenteerd waarbij de rol van de darmmicrobiota bij darmkanker onderzocht wordt. Het doel was om bij patiënten met uitgezaaide darmkanker die

behandeld worden met capecitabine of trifluridine/tipiracil te onderzoeken of de samenstelling van de darmmicrobiota voor de start van chemotherapie geassocieerd is met tumorrespons en/of bijwerkingen. Daarnaast werd onderzocht of capecitabine of trifluridine/tipiracil de samenstelling van de darmmicrobiota beïnvloeden. Voor, tijdens en na drie kuren chemotherapie met capecitabine of trifluridine/tipiracil werden ontlastingsmonsters en vragenlijsten over de kwaliteit van leven, bijwerkingen en therapietrouw verzameld. Tumorrespons werd bepaald door CT- of MRI-scans te beoordelen met de RECIST-criteria. De darmmicrobiota werd geanalyseerd door middel van amplicon sequencing van de 16S rRNA V4 hypervariabele gen-regio.

In **hoofdstuk 4** worden de eerste resultaten uit de studie zoals beschreven in hoofdstuk 3 gepresenteerd. Door longitudinale verzameling van ontlastingsmonsters hebben we de rol van de darmmicrobiota bij patiënten met uitgezaaide darmkanker die behandeld werden met capecitabine onderzocht. In totaal werden er 33 patiënten geïncludeerd en 90 ontlastingsmonsters geanalyseerd. Uit de resultaten bleek dat de samenstelling en diversiteit van de darmmicrobiota voor, tijdens en na drie kuren chemotherapie niet geassocieerd zijn met tumorrespons. Daarnaast werd gevonden dat er gedurende drie kuren capecitabine geen significante veranderingen in de samenstelling of diversiteit van de darmmicrobiota ontstonden. Echter, individuele verschuivingen van specifieke microbiota ten gevolge van antibiotica toediening gedurende de capecitabine behandeling werden wel geobserveerd. Omdat dit de eerste longitudinale klinische studie is die de darmmicrobiota onderzoekt in deze patiëntenpopulatie, zijn aanvullende longitudinale studies met onder andere grotere cohorten, een langere opvolgperiode en functionele microbiota analyses nodig. Hiermee kunnen de bovenbeschreven observaties en microbiota-therapie interacties verder worden geëxploreerd.

De rol van de darmmicrobiota bij borstkanker en de behandeling van borstkanker met chemotherapie

In **hoofdstuk 5** hebben we door middel van het longitudinaal verzamelen van ontlastingsmonsters de rol van de darmmicrobiota onderzocht bij postmenopauzale borstkankerpatiënten. Patiënten die deelname aan het onderzoek hadden een oestrogeen receptor positieve (ER+) en humane epidermale groei factor receptor-2 negatieve (HER2-) tumor die behandeld werd met (neo)adjuvante chemotherapie bestaande uit vier kuren adriamycine (A) en cyclofosfamide (C) gevolgd door vier kuren docetaxel (D). Patiënten verzamelden een ontlastingsmonster en vulden een vragenlijst in over de kwaliteit van leven en bijwerkingen van de chemotherapie voor de start van

AC, tijdens AC, tijdens D en een maand na de laatste D toediening. Er werden hiervoor 44 patiënten geïncludeerd en 153 ontlastingsmonsters verzameld. In totaal werden er van 28 patiënten vier opeenvolgende monsters verzameld. Pathologische tumorrespons na neoadjuvante chemotherapie werd beoordeeld met het scoringssysteem volgens de European Society of Breast Cancer Specialist (EUSOMA). De darmmicrobiota werd geanalyseerd door middel van amplicon sequencing van de 16S rRNA V4 hypervariable gen-regio. De analyses lieten zien dat de samenstelling van de darmmicrobiota voorafgaand aan neoadjuvante chemotherapie niet geassocieerd was met pathologische tumorrespons. Echter, gedurende de chemotherapie behandeling nam de rijkheid aan bacteriesoorten significant af. Tijdens de chemotherapie behandeling namen de relatieve hoeveelheden Proteobacteriën, ongeclassificeerde Enterobacterales en *Lactobacilli* significant toe. Na de chemotherapie behandeling keerden de relatieve hoeveelheden Proteobacteriën en ongeclassificeerde Enterobacterales terug naar een vergelijkbaar niveau als voor de start van de chemotherapie. Daarentegen namen de relatieve aantallen van *Ruminococcaceae NK4A214 group*, *Marvinbryantia*, *Christensenellaceae R7 group*, en *Ruminococcaceae UCG-005* tijdens de chemotherapie behandeling significant af. Patiënten met diarree tijdens de docetaxel behandeling hadden een significant lagere bacteriële soortenrijkheid in vergelijking tot patiënten zonder enige vorm van diarree. Diarree was niet gecorreleerd met antibiotica gebruik. We concludeerden dat dit de eerste longitudinale klinische studie is bij postmenopauzale ER+ en HER2- borstkankerpatiënten die laat zien dat chemotherapie behandeling bestaande uit AC-D het aantal bacteriesoorten in de darm verminderd en voor verschuivingen in de hoeveelheden van specifieke bacteriële taxa kan zorgen. Daarnaast was diarree, tijdens docetaxel behandeling, geassocieerd met een lager aantal bacteriesoorten en een lagere bacteriële biodiversiteit.

