The Microbial Mediators:

Investigating Microbiome-Driven Responses in Cancer Therapy

Inaugural Dissertation

zur

Erlangung des Doktorgrades

philosophiae doctor (PhD) in Health Sciences

der Medizinischen Fakultät

der Universität zu Köln

vorgelegt von

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aus Wittlich

Digital Express 24 GmbH & Co. KG, Köln

2024

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Datum der mündlichen Prüfung: 19.07.2024

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LIST OF ABBREVIATIONS

AIC	Akaike information criterion
AJCC	American Joint Committee on Cancer
AMP	antimicrobial peptide
APC	antigen-presenting cell
AUC	area under the curve
bp	base pair
BV	bacterial vaginosis
CDI	*Clostridioides difficile* infection
CI	confidence interval
CIN	cervical intraepithelial neoplasia
CNS	central nervous system
CST	community state type
CTA	cancer/testis antigen
CTX	cyclophosphamide
CR	complete response
CTLA-4	cytotoxic T-lymphocyte antigen 4
DC	dendritic cell
DCB	durable clinical benefit
DNA	deoxyribonucleic acid
ENS	enteric nervous system
FDA	food and drug administration
GBS	group b streptococcus
GVHD	graft-versus-host disease
HMO	human milk oligosaccharide
HMP	Human Microbiome Project
HPV	human papillomavirus
HSCT	hematopoietic stem cell transplantation
IARC	International Agency for Cancer Research
IBD	inflammatory bowel disease
ICI	immune checkpoint inhibitor
IFN-y	interferon gamma
ITIM	immunoreceptor tyrosine-based inhibitory motif
Ig	immunoglobulin

IL	interleukin
irAE	immune-related adverse event
IVF	*in vitro* fertilization
LAB	lactic acid bacteria
LAG-3	Lymphocyte activation gene-3
LDA	linear discriminant analysis
LPS	lipopolysaccharides
LEfSe	linear discriminant analysis effective size
MAGEA	melanoma-associated antigen
MAP-K	mitogen-activated protein kinase
MHC	major histocompatibility complex
NAFLD	non-alcoholic fatty liver disease
NK cell	natural killer cell
NKT cell	natural killer T cell
NOD	nucleotide-binding and oligomerization domain
NSCLC	non-small-cell lung cancer
NY-ESO-1	new york esophageal squamous cell carcinoma 1
OR	odds ratio
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PCoA	principle coordinate analysis
PD-1	programmed cell death protein 1
PD-L1	programmed cell death protein ligand 1
PVR	poliovirus receptor
PVRL2	poliovirus receptor-related 2
PR	partial response
qPCR	quantitative polymerase chain reaction
Rb	retinoblastoma protein
RCC	renal cell carcinoma
RF	random forest
RFE	recursive feature elimination
RMSE	root mean squared error
ROC	receiver operating characteristic
SCFA	short-chain fatty acids
SD	stable disease

s.d.	standard deviation
TGF	transforming growth factor
Th17 cells	IL-17-producing T-helper cells
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TMAO	trimethylamine-N-oxide
TME	tumor microenvironment
TLRs	Toll-like receptors
TNF-α	tumor necrosis factor-α
Treg	regulatory T cell
UV	ultraviolet
VEGF	vascular endothelial growth factor
VEGF-TKI	vascular endothelial growth factor-tyrosine kinase inhibitors
VISTA	V-domain immunoglobulin suppressor of T-cell activation
WHO	World Health Organization

SUMMARY

The present dissertation explores the critical role of the human microbiota in the context of cancer. With an estimated 19.3 million new cases and nearly 10 million deaths globally in 2020, cancer represents a major global health challenge. In recent years, the study of the human microbiome has emerged as a frontier in biomedical research, revealing its crucial role in the development and treatment of several tumor entities. The research projects of this cumulative dissertation concentrate on the role of the microbiota (intestinal and cervical, respectively) in melanoma and cervical cancer and shed further light on how cancer therapy influences the microbiota and vice versa.

The publication on cutaneous melanoma indicates that specific bacterial populations in the intestine are associated with the response to immune checkpoint inhibitor therapy. These correlations suggest that microbiome profiling could potentially serve as a basis for personalized treatment options. Furthermore, with a combination of two microbiome features and one immune feature, we were able to predict response to immunotherapy already before the initiation of the therapy. As it is still not fully understood why only some patients benefit from immune checkpoint inhibitor therapy, this is an important finding.

In the publication on cervical cancer, we investigated the cervical microbiota's response to chemoradiation therapy. Although the alpha and beta diversity of the cervical microbiota remains relatively stable, the total bacterial load decreases significantly post-treatment. Furthermore, we observed inter-individual differences in the composition of the cervical microbiota in our cohort already before and after treatment with chemoradiation.

Beyond the two studies, the dissertation discusses the broader implications of microbiome research in enhancing cancer therapy efficacy. Potential strategies for future therapeutic interventions aiming at altering the patients' microbiota, such as the use of probiotics and microbiota transfer techniques, are currently under investigation.

Overall, this work makes a relevant contribution to the evolving field of microbiome research in cancer therapy, presenting new pathways for enhancing the effectiveness of treatment protocols and thus paving the way for innovative, microbiome-based personalized treatment approaches that may subsequently lead to better outcomes for patients.

GERMAN SUMMARY

In der vorliegenden Dissertation wird die zentrale Rolle der menschlichen Mikrobiota im Kontext von Krebs untersucht. Mit schätzungsweise 19,3 Millionen Neuerkrankungen und fast 10 Millionen Todesfällen weltweit im Jahr 2020 stellt Krebs ein enormes globales Gesundheitsproblem dar. In den letzten Jahren hat sich die Erforschung des menschlichen Mikrobioms zu einem bedeutenden Gebiet der biomedizinischen Forschung entwickelt und gezeigt, dass das menschliche Mikrobiom eine entscheidende Rolle bei der Entwicklung und Behandlung mehrerer Tumorarten spielt. Die Forschungsprojekte dieser kumulativen Dissertation konzentrieren sich auf die Rolle der Mikrobiota (Darm- bzw. zervikale Mikrobiota) bei Melanom und Gebärmutterhalskrebs und beleuchten, wie Krebstherapien die Mikrobiota beeinflussen und umgekehrt.

Die Publikation über das kutane Melanom deutet darauf hin, dass spezifische bakterielle Populationen im Darm mit dem Ansprechen auf eine Immun-Checkpoint-Inhibitor-Therapie assoziiert sind. Diese Korrelationen legen nahe, dass die Analyse des Mikrobioms potenziell als Grundlage für personalisierte Behandlungsoptionen dienen könnte. Darüber hinaus gelang es, durch die Kombination von zwei Mikrobiom-Merkmalen und einem immunologischen Merkmal das Ansprechen auf die Immuntherapie bereits vor Beginn der Behandlung vorherzusagen. Da noch immer nicht vollständig geklärt ist, warum nur einige Patient*innen von der Therapie mit Immun-Checkpoint-Inhibitoren profitieren, ist dies eine wichtige Erkenntnis.

In der Publikation über Gebärmutterhalskrebs wurde die Reaktion der zervikalen Mikrobiota auf die Chemoradiotherapie untersucht. Obwohl sowohl die Alpha- als auch die Beta-Diversität der zervikalen Mikrobiota relativ stabil blieb, nahm die Gesamtzahl der Bakterien nach der Behandlung signifikant ab. Darüber hinaus beobachteten wir interindividuelle Unterschiede in der Zusammensetzung der zervikalen Mikrobiota in unserer Kohorte bereits vor als auch nach der Chemoradiotherapie.

Neben diesen beiden Studien werden in der vorliegenden Arbeit auch weitere Implikationen der Mikrobiomforschung für die Verbesserung der Wirksamkeit von Krebstherapien diskutiert. Potenzielle Strategien für zukünftige therapeutische Interventionen, die auf eine Veränderung der Mikrobiota der Patient*innen abzielen, wie der Einsatz von Probiotika und Mikrobiota-Transfer-Techniken, werden derzeit untersucht.

Insgesamt leistet diese Arbeit einen wichtigen Beitrag zu den Entwicklungen auf dem Gebiet der Mikrobiomforschung in der Krebstherapie, indem sie neue Ansätze zur Verbesserung der Wirksamkeit von Behandlungsprotokollen aufzeigt und den Weg für innovative, mikrobiombasierte personalisierte Behandlungsansätze ebnet, die in der Folge zu besseren Ergebnissen für die Patient*innen führen könnten.

1. INTRODUCTION

The present work comprises two publications on the subject of microbiome and cancer, the first in patients with cervical cancer and the second in a cohort of melanoma patients. As a basis for a subsequent discussion, this introductory chapter summarizes the current state of human microbiome research, both in general and with regard to the two indications.

1.1. THE HUMAN MICROBIOTA

The human microbiota refers to the complex community of microorganisms that reside in and on the human body. This includes bacteria, archaea, viruses, fungi, and protozoa. The term 'microbiome' is used to describe the collective genetic material of these organisms. Breakthroughs in DNA sequencing and computational tools for microbiome research have significantly enhanced our understanding of its composition [1]. Currently, the preferred method to analyze the microbiota is shotgun metagenomic sequencing, which offers a higher resolution of microbial diversity compared to 16S rRNA gene sequencing. Over the last decades, the study of the human microbiota has gained significant attention, due to its crucial role in human health and disease. The microbiota is involved in numerous biological processes, including digestion, immunity, and protection against pathogens. Emerging research even suggests an influence on the central nervous system [2]. This chapter provides an overview of the human microbiota, including its composition, functions, and implications for health and disease.

Microbial communities establish themselves on all human body sites that interface with the environment. This includes areas such as the skin [3], mouth [4], nose [5], vagina [6], respiratory tract [7], stomach [8], and intestines [9]. There are about as many human cells as bacterial cells (3.8×10^{13}) in a human body, most of them residing in the colon [10]. While the overall quantity of bacteria can vary greatly across these different locations, the predominant bacterial phyla are consistent, with Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, and Cyanobacteria being the most common across all sites [11]. Nonetheless, the relative abundance of these bacterial groups can differ significantly from one body surface to another [12]. Additionally, variations at the genus and species level across different sites underscore the presence of unique habitats and ecological niches within the body. Each niche offers a unique set of conditions like oxygen level, nutrient availability, temperature, or pH, which select for the growth of particular microorganisms. In addition to the difference between the individual parts of the body, the composition of microorganisms varies from person to person, so that each human being has an individual "microbial fingerprint" [13]. The Human Microbiome Project (HMP) has been instrumental in establishing a comprehensive catalog of the

microorganisms that inhabit various regions of the human body. Initiated in 2007, the HMP aimed to characterize the microbial communities found at multiple human body sites and to understand their role in human health and disease [14]. Since then, the field of microbiome research has rapidly developed with numerous studies investigating both the composition of a healthy microbiota and its changes in diseases. While in 2013 nearly 4,000 publications appeared for the key words “microbiome” or “microbiota”, ten years later the number of publications already exceeded 27,000. Most studies focus on the gut microbiota, which accounts for the majority of bacteria in the human body.

1.1.1. THE GUT MICROBIOTA

1.1.1.1. DEVELOPMENT AND STRUCTURE OF THE GUT MICROBIOTA

The establishment of the intestinal microbiota begins very early, when the first substantial exposure to bacteria takes place during birth. The mode of delivery thereby significantly influences the initial colonization of the neonate’s gut. Vaginally delivered infants are primarily colonized by their mothers’ vaginal and fecal microbiota, predominantly with bacteria from the genera *Bacteroides*, *Bifidobacterium*, *Parabacteroides*, *Escherichia/Shigella* [15]. In contrast, the intestinal microbiota of neonates delivered by Caesarian section showed less resemblance to their mothers’ microbiota and rather corresponded to the skin of the mother and the hospital staff or surrounding environment during delivery [16]. These and other factors, including antibiotic use [17], contact to pets [18], maternal health [19] and early-life diet [17] then influence the further maturation of the infant’s microbiota. Besides microorganisms in breast milk that seed the infant gut [20], breast milk contains human milk oligosaccharides (HMOs), which are indigestible by the infant, but serve as prebiotics that selectively stimulate the growth of beneficial bacteria like Bifidobacteria [21]. In contrast, formula-fed infants typically exhibit a more diverse microbiota [22]. Numerous studies have associated breastfeeding with health benefits for the infant, like lower risks of infections [23] or chronic diseases, such as allergies [24], asthma [25] or diabetes [26]. Based on the research findings of recent decades, it is very likely that these effects are partly triggered by the influence on the child's intestinal microbiota. For instance, it was discovered that 1-month-old infants exhibiting changes in their microbial composition (lower relative abundance of particular bacteria, such as *Bifidobacterium* and higher relative abundance of certain fungi, such as *Candida*) and a fecal metabolome enriched for pro-inflammatory metabolites faced a considerably higher risk of developing asthma and atopy during their childhood [27].

During the first years of life, the human gut microbiota undergoes significant changes in composition and function, transitioning from a simple, unstable community to a rather complex and stable ecosystem. In general, the maturation of the gut microbiota after birth can be divided into three

phases: a developmental phase (months 3-14), a transitional phase (months 15-30), and a stable phase (months 31-46) [28]. The introduction of solid foods marks a major shift in the gut microbiota and leads to a rapid expansion of bacterial diversity due to a broader range of substrates for microbial fermentation [29]. The rapid increase in bacterial diversity seen in the first year of life markedly decelerates by the age of three until the composition of the intestinal microbiota stabilizes around the age of five [30], though it still remains less diverse and functionally distinct compared to a healthy adult's microbiome [31]. The level of complexity, resilience and stability rises until adulthood, as different types of bacteria are gradually introduced, resulting in a diverse composition that differs from individual to individual, but which in healthy people is usually characterized by a high diversity. Although the overall individual microbiota composition is relatively stable over time, constant interaction between the microorganisms themselves and their environment dynamically shapes the microbial community on species level. In the course of life, acute perturbations, such as infectious, metabolic or inflammatory diseases can disturb the microbial equilibrium causing a disruption – commonly referred to as "dysbiosis" – that can normally be restored once the illness has passed [32]. However, frequent or longer-lasting disturbances can cause a manifestation of the microbial dysbiosis. One characteristic of a disrupted microbiota is a rise in Gram-negative bacteria associated with conditions of oxidative stress and inflammation [33].

There is still no universal understanding of what characterizes a healthy microbiota composition, except that it is found in a healthy host. A diverse and balanced microbiota is probably more resilient to external disturbances. Research has demonstrated that a higher microbial diversity is associated with beneficial health outcomes, such as reduced risk of chronic diseases including obesity, type 2 diabetes, and inflammatory bowel disease (IBD) [34]. However, healthy individuals differ remarkably in terms of microbiota composition, even though metabolic pathways are more evenly distributed and prevalent across healthy individuals [14]. These interindividual differences can be partly explained by factors such as diet, lifestyle, genetics of the host, and environmental influences, including the before-mentioned early life exposures [35-37]. In 2011, the concept of gut enterotypes was introduced, proposing the existence of three robust clusters consisting of different dominant bacteria in the human gut microbiota: *Bacteroides* (enterotype I), *Prevotella* (enterotype II), and *Ruminococcus* (enterotype III) [38, 39]. Each enterotype harbors different genera that differ in their functional characteristics, for example in the way they generate energy from fermentable substrates in the colon. However, there is an ongoing debate among scientists regarding the concept of discrete enterotypes and some prefer the idea that microbial community types are fluid and continuous [40, 41]. One major criticism is the postulated stability of enterotypes over time. The composition of an individual's gut microbiota has been shown to vary depending on environmental factors such as diet or lifestyle, undermining the idea of robust enterotypes [40]. Furthermore, the different methodological approaches, for example

concerning sampling techniques or bioinformatic approaches, in the studies on enterotypes raise concerns about the reliability and reproducibility of the results.

1.1.1.2. PHYSIOLOGICAL FUNCTIONS OF THE GUT MICROBIOTA

The human gut microbiota has evolved as a symbiotic community that has many regulatory functions far beyond the breakdown of indigestible food compounds and the synthesis of vitamins. One major function of a healthy gut microbiota is the protection against pathogen colonization and overgrowth of pathogenic bacteria. This colonization resistance is maintained by several mechanisms, including direct competition for nutrients, production of metabolites or bacteriocins, that inhibit the growth of pathogenic bacteria, formation of mucus layers serving as a physical barrier, the establishment of intestinal hypoxia by symbiotic bacteria to limit growth of pathogenic facultative anaerobes, and innate or adaptive immune responses that are induced or maintained by the intestinal microbiota [42]. The intestinal microbiota is also involved in the promotion of fat storage [43] and angiogenesis [44], and interacts with the host's immune system via the gut-immune axis [45, 46]. Such functional axes have now been described for almost all organs and are not to be understood exclusively bilateral, but as multiple interactive systems of various regulatory axes.

The enteric nervous system (ENS) of the gut is linked to the central nervous system (CNS) via the gut-brain axis, facilitating bidirectional communication through neural (vagus nerve) [47], endocrine [48], and immunological [49] signaling pathways [50]. The disruption of the gut-brain axis has been associated with a range of psychiatric [51], neurologic [52], and immunologic [53] conditions.

The gut-lung axis involves immune-mediated pathways, but also the exchange of microorganisms and their metabolites, such as short-chain fatty acids (SCFA) [54], impacting various aspects of respiratory health and diseases [55]. Changes in the composition of the gut microbiota can trigger inflammation and enhance intestinal permeability, permitting translocation of intestinal bacteria and microbiota-derived components like lipopolysaccharides (LPS) or pathogen-associated molecular patterns (PAMPs) into the bloodstream and subsequently the lungs [56]. This can lead to alterations in lung immunity marked by dysregulation of T cells and elevated inflammatory markers [57].

The crosstalk between gut and liver is referred to as the gut-liver axis and is mediated through direct and indirect pathways, including blood flow via the portal vein system [58], bile acids [59], metabolic products, and microbial components [60]. This axis is crucial for maintaining metabolic homeostasis and its dysfunction is involved in the pathogenesis of various liver diseases, like non-alcoholic fatty liver disease (NAFLD) [61] or cirrhosis [62]. In response, the liver not only processes microbial inputs but also impacts the intestinal microbiota via the secretion of bile acids and Immunoglobulin A (IgA) antibodies, thereby exerting a significant regulatory effect on the composition of microbial communities [59].

The communication between the gastrointestinal tract and the kidneys is described as the gut-kidney axis. On the one side, disruption of the intestinal microbiota can lead to an increased production of uremic toxins like indoxyl sulphate, *p*-cresyl sulphate, or trimethylamine-N-oxide (TMAO), all potentially relevant for the development of kidney diseases [63, 64]. During the state of intestinal inflammation and epithelial barrier impairment, these bacterial-derived uremic toxins can translocate, leading to oxidative stress injury to the kidneys. Conversely, uremia can lead to alterations in the intestinal microbiota composition resulting from accumulation of uremic toxins, inflammation and malnutrition [65, 66].

1.1.1.3. THE GUT-IMMUNE AXIS

As the gut-immune axis plays a crucial role in the context of cancer, due to its influences on the body's ability to recognize and eliminate tumor cells, a separate chapter is dedicated to it.

The microbiota plays an important role in the development of the host's immune system and as already mentioned in the chapter on the development of the microbiota, early-life perturbations can have long-lasting impacts on immunity [67, 68]. Early studies on germ-free mice demonstrated that T-cell receptor-bearing intraepithelial lymphocytes as well as IgA antibodies and IL-17 producing T-helper (Th17) cells are significantly reduced or absent in these animals but can be induced by microbial colonization [69-71]. Besides, the development of B cells takes place within the intestinal mucosa through regulation by extracellular signals from commensal bacteria that shape gut immunoglobulin repertoires [72].

The intestinal epithelium is a single layer of cells that separates the underlying tissue from the intestinal lumen and as part of the mucosal barrier it is essential for preventing the entry of pathogens and toxins on the one hand and regulating the passage of nutrients and other substances at the other. Trans-epithelial permeability is restricted via tight junctions that can be up- and down-regulated by microbial signals [73, 74]. Other factors that contribute to the mucosal barrier function are mucus-forming mucins like MUC2 [75], antimicrobial peptides (AMPs) [76] and secretory IgA [77] on the extraepithelial side, and immune cells on the inner side [78]. The immune compartment is situated within the lamina propria and Peyer's patches, where specialized dendritic cells (DCs) stimulate naïve T cells to differentiate into either regulatory T cells (Tregs) or pro-inflammatory effector cells. Disruption of the mucosal barrier function can lead to an increased permeability and subsequently to bacterial translocation that may result in several inflammatory conditions [74].

Toll-like receptors (TLRs) play an important role in maintaining gut-immune homeostasis by recognizing conserved molecular patterns associated with microorganisms and regulating immune response in the gut. Thereby, tolerance to commensal bacteria is maintained, while inflammation against pathogens can be induced [79]. TLRs are expressed on various cells in the intestine, including

epithelial cells and immune cells like DCs and macrophages [80]. Another group of pattern recognition receptors that are involved in intestinal homeostasis are nucleotide-binding and oligomerization domain (NOD)-like receptors [81]. Recent findings suggest that abnormal activation of NOD-like receptors may be a major cause of intestinal mucosal barrier dysfunction [82].

