

## Role of the microbiome in non-gastrointestinal cancers

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### Abstract

"The forgotten organ", the human microbiome, comprises

a community of microorganisms that colonizes various sites of the human body. Through coevolution of bacteria, archaea and fungi with the human host over thousands of years, a complex host-microbiome relationship emerged in which many functions, including metabolism and immune responses, became codependent. This coupling becomes evident when disruption in the microbiome composition, termed dysbiosis, is mirrored by the development of pathologies in the host. Among the most serious consequences of dysbiosis, is the development of cancer. As many as 20% of total cancers worldwide are caused by a microbial agent. To date, a vast majority of microbiome-cancer studies focus solely on the microbiome of the large intestine and the development of gastrointestinal cancers. Here, we will review the available evidence implicating microbiome involvement in the development and progression of non-gastrointestinal cancers, while distinguishing between viral and bacterial drivers of cancer, as well as "local" and "systemic", "cancer-stimulating" and "cancer-suppressing" effects of the microbiome. Developing a system-wide approach to cancer-microbiome studies will be crucial in understanding how microbiome influences carcinogenesis, and may enable to employ microbiome-targeting approaches as part of cancer treatment.

**Key words:** Microbiome; Non-gastrointestinal cancers; Carcinogenesis; Dysbiosis; Microbial agent

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**Core tip:** "The forgotten organ", the human microbiome, comprises a community of microorganisms that colonizes various sites of the human body. A complex host-microbiome relationship has emerged in which many functions became codependent. This coupling becomes evident when disruption in the microbiome composition, termed dysbiosis, is mirrored by the development of pathologies in the host. Among the most serious consequences of dysbiosis, is the development of cancer. As many as 20% of total cancers worldwide are caused by a

microbial agent. Here, we will review the available evidences implicating microbiome involvement in the development and progression of non-gastrointestinal cancers. Developing a system-wide approach to cancer-microbiome studies will be crucial in understanding how microbiome influences carcinogenesis, and may enable to employ microbiome-targeting approaches as part of cancer treatment.

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## INTRODUCTION

Bacteria, viruses, archaea and fungi coevolved with the human body for thousands of years. This resulted in diverse and extensive host-microbiome interactions, which influence multiple host physiological processes, including metabolism and the function of the immune system<sup>[1]</sup>. Disruption of the microbial community, termed dysbiosis, is suggested to constitute a major risk factor for an increasing array of diseases including metabolic syndrome and immune disorders as well as several forms of cancer.

Carcinogenesis is a process inflicted and influenced by many mechanisms. However, up to 20% of the cancers worldwide are believed to be caused or modulated by a microbial agent<sup>[2,3]</sup>. Of the various involved microorganisms, viruses are best studied for their role in carcinogenesis. Therefore, multiple mechanisms through which viruses promote development of tumors have been deciphered. The roles of archaea and fungal members of microbiome in cancer formation are much less studied, while only recently studies emerged focusing on bacterial involvement in cancer formation and progression.

This review will provide some conceptual examples of how different organ-specific microbiomes may modulate the carcinogenic processes through involvement of specific members or, alternatively, through changes observed in the microbial community as a whole (summarized in Table 1). An early example of an individual bacterial member that contributes to carcinogenesis is *Helicobacter pylori* (*H. pylori*). *H. pylori* colonizes the gastric mucosa in 50% of humans and causes cancer in 1%-3% of colonized individuals. It thus is recognized by the International Agency for Research on Cancer as a bone-fide carcinogen<sup>[4]</sup>. However, further experiments in germ-free mice showed that infection by *H. pylori* alone was not sufficient to promote neoplastic transformation. Mice mono-associated with the bacteria developed gastritis and subsequent neoplasia at a much slower rate than their fully colonized counterparts, suggests that *H. pylori* may require cooperation by other commensal microbiota members. In other cases an entire dysbiotic microbiome

community was suggested to drive tumor development. One such example is colorectal cancer (CRC) that is transmissible by dysbiotic microbiota<sup>[5,6]</sup>. As such, germ-free mice are partially protected from disease, and treatment with broad-spectrum antibiotics ameliorates cancer development. Identifying the pathogenic bacterial “drivers” of cancer in these cases and differentiating them from secondary microbial alterations remains a major challenge to the field.

Most studies have focused on the effect of the microbiota on gastrointestinal cancers and these are reviewed in detail elsewhere<sup>[7-11]</sup>. In this review, we will discuss research into the carcinogenic properties of microbial agents in the non-gastrointestinal organs. We review these associations per body-site, and highlight the substantial effect microbial involvement may have on all stages of cancer development in the skin, breast, urogenital tract, lung, liver and pancreas.

## SKIN

The human skin is the largest organ in the body and hosts a complex and heterogeneous microbiota. Until recently, the studies of the skin microbiota focused on bacteria, using culture-based assays. However, it has recently been appreciated that the skin is inhabited by a massive, unculturable bacterial ecosystem, as well as by fungi and viruses<sup>[12-14]</sup>. Together, these comprise the skin microbiome, which may have diverse effects on a multitude of skin-specific physiological and pathophysiological processes, including ones that promote the development of skin cancer.

### *Viral involvement in skin cancer*

The skin virome has rarely been investigated, in part because most skin-associated viruses are not culturable and do not display consensus sequences that can be used for high throughput next generation sequencing techniques<sup>[15]</sup>. Several viruses are known to inhabit the healthy skin, but can also induce malignant transformation.