In **hoofdstuk 6** werd onderzocht of er een verschil was in darmmicrobiota tussen postmenopauzale vrouwen met en zonder borstkanker. Hiervoor vergeleken we het aantal bacteriesoorten, de bacteriële biodiversiteit en de algehele samenstelling van de darmmicrobiota van 81 postmenopauzale ER+ en HER2- borstkankerpatiënten en 67 postmenopauzale vrouwen zonder borstkanker. De vrouwen zonder borstkanker werden geïncludeerd in samenwerking met het bevolkingsonderzoek naar borstkanker nadat het mammogram geen aanwijzingen voor borstkanker liet zien. Patiënten die gepland stonden voor (neo)adjuvante (chemo)therapie bestaande uit AC-D of endocriene therapie (tamoxifen) verzamelden een ontlastingsmonster en vulde een vragenlijst in voorafgaand aan de behandeling. In totaal werden er 148 ontlastings-monsters geanalyseerd. De darmmicrobiota werd geanalyseerd door middel van

amplicon sequencing van de 16S rRNA V4 hypervariable gen-regio. Het aantal bacteriesoorten, de bacteriële biodiversiteit en de algehele bacteriesamenstelling waren niet significant verschillend tussen borstkankerpatiënten en gezonde controles. Er was wel een significant verschil in de algehele bacteriesamenstelling tussen patiënten die een recente borstoperatie hadden ondergaan, patiënten zonder recente borstoperatie en gezonde controles zowel op het niveau van bacteriële fyla (p=0.042) als geslacht niveau (p=0.015). De relatieve hoeveelheden van *Dialister* en zijn bijbehorende familie Veillonellaceae (p=0.001) waren hoger bij patiënten die recent een borstoperatie hadden ondergaan in vergelijking tot patiënten zonder recente borstoperatie. Deze hogere aantallen waren niet geassocieerd met intraveneuze profylactische antibiotica toediening met cefazoline. Wij concludeerden dat het aantal bacteriesoorten, de bacteriële biodiversiteit en de algehele bacteriesamenstelling niet geassocieerd waren met postmenopauzale borstkanker. Deze studie liet wel zien dat de aantallen van *Dialister* en Veillonellaceae toegenomen waren bij patiënten die recent een borstoperatie hadden ondergaan en dit was onafhankelijk van intraveneuze profylactische cefazoline toediening voorafgaand aan de borstoperatie. Dit suggereert dat er potentiële antibiotica onafhankelijke darmbacterie modulerende effecten zijn van een recente borstkanker operatie.