The adaptive immune response is also influenced by the intestinal microbiota and vice versa. B cells, for example, produce secretory IgA antibodies in response to commensal bacteria [83]. This adaptive IgA pressure in turn leads to a diversified microbiota, critical for promoting ecological adaptability [84]. SCFAs, such as acetate, butyrate, and propionate, are metabolites that are produced by commensal bacteria during fermentation of dietary fiber and can modulate colonic Treg response [85]. $CD4^+$ Tregs that express the transcription factor Foxp3 play a key role in limiting intestinal inflammation [86]. By inducing the expression of Foxp3, SCFAs contribute to the maintenance of immune homeostasis. Another example for the interaction of immune cells with the intestinal microbiota are DCs, a class of antigen presenting cells (APCs) that are responsible for sampling antigens from the intestinal environment, thereby shaping immune response [87].

These are all examples of how different sets of immune cells interact with the commensal microbiota and there are many more that would go beyond the scope of this thesis. Any dysregulation in this complex interplay, for example triggered by environmental factors, can contribute to the development of various immune-mediated diseases like systemic autoimmune diseases [88], inflammatory bowel disease [89] or cancer [90]. The role of the microbiota in the context of cancer will be discussed in more detail in a later chapter of this thesis.

1.1.2. THE CERVICOVAGINAL MICROBIOTA

The cervicovaginal microbiota refers to the ecosystem of microorganisms present in the vaginal and cervical region of the female reproductive tract. Although less complex compared to the intestinal microbiota, it plays a pivotal role in maintaining a healthy reproductive tract environment and alterations have been associated with diseased states such as viral, bacterial or yeast infections or the development of cancer [91]. The vaginal and cervical microbiota are sometimes summarized under the term “cervicovaginal microbiota”, but more recent research highlights unique microbiota profiles for each region [92, 93]. Due to the local proximity, sampling the cervical microbiota without contact to the vaginal microbiota is methodologically challenging. The following introductory chapter presents the current state of research on both the vaginal and cervical microbiota.

1.1.2.1. STRUCTURE OF THE CERVICOVAGINAL MICROBIOTA

In healthy, premenopausal women, the cervicovaginal microbiota is typically dominated by species of Gram-positive, facultatively anaerobic *Lactobacillus* [94, 95]. In a pioneering study, five different

community state types (CSTs) were identified by analyzing vaginal samples from almost 400 ethnically diverse women using 16S rRNA gene sequencing [95]. Four of these five CSTs are characterized by dominance of *Lactobacillus crispatus*, *Lactobacillus jensenii*, *Lactobacillus gasseri*, or *Lactobacillus iners* respectively with low species diversity. The fifth group was more diverse and characterized by higher proportions of strictly anaerobic bacterial species, such as *Prevotella*, *Dialister*, or *Gardnerella.* Some communities undergo significant changes in a short span of time, while others remain relatively stable [96]. By producing lactic acid, *Lactobacillus* species contribute to the maintenance of a low pH environment (<4.5), thereby inhibiting growth of pathogenic organisms [97, 98]. Furthermore, they produce bacteriocins [99] and hydrogen peroxide (H_2O_2) [100], both having antimicrobial activity and thereby contributing to a healthy vaginal mucosal microenvironment.

The composition of the cervicovaginal microbiota is influenced by numerous factors, including hormonal levels, sexual activity and hygiene practices. Little is known about the cervicovaginal microbiota composition in children prior to puberty. They are less likely to have communities dominated by *Lactobacillus* species [101], but are rather colonized by *Prevotella*, *Porphyromonas*, *Ezakiella*, and *Peptoniphilus* species with a high diversity [102]. Lower levels of estrogen result in thinner vaginal epithelium with less glycogen that may be less promotive for *Lactobacillus* species [103, 104]. The microbiota of adolescent girls resembles that of reproductive-age women [105], indicating a transition between the ages of 6 and 12. During pregnancy, the vaginal microbiome is less diverse and *Lactobacillus* species become even more abundant, supporting the concept that hormonal changes have an impact on the microbiota composition [106]. Decreased estrogen levels during and after menopause may also be responsible for another shift in the microbiota composition, with *Lactobacillus* no longer being the dominant bacteria and an overall lower number of bacteria [107, 108]. The decrease in *Lactobacillus* and lactic acid production results in a higher vaginal pH, possibly leading to higher susceptibility to infections. Besides, smoking and recent sexual intercourse are associated with a reduction of *Lactobacillus* species and an increase in bacterial species diversity [109, 110].

In specimens collected from women with bacterial vaginosis (BV), a vaginal infection caused by an overgrowth of certain bacteria with symptoms like abnormal discharge and pain, the abundance of non-*Lactobacillus* species is higher and they are predominated by *Gardnerella vaginalis* as well as a number of other anaerobic bacterial species. The use of hormonal contraceptives is associated with a reduced risk of BV [111], while vaginal douching has been shown to increase the risk [112]. The infection with human papillomavirus (HPV) is one of the most common sexually transmitted infections and can have major implications for health as oncogenic HPV types are causally related to cervical cancer [113]. It has been shown that the cervicovaginal microbiota composition is associated with HPV

infection and thereby also plays a role in the development of cervical cancer [114]. This relationship is discussed in further detail in the chapter on cervical cancer.

Taken together, the cervicovaginal microbiota is composed of a diverse array of bacterial species, with *Lactobacillus* species being typically predominant in healthy individuals. In the course of a lifetime, the composition undergoes changes and is influenced by environmental as well as host-related factors.

1.1.2.2. PHYSIOLOGICAL FUNCTIONS OF THE CERVICOVAGINAL MICROBIOTA

As already described, the healthy cervicovaginal microbiota establishes a low pH by producing lactic acid and thereby serves as a first line of defense against sexually transmitted infections and other pathogens, including vaginosis-causing bacteria, yeast infections, and even viruses like HPV. Although an infection with HPV does not necessarily lead to the development of cervical cancer, HPV infection is shown to precede cervical intraepithelial neoplasia (CIN), a precancerous condition, potentially leading to cervical cancer [115]. It was shown that features of the cervicovaginal microbiota, namely high abundance of *Gardnerella* as well as an increased microbial Shannon diversity index (a quantitative measure used to characterize species diversity in a community, accounting for both the abundance and evenness of species present), were associated with high-risk HPV progression, whereas *Lactobacillus* showed significant protective effects [116]. The microbial influence on the development of cervical cancer will be discussed in greater detail in a subsequent chapter. The growth inhibitory effect of a healthy cervicovaginal microbiota on pathogens is furthermore facilitated through production of antimicrobial metabolites such as H_2O_2, bacteriocin-like compounds and biosurfactants, amphiphilic agents produced by microbiota that have emulsifying properties [117, 118]. Certain strains of *Lactobacillus* can prevent the adhesion of pathogenic microorganisms by either competitively binding to receptors on the host's epithelium or by breaking down the biofilms formed by pathogenic bacteria [119].

Another important physiological function of the cervicovaginal microbiota lies in the influence on fertility and pregnancy outcomes. For example, a cervical microbiome dominated by *L. crispatus* was shown to be a protective factor for biochemical and clinical pregnancy in *in vitro* fertilization (IVF) patients, while microbiomes enriched in *L. iners* or other bacteria were risk factors for pregnancy failure [120]. A disruption of the cervicovaginal microbiota during pregnancy was associated with a higher risk of spontaneous preterm delivery, the leading cause of neonatal morbidity and mortality [121]. Upon vaginal delivery, neonates are exposed to the maternal cervicovaginal microbiota, thereby acquiring their initial intestinal bacterial communities dominated by *Lactobacillus* or *Prevotella* [122]. As described in the chapter on the development and structure of the gut microbiota, this is a critical stage for the child's future health. On the other hand, if the mother is

colonized by harmful microorganisms, such as group b streptococcus (GBS), there is an increased risk of transmission during vaginal delivery with potentially life-threatening complications [123].

Interaction between the cervicovaginal microbiota and the immune system is facilitated through a number of immune cells and receptors such as toll-like receptors and NOD receptors present on mucosal epithelial cells lining the female genital tract [124]. By recognizing PAMPs produced by bacterial, viral and fungal pathogens, rapid immune defense can be provided as a first line of defense, comparable to the gastrointestinal tract. Upon microbial stimulation, signaling cascades of cytokines and chemokines that recruit immune cells are initiated, such as secretion of interleukin (IL)-6, IL-8, INF-γ or tumor necrosis factor-α (TNF-α) [125]. Studies have shown that levels of induced cytokines and chemokines significantly differ in women depending on the community state types [126]. Besides, the composition of the cervicovaginal microbiota can influence the integrity of the protective mucosal barrier, thereby increasing or reducing susceptibility to infectious agents such as HIV and other sexually transmitted viruses [127]. This effect is caused by an alteration in the diffusion barrier properties of the cervicovaginal mucus. The current state of research suggests that especially *Lactobacillus crispatus* can enhance barrier properties thereby having a protective effect [128, 129].

All the above-mentioned features of the cervicovaginal microbiota provide potential therapeutic approaches, for example in the context of probiotics, which have been the subject of intensive research in recent years [130]. The last chapter of the introduction of this thesis deals intensively with the microbiota as a potential therapeutic target.

1.2. MICROBIOTA IN THE CONTEXT OF CANCER

With nearly 10 million deaths worldwide in 2022, cancer is a leading cause of mortality and due to our aging population and lifestyle, the global incidence of cancer is expected to further increase over the next years [131]. Standard cancer treatments include surgery, chemotherapy, radiation, hormone therapy, and targeted therapy. Due to an often-limited efficacy of monotherapies, combination therapies are well-established. Standard cancer treatments can have severe adverse effects on healthy cells, supporting the need for more targeted and less toxic therapies. In recent years, immunotherapies, such as monoclonal antibodies, engineered T-cell therapy, or immune checkpoint inhibitors (ICIs) have revolutionized cancer treatment [132]. By modifying the patient's immune system, immunotherapies promote the recognition and elimination of cancer cells through various mechanisms. However, due to primary and acquired resistance to ICIs, not all patients benefit from these new therapy options, making biomarkers a promising tool for therapy response prediction [133].

In the last decades, numerous studies have suggested that the microbiota plays an important role in anticancer immune surveillance and can affect the patient's response to immunotherapy, especially ICIs [134]. Through the gut-immune axis, the intestinal microbiota can enhance anti-tumor response,

while microbiota-induced inflammation possibly contributes to cancer progress. The proposed underlying mechanisms are subject of a separate introductory chapter on the gut microbiota as a determinant of immunotherapy efficacy. In addition to the intestinal microbiota, microorganisms colonizing the tumor microenvironment (TME) may be contributing factors to cancer progression and the varying efficacy rates of cancer treatments by shaping the local immune response in the TME [135, 136]. It has been shown that each tumor type has a distinct microbiota composition and that intratumor bacteria are mostly intracellular present in both the cancer and the surrounding immune cells [137]. The role of intra-tumoral microbiota in anti-tumor immunity is highly complex and has not been fully characterized, but it seems that it has the potential to either enhance or suppress immune responses against tumors [138].

There is evidence implying a crucial role of the microbiota in different cancer types. In the context of liver cancer, for instance, intestinal *Clostridium* species-mediated primary-to-secondary bile acid conversion can enhance antitumor effect by accumulating natural killer T (NKT) cells [139]. Regarding colon cancer, the presence of *Fusobacterium nucleatum* in the tumor environment was shown to directly inhibit the ability of NK cells to kill tumor cells. This effect is partly mediated through interaction of the bacterium's Fap2 protein with the human T cell immunoglobulin and ITIM domain (TIGIT) receptor [140]. Gastric cancer can be a long-term consequence of an infection with *Helicobacter pylori*, which was classified as a class I carcinogen by the IARC already in 1994 [141]. *H. pylori* possesses virulence factors that can directly affect host signaling pathways and an infection with *H. pylori* can cause chronic inflammation of the gastric mucosa and subsequent carcinogenesis [142]. Several studies have linked the gut microbiota with cancer development in distant organs, such as the breast. Besides a distinct microbiome in breast cancer tissue compared to healthy breast tissue, changes in the composition of the gut microbiota have been associated with breast cancer, possibly through an altered production of metabolites and disruption of estrogen metabolism in the gut [143, 144]. There is an increasing body of evidence highlighting the significant impact of the intestinal microbiota on clinical outcomes after hematopoietic stem cell transplantation (HSCT). Patients undergoing HSCT, particularly allogeneic HSCT, experience significant changes in the composition of their gut microbiome, which is associated with increased risk of complications like graft-versus-host disease (GVHD), infections and mortality [145, 146].

1.2.1. MELANOMA

Melanoma is a malignancy arising from the pigment-producing cells in the skin (melanocytes) and though less common than other types of skin cancer, melanoma represents the most dangerous one and accounts for the majority (over 80%) of skin cancer-related deaths [147]. In 2020, an estimated number of 325,000 new melanoma cases and 57,000 deaths due to melanoma occurred worldwide

and the global burden is estimated to increase during the next years [147]. The primary risk factor for the development of melanoma is exposure to ultraviolet (UV) radiation, especially for fair-skinned populations of European ancestry. By causing errors in DNA replication, UV light is known to lead to mutations in cell signaling molecules which can subsequently lead to carcinogenesis [148]. In detail, UVB rays damage the DNA in melanocytes in the form of photoproducts between pyrimidine bases and UVA rays cause oxidative stress-induced DNA damage [149]. When left unrepaired by repeated and prolonged exposure to UV radiation, these DNA damages lead to an accumulation of mutations in the cells [150]. The molecular signature of a tumor differs significantly across melanoma subtypes, influencing prognosis and treatment outcomes. Key proteins often affected by these mutations include components of the mitogen-activated protein kinase (MAP-K), like NRAS and BRAF, which play crucial roles in regulating cell growth and differentiation [151]. By disrupting the normal regulation of cell growth and proliferation in this way, these mutations ultimately lead to the development of cancer. Besides, UV radiation can contribute to the development and progression of cancer by impairing the immune response [152].

Treatment options for melanoma depend on the cancer stage at the time of diagnosis and include surgical removal, radiation therapy, chemotherapy, targeted therapy, and immunotherapy. The introduction of the latter two some years ago was a major development in melanoma treatment with improved survival rates for advanced melanoma [153, 154].

1.2.1.1. CHECKPOINT INHIBITOR IMMUNOTHERAPY

Checkpoint inhibitor immunotherapy, which primarily targets the immune system rather than the cancer cells themselves, has become an important systemic treatment option for patients with metastatic melanoma and other malignancies. Immune checkpoints are regulatory pathways in the immune system that hinder it from attacking cells indiscriminately and thereby preventing autoimmune reactions. Immune checkpoint proteins including T-cell surface receptor programmed cell death protein 1 (PD-1), its ligand PD-L1 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) play a key role in maintaining the delicate balance between immune-tolerance and defense [155, 156].

After T-cell receptor engagement and a co-stimulatory signal through CD28, which is also expressed on T cells, CTLA-4 translocates from the intracellular to the T-cell surface, where it binds CD80 and CD86 molecules on APCs, leading to an inhibition of the respective T cell, which can no longer recognize and destroy cancer cells [155]. By blocking CTLA-4 with an antibody (ICI), the T-cell repertoire can proliferate and transition to an active form. The first monoclonal antibody targeting CTLA-4 that was approved by the FDA for the treatment of metastatic melanoma was Ipilimumab in 2011, after a large study showed a significant improvement in overall survival [153]. A response to anti-CTLA-4 therapy is more likely when the tumor has a higher mutational burden [157].

The receptor PD-1, which is expressed mainly on T cells, has two ligands, PD-L1 and PD-L2, that are expressed on the surface of APCs and tumor cells [158]. Upon interaction, an inhibitory signal is triggered, resulting in exhaustion of activated T cells. Exhausted T cells exhibit impaired ability to produce effector cytokines and show upregulation of inhibitory receptors, leading to a dysfunctional state less effective in fighting infections and tumors [159]. Blockade of the interaction of PD-1 with its ligands through therapeutic antibodies results in proliferation of T cells that can then infiltrate the tumor and induce a cytotoxic T-cell response [160]. In 2014, the FDA approved two monoclonal anti-PD-1 antibodies, Pembrolizumab [161] and Nivolumab [162], for the treatment of refractory melanoma and later also for other tumor entities.

It is also possible to block both checkpoint pathways in a combination therapy. A PD-1 inhibitor in combination with Ipilimumab has been shown to have better efficacy (response rates, progression-free survival) compared to monotherapy in patients with advanced melanoma [163, 164]. In various types of cancer, ICIs are furthermore used in combination with other systemic treatments such as chemotherapy or targeted therapy, as well as other modalities like radiation therapy [165].

In the last years, more ICIs have been or are being developed targeting PD-1 (Cemiplimab [166]), PD-L1 (Atezolizumab [167], Avelumab [168], Durvalumab [169]) and other immune checkpoint pathways, including Lymphocyte activation gene-3 (LAG-3) [170], T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) [171], V-domain immunoglobulin suppressor of T-cell activation (VISTA) [172], and TIGIT [173]. TIGIT interacts with its ligands CD155, CD112 and CD113, which are often expressed on tumor cells and APCs in the tumor microenvironment, leading to down-regulation of T-cell and NK-cell function, thereby inhibiting anti-tumor response [174]. Expression of TIGIT on tumor-infiltrating T cells correlates with an increased expression of other inhibitory receptors such as PD-1, leading to the approach of co-inhibition of TIGIT and PD-1 to enhance tumor rejection [175].

Despite important clinical benefits, treatment with ICI is associated with immune-related adverse events (irAEs), including dermatologic toxicities such as rash, gastrointestinal toxicities like diarrhea and colitis, hepatotoxicity, endocrine toxicities like thyroiditis, and other inflammatory events [176]. Although severe irAEs occur only in about 10% of patients treated with monotherapy, they can pose a life-threatening risk [177]. Evidence indicates that the occurrence of irAEs is linked to improved outcomes, including higher response rates and prolonged survival [178, 179]. A meta-analysis has furthermore associated specific microbiome signatures with distinct irAEs [180].

Although ICI therapy has markedly enhanced survival rates, only 40% of patients with advanced melanoma respond to Nivolumab treatment, and 61% respond to the combination of Nivolumab and Ipilimumab [162, 181]. Resistance can be categorized into primary resistance to treatment or acquired resistance after a prior period of response. Factors that contribute to resistance include the immunosuppressive nature of the tumor microenvironment (e.g. tumor-infiltrating lymphocytes),

genetic and molecular features (e.g. neoantigens and mutational burden), adaptive immune escape (e.g. upregulation of alternative immune checkpoints), and genetic evolution of the tumor (e.g. loss of MHC-I expression) [182]. In addition, a growing body of evidence in animals and humans suggests that composition of the intestinal microbiota has an impact on the therapeutic response [183-189]. Identifying biomarkers that can predict at an early stage whether a patient is suitable for a specific immunotherapy is an important aim of current research.

1.2.1.2. THE GUT MICROBIOTA AS A DETERMINANT OF IMMUNOTHERAPY EFFICACY

Numerous studies have associated the gut microbiota with the response to ICI, although the exact underlying mechanisms remain to be fully understood. In 2015, two pioneering publications first linked the gut microbiota to the efficacy of ICI in mouse models [190, 191]. In one study, melanoma growth in mice with different intestinal colonization patterns was compared and differences in spontaneous antitumor activity were identified [190]. After the microbiome differences were eliminated by co-housing and/or a transfer of intestinal microbiota from one group to the other, differences in antitumor activity disappeared. A microbiome analysis based on 16S rRNA gene sequencing identified commensal *Bifidobacterium* as the central bacterial taxon responsible for this effect. The presence of these bacteria induced an increase in the activity of dendritic cells and thus enhanced $CD8^+$ T-cell priming with improved antitumor activity. Based on these findings, oral administration of *Bifidobacterium* was able to reduce tumor growth to the same extent as PD-L1-specific antibody therapy. A combination of the two measures produced an additional additive effect. In another mouse model, the presence of certain *Bacteroides* spp. (*B. thetaiotaomicron* and *B. fragilis*) was shown to significantly enhance the antitumor effect of CTLA-4 inhibitors [191]. In addition, a shift in the microbiome towards the above-mentioned species was observed by transferring feces from patients who had received CTLA-4 inhibitor therapy for non-small cell lung cancer or metastatic melanoma. In this model, the effect was also mediated via the stimulation of dendritic cells and the T cells activated by them.