**The papilloma virus family:** Papilloma viruses (PVs) infect undifferentiated keratinocytes in the basal layer of the stratified squamous epithelia, and in the cutaneous and the mucosal levels. Oncogenic PVs, including the human papilloma virus (HPV-16), are responsible for nearly all cases of cervical and anal cancer<sup>[16]</sup>. PVs are commonly part of the skin and mucosal microbiota of healthy individuals, suggesting commensalism or mutualism between PVs and their host cells<sup>[17]</sup>. Moreover, the majority of HPV infections are subclinical and do not cause any physical lesions<sup>[17]</sup>. However, in some cases chronic inoculation is established through immune escape mechanisms, and a low yet persistent amount of virions is produced. The oncogenic features that allow the virus to induce cell transformation are dependent on the virus’s E5, E6 and E7 oncogenes, which are exclusively present in oncogenic PVs. The E6 protein in

**Table 1** The role of the microbiota in non-gastric cancers

Cancer	Mechanism	Ref.
Protective role		
B16/F10 melanoma and LLC	Microbiota was required for the development of anti-cancer immunity	[87]
HCC	Commensal microbiota was essential for the development and anti-cancer activity of $\gamma\delta$ -Th17 cells Microbiota was required for immune system development Commensal microbiota was needed for the development of the immune system in the liver, which enables mice to clear HBV. A chronic infection with HBV is a major risk factor for HCC	[111]
Tumor-promoting role		
Skin cancer	Dysbiosis causes a cancer-stimulating inflammatory response in the host Microbiota-derived Flagellin stimulates TLR5-MyD88 signaling which promotes skin cancer development	[27]
Breast cancer	Upon injection of a carcinogen, GF mice showed a lower cancer burden than SPF mice	[43]
Lung	Dysbiosis causes a cancer-stimulating inflammatory response in the host <i>E. coli</i> /LPS in the lungs promotes lung injury and inflammation, which lead to an enhanced metastasis from the primary tumor to the lung	[102]
Ovarian and breast cancer	Dysbiosis inhibits anti-tumor immunity: Gut microbiota of TLR5 <sup>-/-</sup> mice promoted the accumulation of MDSCs at the site of breast and ovarian cancers. MDSCs in their turn suppressed anti-cancer immunity	[44]
Breast cancer	Infection with a gastric pathogen promoted cancer-stimulating inflammatory responses In mice, infection with the gastric bacteria <i>H. hepaticus</i> led to an influx of neutrophils in the mammary gland that then promoted cancer. Treatment with antibiotics or the depletion of neutrophils significantly halted cancer development	[30]
Liver	Infection of mice prone to liver cancer with <i>H. hepaticus</i> led to a significant enhancement of carcinogenesis	[114]

LLC: Lewis lung carcinoma; HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; TLR: Toll-like receptor; MDSCs: Myeloid-derived suppressor cells.

oncogenic PVs is able to induce degradation of the p53 cellular protein, thus promoting uncontrolled cell growth. The E5 protein allows for evasion of the host's immune surveillance and decreases the dependence of infected cells on growth factors. Finally, the E7 oncoprotein binds to the tumorsuppressive pRb, dissociating the transcription factor E2F from the pRb/E2F complex. The process induced by these three oncoproteins is slow, with progression from precursor lesions to invasive cancer usually requiring more than a decade<sup>[16]</sup>.

More recently, another HPV member was found to be associated with an unusual form of skin cancer called Merkel cell carcinoma (MCC)<sup>[18]</sup>, concisely reviewed in<sup>[19]</sup>. The Merkel cell polyomavirus (MCPyV) causes a rare but aggressive form of skin cancer and is present in about 80% of MCC tumor specimens. The MCPyV genome was shown to integrate into the cellular DNA of some MCC tumors and their metastases. A majority of MCC tumors also display constitutive expression of the MCPyV large T-antigen oncoprotein<sup>[20]</sup>. This suggests involvement of MCPyV in the oncogenesis of MCC.

### Bacterial involvement in skin cancer

The skin microbiome contains an entrenched bacterial population, forming a microbiome that features a high spatial and temporal stability<sup>[21-24]</sup>. The skin contains different skin microenvironments, defined by sebaceous, moist and dry areas and by the different follicle densities<sup>[25]</sup>. The cutaneous microbiome consists predominantly of 4 bacterial phyla; actinobacteria, firmicutes, proteobacteria and bacteroidetes and six genera, propionibacterium, corynebacterium, staphylococcus, streptococcus and acinetobacter<sup>[23,26]</sup>.

Several studies link commensal skin bacteria to malignant transformation. In one example, antibiotic-

treated mice showed an increased susceptibility to B16/F10 melanoma, as well as lewis lung carcinoma, and exhibited a shortened mean survival time, suggesting a protective role of the skin microbiome in cancer development in these models. In contrast, another experimental setting suggested that an intact commensal bacterial population, and specifically flagellated bacteria, may be required for malignant transformation in the murine skin<sup>[27]</sup>. In this context, toll-like receptor (TLR) 5 and its ligand flagellin linked between chronic inflammation, tissue damage and skin cancer. In the model described, bone-marrow chimeras lacking MyD88 and TLR5 in the hematopoietic cells exhibited protection against a chemical model of wound-induced tumor formation. When mice were treated with a broad-spectrum antibiotic regimen, the skin bacterial load was decreased and wound-induced tumor formation and tumor size were substantially reduced. Topical application of flagellin onto wounds increased tumor incidence in a dose-dependent manner and delayed wound closure. This indicates that MyD88 and TLR-5 signaling on radiosensitive leukocytes is required for tumor formation. Together, these examples suggest that the skin bacterial microbiome can play either a protective or a harmful role in cancerogenesis, depending on the physiological context and microbial composition.