Tot slot wordt in **hoofdstuk 7** een literatuuronderzoek gepresenteerd naar de potentiële rol van de borstmicrobiota als biomarker voor borstkanker en respons op de systemische behandeling van borstkanker. Het is bekend dat borstkankerweefsel een eigen unieke microbiota bevat. Preklinische onderzoeken veronderstellen dat dysbiose van de borstmicrobiota kan bijdragen aan de initiatie en progressie van borstkanker. Daarnaast hebben preklinische studies aangetoond dat de borstmicrobiota een veelbelovende biomarker kan zijn bij het voorspellen van de prognose en het selecteren van een optimaal behandelplan. Er zijn verschillen gevonden in de samenstelling van de borstmicrobiota tussen patiënten met verschillende borstkanker subtypes en verschillende stadia die mogelijk bij kunnen dragen aan het onderdrukken van het immuunsysteem, met als gevolg dat tumorcellen vernietiging door het immuunsysteem kunnen ontwijken. Ook wordt er verondersteld dat er een interactie is tussen de borstmicrobiota, de darmmicrobiota en het immuun systeem. Al deze factoren vormen potentiële aanknopingspunten om de werkzaamheid van de systemische kankerbehandeling te optimaliseren. Om de morbiditeit ten gevolge van kanker te verminderen is het belangrijk om toekomstig onderzoek te richten op het identificeren van samenstellingen van zowel de borstmicrobiota als darmmicrobiota die geassocieerd zijn met een gunstige reactie op systemische kankertherapie.

ADDENDUM

Impact paragraph

Impact paragraph

Colorectal and breast cancer are common types of cancer. Colorectal cancer is the third most common cancer in the world[1]. The incidence of colorectal cancer almost doubled from 1990 to 2016, with 15,000 new patients yearly in the Netherlands. About 20% of colorectal cancer patients already have metastatic disease at diagnosis[2]. Breast cancer is the most common cancer worldwide[3]. One out of seven women (15%) will develop breast cancer during their lifetime[4]. Approximately 70% of the breast cancer patients have an oestrogen receptor positive tumour and this percentage is increasing[5]. In 2018, almost 80% of the breast cancer patients were postmenopausal at diagnosis[6].

Over recent decades, survival rates of colorectal and breast cancer patients have improved substantially due to improved diagnostic and treatment methods and the implementation of the national screening programme for colorectal cancer in 2014 and breast cancer in 1990. As a result, quality of life has become more and more important. Cancer treatment can, besides curation and its survival impact, also negatively impact quality of life. For instance, chemotherapy, which is administered in half of the patients, can induce side effects like diarrhoea, nausea, vomiting, fatigue, hand foot syndrome, peripheral sensory neuropathy, and alopecia. Furthermore, despite improved treatment strategies, it is still not possible to achieve complete tumour response and cure cancer in all patients. In order to optimize tumour response and reduce side effects in cancer patients, factors need to be identified which potentially impact individual tumour response outcomes and the safety profiles of chemotherapy. Besides the already known factors such as genetic predisposition, environmental influences, other factors, such as intestinal microbiota, are thought to influence tumour response and chemotherapy toxicity.

The human intestinal microbiota is a complex and dynamic ecosystem. Trillions of intestinal microorganisms, consisting of bacteria, archaea, fungi, protozoa, and viruses colonize the human gastro-intestinal tract. Intestinal microbiota are essential for the maintenance of metabolism, immune function, and homeostasis[7]. This results in an ecosystem with dynamic host-microbiota-environment interactions. Every human owns a unique microbiota signature, shaped by personal environmental influences. Due to this exclusive character, knowledge on the influence of the intestinal microbiota in cancer treatment might contribute to the next step in personalised medicine for every cancer patient.

To unravel the first steps of the pathway to new predictive, prognostic, and therapeutic targets of cancer treatment, the overall aim of this thesis was to provide clinical evidence on the role of intestinal microbiota in cancer and systemic cancer therapy, focussing on metastatic colorectal and breast cancer patients treated with chemotherapy.

Scientific impact and relevance

Longitudinal faecal sampling to explore the association between chemotherapy and the intestinal microbiota is highly relevant in studying the dynamic alteration in the intestinal microbiota induced by chemotherapy. However, after completing our review in cancer patient treated with systemic cancer therapy, we concluded that the availability of studies with longitudinal faecal sampling is limited. The new insights resulting from our review, should stimulate future longitudinal microbiota research in cancer patients treated with systemic cancer therapy[8].