A few years later, these findings were complemented by human studies [185, 186, 192]. One study showed that the use of antibiotics led to a lower response to ICIs in patients with advanced cancer [192]. Clinical response was associated with the relative abundance of *Akkermansia muciniphila*. This study also transferred fecal microbiota from responder patients into germ-free or antibiotic-treated mice, which improved tumor control and restored response to ICI. Feces transfer from non-responders did not exert this effect. Oral supplementation with *A. muciniphila* after FMT from non-responders could however restore antitumor response. Another study on melanoma patients undergoing anti-PD-1 therapy showed a significantly higher alpha diversity and relative abundance of bacteria of the *Ruminococcaceae* family and Clostridiales order in responders compared to non-responders [185]. In

these patients, antigen presentation was increased and $CD4^+$ and $CD8^+$ T-cell function in the peripheral blood and tumor microenvironment was improved. A difference in the gut microbiota composition between responders and non-responders was also shown in a further study, but this time bacterial species more abundant in responders included *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium* [186]. Transferring fecal material from responding patients into germ-free mice could again enhance tumor suppression, boost T-cell response, and increase the efficacy of ICI therapy. In recent years, more prospective studies confirmed a significant link between the intestinal microbiota and outcomes after ICI therapy in several tumor entities beyond melanoma [193-195]. Furthermore, the attenuating effect of (prior) antibiotic treatment on ICI efficacy has been supported by several studies [196-198].

The proposed molecular mechanisms, by which the intestinal microbiota exerts its influences on ICI efficacy, range from inducing differentiation and proliferation of various immune cell types, over altering antigen presentation, to directly or indirectly regulating the expression and function of immune checkpoint molecules [199]. Many of these mechanisms are mediated through microbial metabolites, such as SCFAs. Patients with high fecal concentrations of SCFAs have longer progression-free survival [200]. On the contrary, high levels of the SCFAs butyrate and propionate have been associated with resistance to ICI therapy [201]. Another microbial metabolite that has been associated with ICI efficacy is inosine. Immunotherapy-induced decreased gut-barrier function can lead to increased systemic translocation of inosine, which in turn reprograms the tumor microenvironment and activates antitumor T cells [202]. Other microbial metabolites with immunomodulatory effects that have been a focus of research are tryptophan metabolites [203] and bile acids [204]. It should be highlighted that some studies have yielded inconsistent results, underscoring the need for further exploration into how gut microbiota metabolites influence the efficacy of ICIs.

1.2.2. CERVICAL CANCER

Cervical cancer represents the fourth most common cancer among women globally, affecting hundreds of thousands of women each year [205]. According to the World Health Organization (WHO), in 2022, there were about 660,000 new cases and 350,000 deaths attributed to cervical cancer worldwide, with highest incidence and mortality rates in Sub-Saharan Africa, Central America and South-East Asia. This variation by geographical region can most likely be attributed to differences in the availability of screening and vaccination programs as well as social and economic determinants [206]. The primary cause of cervical cancer is a persistent infection with specific genotypes of HPV, a common, sexually transmitted, non-enveloped double-stranded DNA virus that infects epithelial cells [207]. In most cases, HPV infections of the cervix are transient and cleared by the host's immune system, but persistent infection with high-risk types of HPV can lead to the

precancerous condition of CIN, and eventually cancer [208]. The International Agency for Cancer Research (IARC) has classified 12 HPV genotypes with sufficient evidence to cause cervical cancer (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) and another eight HPV genotypes (68, 26, 53, 66, 67, 70, 73, 82) as probably or possibly carcinogenic [209]. Globally, HPV types 16 and 18 are found in approximately 70% of all invasive cervical cancers [210]. Fortunately, already six licensed prophylactic vaccines against oncogenic HPV types to prevent HPV infection and development to cervical and other forms of cancer are available since 2006 [211, 212]. Additionally, secondary prevention can be achieved through screening for cervical precancers using cytology testing (Pap smear) or HPV DNA testing [213]. Despite these improvements, the global burden of HPV-mediated cervical cancer remains high and it remains particularly challenging to implement effective vaccination and screening programs in low-resource settings.

1.2.2.1. MICROBIAL INFLUENCE ON TUMOR DEVELOPMENT

The carcinogenic properties of some HPV types are facilitated through several molecular mechanisms, with two viral oncoproteins, E6 and E7, being the key drivers responsible for initiation and progression of cervical cancer. High-risk HPV types, such as HPV-16 and HPV-18, can integrate their viral DNA into the host cell's genome. The viral oncoproteins E6 and E7 are then being expressed by HPV-infected cells and bind and inactivate the tumor suppressor proteins p53 and retinoblastoma protein (Rb), respectively. In detail, the E7 protein binds to Rb and targets it for degradation, leading to uncontrolled cell proliferation [214]. As this induction of hyperproliferation triggers apoptosis through a p53-dependent pathway, E6 proteins have evolved that target p53 and induce its degradation, thereby inhibiting apoptosis and leading to immortalization and genomic instability of the cells that then have a higher tendency for transformation and malignant progression [215]. Other E proteins also play an important role in the early stages of cervical cancer. E1, E2, and E4 have been associated with viral integration, replication, and transcription and E5 facilitates the activity of E6 and E7 by complex mechanisms and regulates cell proliferation and apoptosis [216]. In cells that are persistently infected with high-risk HPV, these pathways result in accumulated cellular mutations over time, ultimately (up to several decades) leading to the development of cancer [217].

Furthermore, HPV can avoid the host's immune system by several mechanisms. By downregulating the expression of major histocompatibility complex (MHC) class I molecules, HPV can prevent recognition and clearance by cytotoxic T cells and evade the host's immune system [218]. Besides, HPV downregulates the expression of interferon and upregulates immunosuppressive cytokines, such as IL-10 and transforming growth factor TGF-β1, to form a local immunosuppressive environment, inhibiting anti-tumor responses [219, 220].

Another mechanism by which HPV can support tumor growth, is the stimulation of the production of angiogenic factors, such as vascular endothelial growth factor (VEGF) to promote the formation of new blood vessels, critical for the growth of tumors [221].

Since infection with HPV is a prerequisite for the development of cervical cancer and many studies have reported on the associations between cervicovaginal dysbiosis and incidence/persistence of HPV, the cervicovaginal microbiota already plays a significant role in the very beginning of cervical cancer development [115]. After first infection with HPV, the cervicovaginal microbiota can still have an impact on the outcome, as it has been shown that local microbial communities also regulate the host's immune response in many ways [222]. Namely, proinflammatory profiles with higher levels of cytokines and chemokines have been shown in patients with BV or microbiomes with a predominance of BV-associated bacteria and increased diversity [223, 224]. These inflammatory conditions can lead to tissue damage, possibly enhancing the cancer-causing potential of HPV. Moreover, *Lactobacillus* species of a healthy cervicovaginal microbiota have antitumoral properties by exerting cytotoxic effects on cervical tumor cells, but not on normal cervical cells [225]. Women with a Lactobacillus-dominant vaginal microbiota are more likely to have a regressive CIN disease compared to women with a vaginal microbiota enriched in anaerobic taxa including *Megasphaera*, *Prevotella timonensis*, and *Gardnerella vaginalis* which was associated with slower regression and persistence of CIN [226]. In the advanced stage of tumor development, CIN disease progression was associated with increased vaginal microbiome diversity and decreased abundance of *Lactobacillus* spp., although longitudinal data are scarce [91, 227]. One study examined the cervical microbiota composition at various stages of cervical cancer and found *Sneathia spp.* and *Fusobacterium spp.* to be predominant bacteria in women with squamous intraepithelial lesions and cervical cancer, respectively, with higher levels of IL-4 and TGF-β1 in the CST dominated by *Fusobacterium spp.* [228]. These results suggest that certain members of the cervical microbiota may be able to modify cytokine profiles within the cervical microenvironment. The complexity of the relationship between the host, the cervicovaginal microbiota and tumor development, makes the cervicovaginal microbiota an important research topic and may be a starting point for future preventive or therapeutic strategies.

1.3. THE MICROBIOTA AS A THERAPEUTIC TARGET

The numerous research results of the past decades on the complex relationships between the various microbiota habitats and the development and therapy of cancer, which were discussed in the previous chapters, suggest that the microbiota could also be an effective therapeutic target. Therapeutic strategies that modify the microbiota composition or function include prebiotics (nutrients, e.g. non-digestible fibers, that are degraded by microbiota), probiotics (live microorganisms with beneficial effect), and fecal or vaginal microbiota transfer (FMT, VMT).

In the context of female reproductive health, probiotics have been successfully used as an adjunctive therapy to antibiotics in patients with BV in order to improve cure rates and prevent recurrence [229, 230]. Especially probiotic lactic acid bacteria (LAB) such as *Lactobacillus* are generally recognized as safe microorganisms for human application. Probiotics are furthermore being explored as an intervention to promote HPV clearance and prevent cervical cancer development [231]. The use of prebiotics, meaning indigestible carbohydrates to promote the growth of healthy bacteria, has been mostly studied in the gastrointestinal tract [232], but several studies have explored the application of prebiotics in the vagina [233, 234]. When combining pre- and probiotics, the term synbiotic is used and this approach may enhance probiotic bacterial growth and secretion of bacteriocins [235]. In the context of prevention or treatment of cervical cancer, a study demonstrated HPV clearance induced by oral intake of probiotic *Lactobacillus crispatus* M247 [236]. Many more experimental studies have supported the idea of probiotics, especially *Lactobacillus*, as a promising alternative non-chemotherapy approach for cervical cancer [231, 237, 238]. Furthermore, probiotics have the potential to reduce the side effects of radiotherapy for cervical cancer, as was shown in a systematic review and meta-analysis including 1508 participants [239].

The earliest records of targeting the intestinal microbiota in the context of a therapeutic approach trace back to China in the fourth century, where human feces material was supposedly used in patients with severe diarrhea [240]. The first case report of an FMT was then published in 1958 [241], the first randomized controlled trial was published in 2013, proving a high efficacy of FMT in the treatment of recurrent *Clostridioides difficile* infection (CDI) [242] and since then the range of FMT applications extended rapidly, also to non-infectious diseases. Given the critical role of the gut-immune-axis, the intestinal microbiota represents a promising potential target for the development of therapeutic strategies aimed at combating cancer. In a preclinical mouse model, mice that received a low-fiber diet or probiotics demonstrated an impaired response to ICI treatment and had lower levels of cytotoxic T cells in the tumor microenvironment [184]. Recent studies by two research groups have demonstrated that modulation of the gut microbiota may help patients overcome resistance to ICI [243, 244]. One study administered FMT from donors who achieved complete response to ten melanoma patients who failed to respond to ICI therapy [244]. FMT was administered concurrently with standard doses of ICI Nivolumab and in three patients, tumor reduction was observed. The intervention was furthermore associated with enhanced immune cell infiltration and upregulation of immune-related genes in both the tumor microenvironment and the gastrointestinal tract. Similarly, in the other study, 15 patients with progressive melanoma and complete resistance to ICI received a FMT from patients who had shown complete or partial response [243]. The FMT was administered alongside Pembrolizumab and in this study, 6 out of 15 patients experienced clinical benefit and exhibited increased abundance of taxa that were previously associated with response to ICI.

Since the administration of donor feces also harbors disadvantages (such as the dependence on reliable donors and limited scalability) and risks (such as the possible transmission of a disease risk), recent research has focused on the development of defined bacterial consortia to enhance the efficacy of ICIs [245]. One recent pre-clinical study reported that oral intake of the probiotic *Lactobacillus reuteri* leads to translocation of the bacteria and colonization of the tumor (melanoma), where they release a dietary tryptophan metabolite promoting anti-tumor $CD8^+$ T cells and subsequently supporting the effect of ICI therapy [246]. Several clinical trials are currently being conducted to evaluate the safety and efficacy of probiotics and microbial consortia in combination with ICI therapy [199]. Therapeutic approaches that involve the modulation of intestinal microbiota to augment the efficacy of ICIs also encompass dietary and lifestyle modifications, the administration of prebiotics, and the avoidance of antibiotics.

2. RESEARCH QUESTIONS AND STUDY DESIGN

2.1. RESEARCH QUESTIONS

Based on the research findings and identified gaps presented in the introductory chapters, the following overarching questions emerged for this cumulative dissertation, which were addressed in two studies. It should be added here that the two studies were already planned in 2017 and the state of research at the time was considerably more incomplete than summarized in the introductory chapter, which reflects the current state of knowledge at the present time.

The primary objective of the first publication "Changes in the cervical microbiota of cervical cancer patients after primary radio-chemotherapy" was to evaluate the quantitative and qualitative changes of cervical microbiota in patients undergoing concurrent chemotherapy and radiation treatment for locally advanced cervical cancer.

The second publication "$TIGIT^+$ NK cells in combination with specific gut microbiota features predict response to CPI therapy in melanoma" investigated the relationship between specific gut microbiota profiles, immune cell markers, and the effectiveness of ICI therapy in melanoma patients. The key research question here was whether and how certain features of the gut microbiota, combined with clinical features and features of the immune system, can predict the response to ICI in melanoma.

Both studies examined the role of microbiota (cervical and gut, respectively) in cancer and had the goal to contribute to a broader understanding of microbiota dynamics in the context of cancer therapy. By gaining further understanding of how the microbiota can be linked to immune responses in cancer therapy and how cancer treatments impact the microbiota, the way is paved for the development of new therapies and biomarkers that may subsequently lead to better outcomes for patients.

2.2. STUDY DESIGN

Both studies employed robust, multidisciplinary methodologies to explore the complex relationship between microbiota and cancer therapy. While the first study in patients with cervical cancer provided a descriptive analysis of cervical microbiota changes post-treatment, the second study in melanoma patients extended this concept by linking microbiota profiles with immune markers to predict treatment outcomes. Together, these studies contribute to a nuanced understanding of how microbiota can influence cancer therapy results and vice versa, underscoring the potential for microbiota-focused strategies in enhancing therapeutic efficacy.

2.2.1. PUBLICATION I ON CERVICAL CANCER

The study was designed as a prospective observational pilot study with the primary objective to evaluate the quantitative and qualitative changes in the cervical microbiota in patients undergoing concurrent chemotherapy and radiation treatment for locally advanced cervical cancer. In order to compare the state after treatment with a baseline, cervical cytobrush samples of 15 patients with histologically proven cervical cancer were collected one day before the start of treatment and on the last day of chemoradiation treatment. In order to avoid contamination, the cytobrush samples were immediately transferred into sterile tubes and stored at -80 °C within one hour. As the cervical microbiota has a lower biomass compared to the gut microbiota, DNA extraction was conducted using a microbial DNA purification kit designed for samples with a low bioburden. 16S rRNA gene sequencing was performed to analyze the microbiome of the cervical samples.

2.2.2. PUBLICATION II ON MELANOMA

The primary objective of this study was to identify intestinal microbiota constellations at baseline (before the start of therapy) associated with the antitumor effect of ICI therapy, in order to confirm the previously published results obtained from mouse models. Furthermore, the aim of the study was to investigate the predictive value of specific gut microbiome features in combination with immune features and clinical features concerning the response to ICI therapy in melanoma patients. By predicting at an early stage which patients will benefit from therapy and which will not, new individualized microbiota-based therapeutic approaches may be developed that could be administered at an early stage in addition to the ICI to increase the response rate to ICI therapy. The study was designed as a prospective non-interventional observational study. For immunophenotyping and gut microbiome analysis, blood and stool samples were collected before the start of ICI therapy. As it is not always possible to plan the collection of a stool sample, patients were given a sample collection kit to take home in order to ensure that a sample was obtained before the start of therapy. The patients

were instructed by the informing clinicians on how to collect the sample and were thus able to collect the stool sample independently at home from two days before the first administration of the ICI. To prevent a shift in the microbiota composition, a collection tube containing a conservation medium was used to fix the condition during sample collection and stabilize it for the transport to the clinic. Samples were then stored at -80 °C until analysis. To achieve a higher resolution in microbiome characterization, shotgun metagenomic sequencing was carried out. Blood samples were analyzed using flow cytometry and FluoroSpot assays to evaluate immune response. Using the Random Forest algorithm, clinical, microbiome, and immune variables were integrated to predict therapy response. For endpoint evaluation, the term durable clinical benefit (DCB) was used and defined as complete response (CR) or partial response (PR) or stable disease (SD) for at least six months, based on RECIST criteria [247]. DCB was chosen as an endpoint because compared to progression-free survival or overall survival it allows an earlier assessment of treatment efficacy referring to the initial therapy. The objective was to find a correlation between microbiota, immune system and response to initial ICI therapy. If a patient does not respond to the initial therapy, a change in protocol is usually performed. Longer survival might then be attributed to a possible change in therapy and confound results.

3. RELEVANT PUBLICATIONS

3.1. PUBLICATION I

Tsakmaklis A, Vehreschild M, Farowski F, et al. **Changes in the cervical microbiota of cervical cancer patients after primary radio-chemotherapy**. *Int J Gynecol Cancer*. 2020;30(9):1326-1330.

Changes in the cervical microbiota of cervical cancer patients after primary radiochemotherapy

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Received 7 August 2019
Revised 6 September 2019
Accepted 10 September 2019
Published Online First
5 May 2020

To cite: Tsakmaklis A, Vehreschild M, Farowski F, *et al. Int J Gynecol Cancer* 2020;**30**:1326–1330.

HIGHLIGHTS

- Cervical microbiota composition differs between patients with histologically proven cervical cancer.
- There is a strong reduction in the cervical bacterial loads after chemoradiation.
- There is no change in terms of the composition of the cervical microbiota under chemoradiation.

ABSTRACT

Objective Several recent studies have identified a potential interaction between the vaginal microbiota and gynecological cancers, but little is known about the cervical microbiota and its changes during cancer treatment. Therefore, the aim of the study was to evaluate the quantitative and qualitative changes of cervical microbiota in patients undergoing concurrent chemotherapy and radiation treatment for locally advanced cervical cancer.

Methods Cervical cytobrush samples of 15 cervical patients undergoing chemoradiation treatment were collected 1 day before starting external beam radiation therapy and on the day of the last fraction of brachytherapy. After DNA extraction, 16S rRNA amplicon sequencing of the V3–V4 region was performed on the MiSeq platform, followed by data processing and statistical analyses concerning the alpha and beta diversity of 16 samples (7 samples were excluded because of incomplete sample sets).

Results The amount of amplicon yield after polymerase chain reaction analysis in post-radiation samples was significantly lower compared with the baseline samples (pre 31.49±24.07 ng/µl; post 1.33±1.94 ng/µl; p=0.007). A comparison of pre-treatment and post-treatment samples did not show significant differences regarding beta diversity (weighted UniFrac). There was no significant difference in alpha diversity, which is used to characterize species diversity within a particular community and takes into account both number and abundance (Shannon Diversity Index pre-treatment samples: 2.167±0.7504 (95% CI 1.54 to 2.79); post-treatment samples: 1.97±0.43 (95% CI 1.61 to 2.33); p=0.38). Interindividual differences in patients could partly explain some variation of the samples (permutational multivariate analysis of variance).

Conclusion There was a strong reduction in cervical bacterial loads after chemoradiation. Neither alpha nor beta diversity varied significantly when baseline samples were compared with post-treatment samples.

INTRODUCTION

The gastrointestinal tract harbors several hundred bacterial phylotypes, which not only play a significant role in aiding digestive processes, but also help maintain a healthy immune system. Chronic inflammation as a reaction to a disrupted microbiota appears to be a key mechanism by which specific bacterial species contribute to the development of cancer.[1] Several studies have emphasized the role of the gut microbiota in carcinogenesis, but data regarding the role of specific bacteria in certain cancers are sparse.

More recent analyses have identified potential interactions between the microbiota and gynecological cancers.[2–4] Although smaller in population, the cervicovaginal microbiota may affect local immune regulation and oncogenesis. It comprises 20–140 bacterial species with *Lactobacillus* species commonly being most abundant. *Lactobacillus* acidifies the vaginal pH by producing lactic acid. While this is well tolerated by *Lactobacillus*, a pH <4.5 inhibits the growth of several other bacterial species, including many pathogenic bacteria. Vaginal microbiota signatures identified in environments with a significantly higher pH are not dominated by *Lactobacillus,* but instead are characterized by high bacterial diversity and a higher prevalence of anaerobic species.[5 6]

In addition to their carcinogenic potential, recent findings in the area of onco-immunology suggest a significant role of the gut microbiota in priming cells of the immune system that improve the endogenous antitumor response, mainly regulatory and CD8+ T cells. This type of immune activation has been shown to enhance the efficacy of checkpoint inhibitors, a class of immunomodulatory drugs that also relies on activation of the T cell response.[7–10] The abscopal effect describes a systemic immune response to tumor metastases as a consequence of localized radiation therapy. It has equally been identified as a potential booster of checkpoint inhibition effects.[11] It could be hypothesized that there should be a synergistic effect between microbiota based and radiation based immune activation. Such synergism might in turn offer the chance of therapeutic options in the field of gynecological oncology.

Table 1 Patients characteristics

Patient No	Age at diagnosis (years)	FIGO stage	Pretreatment tumor size (cm)	Pelvic nodes (removed/infiltrated)	Para-aortic nodes (removed/infiltrated)	Histology	Grading
1	21	IB2	4.1×2.7	1/28	0/12	Adenosquamous	G3
2	36	IIB	4×5	1/16	0/15	Squamous cell	G2
3	44	IIIB	4.4×3.7	1/1	0/7	Squamous cell	G3
4	37	IIB	5×3.2	0/15	0/12	Squamous cell	G3
5	33	IB1	2.5×3	2/16	0/5	Squamous cell	G3
6	52	IIB	4.2×3.6	0/17	0/15	Squamous cell	G3
7	39	IIA1	3.7×2.5	6/36	0/5	Squamous cell	G1
8	30	IA2	0.4×0.7	SN positive; left pelvic SN: 2 mm metastasis; right pelvic SN: isolated tumor cells	0/12	Squamous cell	G2

FIGO, International Federation of Gynecology and Obstetrics; SN, sentinel node procedure.