### BREAST

Breast cancer is the second leading cause of cancer-related deaths in women: One in eight women develop the malignancy in their lifetime<sup>[28]</sup>. Despite considerable and significant progress has been achieved in breast cancer research, in most cases it's etiology remains unknown<sup>[29]</sup>. Mammary glands are colonized by a distinct microbiota<sup>[30,31]</sup>, but the role of microbial involvement in

breast cancer remains at its infancy.

### **Viral involvement in breast cancer**

Until recently, most studies looking into microbial modulation of breast cancer have been focused on specific viruses. The results, however, remain inconclusive. While HPV infection has been reported by some groups are associated with breast cancer development<sup>[32-34]</sup>, others have failed to find such correlation<sup>[35,36]</sup>. Some groups have reported that up to 50% of breast tumors to be EBV-positive<sup>[37-40]</sup>, while others have been unable to detect the virus in breast tumors altogether<sup>[41,42]</sup>. Therefore, additional studies are needed to clarify the potential contribution of viral infections in breast carcinogenesis, and its modulatory mechanisms of activity.

### **Bacterial involvement in breast cancer**

In parallel to viral infections, a number of studies suggest a link between bacterial infections and breast cancer. Involvement of the commensal microbiome was first suggested in a study in which injections of a carcinogen (DMAB) in various body sites of germ free rats resulted in a significantly lower cancer burden in the breast tissue and colon, but not in the skin, as compared to conventionalized rats<sup>[43]</sup>. Of note, this study did not delineate whether the observed effects were linked to the local breast microbiome, or to distal microbial communities such as that of the gut. More recent studies have sought to clarify this issue. Xuan *et al.*<sup>[31]</sup> surveyed the microbiota in tumors or normal adjacent tissues from 20 estrogen receptor (ER)-positive breast cancer patients as well as in tissue from healthy donors. This study indicated that there is a 10-fold decrease in the absolute numbers of bacteria between cancer and control tissues. Moreover, the authors observed changes in the compositional abundance of bacterial species in tumor compared to control tissues. While the genus *Sphingomonas* was found to be more abundant in normal tissues, the tumor tissue hosted *Sphingomonas yanoikuyae* in increased numbers. Other members of the skin microbiome, such *Staphylococcus* and *Corynebacterium*, did not vary significantly between normal and tumor tissues. Nonetheless, these data suggest that mammary tumors bear a different microbial composition than the normal tissue. Significant microbial-associated effects on tumor progression are supported by a recent study, which showed an accelerated mammary malignant progression in TLR5-responsive mice. In this model, malignant progression of mammary tissue in p53-ablated and oncogenic K-ras-activated mice was measured on the background of TLR5 deficient mice<sup>[44]</sup>. Absence of TLR5 signaling in these mice resulted in a divergent microbial composition and reduced tumor progression. In TLR5 proficient mice, on the other hand, microbial signaling through TLR5 increased IL-6 secretion and the number of gamma delta T cells as well as tumor growth. Thus, the commensal microbiome was suggested to be able to induce tumor-promoting

inflammation in a TLR5 dependent manner.

While the above studies focus on the whole microbiome composition and not on specific microbial "drivers" or "modulators" of cancer, a study by Lakritz *et al.*<sup>[30]</sup> implicated a specific bacterium, *Helicobacter hepaticus* (*H. hepaticus*), in the progression of mammary malignancy. In this report, mice with a predisposition for breast cancer were infected with *H. hepaticus*. Compared to non-infected controls, infected mice showed increased mammary tumor burden characterized by extensive neutrophil infiltration. Depletion of neutrophils entirely inhibited tumor development<sup>[30]</sup>. Together, these data suggest that both the whole microbiome composition as well as specific bacteria can contribute to breast tumor progression by promoting inflammation, and that they can do so *via* multiple pathways.

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## **UROGENITAL TRACT**

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Urogenital cancers include cervical, renal, bladder and ovarian carcinomas. Very few studies focusing on the roles of the microbiota in urogenital tract tumors have been published to date<sup>[45]</sup>. Nonetheless, there is some emerging evidence towards the possibility that chronic viral infections may promote the development of renal cell carcinoma and bladder cancer.

### **Cervical cancer**

The most frequently occurring and the best studied of cancers of the female urogenital tract is cervical cancer. The most highly associated risk factor for cervical cancer is viral infection by the HPV family. Mucosal HPV serotypes infect the basal epithelial cells of the anogenital mucosa *via* micro-abrasions in the epithelial lining<sup>[46]</sup>. Vulval, vaginal, penile, cervical, and anorectal areas are affected. Cervical and anal squamous cell carcinoma develop at sites of squamous metaplasia; cervicovaginal and anorectal squamous columnar junctions are therefore especially vulnerable to HPV infection leading to malignant transformation<sup>[47]</sup>. Although data are limited, antibodies developed during natural infection do not seem to offer full protection against reinfection, possibly because of low or waning titers of the virus<sup>[48,49]</sup>. By contrast, the available prophylactic HPV vaccines induce high concentrations of neutralizing antibodies - at least two-log scale higher as compared to natural infection-induced concentrations, leading to a better immune memory<sup>[50,51]</sup>. The efficacy of the anti-HPV vaccine in cervical intraepithelial neoplasia associated with HPV 16 and 18 in women naive for infection is high: 93% (95%CI: 79.9-98.3) for the bivalent vaccine (HPV 16/18) after 35 mo of follow-up and 98% (95%CI: 93.3-99.8) for the quadrivalent vaccine (HPV 6/11/16/18) after 42 mo of follow-up<sup>[52-54]</sup>.