In addition, from our own cohort study in metastatic colorectal cancer (mCRC) we concluded that after three cycles of capecitabine, the intestinal microbiota of mCRC patients showed no significant changes. This could be due to the relatively mild impact of capecitabine on the intestinal microbiota, and the low number of treatment cycles that were evaluated. In addition, it could be hypothesized that the intestinal microbiota is already altered due to previous extensive medical treatment of mCRC patients, which includes for example abdominal surgery and/or chemotherapy, earlier administered in 48% of the patients. These new hypotheses will lead to the formulation of additional endpoints and the design of a new research line. In more detail, a follow up period until tumour progression and/or discontinuation of chemotherapy due to chemotherapy toxicity is advised. To further compare microbiota composition of mCRC with primary diagnosed patients, it is necessary to include a cohort with primary diagnosed CRC patients. Therefore, the first steps for a new collaboration between Wageningen University and Maastricht University have already been initiated. The aim is to compare the intestinal microbiota of primary diagnosed non-metastatic CRC patients with our mCRC patient cohort. If the intestinal microbiota of mCRC patients is already depleted or affected by previous antibiotic or chemotherapy treatments and/or operations, early intervention to restore the dysbiotic stage, may be more appropriate. In addition, the type of intervention will depend on the intestinal microbiota composition. Probably, it will not be effective to feed eradicated microbiota with prebiotics. It will be more appropriate to supply eradicated bacteria by probiotic interventions or even provide a faecal microbiota transplantation (FMT). However, at this moment, FMT is a subject of

debate, since the safety in cancer patients has not been fully established[9,10]. As a consequence, primary FMT intervention studies will have to be established in an acceptable ethical situation, for example in patients with metastatic disease without further anti-tumour treatment options. To limit time-consuming faecal donor screenings and minimize the risk of adverse events, a more standardised approach of FMT is attractive. By combining a selection of bacteria from a (single) donor into a capsule[11], a more consistent, safe, and, acceptable approach can be created in this way. This synthetic FMT provides opportunities to manipulate microbial compositions and could be one step towards personalised microbial-based medicine.

To summarize, the scientific impact and relevance of this thesis is mainly based on the design and planning of upcoming clinical microbiota related research. This thesis provides the first steps for the desired longitudinal studies and provides resources for upcoming metabolite analysis and cohort comparisons of colorectal and breast cancer patients.

Implementation and target population

In the first place, the ideas and results of this thesis are and will be published in peer reviewed (inter)national journals. Given the translational character of the data, and lack of primary clinical knowledge, results are submitted to clinical oncologic translation journals. To bridge the knowledge gap between fundamental and clinical research, it is highly relevant to mainly target breast and colorectal cancer physicians and microbiology specialists. By presenting research ideas, oncology specialist should be inspired and informed of the potential role of the intestinal microbiota in systemic cancer therapy. The focus on chemotherapy is of great importance, since chemotherapy remains the backbone of most systemic cancer treatments.

Clinical implementation should be based on the integration of this microbiota research in current and future research. Therefore researchers should invest in long lasting collaborations between departments of microbiology and oncology and join (inter)national collaborations. These collaborations are also of added value for validation studies and exchange of knowledge, materials, and expertise. In more detail, the first steps for a new collaboration with Wageningen University have already been initiated. Furthermore, we are considering the idea of sharing our faecal samples of the breast cancer patients treated with chemotherapy for a validation study of the microbiota research group at the Gustave Roussy Institute in Villejuif, France. By using

metagenomic shotgun sequencing, this collaboration will also provide the opportunity to further reveal associated metabolic microbial functions.

At this stage of research, it is too early to start clinical intervention studies. Further research in the TNO *in vitro* model of the colon (TIM-II), a model that mimics the colon, will bridge the knowledge gap between clinical and fundamental pre-clinical research. During these experiments potential prebiotic compounds will be used to assess the effect of prebiotics on human intestinal microbiota treated with chemotherapy. After completing these pre-clinical experiments, existing multicentre clinical collaborations might be used to set up a clinical intervention study with prebiotics.

In the long term, the ultimate goal will be to integrate the intestinal microbiota as predictive, prognostic, and therapeutic target to improve personalised medicine. Besides the easily accessible and low invasive procedures to obtain non-invasively blood or urine, the faecal microbiota might be an easily accessible tool. Due to the low invasive character, ethical drawbacks of using the faecal microbiota as markers are not expected.