Few data, however, are available on the cervical microbiota of affected patients over the course of their treatment. Hence the aim of this study was to evaluate the quantitative and qualitative changes in the cervical microbiota in patients undergoing concurrent chemotherapy and radiation treatment for cervical cancer.

METHODS

Fifteen patients with histologically proven cervical cancer and an indication for primary chemoradiation treatment were included. Because of incomplete sample sets, seven patients were excluded from the analysis. Patient characteristics are shown in Table 1. The analysis was approved by the local ethics committee (ISI Study, ethics committee No 08–160). After CT planning in the supine position with an emptied rectum and filled bladder with kneefix and footfix on a big bore TOSHIBA CT, radiation was performed. It included external beam radiation with 6/10 MV photons using volumetric arc techniques on a linear accelerator (TrueBeam, Varian) and daily cone beam CT with 5 weekly single doses of 1.8 Gy to the primary tumor, including the uterus, pelvic and, in case of histologically confirmed para-aortic lymph nodes including the para-aortic node, up to the renal vessels, to a total dose of 50.4 Gy in 28 fractions. A simultaneous boost was given with 5 weekly single doses of 2.12 Gy to both parametric regions, to a total dose of 59.36 Gy in 28 fractions. Brachytherapy started in the third–fourth week of external beam with MRI planned three dimensional generated plans and five fractions of 5 Gy single doses to the cervix and residual tumor volume. Concomitant chemotherapy was given to every patient once a week (cisplatin 40 mg/m^2 body surface area) for 5–6 applications. At the time of chemotherapy, comedication with dexamethasone 8 mg on day 1 of cisplatin and 4 mg on days 2 and 3 were administered. Proton pump inhibitor treatment was prescribed with oral pantoprazole 40 mg/day concomitantly with chemoradiation.

Cervical cytobrush samples of each patient were collected 1 day before starting external beam radiation (baseline) and on the day of the last fraction of brachytherapy. Each sample was transferred into a clean collection tube and stored at −80°C within 1 hour. For microbiome analysis, samples were thawed and directly subjected to DNA extraction using the ZymoBIOMICS DNA miniprep Kit (Zymo Research Corp, Irvine, USA) following the manufacturer's instructions. Afterwards, 16S rRNA amplicon sequencing of the V3–V4 region was performed as described in the Illumina 16S Sample Preparation Guide on the MiSeq platform (Illumina, San Diego, USA) in a 300 bp paired end run.[12] The sequencing data were processed using the DADA2 pipeline and analyzed using QIIME 2.[13 14] Quality profiles of the reads were analyzed. Reads were trimmed (trunc_len_f=290, trunc_len_r=220) and processed by the QIIME DADA2 plugin with the denoise paired option and standard parameters (trunc_q=2, max_ee=2, chimera_method=consensus). Taxonomic classification was performed by a Naïve Bayes classifier (sklearn),

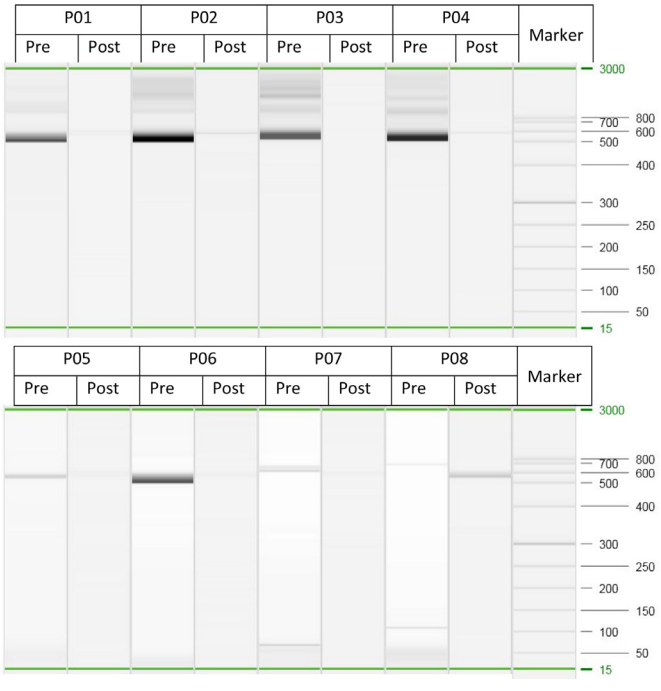

Figure 1 Polymerase chain reaction (PCR) product of the amplicon PCR. Capillary electrophoresis 'gel' showing the PCR product obtained after 25 cycles, targeting the 16S V3 and V4 region (Klindworth 2013) followed by clean-up using AMPure XP beads. The expected size of the PCR product is approximately 550 bp.

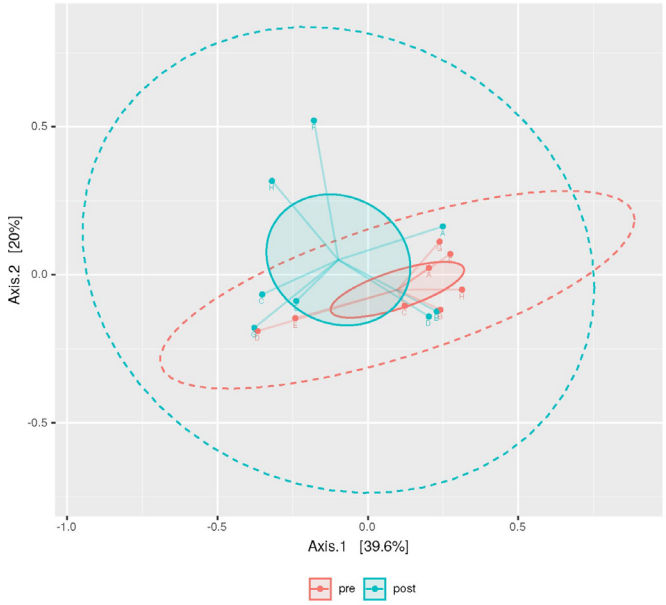

Figure 2 Principal coordinate analysis of bacterial community structures on the basis of weighted UniFrac distances of all samples; 95% confidence levels assuming normal (- - -) distribution and 95% confidence ellipses (---). Red dots represent the samples collected before chemoradiation and blue dots represent the samples collected after chemoradiation.

who was trained on the SILVA database release 128.[15 16] Rarefaction curves were determined based on the feature table, and analysis of the relative proportion of each bacterial taxon was made after the data were rarefied at a depth of 3500 sequences per sample. Statistical analyses were carried out using R for Statistical Computing (V.3.2.5, R Foundation for Statistical Computing, Vienna, Austria).[17] Alpha and beta diversity scores were calculated using the R package phyloseq.[18] Beta diversity, in this case the weighted UniFrac distances between the samples, was visualized using principal coordinate analysis. The effect of radiation status on beta diversity was tested by a permutational multivariate analysis of variance.

RESULTS

During the entire sample collection period, 23 cervical cytobrush samples were collected, 16 of which were included in further microbiome analyses. Seven samples were excluded from analysis because of incomplete sample sets, (ie, either the pre-treatment or post-treatment sample was missing because sampling had to be stopped due to pain). The mean concentration of the (total) extracted genomic DNA was 43.6±12.1 ng/µl (Qubit fluorometer). The product of the amplicon polymerase chain reaction—that is, of the 16S V3–V4 region—was checked by capillary electrophoresis. Although equal amounts of DNA template were used, the yield of the amplicon was much less in post-radiation samples (Figure 1). The QIAxcel Software was used to quantify the V3–V4 amplicons. DNA concentration of the pre-radiation amplicons was significantly higher compared with post-radiation amplicons (pre 31.49±24.07 ng/µl; post 1.33±1.94 ng/µl; p=0.007).

There was no significant difference in alpha diversity, which is used to characterize species diversity within a particular community and takes into account both number and abundance (Shannon Diversity Index pre-treatment samples 2.167±0.7504 (95% CI 1.54 to 2.79); post-treatment samples: 1.971±0.4296 (95% CI 1.61 to 2.33); p=0.38) when we compared baseline samples with samples after chemoradiation samples. The same was true for beta diversity, which refers to diversity between two communities by measuring variation between multiple samples. In terms of beta diversity, principal coordinate analysis of the weighted UniFrac distances did not show any shift in the cervical microbiota composition after radiation therapy (permutational multivariate analysis of variance F=1.42, p=0.197)—that is, the 95% confidence ellipses around the centeroids of both groups overlapped (Figure 2).

Another permutational multivariate analysis of variance revealed that there was some spread of the samples that (in part) could be explained by interindividual differences in patients (unweighted UniFrac F=2.15, p<0.001; weighted UniFrac F=1.42, p=0.106). The effect of the patient identification number (PID) on several dissimilarity indices is listed in Table 2.

The relative abundances of the bacterial families detected within the patients' cervical microbiota is shown in Figure 3.

DISCUSSION

There is a growing body of data which suggests that the microbiota plays an underestimated role in the development of chronic inflammation, human papillomavirus infection, and human papillomavirus clearance, progressing to invasive cancer, and may also play a role in prevention of cancer.[2–6 19–25] This pilot study is the first to analyze the effects of chemoradiation for cervical cancer on the cervical microbiota. Previous studies performed in this patient population have focused on analysis of the gut microbiota and its correlation with radiation related gastrointestinal toxicity. A systematic review[26] analyzed data from three cohort studies[27–29] on changes in the gut microbiome of 23 women with gynecological cancer. It has been shown that there is a change in microbiota quality in patients who developed gastrointestinal toxicity. An increase in unspecified bacterial species was seen in those with diarrhea, but patients without diarrhea maintained their initial bacterial profiles.[26] Supplementation with *Lactobacillus acidophilus* and *Bifidobacterium bifidum* before and during radiation decreased

Table 2 Effect of the patient identification number (PID) on the dissimilarity indices

Dissimilarity index	Bray–Curtis	Jaccard	Unweighted UniFrac	Weighted UniFrac
Test statistic	1.71	1.54	2.15	1.42
P value	0.004	0.003	<0.001	0.106

Test=pseudo-F; sample size=16; No of groups=8; No of permutations=999.

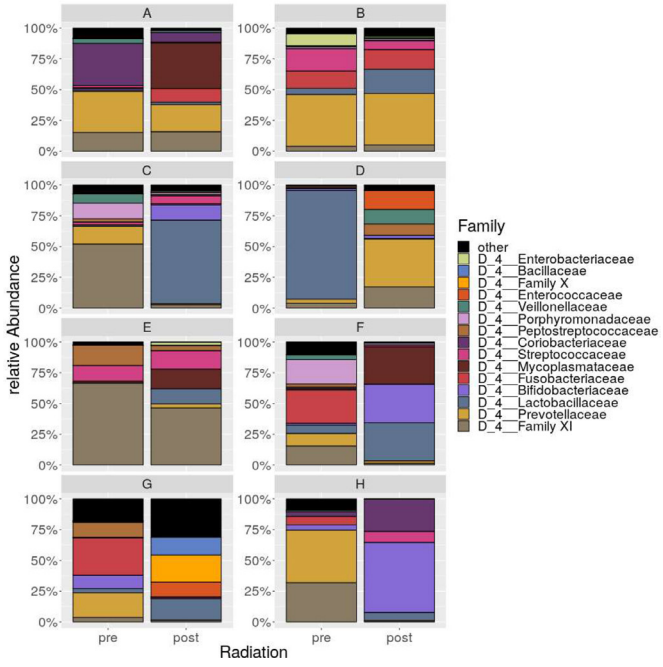

Figure 3 Relative abundance of different bacterial families in each sample. (A–H) Different patients, family XI=Clostridiales.

the rate of grade 2 and 3 diarrhea significantly (treatment group 9% vs no treatment group 45%).[30]

Our results suggest that there is an effect of chemoradiation therapy on the cervical microbiota. Although the DNA concentration measurement after DNA extraction from the swab samples showed high values, we have to consider that this could in part be due to high levels of simultaneously extracted host DNA. The results of electrophoresis nevertheless indicated a strong reduction in cervical bacterial loads after radiation therapy, as expected, as chemotherapy and radiation both lead to damage or destruction of cells.

Although the cervical microbiota of all patients considerably changed in terms of quantity, as revealed by electrophoresis, this conclusion cannot be drawn concerning quality—that is, the composition of the microbiota. Neither alpha nor beta diversity differed significantly when we compared pre-radiation with post-radiation samples. Clearly, this conclusion is limited by the small sample size of our study. What we could see, however, was inter-individual differences in the composition of the cervical microbiota. Some women showed a stronger dominance of certain families, such as *Clostridiales*, *Lactobacillaceae*, and *Prevotellacecae*, while others showed a more diverse composition.

To our knowledge, no data on changes to the cervical microbiota during chemoradiation treatment have been published previously. As a first step, we analyzed eight patients before and after treatment. We demonstrated that chemoradiation results in quantitative, but not qualitative, changes in the cervical microbiota. Quantification is an aspect that has been under appreciated in microbiome research to date, but may have relevant clinical implications. It remains to be determined in future studies whether there is any impact of local bacteria on the response to treatment of cervical tumor or whether such responses are mediated by the much more abundant microbiota colonizing the gut.

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Funding This study was funded by Deutsche Forschungsgemeinschaft (DFG grant HE-6810/3-1) to JH.

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval The analysis was approved by the local ethics committee (ISI Study, ethics committee No 08–160).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or available upon reasonable request.

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3.2. PUBLICATION II

Tsakmaklis A, Farowski F, Zenner R, et al. **TIGIT+ NK cells in combination with specific gut microbiota features predict response to checkpoint inhibitor therapy in melanoma patients**. *BMC Cancer*. 2023;23:1160.

BMC Cancer

RESEARCH

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TIGIT^{+} NK cells in combination with specific gut microbiota features predict response to checkpoint inhibitor therapy in melanoma patients

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Abstract

Background Composition of the intestinal microbiota has been correlated to therapeutic efficacy of immune checkpoint inhibitors (ICI) in various cancer entities including melanoma. Prediction of the outcome of such therapy, however, is still unavailable. This prospective, non-interventional study was conducted in order to achieve an integrated assessment of the connection between a specific intestinal microbiota profile and antitumor immune response to immune checkpoint inhibitor therapy (anti-PD-1 and/or anti-CTLA-4) in melanoma patients.

Methods We assessed blood and stool samples of 29 cutaneous melanoma patients who received immune checkpoint inhibitor therapy. For functional and phenotypical immune analysis, 12-color flow cytometry and FluoroSpot assays were conducted. Gut microbiome was analyzed with shotgun metagenomics sequencing. To combine clinical, microbiome and immune variables, we applied the Random Forest algorithm.

Results A total of 29 patients was analyzed in this study, among whom 51.7% ($n=15$) reached a durable clinical benefit. The Immune receptor TIGIT is significantly upregulated in T cells ($p=0.0139$) and CD56^{high} NK cells ($p=0.0037$) of responders. Several bacterial taxa were associated with response (e.g. *Ruminococcus torques*) or failure (e.g. *Barnesiella intestinihominis*) to immune therapy. A combination of two microbiome features (*Barnesiella intestinihominis* and the Enterobacteriaceae family) and one immune feature (TIGIT^{+} CD56^{high} NK cells) was able to predict response to ICI already at baseline (AUC = 0.85; 95% CI: 0.841–0.853).

Conclusions Our results reconfirm a link between intestinal microbiota and response to ICI therapy in melanoma patients and furthermore point to TIGIT as a promising target for future immunotherapies.

Keywords Microbiome, Melanoma, Immune checkpoint inhibitors, NK cells, TIGIT, Response, Random Forest

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Background

Immune checkpoint proteins including T-cell surface receptor programmed cell death protein 1 (PD-1), its ligand PD-L1 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) play a key role in maintaining the delicate balance between immune-tolerance and defense. As a mechanism of immune evasion, however, tumor cells are able to stimulate these checkpoints, resulting in downregulation of the T cell-mediated anti-tumor response [1]. During immune checkpoint inhibitor (ICI) therapy, monoclonal antibodies are used individually or in combination to block these receptors, thus initiating a restimulation of the T-cell response [2]. Since first approval of an ICI for the treatment of melanoma by the FDA in 2011, numerous compounds have been introduced and initiated a revolution in the field of immunotherapy [3–7]. Despite significant improvement in survival rates under ICI therapy, only 40% of patients with advanced melanoma respond to Nivolumab treatment and 61% respond to the combination of Nivolumab and Ipilimumab [4, 8]. A T-cell-infiltrated tumor microenvironment at baseline seems to have a favorable effect on the therapeutic outcomE [9, 10]. In addition, a growing body of evidence in animals and humans suggests that composition of the intestinal microbiota has an impact on the therapeutic response [11–17]. While all analyses conclude that specific taxa were associated with response to treatment, the taxonomic overlap is limited. The largest cohort so far, uniting data and samples from 293 patients, identified members of the family Ruminococcaceae as the main microbiome-based driver of response to ICI [12]. None of these cohorts, however, combined microbiota signature analysis with immune analysis to predict treatment response in patients.

To close this gap, we conducted a study using clinical metadata in combination with functional and phenotypical immune analyses to determine antitumor effects, as well as shotgun metagenomics sequencing for gut microbiome analysis.

Methods

Study design

This prospective, non-interventional study was conducted at the Department of Dermatology at the University Hospital of Cologne. From 07/2017 to 08/2019, all adult patients diagnosed with melanoma and scheduled to receive ICI therapy, were screened for study inclusion. The study was approved by the local ethics committee (Cologne-ID #17–269) and written informed consent was obtained from all patients.

Clinical data, fecal and blood samples were obtained at baseline (range: two days before until the day of first ICI infusion), as well as at 3, 6 and 9 months after initiation of ICI therapy. Only baseline samples were used in the present analysis. Fecal samples were obtained using OMNIgene GUT OMR-200 (DNA Genotek, Ottawa, Canada) and stored at -80 °C. Blood draws were conducted alongside clinical routine diagnostics. Serum was immediately frozen at -80 °C and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Ficoll Hypaque) and stored in liquid nitrogen until analysis. For the purposes of the present analysis, only data from the baseline visit was analyzed.

Documented clinical parameters are shown in Table S1 [18].

For endpoint evaluation, the term durable clinical benefit (DCB) was used and defined as complete or partial response or stable disease for at least six months, based on RECIST criteria [18]. DCB was chosen as endpoint because compared to progression-free survival or overall survival it allows an earlier assessment of treatment efficacy referring to the initial therapy. The objective was to find a correlation between microbiota, immune system and response to initial immunotherapy. If a patient does not respond to the initial therapy, a change in protocol is usually performed. Longer survival might then be attributed to a possible change in therapy and confound the results.

Microbiome analysis

Fecal samples were subjected to genomic DNA extraction using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA), quantification of DNA was performed using the Qubit 2.0 Fluorometer with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and purity checked by spectrophotometry (NanoDrop, Thermo Fisher Scientific, Waltham, MA, USA).

For metagenomic sequencing, the frozen DNA samples were sent to the Helmholtz Centre for Infection Research in Braunschweig on dry ice. The DNA library for metagenomics sequencing was generated using NEBNext® Ultra™ II FS DNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA) for Illumina with parameters as followed: 500 ng input DNA and 5 min at 37 °C for fragmentation; > 550-bp DNA fragments for size selection; primers from NEBNext Multiplex Oligos for Illumina Kit (New England Biolabs, Ipswich, MA, USA) for barcoding. The libraries were sequenced on the Illumina NovaSeq (2×150 bp). Raw reads were pre-processed using KneadData (v0.7.4), trimmomatic (v0.39, SLIDINGWINDOW:4:20 MINLEN:50), and BowTie2 (v2.4.2, with hg37dec_v0.1). Taxonomic species profiling of the cleaned reads was done using MetaPhlAn4 (v4.0.3, mpa_vJan21_CHOCOPhlAnSGB_202103) with default

parameters [19, 20]. Data was summarized into biom format and analyzed using phyloseq [21, 22].

Immunophenotyping and functional analyses

For the phenotypical quantification of the immune response, PBMCs were characterized by 12-color flow cytometry (CytoFLEX LX, Beckman Coulter, Brea, CA, USA). Using fluorescence-labeled antibodies, characteristic surface and intracellular proteins of PBMCs were labeled and measured with the CytoFLEX LX Flow Cytometer. The proportions of different lymphocyte subpopulations in the PBMCs and expression levels of the investigated markers on different T-cell populations were then analyzed using Kaluza Analysis Software (Beckman Coulter, Brea, CA, USA).