### **Vaginal cancer**

The vagina harbors a unique microbiota that serves as an important line of defense against pathogens, including sexually transmitted infections<sup>[55]</sup>. The dominant

members of the vaginal microbiome *Lactobacillus spp.* were shown to provide broad-spectrum protection from pathogens through their production of lactic acid<sup>[56]</sup>, bacteriocins (bactericidal proteinaceous molecules)<sup>[57]</sup>, antagonistic bacteriocin-like substances<sup>[58]</sup>, and biosurfactants<sup>[59]</sup>, that can adhere to mucus, a component of the barriers against pathogens<sup>[60]</sup> and disrupt biofilms<sup>[61]</sup>. Disruption of the protective microbiota configuration, termed bacterial vaginosis (BV) was shown in numerous studies to correlate with cervical cancer-inducing HPV infections<sup>[62-69]</sup>. BV affects one in three United States women<sup>[70]</sup> and is characterized by decrease in protective *Lactobacillus spp.*, increased specie richness, and elevated numbers of anaerobic bacteria, including species of *Gardnerella*, *Prevotella*, and *Clostridiales*<sup>[71]</sup>.

While numerous association studies showed a strong association between dysbiotic disruption of vaginal microbiota (BV) and HPV infections, the mechanistic link between the two events is yet to be explored.

### Ovarian cancer

Chronic inflammation was previously suggested to be involved with the pathogenesis of Ovarian carcinogenesis<sup>[72]</sup>, yet this evidence remains sparse. Specific pathogens suggested to be indirectly associated with human ovarian cancer include Chlamydial HSP60-1 IgG and *M. genitalium*, with IgG antibodies specific to these bacteria suggested to be associated with epithelial ovarian tumors in some patient subsets<sup>[73]</sup>. Possible involvement of impaired host-microbiome interactions in ovarian cancer was suggested from a study utilizing TLR-5 deficient mice that feature a dysbiotic microbiome configuration. These mice showed increased survival rates compare to WT controls when injected with syngenic ID8 ovarian tumor cells<sup>[44]</sup>. In addition, ovarian tumors of patients who were heterozygous for the dominant TLR5<sup>R392X</sup> polymorphism showed negligible induction of IL-8 transcript levels but significantly higher IL-17A transcript levels in response to flagellin as compared to control population. Furthermore, the proportion of long-term survivors was significantly higher among TLR5<sup>R392X</sup> carriers, all suggesting that host TLR5 microbe interactions may play a role in ovarian tumor pathogenesis<sup>[44]</sup>.

### Bladder cancer

Until recently, the healthy urinary tract was considered sterile and bacterial presence in the urine of patients identified *via* culture-based methods was considered a sign of a urinary tract infection<sup>[45,74]</sup>. In recent years, however, the emergence of next generation sequencing of the microbiome has established the presence of a urinal microbiome in the healthy humans urinary tract<sup>[75-85]</sup>. Among them, several works describe the presence of a complex bacterial community with the predominant genera *Lactobacillus*, *Prevotella* and *Gardnerella*, with a considerable variation featured between individuals<sup>[79,83]</sup>.

Bladder cancer is the most prevalent malignancy of the urinary system. In 2015, it is estimated that 75000

new cases will be diagnosed, and more than 15000 patients will die due from Bladder cancer in the United States<sup>[86]</sup>. The most important risk factors known for urothelial carcinoma are cigarette smoking and various occupational exposures. The nematode, *Schistosoma haematobium* infection was also associated with the development of squamous cell carcinoma of bladder due to chronic inflammation. Regarding microbiome involvement in urothelial carcinoma, a study comparing the microbiome of urine specimens from healthy individuals and urothelial carcinoma revealed that *Streptococcus* was nearly undetected in normal samples but significantly elevated in 5 out of the 8 cancer samples. *Pseudomonas* or *Anaerococcus* were the most abundant genus in 2 out of the 3 cancer samples where *Streptococcus* abundance was low<sup>[87]</sup>. While descriptive in nature, this study suggests that urothelial carcinoma may be associated with altered microbiota of the urinary tract. More studies are needed to establish whether microbiome composition plays a causative role in bladder cancer.

### Renal cancer

The role of the healthy microbiome on kidney cancer has not been studied to date. However, a number of studies suggested an association between viral infections and risk of renal cancer, yet these remain controversial and at times contradictory to each other<sup>[72,88,89]</sup>. One virus that has been implicated in RCC pathology is HPV. One study reported that 7 out of 49 RCC samples to be HPV positive<sup>[90]</sup>. A second study, including histology samples of 122 patients, found 30.3% of RCC tumor tissues to be HPV positive. Of these, 45% were positive for high-risk (HR)-HPVs such as HPV-16 and HPV-18. Moreover, HR-HPV infection correlated with the expression of p16INK4a, a viral immunosuppressant. The authors hypothesized that HR-HPV infection may precede RCC and promote oncogenesis<sup>[91]</sup>. However, more research is required to test this hypothesis.

In contrast, Newcastle disease virus has been suggested in *in vitro* studies to play a therapeutic role in RCC. This virus preferentially infects cancer cells and, upon infection induces apoptosis *via* the p38 MAPK/NF- $\kappa$ B/I $\kappa$ B $\alpha$  pathway. Similar outcomes were obtained in other cancer types<sup>[92]</sup>. Future studies are needed to translate these *in vitro* findings to the clinical context.