Finally, the results of this thesis are the starting point for future research of the expanding scientific team of Prof. Marjolein Smidt. These data can be used to support upcoming grant applications. In addition, the team will further study and share data with national and international partners. Moreover, it is highly relevant to educate our future scientists at primary schools to make them enthusiastic for microbiota research. Therefore, it is highly encouraged to also participate in for example "de Klokhuis Wetenschapsprijs" contest. In the 2020 edition, our microbiota research ended up in the top three of this contest.

ADDENDUM

Dankwoord

Dankwoord

Prof. dr. M.L. Smidt, beste **Marjolein,** bedankt dat u mij uitkoos om het microbiota avontuur aan te gaan. Er was nog helemaal niets. Dat maakte het enerzijds makkelijk, want we konden nog alle kanten op. Anderzijds moesten er veel ontdekkingstochten worden gemaakt door het ziekenhuis, de universiteit, maar ook daarbuiten. Wie zijn geschikte partners, welke bestaande connecties kunnen we gebruiken, wie willen mee op avontuur, wie kunnen out of the box denken en hoe kunnen we het microbiota onderzoek introduceren bij de professionals van de afdeling oncologie? Er waren periodes dat er elke week weer nieuwe ideeën, kansen en geschikte subsidie aanvragen op ons pad kwamen. We hebben keuzes gemaakt. Met welke stappen zouden we het meeste kunnen bereiken? Als je het mij vraagt, zijn er hele mooie samenwerkingen tot stand gekomen. Ik heb er onwijs veel van geleerd. En het mooie is, deze ervaringen neemt echt niemand meer van mij af! Ik ben dankbaar en blij dat je mijn promotor bent. Je was altijd snel bereikbaar voor advies, feedback en een luisterend oor. Het leuke is, dat je bij wat voor tegenslag dan ook, er altijd weer een positieve draai aan probeert te geven. Zowel in wetenschappelijk zin als op privé vlak. Uiteraard ook bedankt **Ivo**, **Linde, Nienke** en **Gijs**, wat kunnen jullie trots zijn op je echtgenoot/ moeder. Want als ik het zo van een afstandje hoorde, was het thuis of op vakantie nooit saai. Marjolein, jouw tomeloze energie is op allerlei vlakken terug te vinden, en dat maakt jou bijzonder.

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Lars, jij was een van de eerste studenten die ik mocht begeleiden. Je volgde je wetenschap stage voor de opleiding geneeskunde. Samen met **Aurelia**, die haar laatste jaar van je opleiding tot arts-klinisch onderzoeker volgde, zijn jullie in het microbiota onderzoek gedoken. Lars ging op ontdekkingstocht naar de darmmicrobiota van

gezonde vrouwen en Aurelia analyseerde de darmmicrobiota van patiënten met uitgezaaide darmkanker. Dit was voor jullie een mooi startpunt om jullie eigen promotietraject te volgen. Het is zo mooi om te zien dat jullie beiden nu in de voetsporen van het microbiota onderzoek zijn gedoken! Lars in het bijzonder, jij bent mijn opvolger en wat doe je dit goed! Je wist in no time mijn gestructureerde afvinklijstjes, mappen logica en werkwijze onder de knie te krijgen. Behoorlijk knap om de organisatie van een ander over te nemen en hiermee verder te werken. Hopelijk durf je de komende tijd ook je eigen ideeën te verwerken in deze organisatie. Want achteraf is toch niet alles even logisch :-). En Aurelia, bedankt dat je mij af en toe tijdens de thuiswerkperiode in corona tijd even op de hoogte bracht van de ontwikkelingen op de afdeling :-).
Na jullie zijn nog vele andere studenten gevolgd. Onder andere, **Anne Vievermans**, **Anne Heuft**, **Saskia**, **Elvira**, **Sabine Dieleman**, **Lisa Dohmen**, **Lisa Coolen**, **Coco, Demi, Sanne en Janneke**. Er waren zelfs een aantal van jullie met dezelfde voornamen. Dit zorgde soms voor de nodige verwarring, dus werd het noodzakelijk om jullie achternamen te gebruiken. Gelukkig is alles tot nu toe goed gekomen.