For functional analysis of lymphocytes, tumor antigen-specific T-cell responses were determined by performing a FluoroSpot assay (Mabtech, Nacka, Sweden) to measure specific release of cytokine Interferon gamma (IFN-γ) upon stimulation with Cancer Testis Antigens (CTAs). PBMCs were stimulated for 20 h at 37 °C with five different CTAs (NY-ESO-1, MAGEA1, MAGEA3, MLANA, SURVIVIN), as well as the biological control peptide CEF and a technical positive control (CD3/CD28 activation). Fluorescent spots as indicators for specific responses were counted on an AID FluoroSpot reader.

Statistics

Statistical analyses were conducted using R for Statistical Computing (version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism V.9.0.2 (GraphPad, USA) [23]. Continuous variables were presented as mean (± standard deviation) or median (interquartile range), while categorical variables were presented as number and percentage. Mann-Whitney t-test was used to compare continuous variables. The beta diversity, in this case the weighted UniFrac distances between the samples, were visualized using Principal Coordinate Analysis (PCoA) and differentially abundant taxa were identified using Linear Discriminant Analysis (LDA) Effective Size (LEfSe, LDA score (log 10) > 3 for comparison) [22].

A multivariate logistic regression analysis and stepwise regression in both directions using the Akaike Information Criterion (AIC) was performed to identify independent predictors for response to ICI treatment. Odds ratios (ORs) and 95% Confidence Intervals (CI) were calculated on the basis of the respective coefficients for these predictors. The analysis was performed entering 84 preselected variables (clinical parameters that have previously been shown to influence response, immune parameters with a variance inflation factor below 10, and microbiome parameters based on LEfSe, see S2 + S3) as input features.

In a sensitivity analysis, the 84 preselected variables (as mentioned above) were entered used as input into a Random Forest regression model, utilizing the caret package in R [24]. To mitigate the risk of overfitting and to eliminate possible collinearities and dependencies in the model, a recursive feature elimination (RFE) algorithm with a leave-group out (Monte Carlo) cross validation (1000 iterations) was applied to select up to 20 features according to the best accuracy and discard those with the lowest rank. The remaining (most informative) features, i.e. those with the lowest root mean squared error (RMSE), were included in the final Random Forest model. To evaluate the performance of our Random Forest model, we employed a rigorous cross-validation strategy. The dataset was randomly split into training and test subsets 1000 times, ensuring a diverse set of training and test data combinations for robust assessment. In each iteration, the model was trained on the respective training subset incorporating the selected features and the area under the receiver operation characteristic curve (ROC-AUC) was computed, offering insights into the model's discriminative capabilities on the test subset. Finally, the mean of all receiver operation characteristic curves (from the 1000 iterations) and its respective AUC was computed to provide an overall measure of the model's performance All statistical tests were two-tailed, and a p-value of < 0.05 was considered statistically significant.

Results

Patients

A total of 93 patients was screened and 40 enrolled into the study. Due to suspected immunological differences between melanoma types, which may have confounded our analysis, we decided to focus on patients with cutaneous melanoma ($n = 29$; Fig. 1).

Detailed characteristics at baseline are shown in Table 1.

Follow up documentation was closed after the last staging (9 months after baseline) of the latest included patient. Mean age was 64.76 ± 14.24 years (range: 34–86) and about two thirds of the patients ($n = 19$, 65.5%) were male. The mean age in the group of patients with a durable clinical benefit (DCB), which was 65.5 years, is comparable to the mean age of patients without DCB, which was 63.9 years. The cohort consisted of 86.2% ($n = 25$) patients with metastatic melanoma stage IV (AJCC 2017). Three different ICI regimens were applied with 51.7% ($n = 15$) of the patients receiving anti-PD-1 therapy, 44.8% ($n = 13$) a combination of anti-PD-1 and anti-CTLA-4, and 3.5% ($n = 1$) anti-CTLA-4 therapy.

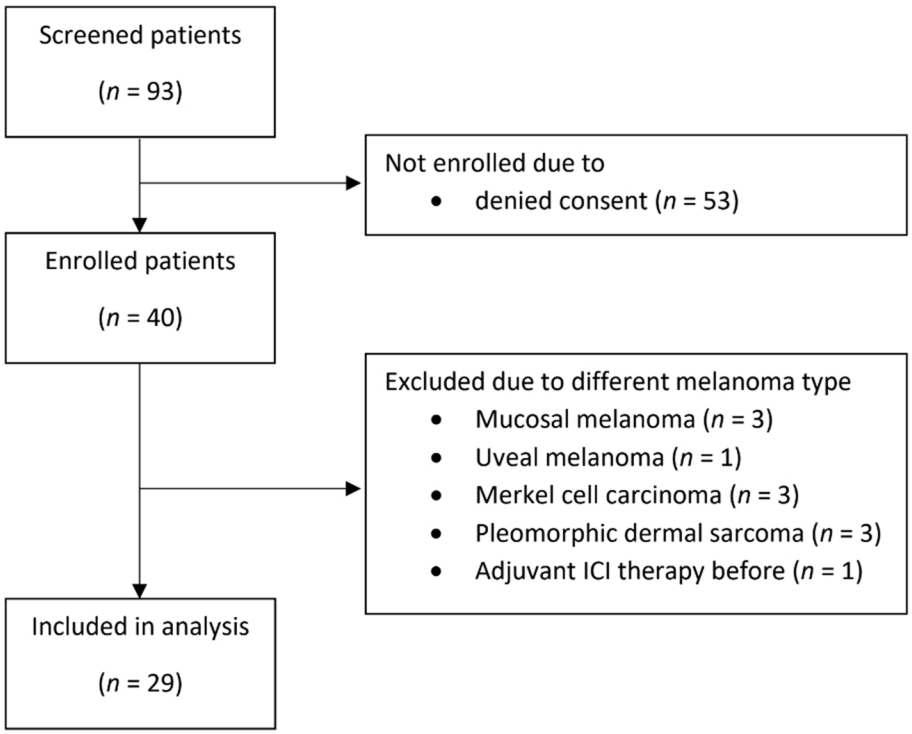

Fig. 1 Total numbers of patients screened, enrolled and included in the final analysis

DCB was reached in 51.7% ($n=15$) of all patients. Out of the remaining patients, 10 (34.5%) did not show DCB and 4 (13.8%) died prior to reaching the six months follow-up. Overall, 44.8% ($n=13$) of the patients had an immune-related adverse event, e.g. colitis, that had to be treated with steroids.

The microbiome profile differs between responders and non-responders

Based on metagenome sequencing, there was no significant difference concerning alpha diversity between patients with and without DCB (Fig. 2A). In terms of beta diversity, principal coordinate analysis of the weighted UniFrac distances did not show a separate clustering of the two groups (Fig. 2B).

To identify bacteria that are differentially abundant and thus potentially suitable as biomarkers, LEfSe analysis was performed. There was an increased abundance of *Ruminococcus torques* ($p=0.017$), Lacrimispora ($p=0.014$), and *Lacrimispora amygdalina* ($p=0.022$) in patients with DCB (Fig. 2C). In patients without DCB, abundances were increased for Odoribacteraceae ($p=0.049$), *Butyricimonas paravirosa* ($p=0.031$), Butyricimonas ($p=0.031$), Barnesiellaceae ($p=0.003$), *Akkermansia muciniphila* ($p=0.036$), Barnesiella ($p<0.001$), *Barnesiella intestinihominis* ($p<0.001$), Akkermansiaceae ($p=0.014$), Akkermansia ($p=0.014$), and Enterobacteriaceae ($p=0.040$).

We further explored the abundance of species of the family Enterobacteriaceae, as these Gram-negative bacteria have previously been associated with disease states [25, 26]. Only one out of 15 patients (6.7%) with DCB had an abundance > 1% of Enterobacteriaceae compared to 8 out of 14 patients (57.1%) without DCB (Fig. 2D).

Phenotypic and functional immune analysis

Of the 29 patients who underwent microbiome analysis, one patient had to be excluded from immune analyses due to a missing baseline sample and another patient had to be excluded from the phenotypic analysis due to a previous leukemic disease.

There were no significant differences in the proportions of several analyzed circulating lymphocytes between patients with or without a DCB at baseline. The main subsets are shown in Fig. 3A. Furthermore, no significant differences in any activation marker could be found, as shown exemplarily in Fig. 3B. To evaluate checkpoint marker expression on lymphocytes prior to ICI treatment, a broad range of 25 surface checkpoint molecules was analyzed. TIGIT (T-cell immunoreceptor with Ig and ITIM domains) expression on T cells and $CD56^{high}$ NK cells was significantly higher in patients with DCB

Table 1 Patient characteristics

Variable	Cohort ($n=29$)
Age (years) — mean ± s.d. (range)	64.76 ± 14.24 (34–86)
Sex (male) — n (%)	19 (65.5)
Underlying cancer — n (%)	
Cutaneous melanoma	24 (82.8)
Melanoma of unknown primary (most likely of cutaneous origin)	5 (17.2)
ECOG performance status — n (%)	
1 or 2	27 (93.1)
3 or 4	2 (6.9)
Cancer staging (AJCC 2017) — n (%)	
IIIC	3 (10.4)
IVM1a	3 (10.4)
IVM1b	7 (24.1)
IVM1c	11 (37.9)
IVM1d	4 (13.8)
Unknown	1 (3.5)
Checkpoint-Inhibitor — n (%)	
anti-CTLA-4	1 (3.5)
anti-PD-1	15 (51.7)
anti-PD-1 + anti-CTLA-4	13 (44.8)
Durable clinical benefit — n (%)	
Yes	15 (51.7)
No	10 (34.5)
Not reached	4 (13.8)
irAE treated with steroids — n (%)	
Yes	13 (44.8)
No	16 (55.2)
irAE toxicity grade — n (%)	
0	9 (31.0)
1	3 (10.4)
2	4 (13.8)
3	9 (31.0)
4	4 (13.8)
Serum LDH level — n (%)	
Normal (< 250 U/l)	15 (51.7)
High (> 250 U/l)	14 (48.3)
Death — n (%)	
Yes	13 (44.8)
No	16 (55.2)

Durable clinical benefit: CR (complete response), PR (partial response) or SD (stable disease) for at least 6 months, *irAE* Immune-related adverse event, *LDH* Lactate dehydrogenase; Death: Follow up documentation was closed after the last staging (9 months after baseline) of the latest included patient

compared to patients without DCB at baseline (T cells: DCB + = 19.22 ± 7.03%; DCB- = 13.01 ± 4.16%; $p=0.014$; $CD56^{high}$ NK cells: DCB + = 14.74 ± 8.91%; DCB- = 6.119 ± 6.34%; $p=0.0037$; Fig. 3C).

Regarding the tumor antigen-specific interferon-γ release (Fluorospot Assay), there were no significant differences between patients with and without DCB at baseline (4 patients (28.6%) from each group; Fig. 3D).

A combined analysis for prediction of the benefit of ICI therapy

In our multivariate regression analysis, the baseline variables "*Barnesiella intestinihominis*" (OR > 1000; 95% CI 3e + 69 – Infinity; $p=0.007$) and "$TIGIT^{+}$ $CD56^{high}$ NK cells" (OR 0.74; 95% CI 0.583–0.918; $p=0.009$) were significantly associated with treatment failure (see Supplements Table S2 + Figure S1).

Based on the available clinical, microbiome, and immunological data, our Random Forest model with recursive feature elimination (RFE) for the prediction of response to ICI therapy achieved a mean area under the curve (AUC) of 0.85 (95% CI: 0.841–0.853; Fig. 4A) by combining three baseline variables (TIGIT expressing $CD56^{high}$ NK cells, *Barnesiella intestinihominis*, and the Enterobacteriaceae family; Fig. 4B).

Discussion

To our knowledge, our analysis is the first to combine clinical metadata, metagenomic microbiome analyses and immune profiling for the prediction of the benefit of ICI treatment in patients with cutaneous melanoma. Based on our integrated analysis, with the combination of two microbiome features and one immunological feature we were able to predict response to ICI treatment already before the first infusion with a mean area AUC of 0.85. All three variables can be determined by analyzing a blood sample and a stool sample, and together they provide a predictive model that could be used to identify patients who might benefit from ICI therapy as part of the treatment decision process.

Our LEfSe analysis identified several bacterial taxa that were more abundant at baseline in patients with a DCB (e.g. *Ruminococcus torques)* and several other bacterial taxa elevated in patients with no DCB (e.g. Enterobacteriaceae). Yet, not all of these taxa seem to have a predictive value. While previous studies performed in melanoma patients identified microbiome signatures as predictors of treatment success or failure, there is no taxonomic consistency across cohorts [13–17]. This may be explained by the methodological heterogeneity of these studies. Fecal sampling and storage techniques differ and sampling timepoints were inconsistent across studies. Further down the pipeline there were differences in DNA extraction, sequencing technology and bioinformatics [27]. Another important factor that may contribute to the divergence are the varying disease stages of patients analyzed. Although some researchers suggest the existence of a microbiome signature inherent across different cohorts [28], these results of

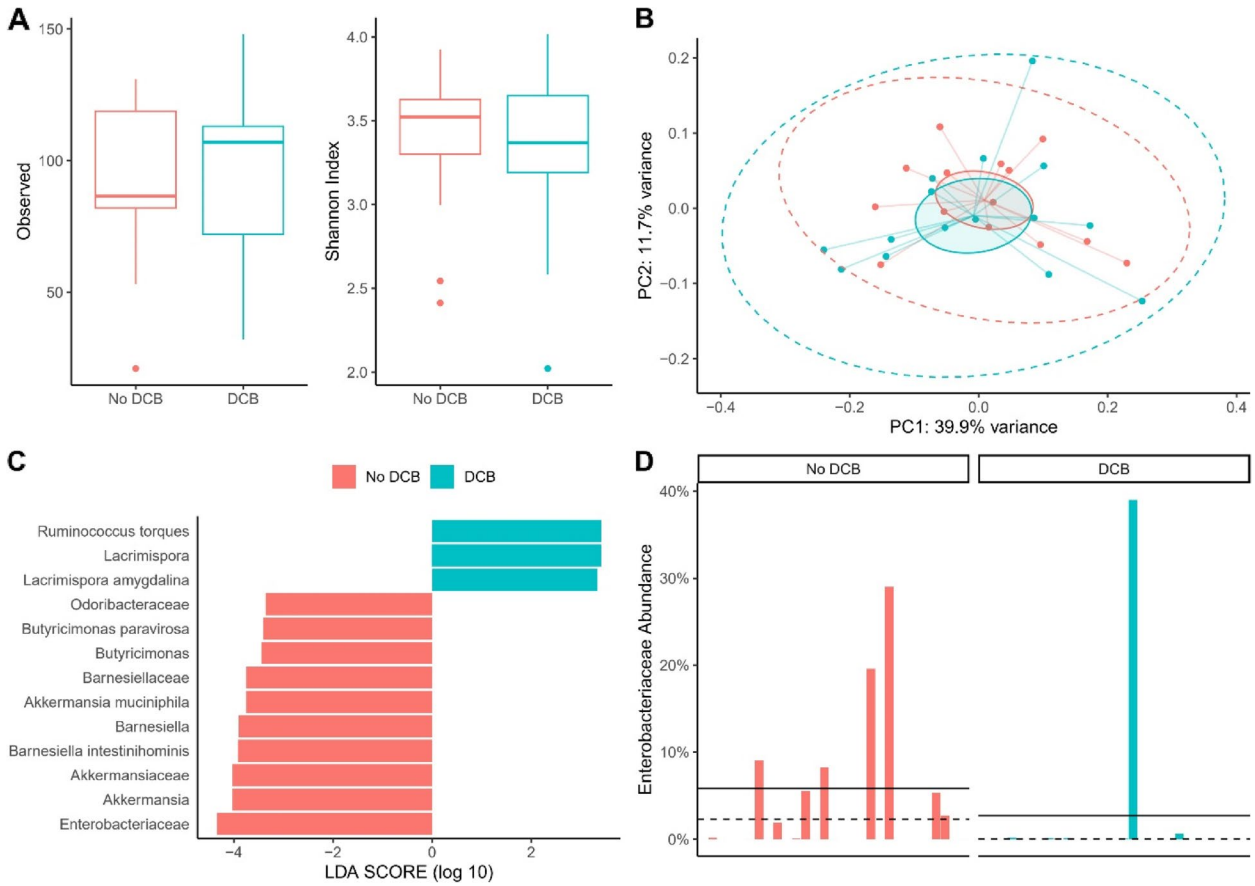

Fig. 2 Differences in fecal microbiota of patients with or without DCB. **A** Alpha diversity indices (Observed species and Shannon diversity index): Shannon diversity index: DCB+: 3.344 ± 0.530 (95% CI 3.050–3.638); DCB-: 3.377 ± 0.445 (95% CI 3.121–3.634); $p = 0.86$; Observed: DCB+: 94.13 ± 31.12 (95% CI 76.90–111.40); DCB-: 90.50 ± 31.34 (95% CI 72.40–108.60); $p = 0.76$. **B** Principal Coordinate Analysis (PCoA) of bacterial community structures on the basis of weighted UniFrac distances of 29 baseline samples. PERMANOVA $F = 0.58$, $R^2 = 0.02$, $p = 0.819$. Blue dots represent the samples of patients with DCB and red dots of patients without DCB. **C** Linear discriminant analysis (LDA) effect size LEfSe analysis after shotgun metagenomic sequencing. **D** Relative abundance in % of the family Enterobacteriaceae, each column represents a patient

a meta-analysis were not confirmed in a large cross-cohort study clearly showing that the microbiome associations are very much cohort-dependent, even when focused on only one cancer type [15]. In this sense, our findings are in line with the previous inconsistency of predictive microbiome signatures.

While it is recognized that advanced cancer stages can imply an altered composition of the gut microbiome, especially as an adverse effect of more medication such as chemotherapy or antibiotics, the specific implications of different cancer stages on the efficacy of ICI is still subject of ongoing investigation [29, 30]. The response to ICI is attributed to various factors, including increased tumor burden, immune exhaustion, and immunosuppressive tumor microenvironments [31]. As most of the patients in our cohort were already in stage IV cancer, and only two patients received antibiotics before start of ICI, our distribution was too inhomogeneous for further subgroup analysis.

Another factor that can have an impact on the microbiome is age. Age-related alterations in the gut microbiome are related to factors like progressive physiological changes, lifestyle-related factors such as diet or medication, and decreased social interaction [32]. Nonetheless, our primary focus in this study was to investigate the potential impact of microbiome composition on treatment outcomes. To account for the influence of "age" itself, we initially included it as a preselected input variable in both our regression and Random Forest analyses (see Table S3). However, it's noteworthy that in the final regression model (see Figure S1), the variable "age" was not included as a significant risk factor. This suggests that, within our dataset, age may not be the primary driver of the observed treatment outcomes. Importantly, the mean age in the group of patients who experienced a

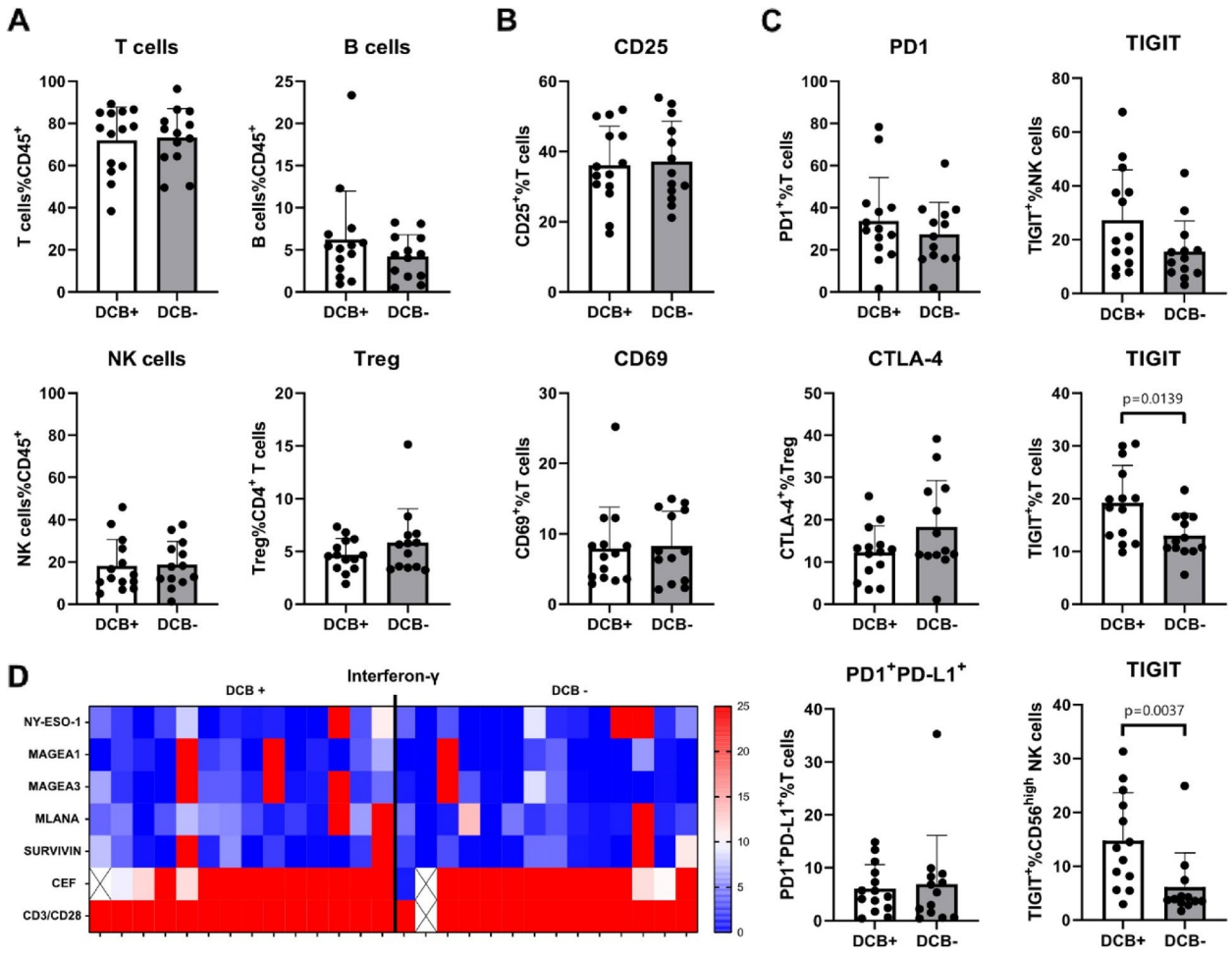

Fig. 3 Phenotypical flow cytometry analysis and functional analysis at baseline. **A** Proportions of T cells, natural killer cells (NK cells) and B cells in percentage of $CD45^+$ lymphocytes and regulatory T cells (Tregs) in percentage of $CD4^+$ T cells. **B** CD25 and CD69 expression as markers of T-cell activation. **C** Expression of the checkpoint molecules PD-1, CTLA-4 and TIGIT on different immune cells (TIGIT expression on T cells of patients with DCB = 19.22% ± 7.03; patients with no DCB = 13.01% ± 4.16; $p = 0.0139$; TIGIT expression on $CD56^{high}$ NK cells of patients with DCB = 14.74 ± 8.91%; patients with no DCB = 6.119 ± 6.34%; $p = 0.0037$). Bar charts show mean percentage ± SD. **D** Functional analysis of the immune response (Fluorospot Assay). Colorcode indicates spot number of IFNγ release by T cells. Ten or more spots is considered as tumor antigen-specific response

DCB was comparable to the mean age of patients without DCB.