## LUNG

The human microbiota is the body's first interface with environmental exposures. In this sense, the lung microbiota may play an important role in the body's response to airborne carcinogens. The mechanisms of lung carcinogenesis are still not fully understood. The current most important risk factor for lung cancer is smoking. In non-smokers, suggested risk factors include environmental tobacco smoke, exposure to radon gas, cooking oil vapors, indoor coal and wood burning, asbestos, genetic factors, parasitic infections as well as viral and bacterial agents<sup>[93]</sup> that will be described below.

### **Viral involvement in lung cancer**

As in skin carcinoma, HPV has been associated with lung cell malignancies<sup>[94]</sup>. Studies suggest a link between HPV infection and lung cancer in non-smoking patients; it has been shown that epithelial changes in bronchial carcinoma closely resembled HPV-induced genital lesions<sup>[95]</sup>. Similarly to the mechanisms by which HPV contributes to skin cancer susceptibility, it is suggested that the molecular mechanism of transformation by HPV is mediated by its oncoproteins E5, E6 and E7. In addition, *in vivo* data show HPV integration, E6/E7 expression and down regulation of p53 in lung cancers, further supporting this classical oncogenic mechanism<sup>[96]</sup>.

### **Bacterial involvement in lung cancer**

*C. pneumoniae* is a gram-negative obligatory intracellular bacterium and a common cause of pneumonia<sup>[69]</sup>. *C. pneumoniae* can also cause other conditions, such as sinusitis, bronchitis, rhinitis and worsening of chronic obstructive pulmonary disease (COPD). However, infection can also be asymptomatic. The involvement of *C. pneumoniae* infection in lung cancer development and risk has been suggested by several studies<sup>[97-99]</sup>. However, the mechanisms for this association remain unclear<sup>[100,101]</sup>. Pulmonary infections with gram-negative bacteria have also been suggested to contribute to lung metastasis. Acute lung infection models induced by either infection with *E. coli* or administration of LPS increased cancer cell homing to the lung and enhanced lung metastasis<sup>[102]</sup>. Moreover, the broncho-alveolar lavage fluid from LPS or *E. coli*-injected mice induced the migration of transformed cells *in vivo*. The tumor cells migratory activity was blocked by AMD3100, a chemokine receptor-4 inhibitor, as well as by amoxicillin, an antibacterial agent. In addition, tracking of the metastatic tumor cell line in the mouse showed that bacteria injection enhanced early localization of the tumor cells to the lung.

The bacterium *C. pneumoniae* is a gram-negative obligatory intracellular bacterium and a common cause of pneumonia<sup>[101]</sup>. In addition to pneumonia, *C. pneumoniae* can cause other conditions, such as sinusitis, bronchitis, rhinitis, exacerbation of COPD. However, infection can also be asymptomatic. The association of *C. pneumoniae* infection with lung cancer risk has been suggested by multiple studies<sup>[97-99]</sup>, although the mechanisms for this association remain unclear<sup>[100,103]</sup>.

Pulmonary infections with gram-negative bacteria have also been suggested to contribute to lung metastasis. Acute lung infection models induced by either infection with *E. coli* or administration of LPS increased cancer cell homing to the lung and enhanced lung metastasis<sup>[102]</sup>. Moreover, the broncho-alveolar lavage fluid from LPS or *E. coli*-injected mice stimulated migration of tumor cells *in vivo*. The tumor cells migratory activity could be blocked by AMD3100, a chemokine receptor-4 inhibitor, as well as by the antibacterial agent amoxicillin. In addition, *in vivo* tracking of the metastatic

tumor cell line showed that bacterial injection enhanced early dissemination of the tumor cells to the lung.

In contrast, antibiotics-treated mice were shown to be more susceptible to tumor development in the lungs after inoculation with B16/F10 melanoma or a lung carcinoma cell-line. In this model, commensal bacteria were found to be essential for the function of  $\gamma\delta$ -Th17 cells in the lung, and the absence of these cells increased the susceptibility to lung carcinoma and B16/F10 melanoma development<sup>[87]</sup>. This indicates, that the antitumor defense of the host through  $\gamma\delta$ -Th17 cells is dependent on an intact microbiome composition. Therewith, lung cancer is an excellent example of the healthy microbiome playing a protective role in tumorigenesis, whereas when dysbiosis develops, pathogenic or pathobiont bacteria may promote, in certain contexts, cancer development.

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## **LIVER**

Primary liver cancer is the fifth most diagnosed form of cancer in males, and the second most frequent cause of cancer death worldwide. Seventy percent to ninety percent of the primary liver cancer cases can be classified as hepatocellular carcinoma (HCC)<sup>[104,105]</sup>. With mortality to incidence ratio of 0.95, the prognosis for patients with HCC is extremely poor<sup>[105]</sup>. In developed countries, chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections account for approximately 43% of cases. However, the majority of patients develop HCC secondary to alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD)<sup>[104]</sup>. It has recently been suggested that the microbiota plays an important role in HCC development. Although the liver, under normal conditions, is considered sterile, its environment is greatly influenced by the nutrients, metabolites and also toxins and pathogens derived from the gut *via* the portal vein. Therefore, the composition in the gut microbiota can greatly influence the functioning of the liver, by its myriad metabolic activities regulating the gut liver axis. Indeed, recent studies have suggested that the composition of the gut microbiota can both influence the development of diseases predisposing to HCC such as chronic HBV and HCV infections, ALD and NAFLD, and the transition from these diseases into HCC<sup>[106,107]</sup>, yet the mechanisms driving these effects remain elusive.