Ook de artsen en verpleegkundigen uit de ziekenhuizen waarmee ik samenwerkte wil ik graag bedanken. Om te beginnen natuurlijk het **Maastricht Universitair Medisch Centrum+**. In het speciaal wil ik de afdelingen chirurgie, oncologie, microbiologie, radiologie en pathologie bedanken. Hiervan is een groot deel ook betrokken bij het zogeheten "mammateam". Het bijwonen van de "mamma MDO's" of terwijl multidisciplinaire overleg over borstkankerpatiënten (voor degene die niet dagelijks met deze afkortingen te maken hebben) was een leerzame bezigheid. Daarom bedank ik alle aanwezige specialisten voor het voeren van interessante discussies tijdens deze overleg momenten. In het bijzonder, **Elly, Jeanine, Conny, Christel, Mieke** en **Femke**, van jullie heb ik veel over het reilen en zeilen op de oncologie afdeling geleerd. Super handig om voort te kunnen bouwen op jullie jarenlange ervaring! **Maud**, **Joyce**, **Kim** en **Nicol**. Jullie hulp en ondersteuning bij het includeren van patiënten is van onschatbare waarde. En sorry dat het onderwerp "ontlasting" bij oncologie patiënten niet zo aantrekkelijk klinkt als als die andere bekende onderzoeken, zoals de BOOG, RISAS of PET-MRI.

Naast de inzet van de darm- en borstkankerpatiënten had dit onderzoek ook niet uitgevoerd kunnen worden door de sterke samenwerking met de oncologie. Samen met dokter **Judith de Vos-Geelen**, **Liselot Valkenburg** en **Maaike de Boer** hebben we de onderzoeken opgezet in Maastricht en uitgerold naar externe ziekenhuizen. Ik hoop in de toekomst nog veel meer van jullie te kunnen leren. Uiteraard wil ik ook alle

oncologen in Maastricht bedanken die de moeite hebben genomen om patiënten te informeren en motiveren om na, een vaak beladen gesprek, toch nog met een van de onderzoekers te praten over deelname aan het onderzoek.

Maar daarnaast bedank ik natuurlijk ook, de altijd behulpzame collega's bij de microbiologie. Onder de vleugels van **dr. John Penders** en **Prof. dr. Koen Venema** ontwikkelen jullie allen je eigen kwaliteiten. Koen, bedankt dat u betrokken wilt zijn bij de onderzoeken en de onderzoekslijnen samen met Marjolein, Janine en Lars verder wilt uitbouwen naar het TIM-II model (de kunstdarm). Ik kijk uit naar de toekomstige resultaten. **Heike** je hebt de laboratorium experimenten voor het TIM-II onderzoek van Janine al goed op weg geholpen. Dank daarvoor! **Giang** and **David**, you were both the best to assist in the last step to analyse our microbiota data! Without your help, this work was not finished yet! **Christel** en **Wesley**, jullie wil ik graag bedanken voor al jullie kennis en kunde die jullie hebben gebruikt om ons te ondersteunen bij de laboratorium analyses.

Loes, altijd in voor onderzoek, als het maar met pathologie te maken heeft. Ik bewonder je visie en enthousiasme bij het opzetten en uitvoeren van out of the box onderzoeken. Samen met **Sabine Dieleman en dr. Annemarie Boleij** onderzoek je de borstmicrobiota. Ik ben benieuwd wat jullie bevindingen zullen zijn inclusief de TIL's metingen. Heel veel succes bij jullie eigen promotieonderzoeken!

Daarnaast was het onderzoek nog niet zover geweest als externe ziekenhuizen niet hadden geparticipeerd. In het bijzonder wil ik de specialisten bedankten de tevens al betrokken zijn als co-auteur op de stukken die in dit proefschrift terug te vinden zijn. **Geert-Jan Creemers**, **Birgit Vriens en Yvonne van Riet** uit het **Catherina Ziekenhuis**, **Arnold Baars** uit de **Gelderse Vallei**, **Hanneke Vestjens** en **Yes van de Wouw** uit het **Viecuri Medisch Centrum** en **Jeroen Vincent** uit het **Elkerliek Ziekenhuis.** Daarnaast bedankt ik ook alle betrokken **verpleegkundigen** voor hun tijd, geduld, uitleg aan patiënten, en alle administratieve handelingen die ervoor gezorgd hebben dat er zoveel mogelijk patiëntengegevens en materiaal verzameld kon worden.