Besides the discussed methodological confounders, it is also conceivable that the heterogeneity in associated microbiome signatures may reflect a functional overlap. Following this hypothesis, different microbiome signatures would be able to trigger similar immunological effects. To tackle this problem, we included immune analyses into our assessment. Based on our results, the inhibitory immune receptor TIGIT was significantly upregulated in T cells and $CD56^{high}$ NK cells of patients with a DCB. In our Random Forest analysis, TIGIT expressing $CD56^{high}$ NK cells remained in the final prediction model as the most important feature. TIGIT expression is observed on peripheral memory and regulatory $CD4^+$ T cells and NK cells, and its expression can be augmented following the activation of these cells, including naïve T cells. By promoting the generation of mature immunoregulatory dendritic cells, TIGIT suppresses T cell activation [33]. Similar to CTLA-4 or PD-1, TIGIT is a co-inhibitory molecule that prevents overactivation of the immune system. Upregulation of TIGIT in patients with a DCB may be an indicator of a more activated immune cell phenotype prior to ICI therapy, which in turn tends to benefit more from the therapy. These findings support the idea of TIGIT as a promising target in cancer immunotherapy, especially by dual PD-1/TIGIT blockade [34]. Concerning a microbiota-mediated

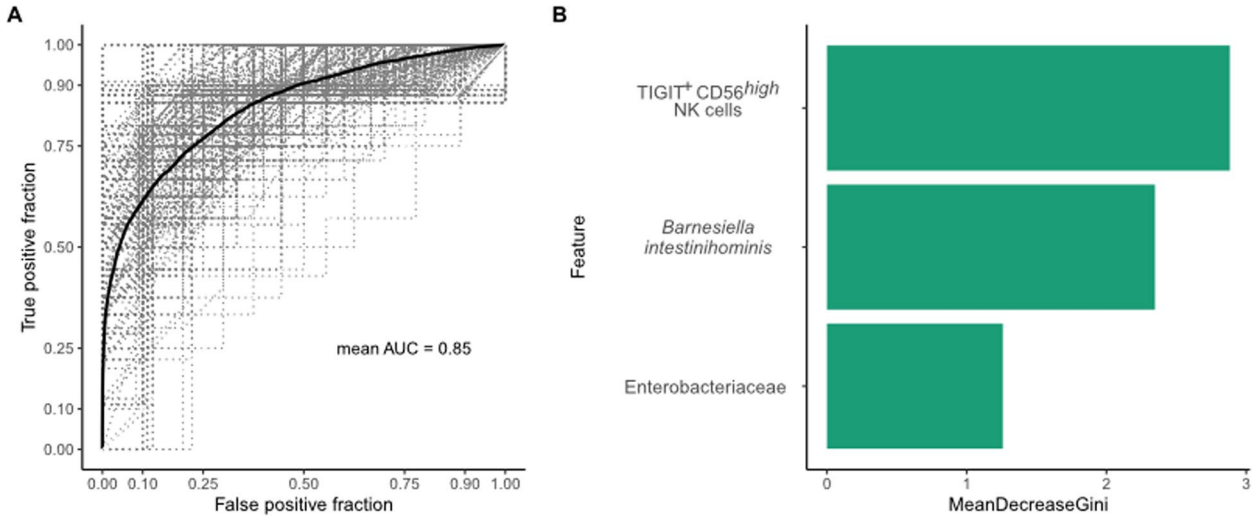

Fig. 4 Random Forest model to predict clinical outcome (no DCB) after ICI therapy. **A** Area under the curve (AUC) for our Random Forest model based on top 3 of 84 features selected by Random Forest Recursive Feature Elimination (RF-RFE). Dashed grey lines indicate the receiver operating characteristic (ROC) curves for the different random splits between training and test dataset, the thick black line represents the median over all ROC curves. **B** Features ranked according to importance based on mean decrease in Gini index

immune regulation, a previous study could show that *Fusobacterium nucleatum*, a commensal bacterium in the tumor microenvironment associated with colorectal cancer, can directly bind TIGIT leading to suppression of antitumor NK-cell and T-cell response [35].

T and NK cells play pivotal roles in the tumor immune microenvironment in the context of ICI therapy. The expression of immune receptors on T and NK cells can vary significantly, leading to different effects on antitumor activity [36]. This is reflected in an intra- and intertumoral heterogeneity [37]. An association between specific immune cell populations and the patients' prognosis has also been shown in other malignancies like cervical cancer [38]. In a recent study, a T-cell and a NK-cell subpopulation were found to express high levels of cytotoxic effector molecules and low levels of inhibitory markers including TIGIT [39]. Both signatures were associated with a favorable prognosis in a large cohort of cervical cancer patients. Unfortunately, in our study, no tumor samples were available for analysis of the tumor microenvironment.

Of 93 screened patients, only 40 enrolled into the study. Reasons for the high loss included that some patients perceived the collection of stool as too uncomfortable, as well as the advanced age of many patients for whom the collection of fecal samples would have been difficult. Due to its small sample size, the statistical power of our analyses and especially the predictive power of the Random Forest model were limited. The architecture of the Random Forest model builds on various fundamental concepts, such as ensemble learning, bootstrap aggregation (bagging) and random feature selection. In Random Forests, ensemble learning is achieved through bagging, where individual trees are trained on separate bootstrap samples from the training dataset. This ensemble approach fosters diversity among trees and enhances prediction accuracy by mitigating model variance and overfitting tendencies. Nevertheless, with only 29 samples, there is still a risk of overfitting. Random feature selection enhances diversity by considering only subsets of features at each decision tree node. Instead of evaluating all available features for the best split, only a random subset is evaluated. This enhances the forest's diversity and generalization capability and results in a more robust model that excels in classification, can handle imbalanced data, and provides insights into feature importance. Due to the small sample size, our prediction may have a higher uncertainty, limiting the predictive value of our analysis. By using a cross-validation and careful consideration of the data, we tried to counteract this limitation. Nevertheless, an additional validation cohort would be of high importance.

In addition, our lack of data on the patients' dietary habits, smoking habits and comedications besides antibiotics limit our understanding of nutritional and environmental effects on microbial composition and therapy outcome. Especially the amount of dietary fiber intake and supplementation with probiotics or prebiotics may have modified the microbiota and could be an undetected confounder [12].

The current study analyzed a relatively modest sample size. Future research should involve the inclusion of

larger and more diverse cohorts of melanoma patients to further validate and generalize our findings. A deeper understanding of the dynamics between the gut microbiome, immune response, and ICI therapy could be gained through longitudinal studies that track changes over time in response to treatment. Investigating the mechanisms underlying the observed associations, such as the role of specific microbial metabolites or immune pathways, could provide a more in-depth understanding of the interactions involved. A multifaceted approach, including in vitro and in vivo models to experimentally validate the observed associations would provide a more comprehensive understanding of the microbiome-immune relationship. Furthermore, expanding our research to include other cancer types beyond melanoma could help uncover broader implications and commonalities in the relationship between the gut microbiome, immune response, and ICI therapy. Finally, as TIGIT is a promising target for future immunotherapies, several clinical studies investigating TIGIT inhibitors are underway.

In conclusion, our study reaffirms the significance of the gut microbiota in influencing the response to ICI therapy in melanoma patients. The association between specific bacterial taxa, such as *Barnesiella intestinihominis*, and treatment response underscores the role of microbial composition as a potential biomarker for predicting clinical outcomes. Furthermore, we have identified the immune receptor TIGIT as significantly upregulated in T cells and $CD56^{high}$ NK cells of responders to ICI therapy. This novel finding highlights TIGIT as a crucial immune checkpoint and potential target for therapeutic intervention. In the context of melanoma, the tumor microenvironment is often characterized by an immunosuppressive milieu. TIGIT expression on tumor-infiltrating T cells has been associated with T-cell exhaustion, a state of functional impairment that limits anti-tumor immune responses [40]. Furthermore, the competition between TIGIT and the co-stimulatory receptor CD226 (DNAM-1), which recognizes the same ligands, for ligand binding can tilt the balance towards immune suppression, inhibiting effector T-cell responses [41]. Further exploration of TIGIT modulation may yield promising strategies to enhance ICI responses.

The insights gained from this study have important clinical implications. They underscore the potential for personalized therapeutic approaches based on individual gut microbiota profiles and immune receptor expression. Future research should focus on expanding our understanding of the mechanisms underlying these associations and translating them into clinical practice. Importantly, future analyses should be applied to larger samples and integrate analysis of an independent validation cohort.

Abbreviations

AIC	Akaike information criterion
AJCC	American joint committee on cancer
AUC	Area under curve
Bp	Base pair
CI	Confidence interval
CTA	Cancer testis antigen
CR	Complete response
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DCB	Durable clinical benefit
DNA	Deoxyribonucleic acid
FDA	Food and drug administration
ICI	Immune checkpoint inhibitor
IFN-y	Interferon gamma
ITIM	Immunoreceptor tyrosine-based inhibitory motif
Ig	Immunoglobulin
irAE	Immune-related adverse event
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effective size
MAGEA	Melanom-associated antigen
NK	Cell natural killer cell
NY-ESO-1	New york esophageal squamous cell carcinoma 1
OR	Odds ratio
PBMC	Peripheral blood mononuclear cell
PCoA	Principle coordinate analysis
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death protein ligand 1
PR	Partial response
RF	Random forest
RFE	Recursive feature elimination
RMSE	Root mean squared error
ROC	Receiver operating characteristic
SD	Stable disease
s.d.	Standard deviation
TIGIT	T cell immunoreceptor with Ig and ITIM domains

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-023-11551-5.

Additional file 1: Table S1. Variables collected as patient data. **Table S2.** Variables included in full regression model. **Table S3.** Preselected variables for Regression and Random Forest Model. **Figure S1.** Stepwise (forward and backward) regression analysis assessing risk factors for the response to ICI treatment; The variables "Barnesiella intestinihominis" (OR >1000; 95% CI 3e+69 – Infinity; p=0.007), "$TIGIT^+$ $CD56^{high}$ NK cells" (OR 0.74; 95% CI 0.583 – 0.918; p=0.009), and "$CD86^+$ Antigen-Presenting B Cells" (OR 0.74; 95% CI 0.549 – 1.01; p=0.046) were significantly associated with treatment failure, the latter not having biological relevance, because BAPCs are per definition $CD86^+$.

Acknowledgements

We would like to thank the patients who participated in this study and the clinical and nursing staff as well as all participating investigators.

Authors' contributions

All authors contributed to the publication according to the International Committee of Medical Journal Editors (ICMJE) guidelines for the authorship. A.T., M.J.G.T.V., M.B., M.S., and C.M. contributed to conceptualization, methodology, and funding acquisition of the study; A.T. and M.J.G.T.V. contributed to project administration; M.J.G.T.V. contributed to supervision; A.T., J.K., V.S., T.S. L.M.H., and H.S. contributed to resources; A.T., R.Z., T.R.L., J.L., and F.F. contributed to investigation; F.F. and T.R.L. contributed to validation and data curation; A.T., F.F., T.R.L., T.S., J.L., and H.S. contributed to formal analysis; A.T., F.F., J.L., and T.R.L. contributed to visualization; A.T. contributed to writing (original draft and review) the article and all authors reviewed, edited and approved the final manuscript.

Funding
Open Access funding enabled and organized by Projekt DEAL. This study was funded by a grant from the German Cancer Aid (Stiftung Deutsche Krebshilfe; grant number: 70112696).

Availability of data and materials
The datasets generated and analyzed during the current study are available in the Sequence Read Archive (SRA) repository (NCBI), [PRJNA1011235].

Declarations

Ethics approval and consent to participate
The study was approved by the Ethics Committee of the Medical Faculty of the University of Cologne (Cologne-ID #17–269) and was conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all patients.

Consent for publication
Not applicable.

Competing interests
MJGTV reports grants and personal fees from 3M, Arderypharm, Astellas Pharma, Basilea, Bio-Mérieux, Biontech, DaVolterra, Evonik, FarmakInternational Holding GmbH, Ferring, Gilead Sciences, Glycom, Heel, Immunic AG, MaaT Pharma, Merck/MSD, Organobalance, Pfizer, Roche, Seres Therapeutics, SocraTec R&D GmbH, Takeda Pharmaceutical, Tillots Pharma GmbH.
MS has received consultant or speaker fees or travel grants from BMS, MSD, Roche, Kyowa Kirin, Novartis, Sanofi Genzyme, Pierre Fabre, Sun Pharma, Immunocore.
All remaining authors have declared that they have no competing interests.

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Received: 29 July 2023 Accepted: 20 October 2023
Published online: 28 November 2023

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4. DISCUSSION

The two publications of this cumulative dissertation contribute to the further understanding of the complex interactions between the human microbiome and cancer therapy, proposing novel insights into potential therapeutic strategies. In the subsequent chapters, the outcomes of both empirical investigations are rigorously examined in a consolidated manner. This includes a detailed discussion of the results including novel findings in the research field, identification of the limitations of each study, and highlighting of areas where further research may be necessary. Additionally, the discussion extends to provide an outlook, proposing future avenues for analyses and suggesting how subsequent research might build upon the established results to enhance the knowledge in the field.

4.1. CHEMORADIATION THERAPY REDUCES THE BACTERIAL LOAD OF CERVICAL MICROBIOTA

The first study of this cumulative dissertation was the first to analyze the effects of chemoradiation for cervical cancer on the cervical microbiota and examined 8 complete sample sets (pre- and post-treatment) of patients with histologically proven cervical cancer, undergoing primary chemoradiation. Quantitatively, a significant reduction in cervical bacterial loads was observed post-treatment, suggesting an effect of chemoradiation therapy on the cervical microbiota. This was expected as chemoradiation is known to cause a reduction in both malignant and healthy cells, including bacterial communities [248, 249]. Nevertheless, the qualitative analysis of the microbiome disclosed surprising findings: despite the drastic reduction in bacterial loads, the diversity of the cervical microbiome – both in terms of alpha diversity (within-sample diversity) and beta diversity (between-sample diversity) – remained largely unchanged. This persistence of microbial diversity despite aggressive treatment raises further questions on the role of the remaining bacteria. Could they be protective, maintaining essential functions during the physiological disturbances induced by cancer therapy? Or might they hinder treatment by preserving a microbial environment that supports disease progression? There are studies suggesting that the post-radiation shift in the (gut) microbiome may exert a proinflammatory effect on epithelial cells by host cytokine induction, leading to tissue damage [250], while others did not find evidence of increased inflammatory cytokines, but nevertheless describe structural and functional changes in the microbiome [251]. In the overall view, it appears to be a bidirectional interplay between (chemoradiation) therapy and the (gut) microbiota, in which there is a therapy-induced change in microbiome composition, yet, these alterations in the microbiota may also have an impact on the efficacy of the therapy [252, 253]. Whether these results can also be transferred to the cervical microbiota remains unclear. There are only two other studies that examined the changes in the cervical microbiota of patients undergoing chemoradiation therapy for cervical cancer [254, 255].

One study included a comparison of pre- and post-radiation samples of 16 cervical cancer patients and did not find significant changes in cervical microbiota diversity or richness, in line with our results [254]. However, a significantly higher percentage of anaerobic bacteria was observed in pre-treatment samples compared to post-treatment samples, whereas more facultatively anaerobic bacteria were found in post-treatment samples. Furthermore, this study compared cervical cancer patients with healthy individuals and observed a significantly higher microbiome diversity in cancer patients compared to healthy controls, as described before [91, 254]. The second study did not observe significant differences between pre- and post-treatment samples concerning diversity or richness either, but nevertheless found changes in the relative abundance of several taxa, as was also observed in our study [255]. The fact that all three studies did not observe any significant changes in diversity might be due to inter-individual differences in the cervical microbiota composition, and the small number of samples. One more publication analyzed the effects of radiotherapy on the vaginal microbiota in a larger cohort of patients and observed a slightly increased diversity in post-treatment samples compared to pre-treatment samples in terms of Shannon and phylogenetic diversity [256]. In the post-treatment samples, this study also found decrease of *Lactobacillus* and enrichment of species that have been associated with BV or genital tract inflammation. Taken together, all studies point to a cancer-related state of cervicovaginal changes in the microbiota, though the knowledge regarding the impact on the onset and advancement of cervical cancer remains limited. Furthermore, changes in the microbiota may play a role in mitigating some of the side effects of chemoradiation, such as vulvovaginal atrophy [257].

Our study highlights a significant inter-individual variability in the microbiota's response to cancer treatment. Some women showed a stronger dominance of certain families, such as *Lactobacillaceae*, and *Prevotellacecae*, while others showed a more diverse composition. This variability underscores the complexity of microbial ecosystems and suggests that personalized microbial management strategies might be necessary to optimize treatment outcomes for each patient.

Our pilot study has several limitations. First of all, the modest cohort size restricts the generalizability of our findings. Due to incomplete sample sets, seven out of 15 patients had to be excluded from the analysis. Furthermore, the comparatively low resolution of 16S rRNA gene sequencing precluded precise identification of bacteria at species or strain level and impeded differentiation between pathogenic and commensal strains within the same species or genus. Different *Lactobacillus* species have been ascribed different roles in reproductive health. A recent study associated *L. iners* with poor tumor response to chemoradiation and showed that *L. iners* can alter tumor metabolism, causing therapeutic resistance [258]. In contrast, *L. crispatus* has been shown to have several beneficial effects [128, 236].

To substantiate these preliminary observations and elucidate the underlying mechanisms, a larger cohort and, ideally, longitudinal sampling would be necessary. Due to the already more intensively described role of the much more abundant gut microbiota in relation to immune response in cancer, future studies should also consider a combination of gut and cervical microbiome analyses.

Possible future applications include the development of novel therapeutic interventions complementing conventional cancer treatment, such as probiotics or vaginal microbiota transfer (VMT), in order to restore cervicovaginal homeostasis and potentially mitigate side effects or even enhance the efficacy of cancer therapy.

4.2. GUT MICROBIOME CHARACTERISTICS IN MELANOMA PATIENTS UNDER ICI THERAPY DIFFER BETWEEN RESPONDERS AND NON-RESPONDERS

The second study that is part of this cumulative dissertation explored the predictive value of gut microbiota composition and immune variables in 29 cutaneous melanoma patients undergoing ICI therapy targeting PD-1 and/or CTLA-4. Only about half of the patients achieved a DCB, in line with previously reported results, highlighting the challenge of predicting treatment response with current methods. Based on whole metagenomic sequencing, there was no significant difference concerning alpha or beta diversity between patients with and without DCB, but LEfSe analysis revealed an increased abundance of several bacterial taxa in responders and non-responders. The gut microbiota of patients with a DCB was significantly enriched in the species *Ruminococcus torques*, and *Lacrimispora amygdalina*, whereas in patients without a DCB, species abundances were increased for *Butyricimonas paravirosa*, *Akkermansia muciniphila*, and *Barnesiella intestinihominis*.