### **Viral contribution to HCC**

The majority of HCC cases occur in patients previously suffering of chronic hepatotropic viral infection, mainly HBV and HCV<sup>[108]</sup>. A unique feature of HBV infection is that while 95% of adults are able to spontaneously clear the virus, over 90% of neonates and approximately 30% of children aged 1-5 develop persistent infection<sup>[109,110]</sup>. Possible involvement of the microbiota in this phenomenon was suggested from a study in which mice treated with oral antibiotics for 6 wk prior to HBV infection were no longer able to rapidly clear the virus<sup>[111]</sup>. Further experiments indicate that the microbiota in

young mice may induce HBV tolerance in the liver *via* LPS-TLR4 mediated secretion of IL-10 by Kupffer cells (KC), whereas the mature microbiota shifts this balance towards clearance of the virus by stimulating KC-dependent lymphoid organization and tissue priming in the liver<sup>[111,112]</sup>. Future studies are needed to further validate this interesting association, and to determine possible roles of the gut microbiota in the clearance of other hepatotropic viruses such as HCV.

Both HBV and HCV infections contribute to the development of HCC by promoting a pro-inflammatory liver micro-environment, affecting cell cycle regulation and inducing ER stress, as has been extensively reviewed elsewhere<sup>[113]</sup>. However, a study by Fox *et al.*<sup>[114]</sup> indicated that colonization with *H. hepaticus* in the gut was sufficient to promote HCC in HCV-transgenic mice, in the absence of either translocation of *H. hepaticus* to the liver or overt hepatitis. From its niche in the intestinal mucosa, *H. hepaticus* activated NF- $\kappa$ B dependent networks associated with innate and T-helper 1 (Th-1)-type adaptive immunity, both in the intestines and in the liver. The resultant transcriptional changes promoted the development of pre-neoplastic and neoplastic liver foci in mice bearing an HCV transgene, while neither factor by itself was sufficient to induce tumor formation<sup>[114]</sup>. This demonstrates that *H. hepaticus* can alter hepatic immune regulation from its intestinal niche, in a manner that synergizes with viral tumorigenic factors. More research is warranted to uncover whether other changes in the intestinal microbiota can induce similar effect.

### **Bacterial contribution to HCC**

Chronic alcoholic consumption is considered a major risk factor for chronic liver disease and HCC. Already in 1991 it was noted that patients with alcoholic cirrhosis displayed far higher levels of serum endotoxin than those with non-alcoholic cirrhosis, suggesting that alcoholic cirrhosis is associated with impaired intestinal barrier function<sup>[115]</sup>. Likewise, treatment of rats with antibiotics targeting Gram-negative bacteria drastically decreased serum endotoxin levels and liver injury in ethanol-fed rats<sup>[116]</sup>. These suggested that some of the features of chronic alcohol toxicity may be mediated by gut-associated pathogen-induced molecular patterns released by the gut microbiota. These findings were further confirmed, with the finding that germ-free mice, which are devoid of a microbiota, are protected from ethanol-induced liver disease<sup>[117]</sup>. Moreover, transplanting microbiota from alcohol-fed mice into naive germ-free mice was sufficient to induce liver injury and inflammation. Furthermore, excessive alcohol intake lead to dysbiosis by an overgrowth of Gram-negative bacteria, that caused increased gut permeability. As leaky gut leads to increased availability of bacterial metabolites to the liver, as well as pro-inflammatory molecules such as bacterial toxins, LPS and even living microbes, this may explain how alcohol-induced dysbiosis could lead to ALD. Indeed, feeding mice a high-fiber diet partially prevented alcohol-induced dysbiosis, decreased gut

permeability and mitigated the damage to the liver<sup>[117]</sup>. This model is supported by preliminary data from patients with alcoholic cirrhosis that show an increase in Gram-negative bacteria as well as an increased bacterial translocation to the liver<sup>[118]</sup>, suggesting that microbiota-targeting interventions may potentially mitigate alcohol-induced liver damage.

Alcohol-induced liver cirrhosis is characterized by cellular injury, inflammation, and fibrosis coupled with compensatory cell growth and proliferation, conditions promoting tumor development<sup>[119]</sup>. Furthermore, ethanol can induce epigenetic changes in hepatocytes that lead to tumor formation<sup>[120]</sup>. There is evidence that ethanol-mediated TLR4 signaling is crucial in the dedifferentiation of hepatocytes seen in HBV/HCV- and ALD-associated HCC. Ethanol-induced hepatic translocation of LPS and gram-negative bacteria may further synergize with these direct effects in activating the innate immune response. In agreement, diethylnitrosamine-induced liver cirrhosis was accompanied by dysbiosis. When treated with probiotics, a reduction in gut permeability and intestinal inflammation was observed together with a reduced incidence of cirrhosis and HCC in this model<sup>[121]</sup>. Together, these studies suggest that ALD-associated dysbiosis may contribute to HCC susceptibility.