Ook ben ik dankbaar dat **Arnold Smals** uit het **St. Anna Ziekenhuis** en **Anne-Marie Dietvorst** uit het **van Weel Bethesda Ziekenhuis** en **Fabienne Warmerdam** van het **Zuyderland Medisch Centrum** participeren om meer patiënten te includeren om de onderzoekscohorten verder uit te breiden, zodat vervolgens additionele onderzoeks-vragen beantwoord kunnen worden.

Prof. dr. Frans Ramaekers, beste Frans, als voormalig voorzitter van **GROW** heb ik u leren kennen als een intelligente, enthousiaste man. Ik wist dat we u, na de ingangsdatum van uw pensioen, toch nog vaker terug gingen zien. U heeft laten zien dat de passie voor wetenschap blijft! **Prof. dr. Manon van Engeland**, Beste Manon, u bedank ik dubbel. Niet alleen als lid van de beoordelingscommissie, maar ook als opvolgend voorzitter van GROW heeft u de afgelopen jaren mij en mijn mede promovendi gesteund en kansen gegeven voor een optimale ontwikkeling op wetenschappelijk gebied. Dank daarvoor!

En natuurlijk de ondersteunende medewerkers van de afdeling chirurgie en GROW, **Livia**, **Sabeth, Joyce, Birgitte, Marion en Judith Doomen**. De ene keer nog sneller dan de andere keer stonden jullie klaar voor vele administratieve vragen, budgettaire zaken, handtekeningen of agendaverzoeken. Bedankt voor alle hulp!

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Daarnaast, wil ik graag de **financierders** van dit onderzoek bedanken. Gedurende het eerste jaar van mijn promotietraject ontving ik de **Kootstra Talent Fellowship**. Ik bedank de organisatie en de beoordelingscommissie voor het vertrouwen en de steun die ze mij hebben gegeven om het begin van dit onderzoek mogelijk te maken. Daarnaast heeft **Hessam Tabeian** een belangrijke bijdrage geleverd aan de uitbreiding van de onderzoekslijnen. Hessam heeft namens Servier Farma B.V. het mogelijk gemaakt om darmmicrobiota onderzoek bij patiënten met uitgezaaide darmkanker mogelijk te maken. Hierdoor konden patiënten die behandeld worden met tabletten chemotherapie ook deelnemen aan het onderzoek. Als derde wil ik ook de **Stichting J. Coenegracht Sr.** bedanken voor de financiële bijdrage aan het onderzoek naar de darmbacteriën bij patiënten met uitgezaaide darmkanker. Tot slot, vind ik het ook belangrijk om alle individuele mensen te bedanken die een financiële bijdrage leveren aan wetenschappelijk onderzoek.