Daillère et al. identified *Barnesiella intestinihominis* to ameliorate the anticancer effects of Cyclophosphamide (CTX) in a murine cancer model [259]. CTX is used as chemotherapy to treat cancers, but in contrast to ICI therapy, CTX suppresses the immune system. Because of the different mechanisms of action, the results of the study cannot be directly applied to patients receiving ICI therapy. CTX therapy has the side effect of making the intestinal barrier more permeable to bacteria and to fight the bacteria, an immune response is triggered that also targets the tumor cells. In our cohort of melanoma patients treated with ICI therapy, there is no reasonable assumption that the patients' intestinal mucosa was impacted before the start of ICI therapy (baseline). Only two patients received antibiotics within the three months before their baseline stool sample that could have harmed the intestinal barrier [260]. Two more patients were exposed to irradiation before baseline (femoral metastases and postoperative irradiation of lymphatic drainage), which, however, most likely did not affect the integrity of the intestinal microbiota. This is also supported by the fact that alpha diversity was rather high in almost all patients and that the microbiota composition at baseline did not

show signs of dysbiosis in most of the patients. Only one patient showed bacterial dominance, defined as relative abundance of one species > 30%. Another recent study also identified an association between *Barnesiella intestinihominis* and clinical benefit after vascular endothelial growth factor-tyrosine kinase inhibitors (VEGF-TKIs) in 20 metastatic renal cell carcinoma (RCC) patients [261]. However, because the mechanism of action is different from ICI, these results are not generalizable to our cohort and thus not necessarily inconsistent with our results.

Furthermore, in our study, *Enterobacteriaceae*, a family within the Proteobacteria phylum, were present in 8 out of 14 (57.1%) non-responders, while only one out of 15 responders (6.7%) had an *Enterobacteriaceae* abundance of over 1%. These Gram-negative bacteria have previously been associated with inflammation and dysbiosis [262-264]. It has been shown that *Enterobacteriaceae* can induce an inflammatory state by modulating host production of anti-inflammatory steroid corticosterone [264]. Other mechanisms by which *Enterobacteriaceae* may promote intestinal inflammation include secretion of IL-8 and TNF-α, disruption of intestinal mucosa tight junctions, and the existence of a more immunostimulatory version of LPS in *Enterobacteriaceae* [265].

Several other studies performed in melanoma patients have identified microbiome signatures associated with treatment outcomes, though there is no taxonomic consistency across cohorts [185-189]. This may be explained by the methodological heterogeneity of these studies, which are discussed in more detail in the following.

Chaput et al. analyzed anaerobically collected stool samples and blood samples from 26 metastatic melanoma patients treated with anti-CTLA-4 [188]. 35% had a long-term clinical benefit, according to immune related response criteria, whereas many other studies used the gold standard RECIST. Baseline 16S rRNA gene sequencing revealed three clusters: Stool samples of patients with a long-term clinical benefit were enriched with *Faecalibacterium* genus and other bacteria of the phylum Firmicutes. These patients were also more likely to develop colitis and had a lower frequency of baseline regulatory T cells. Conversely, patients with a higher abundance of Bacteroides showed a poor treatment response but remained colitis-free. This result confirms the findings of another study, in which non-colitis patients showed higher levels of Bacteroidetes phylum [266]. A third cluster was enriched with *Prevotella* but was excluded from further analyses due to small sample size. This study excluded four patients because they died soon after their first and single infusion of ICI therapy, leaving a healthier-than-average patient cohort for analysis.

In 2017, Frankel et al. published their analysis of 39 metastatic melanoma patients treated with three different types or combinations of ICI [189]. Fecal samples were collected and frozen at home by the patient before start of ICI treatment, transferred to the lab on ice and analyzed by metagenomic shotgun sequencing and metabolomic profiling. Taken together, 62% of all patients showed a treatment response, defined as complete or partial response or stable disease using the RECIST

criteria. The intestinal microbiota of these patients had a higher abundance of *Bacteroides caccae* and *Streptococcus parasanguinis* compared to patients with disease progression. Responder microbiome was furthermore significantly enriched with genes encoding for bacterial enzymes associated with fatty acid synthesis. Subgroup analyses revealed that stool of responders treated with a combination of anti-CTLA-4 and anti-PD-1 was enriched with other bacterial species compared to responders treated with anti-PD-1 only. There were no differences in overall gut microbiome diversity of responders versus patients with progressive disease, which is supported by the findings of our study, and no effect of antibiotics or probiotics, although this might be due to the low number of patients exposed to antibiotics.

Gopalakrishnan et al. analyzed stool samples (collected with a DNA stabilizing kit) of 43 metastatic melanoma patients treated with anti-PD-1 therapy using 16S rRNA gene sequencing and metagenomic shotgun sequencing in a subgroup [185]. With a range of -481 to +14 days in relation to the start of the ICI therapy, the time span over which the baseline samples were collected was very long. 70% of the patients responded to ICI therapy (complete or partial response or stable disease lasting at least 6 months using the RECIST criteria) and 30% were non-responders. Compared to our cohort and other publications, this is a very high response rate and it is questionable to what extent these results are transferrable to other cohorts. The gut microbiota of responders was enriched with Clostridiales/*Ruminococcaceae* and *Faecalibacterium* and non-responders showed an overabundance of *Bacteroides thetaiotaomicron*, *Escherichia coli* and *Anaerotruncus colihominis*. Responders had a significantly higher alpha diversity compared to non-responders and diversity was positively correlated with a prolonged progression-free survival. When this analysis was repeated recently with a larger, newly recruited cohort (n = 132), no significant differences in the alpha and beta diversity could be detected, which is consistent with our results [184]. However, the enrichment of Ruminococcaceae family and *Faecalibacterium* genus in responders could be replicated.

Matson et al. studied 42 metastatic melanoma patients under anti-PD1 or anti-CTLA4 therapy and analyzed pre-treatment stool samples through an integration of 16S rRNA gene sequencing, metagenomic sequencing, and quantitative polymerase chain reaction (qPCR) for selected bacteria [186]. Response (38.1% of the patients, assessed with RECIST) was significantly associated with a high abundance of eight species, such as *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium*, whereas in non-responders *Ruminococcus obeum* and *Roseburia intestinalis* were more abundant. Results of metagenomic sequencing compared to 16S rRNA gene sequencing revealed a smaller number of species differently abundant in responders versus non-responders.

Another study by Routy et al. analyzed the gut microbiota composition of 100 patients diagnosed with non-small-cell lung cancer (NSCLC) or RCC treated with PD-1 based ICI [192]. It is uncertain to what extent the results can be transferred to completely different cancer types like cutaneous melanoma in

our cohort. Stool was collected by patients at home using an anaerobic generator and transferred to -80 °C within 24 hours. Clinical response was defined as absence of disease progression after 6 months based on RECIST criteria. The microbiota of responders was enriched with *Akkermansia muciniphila* as well as unclassified and classified Firmicutes. Furthermore, feces culturomic analyses of 32 patients revealed a higher incidence of cultivable *Enterococcus hirae* in responders compared to non-responders.

Although the observations made in these clinical studies point to a relationship, they do not establish a causal link between intestinal microbiota and the efficacy of ICI therapy. Due to several reasons the comparability of these studies is limited. First of all, the techniques for stool sample collection vary widely, including the use of anaerobic generators, preservation media, and aerobic collection methods, coupled with sometimes extensive variability in the timing of sample collection and storage. The method of collection can significantly impact the sequencing results, as the bacterial composition of a stool sample may rapidly alter before it is preserved. Additionally, the studies differ in their approaches to DNA extraction, sequencing technologies, and bioinformatic analyses. This issue of bioinformatics was addressed in a recent analysis that reanalyzed the sequencing data for three of the above-mentioned studies using a common analytical pipeline [267]. The results confirmed that there are unique microbial signals in each study, emphasizing the poor transferability to other cohorts. For microbiome analysis, patient cohorts were stratified into responders and non-responders, with response rates across the studies varying significantly from 35% to 70%. Another important factor that may contribute to the divergence in the findings are the varying disease stages of the patients at the start of ICI therapy. While it is recognized that advanced stages of cancer can imply an altered composition of the gut microbiome, particularly as a negative consequence of increased medication use, such as antibiotics or chemotherapy, the specific impact of varying cancer stages on the efficacy of ICI is still subject of ongoing investigations [268, 269]. In our cohort, most of the patients were already stage IV cancer, and only two patients received antibiotics before start of ICI therapy. Due to this inhomogeneous distribution, no further subgroup analysis was done. Besides, the intestinal microbiota profiles seem to be quite distinct comparing different cancer types like RCC, NSCLC and metastatic melanoma. Although certain researchers propose the existence of a consistent microbiome signature across various cohorts [270], these results from a 2020 meta-analysis were not confirmed by a subsequent extensive cross-cohort study clearly. This study demonstrated that the microbiome associations are highly dependent on specific cohorts, even when examining a single type of cancer, and highlighted the limited reproducibility of microbiome-based signatures across different cohorts [187]. The geographical location has also been shown to influence the composition of the intestinal microbiota and further limits the transferability from one cohort to another [271]. In this

sense, our results align with the previously observed inconsistencies in the predictive power of microbiome signatures.

Over the last several years, many studies have identified different microbial species as modulators of checkpoint inhibitor therapy efficacy and toxicity. The link between intestinal microbiota and the immune system is undisputed [272]. However, it is unlikely that the abundance of individual microbial species exerts substantial influences on cancer therapies or serves as a uniformly reliable biomarker. Instead, the effects observed are likely attributable to a multifaceted interaction among a diverse array of bacteria, fungi, viruses, and their metabolites, such as SCFA. In addition to the methodological confounders mentioned, it is also plausible that the heterogeneity observed in microbiome signatures across different studies might indicate a functional overlap. In order to answer this question, larger mechanistic studies are necessary, which also include functional assays and meta-omics approaches such as metabolomics, -proteomics, -transcriptomics, and -peptidomics. While the analysis of stool samples provides a useful approximation, a more insightful investigation would involve examining samples from the intestinal mucosa and its microbial colonization. However, such studies are not feasible in human cohorts due to practical and ethical constraints.

4.3. A COMBINATION OF MICROBIOME AND IMMUNE FEATURES CAN PREDICT RESPONSE TO ICI THERAPY

To our knowledge, this study was the first to integrate clinical metadata, metagenomic microbiome analyses and immune profiling to forecast the efficacy of ICI therapy in patients with cutaneous melanoma. Utilizing a model that incorporates two microbiome features and one immunological feature, we successfully predicted the response to ICI treatment prior to the initial infusion, achieving a mean area under the curve (AUC) of 0.85. These three variables can be determined through the analysis of a blood sample and a stool sample and collectively, they provide a predictive model that could potentially be employed to identify patients likely to benefit from ICI therapy, thereby informing the treatment decision-making process.

A key finding in our study is the significant upregulation of the immune checkpoint receptor TIGIT in both T cells and $CD56^{high}$ NK cells in patients who responded to treatment compared to non-responders. TIGIT, a receptor involved in regulating innate and adaptive immune responses through multiple mechanisms, has emerged as a promising therapeutic target in the context of cancer. It is expressed on various immune cells, including $CD4^+$ T cells, $CD8^+$ T cells, Tregs, and NK cells [273, 274]. NK cells belong to the primary agents of innate immunity, whereas T cells are a main force of adaptive immune response, both playing essential roles in anti-tumor immunity. The binding of TIGIT and its ligands leads to inhibition of effector T cell function and decreased cytotoxicity of NK cells, while promoting the immunosuppressive activity of Tregs [274]. The ligands of TIGIT, CD155 (poliovirus

receptor, PVR) and CD112 (PVR-related 2, PVRL2), are abundantly expressed on dendritic cells and activated T cells, and also by various tumors, including melanoma, that use this mechanism for immune escape [275-277]. In melanoma patients, high baseline tumor expression levels of CD155 correlate with poor response to anti-PD1 therapy [278]. Furthermore, it has been shown that TIGIT is upregulated on tumor antigen-specific $CD8^+$ T cells and $CD8^+$ tumor-infiltrating lymphocytes of melanoma patients, often co-expressed with PD-1 [279]. While (pre-)clinical findings concerning anti-TIGIT monotherapy are mixed, the combination of TIGIT- and PD-1-blockade shows promising results [280, 281]. The influence of TIGIT-blockade on tumor-infiltrating NK cells seems to be critical for the therapeutic effect [281]. Currently, many clinical trials are registered to evaluate dual PD-1/TIGIT blockade for treatment of a wide range of solid tumors and hematological malignancies [175]. In our cohort, the upregulation of TIGIT in peripheral blood cells of responders may indicate a more activated, competent immune cell phenotype prior to ICI therapy, which in turn tends to benefit more from the therapy. It is however unclear if these changes in the peripheral blood reflect the situation in the tumor microenvironment [282]. Within the tumor microenvironment, T cells and NK cells are integral to the immune dynamics, particularly in the context of ICI therapy. The variability of the expression of immune receptors like PD-1 or TIGIT on these cells can influences their ability to recognize and destroy tumor cells [283], and is reflected in both intra- and inter-tumoral heterogeneity [284].

The surface molecule CD56 is differently expressed on NK cell subsets and used as a phenotypic marker to define different states of maturation and function. NK cells with a high expression of CD56 have immunoregulatory functions and produce high levels of cytokines and chemokines, whereas NK cells with a lower CD56 expression are considered to have greater cytotoxic abilities [285]. In the peripheral blood, most of NK cells express low levels of CD56 and only up to 10% express high levels of CD56 [286]. The role of TIGIT in the latter NK cell subpopulation is poorly understood, but as in our study TIGIT was upregulated in $CD56^{high}$ NK cells of responders, it is conceivable that binding to TIGIT leads to an inhibition of the immunoregulatory function of these cells, thereby enhancing the body's ability to fight the tumor.

Regarding microbiota-mediated immune regulation, a previous study demonstrated that *Fusobacterium nucleatum*, a commensal bacterium in the tumor microenvironment associated with colorectal cancer, can bind directly to TIGIT, resulting in the suppression of anti-tumor responses from NK cells and T cells [140]. This result shows that microbiome analyses based on fecal samples may not be sufficient for identification of immunologically relevant bacteria. The analysis of intra-tumoral microbiota might be better suited to identify a linkage between microbiome signatures and immune function. A recent study showed that peptides of microorganisms residing in the tumor microenvironment are presented by tumor cells and thus elicit immune reactivity [287]. Another study demonstrated an association between intra-tumoral gut bacteria (e.g. *Lachnoclostridium*) and

infiltrating CD8+ T cells in cutaneous melanoma [288]. When analyzing tumor material, a low bacterial biomass is to be expected, posing the risk of misidentifying contaminants as relevant bacteria. Unfortunately, in our study, no tumor samples were available for analysis of the tumor microenvironment.

Although the value for the AUC in our model (0.85) is good, the predictive power and reliability of the Random Forest model is limited, due to its small sample size. The algorithm builds multiple decision trees on bootstrap samples of the training data. With a small sample size, there is less diversity among the bootstrap samples, leading to more correlated trees and a reduced advantage of the ensemble over a single tree. Furthermore, with a small dataset, the risk of overfitting increases, diminishing the ability to generalize. To address the limitations inherent in our study, we implemented cross-validation and meticulously analyzed the data, aiming to enhance the robustness and reliability of our findings. Despite these efforts, the inclusion of an additional validation cohort would be crucial to further substantiate our results and confirm their generalizability across different populations. Furthermore, our study was constrained by incomplete data regarding the patients' broader clinical and lifestyle contexts. Specifically, we lacked comprehensive information on patients' dietary habits, comedication use beyond antibiotics, and other environmental factors that could significantly impact both the microbiome and immune response dynamics. Particularly, the level of dietary fiber consumption and the use of pro- or prebiotics might have altered the microbiota composition, potentially acting as an unrecognized confounding factor [184]. This lack of detailed patient data potentially limits our ability to fully understand the complex interactions that influence the efficacy of ICI therapy. Moreover, integrating this information could help develop more personalized approaches to cancer therapy, tailoring treatments not only to genetic and biomarker profiles but also to individual environmental and lifestyle contexts.

4.4. CHALLENGES AND LIMITATIONS

In the field of microbiome research, there are a number of methodological challenges. Consistency in the initial step, sample collection and handling, is crucial for accurate microbiome analysis. Variations in these protocols can lead to discrepancies in data, affecting the reproducibility and reliability of findings. A major challenge is ensuring that the collected samples accurately represent the microbial community without contamination (especially for low biomass samples) or shift. Factors such as the site of collection, the timing and material or method used (swabs, biopsies, preservation medium, etc.) can introduce variability. Post-collection, the proper storage of samples is important to prevent changes in the microbial community composition due to cell growth or cell death. Most microbiome samples require immediate freezing and storage at -80 °C. Freeze-thaw cycles can have a significant impact on the samples and should be avoided [289]. For DNA extraction, different kits and protocols

exist and preferentially lyse certain bacteria over others, leading to biased results that do not accurately reflect the original microbial community [290]. Next, the choice of a sequencing pipeline impacts what information is obtainable. Whereas 16S rRNA gene sequencing targets only one gene and thereby can identify bacteria and archaea only up to the genus level, metagenomic shotgun sequencing analyzes the entire genomic content of all cells (bacteria, archaea, fungi, etc.) present in a sample and thereby provides species and strain-level resolution. This can be very important as certain strains of a species may have totally different characteristics, being either pathogenic or harmless and potential probiotics. However, metagenomic shotgun sequencing is more expensive and requires more complex data processing and interpretation. The choice of reference database for aligning sequencing data significantly influences the outcomes of microbiome research as no database comprehensively covers all microbial sequences and many are biased towards well-studied organisms. This limitation can lead to a substantial underrepresentation of less-studied or novel microorganisms. Interpreting microbiome data requires careful consideration of various confounding factors such as diet, genetic predisposition, or lifestyle of the host. Disentangling these effects to identify true microbial influences remains a substantial challenge. Patient microbiome studies often suffer from small sample sizes due to the high cost of sequencing and additional effort concerning high quality sample collection. This is particularly problematic in studies attempting to link microbiome composition to health outcomes, where large, diverse cohorts are necessary to draw robust conclusions. Furthermore, many studies are not sufficiently powered to detect subtle but potentially important associations. Due to the many possibilities during each analysis step and potential biases, variability in experimental protocols, sample processing, sequencing methods, bioinformatic pipelines, and data analyses approaches across studies is high. This lack of standardization makes it difficult to compare and integrate findings.

4.5. FUTURE DIRECTIONS

In the last decades, the field of microbiome research has unveiled numerous associations between microbial communities and cancer, significantly enhancing our understanding, yet also highlighting the complex nature of these relationships. Despite these advances, much remains unknown about the intricate dynamics at play, prompting a continued need for innovative and thorough research approaches.

To gain a deeper understanding of the causal relationships and mechanisms underlying the observed correlations between the microbiome and cancer, it is essential to perform detailed mechanistic studies, integrating advanced functional assays and meta-omics approaches to provide a multi-dimensional view of the biochemical interactions occurring between the microbiota and the host during cancer development and treatment. This integration will help in identifying specific microbial metabolites that could influence therapeutic outcomes or serve as biomarkers.

Moreover, humanized animal models offer an additional opportunity to study mechanisms in controlled experimental settings. Such models allow researchers to manipulate the microbiota and observe resulting effects on immune response or therapy efficacy, providing insights that are often unattainable in human studies due to ethical or practical constraints. Larger cohorts are needed to identify robust microbiome and immune signatures that may predict therapeutic outcomes. For a better generalizability, these studies should be conducted across different geographical locations. Future research should furthermore focus on longitudinal studies that follow patients over time to observe dynamic changes throughout the course of a treatment and beyond.

Other components of the microbiome such as the virome and the mycobiome gain more and more scientific attention and future studies should consider this, as these lesser-studies microorganisms could play critical roles in modulating immune responses and influencing the efficacy of cancer therapies. Finally, clinical trials focusing on the microbiota as a therapeutic target have begun to show promising results, indicating potential pathways to modulate the microbiota to improve cancer treatment outcomes. These trials not only contribute to a greater understanding of the microbiome's role in cancer but also open new avenues for microbiome-based therapies, which could lead to more personalized and effective treatment protocols.

By addressing these areas, future research can build on the foundational knowledge of the past decades, moving from correlation to causation, and from broad observational studies to targeted, mechanism-based interventions that leverage the full therapeutic potential of the microbiome in cancer treatment.