NAFLD, a component of the "metabolic syndrome", is rapidly becoming a common cause of chronic liver disease in both developed and developing countries<sup>[122]</sup>. While most patients with NAFLD feature isolated liver steatosis, in approximately 20% of cases NAFLD evolves into non-alcoholic steatohepatitis (NASH), a progressive liver disease involving a combination of steatosis, hepatocellular damage, inflammation and fibrosis. NASH has the potential to develop into cirrhosis, which is a major risk factor for HCC as described above. In addition, there has been also a rising incidence of NAFLD-associated HCC in the absence of cirrhosis<sup>[122,123]</sup>. It is unclear to what extent the pathophysiology for NAFLD-associated HCC in the absence of cirrhosis differs from that in a cirrhotic liver. The microbiota has been described to contribute to this *via* two distinct pathways, which may play a role in either situation.

Obesity has been associated with dysbiosis and increased gut permeability, allowing for more LPS to translocate into the liver, where it can trigger TLR signaling resulting in NF- $\kappa$ B-dependent transcription of TNF- $\alpha$ , which drives NAFLD and NASH progression<sup>[106,124]</sup>. This state is further aggravated as leptin-mediated up-regulation of CD14 leads to hypersensitivity to LPS-signals in obese patients<sup>[125]</sup>. Furthermore, microbiota-induced TLR9 signaling and dysbiosis-induced repression of inflammasome signaling can further promote the development of NAFLD and NASH<sup>[106]</sup>. In this context, increased activation of Kupffer cells *via* TLR4 further exacerbates steatohepatitis<sup>[126]</sup> and promotes the activation of hepatic stellate cells (HSCs). Activated HSCs, in turn, can contribute to liver fibrosis as well as secrete EGFR, which leads to increased proliferation of HSCs and may promote tumor formation<sup>[127]</sup>. Nonetheless, a study

by Dapito *et al.*<sup>[128]</sup> suggested that the gut microbiota may not be required for HCC initiation. Instead, it plays a major role in the progression of the disease, as TLR4 signaling was able to increase the expression of the hepatomitogen epiregulin as well as to promote proliferation and prevent apoptosis. Both sterilization of the gut during late stages of HCC as well as using a TLR4<sup>-/-</sup> model greatly reduced the progression of HCC, suggesting new avenues of treatment<sup>[128]</sup>.

Whereas mainly gram-negative bacterial LPS drives the pathways above, a second and independent process has been suggested to primarily depend on Gram-positive bacteria. Yoshimoto *et al.*<sup>[129]</sup> showed that an HFD-induced overgrowth of Gram-positive bacteria with the ability to produce the secondary bile acid deoxycholic acid (DCA) *via* 7 $\alpha$ -dehydroxylation of primary bile acid lead to a marked rise in serum DCA levels<sup>[129]</sup>. DCA is known to cause DNA damage through the production of reactive oxygen species, as well as to promote liver carcinogenesis<sup>[130,131]</sup>. Furthermore, DCA can induce a state of senescence accompanied by the secretion of specific chemokines, called the senescence-associated secretory phenotype (SASP)<sup>[132,133]</sup>. Indeed, the authors showed DCA is able to induce SASP in HSCs *in vivo*. This phenotype then promoted HCC development in mice treated with a chemical carcinogen. By blocking DCA production or treating mice with vancomycin, an antibiotic preferentially targeting Gram-positive bacteria, the induction of SASP and the progression of HCC could almost completely be blocked. When antibiotic treatment was supplemented with DCA, the beneficial effect was lost. It should be noted, however, that treating lean mice with a carcinogen and DCA was not sufficient to enhance HCC development. This suggests that additional, obesity-associated tumor-promoting factors may be required<sup>[129]</sup>. Nonetheless, some preclinical studies have showed that probiotic treatment can substantially alter bile acid levels by increasing fecal secretion and enhancing hepatic bile synthesis<sup>[134]</sup>. In humans, who unlike rodents cannot revert DCA into cholic acid, DCA can accumulate until it represents > 50% of the total bile pool<sup>[135]</sup>. Enhanced secretion of bile acids accompanied by hepatic bile synthesis might be a clean way to lower DCA levels, which in turn may substantially decrease the progression of HCC.

Increasing evidence suggests that the microbial composition plays a crucial role mediating liver damage in response to hepatitis infections, excessive alcohol intake or obesity. As HCC rarely occurs without previous liver disease, modulating the microbiota to prevent primary damage would be a potentially effective method of HCC prevention. However, even after liver disease has developed, the microbiota plays an important role in its progression and in creating a tumorigenic environment, through bacterial signaling *via* toxins, LPS and metabolites. Although the bile acid-driven pathophysiology seems specific to NAFLD-associated HCC, the LPS-TLR4 pathway appears common to all cirrhosis-associated HCC entities. While human microbiome-HCC

clinical correlations remain preliminary, they represent a potential new avenue for HCC prevention and treatment, which merits further studies.

## PANCREAS

Pancreatic cancer is associated with a poor outcome due to its rapid dissemination through the lymphatic system. This aggressive biology combined with a lack of biomarkers for early detection and resistance to conventional therapy results in a 5-year survival rate of only 5%<sup>[136]</sup>.

Suggestions for possible microbial involvement in pancreatic cancer comes from studies that found an epidemiological association between periodontitis and tooth loss, and the risk for pancreatic cancer<sup>[137,138]</sup>. However, a study by Stolzenberg-Solomon *et al.*<sup>[139]</sup> trying to correlate this with a specific bacterium known to play a role in tumor formation, *Helicobacter pylori*, was unable to validate the association. Unlike Stolzenberg, Farrell *et al.*<sup>[140]</sup> decided to study the entire composition of the oral microbiome in relation to pancreatic cancer. In this work, researchers identified a total of 56 clusters of bacterial species to be changed significantly between patients with pancreatic cancer and healthy controls. A combination of two bacteria, *N elongata* and *S mitis*, was suggested as a possible biomarker for the detection of pancreatic cancer. Although these results may allow for a better detection of pancreatic cancer, further research is required to understand whether these changes in the oral microbiota are causative and contribute to the pathogenesis of pancreatic cancer.