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Onvergetelijk zijn natuurlijk de **Smidties** ..... goed, jullie weten wel hoe de app-groep heet. Naast de hulp van alle studenten heb ik ook veel steun en gezelschap gehad van de collega's op de gang. Alhoewel ik af en toe ook wel de kloof voelde tussen de feestende promovendi en mij als moeder van twee kinderen. Zeker toen de overweldigende corona crisis uitbrak. Gelukkig is het contact met de **Smitties** ..... altijd intens gebleven en hebben we een mooie tijd gehad. Naast Janine Ziemons en Lars, wil ik ook jullie graag bedanken. Met aan aantal van jullie zijn we zelfs op congres geweest in o.a. Barcelona en Seattle. Cocktails drinken in de rooftopbar zat er voor mij niet in, want ja, ik had een dikke buik.... Het was er niet minder gezellig om, met mijn alcoholvrij cocktail. Tussen de belangrijke praatjes en abstract presentaties door gingen we naar de Boeingfabriek. **Janine Simons** (ja, alweer bijna dezelfde naam), bedankt dat je ons heelhuids naar het appartement terugbracht in die enorme bolide. **Thiemo**, jij was altijd vast beraden, rustig en weloverwogen. Van jou heb ik geleerd om te focussen. **Briete**, het was voor mij een geruststelling om te zien dat je ook met kinderen uitgerust op het MDO kon verschijnen. Het viel toch best mee die slapeloze nachten.... Zeker als je ze verdeeld met je echtgenoot ;-). **Sanaz**, jij werkte je een slag in de rondte en sloeg nogal eens een lunch over. Ik bewonder je vastberadenheid. **Marissa**, jou ken ik al bijna 10 jaar, dus het is teveel om hier op te schrijven. Maar mede dankzij jou, heb ik gekozen om te solliciteren bij Marjolein. Bedankt voor al je verhalen en inzichten. Succes met het afronden van jouw promotieonderzoek en verdediging! **Renée**, de nuchterheid zelve. Hier houd ik van. Bedankt voor de ingewikkelde R ondersteuning aan Janine en fijn dat we af en toe samen konden sparren over hoe we het onderzoek het beste konden afronden. **Evie**, bedankt voor je kritische blik, gezellige borrelavonden/ middagen, kletsmiddagen, koffiemomenten en ideeën. Ook jou bedankt ik dubbel, omdat je ook je zojuist genoemde expertises wil inzetten als paranimf. **Kees**, de barbecue master! Jij bracht wat meer balans tussen al die vrouwen in het borstkankerteam! Bedankt voor de heerlijke diners en hopelijk mogen er nog een aantal volgen! Top chef! **Sabine de Wild**, bedankt voor je regelmatig bezoekjes voor een gezellig film- of borrelavond. Hopelijk leert Rens snel dat je Sabine heet ipv Sabina ;-). **Lidewij,** jou droge humor maakte mijn dag altijd goed. Je verassende uitspraken of kaartje bleven mij altijd lang bij. Bedankt voor deze geest verruimende inzichten. **Veerle en Roxanne**, jullie heb ik met name "online" leren kennen, gelukkig leven we in een prachtig digitaal tijdperk, zodat jullie humor mij ook niet ontgaan is. Gelukkig hebben we de laatste weken al redelijk ons best gedaan om de live bijeenkomsten in te halen. Succes bij al jullie onderzoek!

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ADDENDUM

List of publications

List of publications

This thesis

Aarnoutse R, Hillege LE, Ziemons J, de Vos-Geelen J, de Boer M, Aerts EMER, Vriens BEPJ, van Riet Y, Vincent J, van de Wouw AJ, Le GN, Venema K, Rensen SS, Penders J, and Smidt ML.
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* Both authors contributed equally to this work.

ADDENDUM

Curriculum vitae

Curriculum vitae

Romy Aarnoutse was born on 22th April 1990 in Vlissingen, the Netherlands. She grew up in Koudekerke. In 2008, she graduated from high school (Christelijke Scholen Gemeenschap van de Perre) and started her Bachelor's in Nutrition and Health at Wageningen University. She received her Bachelor's degree in 2011, and subsequently moved to Maastricht to become both a medical doctor and a scientist. During her Master's program, she worked for the Clinical Trial Centre Maastricht, and was involved in a study on sacral pressure ulcers under the supervision of prof. dr. Nicole Bouvy. For her last internship, she was involved in a clinical trial on groin wound infections under supervision of dr. Jan Willem Daemen. She presented the results of this trial at the Vascular Rounds in Thorn. Directly after her graduation in 2015, she started working at the emergency department at Zuyderland Medical Centre in Sittard and Heerlen.

In September 2016, she started her PhD project under the supervision of prof. dr. Marjolein Smidt, dr. John Penders, and dr. Judith de Vos-Geelen at the surgery department of Maastricht University Medical Centre+ in collaboration with the department of microbiology and medical oncology. Her PhD project aimed to further explore the role of intestinal microbiota in cancer treatment, in order to bridge the translational gap between pre-clinical and clinical studies. During the first year, she was awarded with a personal grant from the Kootstra Talent Fellowship (€42,000). Shortly after that, one of the studies received additional financial support from the Stichting J. Coenegracht Sr. (€112,273). After the publication of the study protocol of the colorectal cancer research line, Servier Nederland Farma B.V. declared an interest in supporting this study. This resulted in an Investigator Initiated Study agreement (€150,000).

Throughout her PhD trajectory, she attended numerous conferences and gave multiple presentations. During the COVID-19 pandemic, she worked for a short period at the Intensive Care department in Maastricht. Romy will pursue her clinical career as a medical doctor at the department of gastroenterology, hepatology, and internal medicine at Zuyderland Medical Centre in Sittard and Heerlen.

Romy is married to Ramon Meijers and they live together in Maastricht with their children Rens (5th June 2017) and Roos (15th June 2019).