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APPENDIX

Supporting Information Publication 2

Table S1: Variables collected as patient data

Variables
Age
Sex
Underlying disease
Melanoma details
Initial diagnosis
Tumor stage
Localization of metastases
Presence of driver mutations
Previous therapies
PD-L1 status
Stool characteristics
Eastern Cooperative Oncology Group (ECOG) performance status
Charlson Comorbidity Index
Antibiotic use
Response to therapy according to Response Evaluation Criteria in Solid Tumors (RECIST)
Lactate dehydrogenase (LDH)
Drug-related adverse events
Death
Type of Checkpoint Inhibitor
Radiation therapy
Immune-related adverse events
Steroid use in the case of immune-related adverse event

Table S2: Variables included in full regression model

Variables	
1	Age: [<65, 65-75, 76-86]
2	Checkpoint Inhibitor combination [yes]
3	Sex [male]
4	CK2_CD45+Lymphocytes
5	CK2_CD56highNKcells
6	CK2_Tigit+%Tcells
7	CK2_Tigit+%Bcells
8	CRP [high]
9	D4__Enterobacteriaceae
10	D6__Akkermansia_muciniphila
11	D6__Barnesiella_intestinihominis
12	D6__Butyricimonas_paravirosa
13	D6__Lacrimispora_amygdalina
14	D6__Ruminococcus_torques
15	Driver mutation [yes]
16	IFNg_MLANA
17	Therapy before [yes]
18	Bcell_CD40+%Binact
19	Bcell_CD40+%CD24hiCD38hi
20	Bcell_CD45+%Lymphocytes
21	Bcell_CD86+%activated.B.cells
22	Bcell_CD86+%BAPC
23	Bcell_CD86+%Binact
24	Bcell_CD86+%CD24hiCD38hiBcells
25	Bcell_CD86+%plasmablasts
26	Bcell_IgG.CD27+%.CD19.CD20.
27	Bcell_IgG.IgD+%CD19.CD20.
28	Bcell_lymphocytes
29	Bcell_Plasmablast.CD19.Bcells
30	NKcell_CD126+%IL6R+%+%Tcells
31	NKcell_CD16+%CD56highNKcells
32	NKcell_CD45%Lymphocytes
33	NKcell_lymphocytes
34	NKcell_TIGIT+%CD56hiNKcells
35	NKcell_TIGIT+%CD56interNKcells
36	Treg_CD137+%CD45
37	Treg_CD25+%CD8+Tcells
38	Treg_CD39+CD73+%Tcells
39	Treg_CD39+CD73+%Tregs+
40	Treg_CD45+Lymphocytes
41	Treg_CTLA+4+%CD45
42	Treg_GITR+%CD45

Table S3: Preselected variables for Regression and Random Forest Model

Variables			
1	Bcell_CD40+%BAPC	43	NKcell_CD69+%CD45
2	Bcell_CD40+%Binact	44	NKcell_lymphocytes
3	Bcell_CD40+%Breact	45	NKcell_TIGIT+%CD56hiNKcells
4	Bcell_CD40+%CD24hiCD27+	46	NKcell_TIGIT+%CD56interNKcells
5	Bcell_CD40+%CD24hiCD38hi	47	Observed
6	Bcell_CD40+%plasmablasts	48	Shannon
7	Bcell_CD45+%Lymphocytes	49	Treg_CD137+%CD45
8	Bcell_CD86+%activated+B+cells	50	Treg_CD137+%Tcells
9	Bcell_CD86+%BAPC	51	Treg_CD25+%CD4+Tcells
10	Bcell_CD86+%Binact	52	Treg_CD25+%CD8+Tcells
11	Bcell_CD86+%Breact	53	Treg_CD39+%Tregs
12	Bcell_CD86+%CD24hiCD27+Bcells	54	Treg_CD39+CD73+%Tcells
13	Bcell_CD86+%CD24hiCD38hiBcells	55	Treg_CD39+CD73+%Tregs+
14	Bcell_CD86+%plasmablasts	56	Treg_CD4+%T+cells+Tcells
15	Bcell_IgG+CD27+%+CD19+CD20+	57	Treg_CD45+Lymphocytes
16	Bcell_IgG+IgD+%CD19+CD20+	58	Treg_CD73+%Tcells
17	Bcell_lymphocytes	59	Treg_CD73+%Tregs
18	Bcell_Plasmablast+CD19+Bcells	60	Treg_CTLA+4+%CD45
19	CK2_CD155+%NKcells	61	Treg_GITR+%CD45
20	CK2_CD226+%Bcells	62	Treg_Lymphocytes
21	CK2_CD226+%Tcells	63	Age
22	CK2_CD45+Lymphocytes	64	Antibiotic_before
23	CK2_CD56highNKcells	65	BRAF Wildtype
24	CK2_CD96+%Bcells	66	Bristol Stool Scale
25	CK2_lymphocytes	67	Charlson-Comorbidity Index
26	CK2_Tigit+%Bcells	68	Checkpoint-Inhibitor combination
27	CK2_Tigit+%Tcells	69	CRP
28	IFNg_MAGEA1	70	Distant metastases
29	IFNg_MAGEA3	71	Driver mutations
30	IFNg_MLANA	72	ECOG
31	IFNg_NY+ESO+1	73	LDH (<250 U/l)
32	IFNg_SURVIVIN	74	Lymphocytes absolute
33	NKcell_CD126+%IL6R+%+%CD56interNKcells	75	Lymphocytes relative
34	NKcell_CD126+%IL6R+%+%Tcells	76	Neutrophils relative
35	NKcell_CD158a+%CD56hiNKcells	77	Sex
36	NKcell_CD158a+%CD56interNKcells	78	Therapy before
37	NKcell_CD158b+%CD56highNKcells	79	D4__Enterobacteriaceae
38	NKcell_CD158e_interCD56highNKcells	80	D6__Akkermansia_muciniphila
39	NKcell_CD158e_interCD56interNKcells	81	D6__Barnesiella_intestinihominis
40	NKcell_CD158e+%CD56interNKcells	82	D6__Butyricimonas_paravirosa
41	NKcell_CD16+%CD56highNKcells	83	D6__Lacrimispora_amygdalina
42	NKcell_CD45+Lymphocytes	84	D6__Ruminococcus_torques

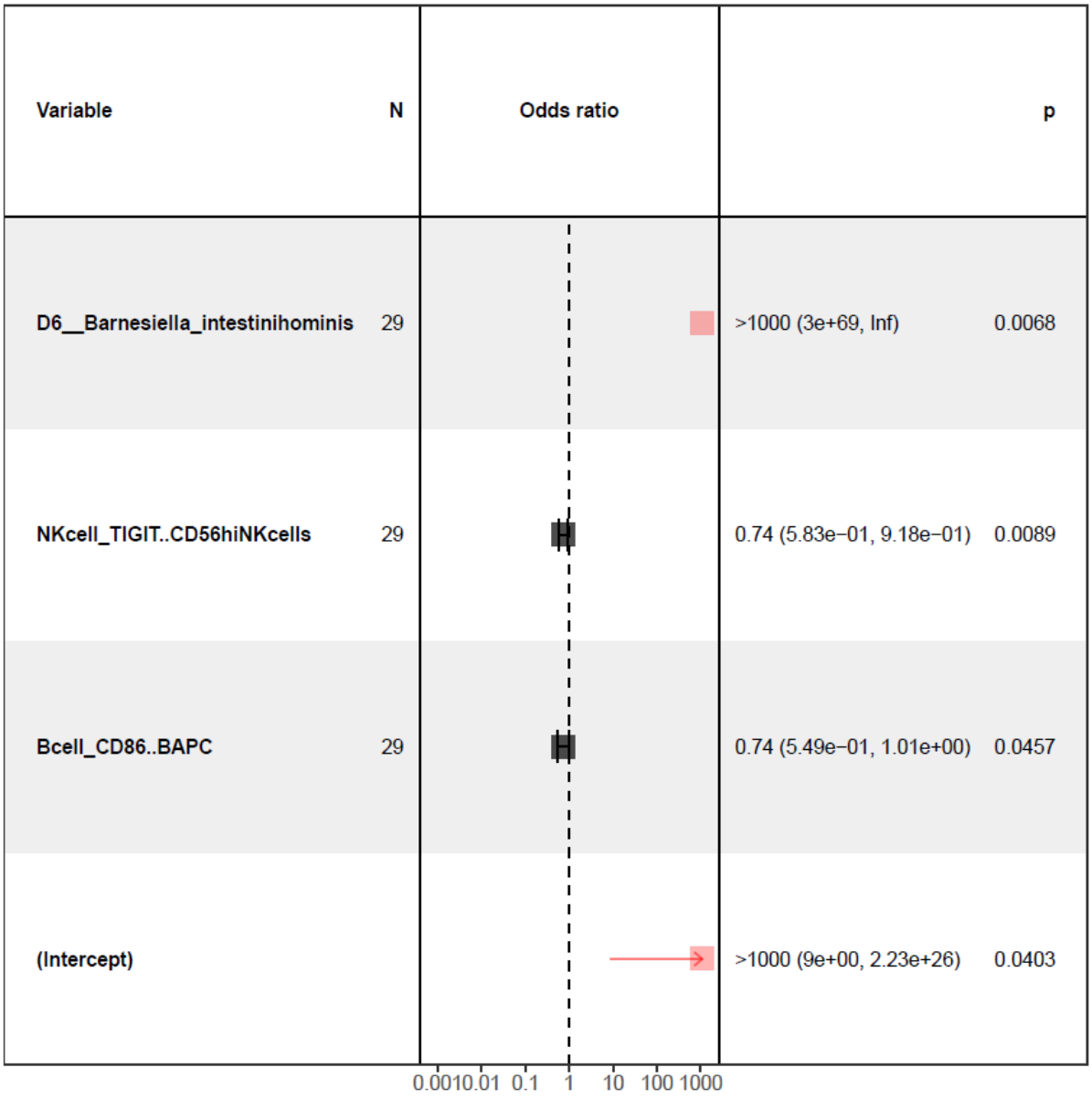

Figure S1: Stepwise (forward and backward) regression analysis assessing risk factors for the response to ICI treatment; The variables "Barnesiella intestinihominis" (OR >1000; 95% CI 3e+69 – Infinity; p=0.007), "TIGIT^{+} CD56^{high} NK cells" (OR 0.74; 95% CI 0.583 – 0.918; p=0.009), and "CD86^{+} Antigen-Presenting B Cells" (OR 0.74; 95% CI 0.549 – 1.01; p=0.046) were significantly associated with treatment failure, the latter not having biological relevance, because BAPCs are per definition CD86^{+}.

PUBLICATION LIST AND CONTRIBUTION

PUBLICATION 1	
Reference	**Tsakmaklis** A, Vehreschild M, Farowski F, et al. Changes in the cervical microbiota of cervical cancer patients after primary radio-chemotherapy. *Int J Gynecol Cancer*. 2020;30(9):1326-1330.
Journal	International Journal of Gynecological Cancer
Impact Factor	3.437 (2020)
Date of Acceptance	10 September 2019
Abstract	**Changes in the cervical microbiota of cervical cancer patients after primary radio-chemotherapy** **Objective:** Several recent studies have identified a potential interaction between the vaginal microbiota and gynecological cancers, but little is known about the cervical microbiota and its changes during cancer treatment. Therefore, the aim of the study was to evaluate the quantitative and qualitative changes of cervical microbiota in patients undergoing concurrent chemotherapy and radiation treatment for locally advanced cervical cancer. **Methods:** Cervical cytobrush samples of 15 cervical patients undergoing chemoradiation treatment were collected 1 day before starting external beam radiation therapy and on the day of the last fraction of brachytherapy. After DNA extraction, 16S rRNA amplicon sequencing of the V3-V4 region was performed on the MiSeq platform, followed by data processing and statistical analyses concerning the alpha and beta diversity of 16 samples (7 samples were excluded because of incomplete sample sets). **Results:** The amount of amplicon yield after polymerase chain reaction analysis in post-radiation samples was significantly lower compared with the baseline samples (pre 31.49±24.07 ng/µl; post 1.33±1.94 ng/µl; p=0.007). A comparison of pre-treatment and post-treatment samples did not show significant differences regarding beta diversity (weighted UniFrac). There was no significant difference in

	alpha diversity, which is used to characterize species diversity within a particular community and takes into account both number and abundance (Shannon Diversity Index pre-treatment samples: 2.167±0.7504 (95% CI 1.54 to 2.79); post-treatment samples: 1.97±0.43 (95% CI 1.61 to 2.33); p=0.38). Interindividual differences in patients could partly explain some variation of the samples (permutational multivariate analysis of variance). **Conclusion:** There was a strong reduction in cervical bacterial loads after chemoradiation. Neither alpha nor beta diversity varied significantly when baseline samples were compared with post-treatment samples.
Contribution	**Anastasia Tsakmaklis** designed the study in cooperation with Simone Marnitz and Maria Vehreschild. **Anastasia Tsakmaklis** coordinated the project and carried out the sample collection together with clinicians from the Clinic and Polyclinic for Radiooncology, Cyberknife and Radiotherapy at Cologne University Hospital. **Anastasia Tsakmaklis** carried out the sample processing. **Anastasia Tsakmaklis** and Fedja Farowski analyzed the samples and evaluated the data. **Anastasia Tsakmaklis** prepared the manuscript draft and all co-authors read, revised and approved it for publication.
Cooperation	<u>Co-operation partner:</u> Clinic and Polyclinic for Radiooncology, Cyberknife and Radiotherapy at Cologne University Hospital. <u>Scope of co-operation</u>: The study was designed in cooperation with Prof. Dr. Simone Marnitz-Schulze, who was head of the clinic at the time. The patients were informed and the cervical samples were taken by physicians at the clinic. Anastasia Tsakmaklis was responsible for the entire sample processing, analysis and evaluation as well as the preparation of the manuscript.

PUBLICATION 2	
Reference	**Tsakmaklis** A, Farowski F, Zenner R, et al. TIGIT+ NK cells in combination with specific gut microbiota features predict response to checkpoint inhibitor therapy in melanoma patients. *BMC Cancer*. 2023;23:1160.
Journal	BMC Cancer
Impact Factor	4.638
Date of Acceptance	20 October 2023
Abstract	**TIGIT$^+$ NK Cells in Combination with Specific Gut Microbiota Features Predict Response to Checkpoint Inhibitor Therapy in Melanoma Patients** **Background:** Composition of the intestinal microbiota has been correlated to therapeutic efficacy of immune checkpoint inhibitors (ICI) in various cancer entities including melanoma. Prediction of the outcome of such therapy, however, is still unavailable. This prospective, non-interventional study was conducted in order to achieve an integrated assessment of the connection between a specific intestinal microbiota profile and antitumor immune response to immune checkpoint inhibitor therapy (anti-PD-1 and/or anti-CTLA-4) in melanoma patients. **Methods:** We assessed blood and stool samples of 29 cutaneous melanoma patients who received immune checkpoint inhibitor therapy. For functional and phenotypical immune analysis, 12-color flow cytometry and FluoroSpot assays were conducted. Gut microbiome was analyzed with shotgun metagenomics sequencing. To combine clinical, microbiome and immune variables, we applied the Random Forest algorithm. **Results:** A total of 29 patients was analyzed in this study, among whom 51.7% (n = 15) reached a durable clinical benefit. The Immune receptor TIGIT is significantly upregulated in T cells (p = 0.0139) and CD56high NK cells (p = 0.0037) of responders. Several bacterial taxa were associated with response (e.g. *Ruminococcus torques*) or failure (e.g. *Barnesiella intestinihominis*) to immune therapy. A combination of two microbiome features (*Barnesiella intestinihominis* and the Enterobacteriaceae

	family) and one immune feature (TIGIT+ CD56high NK cells) was able to predict response to ICI already at baseline (AUC = 0.85; 95% CI: 0.841-0.853). **Conclusions:** Our results reconfirm a link between intestinal microbiota and response to ICI therapy in melanoma patients and furthermore point to TIGIT as a promising target for future immunotherapies.
Contribution	**Anastasia Tsakmaklis**, Maria Vehreschild, Michael von Bergwelt-Baildon, Max Schlaak and Cornelia Mauch contributed to the conceptualization, methodology and fundraising of the study. **Anastasia Tsakmaklis** coordinated and Maria Vehreschild supervised the project. **Anastasia Tsakmaklis** and Rafael Zenner carried out the sample collection and processing of the samples and documented the clinical data for all patients in an eCRF. **Anastasia Tsakmaklis** and Fedja Farowski performed the DNA extraction, PCRs and 16S rRNA gene sequencing of the stool samples. Till Robin Lesker carried out the genome sequencing of the stool samples. Jonas Lehmann carried out the analyses of the blood samples. Fedja Farowski and Till Robin Lesker contributed to the validation and data curation. **Anastasia Tsakmaklis**, Fedja Farowski, Till Robin Lesker and Till Strowig analyzed and evaluated the microbiome data. Jonas Lehmann and Hans Schlösser carried out the data evaluation of the blood analyses. **Anastasia Tsakmaklis** and Fedja Farowski calculated the prediction model. **Anastasia Tsakmaklis**, Fedja Farowski and Jonas Lehmann visualized the results. **Anastasia Tsakmaklis** prepared the manuscript draft and all co-authors read, revised and approved it for publication.
Cooperation	<u>Co-operation partner 1</u>: Tumor Immunology Group (CMMC, University Hospital Cologne, Head PD Dr Schlößer) <u>Scope of co-operation 1</u>: The planning of the immune analysis (e.g. selection of antibodies) was carried out in cooperation with PD Dr Schlößer. Jonas Lehmann from the working group analyzed the blood samples (FACS and FluoroSpot) and visualized the results.

	Anastasia Tsakmaklis and Fedja Farowski received the analyzed data and used it for the prediction model. _____ Co-operation partner 2: Clinic and Polyclinic for Dermatology and Venereology (University Hospital Cologne, Head Prof Dr von Stebut-Borschitz) Scope of co-operation 2: Michael von Bergwelt-Baildon, Max Schlaak and Cornelia Mauch were involved in the design of the study and the application for funding. Potential study participants were screened and informed by physicians at the clinic (Viola Schweinsberg and Jana Knüver). _____ Cooperation partner 3: Working Group Microbial Immune Regulation of the Helmholtz Centre for Infection Research, Braunschweig (Head Prof. Dr. Till Strowig) Scope of cooperation 3: Till Robin Lesker from the AG performed the shotgun metagenome microbiome analysis under the supervision of Prof. Dr Till Strowig using extracted DNA sent to the Helmholtz Centre by **Anastasia Tsakmaklis**. The fastq files were then analyzed and visualized by **Anastasia Tsakmaklis** and Fedja Farowski and used for the prediction model. **Anastasia Tsakmaklis** previously carried out the DNA extraction from the collected stool samples and sent the extracted DNA to the Helmholtz Centre. **Anastasia Tsakmaklis** also carried out PCRs and library preparations for 16S rRNA gene sequencing, which was used to analyze the samples beforehand. The 16S sequencing data has not been published.

Further publications	
1	Farowski F, Els G, **Tsakmaklis** A, et al. Assessment of urinary 3-indoxyl sulfate as a marker for gut microbiota diversity and abundance of Clostridiales. *Gut Microbes*. 2019;10(2):133-141.
2	Rub AM, **Tsakmaklis** A, Grafe SK, Simon MC, Vehreschild MJ, Wuethrich I. Biomarkers of human gut microbiota diversity and dysbiosis. *Biomark Med*. 2021;15(2):137-148.
3	Dimitriou V, Biehl LM, Hamprecht A, et al. Controlling intestinal colonization of high-risk haematology patients with ESBL-producing Enterobacteriaceae: a randomized, placebo-controlled, multicentre, Phase II trial (CLEAR). *J Antimicrob Chemother*. 2019;74(7):2065-2074.
4	Hoelz H, Heetmeyer J, **Tsakmaklis** A, et al. Is Autologous Fecal Microbiota Transfer after Exclusive Enteral Nutrition in Pediatric Crohn's Disease Patients Rational and Feasible? Data from a Feasibility Test. *Nutrients*. 2023;15(7).
5	Stein-Thoeringer CK, Nichols KB, Lazrak A, et al. Lactose drives Enterococcus expansion to promote graft-versus-host disease. *Science*. 2019;366(6469):1143-1149.
6	Biehl LM, Farowski F, Hilpert C, et al. Longitudinal variability in the urinary microbiota of healthy premenopausal women and the relation to neighboring microbial communities: A pilot study. *PLoS One*. 2022;17(1):e0262095.
7	Farowski F, Solbach P, **Tsakmaklis** A, et al. Potential biomarkers to predict outcome of faecal microbiota transfer for recurrent Clostridioides difficile infection. *Dig Liver Dis*. 2019;51(7):944-951.
8	Svacina MKR, Sprenger-Svacina A, **Tsakmaklis** A, et al. The gut microbiome in intravenous immunoglobulin-treated chronic inflammatory demyelinating polyneuropathy. *Eur J Neurol.* 2023;30(11):3551-3556.
9	Spiertz A, **Tsakmaklis** A, Farowski F, et al. Torque teno virus-DNA load as individual cytomegalovirus risk assessment parameter upon allogeneic hematopoietic stem cell transplantation. *Eur J Haematol.* 2023;111(6):963-969.

ERKLÄRUNG

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Übersicht der Publikationen:

(1) **Tsakmaklis A**, Vehreschild M, Farowski F, et al. Changes in the cervical microbiota of cervical cancer patients after primary radio-chemotherapy. Int J Gynecol Cancer. 2020;30(9):1326-1330.

(2) **Tsakmaklis A**, Farowski F, Zenner R, et al. TIGIT+ NK cells in combination with specific gut microbiota features predict response to checkpoint inhibitor therapy in melanoma patients. BMC Cancer. 2023;23:1160.

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