Mitsuhashi *et al.*<sup>[141]</sup> took an entirely different approach in studying the role of the local pancreatic microbiota in cancer development. From a large databank of pancreatic cancer tissue specimens, they tested samples for the presence of an oral microbe group, *Fusobacterium*, in the pancreatic tissue. Members of *Fusobacterium* have been implicated in periodontitis as well as pancreatic abscesses and CRC<sup>[142,143]</sup>. Mitsuhashi *et al.*<sup>[141]</sup> detected *Fusobacterium* in 8.8% of the samples. Despite a lack of correlation between these taxa and the molecular characteristics of the tumor tissue, the *Fusobacterium*-positive patients featured a higher rate of cancer-associated mortality than those without detectable microbial inoculation<sup>[141]</sup>.

While these two studies suggest there may be some role for the oral and/or local pancreatic microbiota in the pathology of pancreatic cancer, they remain purely correlative. Further research is required to determine the causative role and mechanisms of activity through which microbial infection influences the occurrence or progression of pancreatic cancer.

## CONCLUSION

Among the microbes affecting cancer development and progression, viruses are a major pathogenic cause of carcinogenesis in non-gastrointestinal tumors, through



some well-established molecular mechanisms<sup>[144]</sup>. In addition, specific bacterial pathogens have been described to induce or contribute to carcinogenesis in these entities. Bacterial mechanisms implicated in carcinogenesis include directly DNA-damaging toxin secretion, induction of chronic inflammation and suppression of immune cell activation<sup>[4,145,146]</sup>. However, the promotion of cancer formation can also result from compositional and functional changes in the microbiome configuration as a whole. When considering the role of the microbiome in cancer, a distinction should be made between local and systemic microbiome-associated effects. Currently, most studies focus on local effects of organ-specific microbiomes, such as those illustrated for the lung microbiome that may contribute to the development of lung cancer. Nonetheless, research into HCC revealed that the gut microbiota might influence carcinogenesis at distal organs, such as the sterile liver. In this particular case, the liver is anatomically linked to the gut *via* the portal vein, thereby efflux of gut microbiome-secreted or modulated metabolites may provide a mechanism linking gut microbes to hepatic carcinogenesis. However, multiple other studies<sup>[147,148]</sup> recently suggested that the gut microbiome is also able to induce systemic and long-term changes in the immune system, thereby providing possible mechanisms by which one microbiome may contribute to cancer pathogenesis even in anatomically distinct organs.

Another focus of intense research is aimed at deciphering the mechanisms governing microbiota composition, which are currently thought to be determined by a balance between the state of the host and particularly its immune system and the microbial configuration that inhabits it<sup>[106,107,149]</sup>. When one of these components is disturbed the altered “dysbiotic” microbiota may contribute to the emergence of multifactorial disease, yet the mechanisms regulating these alterations and their consequences remain elusive. Indeed, in this review, we show examples for how alterations in host microbiota interactions may be involved in cancer promotion and progression. For example, Host immune alterations, such as TLR-5 deficiency may lead to tumor progression by its altered microbiota components<sup>[128,150]</sup>. In other cases, a single microbial component, such as Hepatitis B or C virus, may directly promote carcinogenesis<sup>[108]</sup>. Finally, we provide examples suggesting that a pathogen or a pathobiont may alter the whole microbiota composition and function, thereby indirectly promoting cancer development. For instance, multiple independent studies showed that the composition of the vaginal microbiota differs between individuals infected or uninfected with HPV<sup>[65,112]</sup>. Future studies merit elucidation of causality of these associations in promoting carcinogenesis, as well as delineating the mechanisms driving these effects.

Another currently unanswered question relates to the nature of microbe-microbe interactions in driving homeostasis or cancer susceptibility. Until recently, involvement of microorganisms in cancer development was an area of research dominated by studies implicating

viral agents. This has recently changed as studies focusing on bacterial composition suggested that the bacterial microbiome may be involved, at steady state, in prevention of tumor development and when altered may participate in carcinogenesis. Studies focusing on the roles of interactions between the viral and bacterial microbiome components (such as phages affecting the composition of the bacterial microbiome) will add yet another complexity to our understanding of host-microbe interactions in cancer and merit further studies.

In summary, the host and the microbiome are increasingly regarded as two integral components of the “holobiome”, and extensively interact through a complex communication network. As such, the host and its microbiome continuously affect each other and cooperate in inducing and maintaining a healthy steady state homeostasis. Alterations of the host-microbiome communications results in breach of normal interactions, and when coupled to host germ-line encoded disease susceptibility risks, may lead to emergence of multifactorial diseases, such as cancer. A more thorough understanding of the underlying mechanisms that govern this balance of protective and cancer-promoting effects of the host and its microbiome will highlight new therapeutic targets, offering novel avenues of therapy. In years to come extensive research will likely focus on the roles of the tumor and organ-specific microbiome in cancer development and progression, effects of one microbiome (such as the gut microbiome) on tumorigenesis in other locations, the effects of microbiome alterations (dysbiosis) on immune function and hence tumor immunity, and the possible roles of other commensal microbial kingdoms, such as fungi, archaea and parasites, and of environmental triggers in cancer biology.

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