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Jun Sun *Editor*

Inflammation, Infection, and Microbiome in Cancers

Evidence, Mechanisms, and Implications

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Preface

The complex microbial communities that inhabit most external human surfaces play a key role in health and diseases. Perturbations of host–microbe interactions by pathogens can lead to altered host responses that promote cancers. My research interests are host–microbiome interactions in inflammation and cancer. I have been working on *Salmonella* infection and the risk of colon cancer for years. We have identified *Salmonella* protein AvrA that manipulates host–bacteria interactions in inflammation and infection. Our research has characterized *Salmonella* in regulating intestinal stem cells and leading to cancer. Our ongoing studies also include investigating vitamin D receptor regulation of microbiome in intestinal homeostasis and cancer and identifying dysbiosis and intestinal dysfunction in amyotrophic lateral sclerosis (ALS).

In June 2017, I was contacted by Becky Zhan, a Senior Editor at Springer Nature. She was interested in my research on host–bacteria interactions and asked if I had any proposal suitable for a new Nature/Springer book. To my knowledge, there is no book combining the topics on infection, inflammation, and microbiome in various cancers and focusing the mechanisms and implications. About 20% of human cancers are linked to infection by virus, bacteria, or parasites. However, the majority of the research papers and books are focused on viral infection and cancer; limited topics of bacterial infection in cancer are mainly about *H. pylori* and gastric cancer. It would be very novel to highlight the progress of infection, inflammation, and emerging roles of microbiome in the pathophysiology of cancers and outcomes of therapy. Thus, I proposed a book project entitled *Inflammation, Infection, and Microbiome in Cancers: Evidence, Mechanisms, and Implications* in 2018. This book proposal was well received by peers and approved from the publisher. Later, it was recommended to the American Physiological Society (APS) and approved as an APS e-book.

I am very motivated by this opportunity to present the progress made in the field by combining basic research and clinical application with tools of microbiology and bioinformatics to address many fundamental and applied questions in infection and cancer. It took me about 2 years to finalize the content. I have contacted peers around

the world to get them excited about the book project and to become contributors to the book. I identified some authors when attending the international meetings on microbiome and cancer. Coincidently, in October 2019, I was invited to present my research on *Salmonella* infection and colon cancer at an EMBO workshop entitled "The impact of bacterial infections on human cancers." This meeting is considered as the very first meeting to bring researchers together in the bacterial infection and cancer field. It also gave me an opportunity to recruit more authors for the ongoing book project.

Our book is unique as it combines the global expertise and perspective from basic researchers and physician scientists from American, Asian, and European countries. In the final book, we are able to offer summary and discussion on the advances of inflammation and infection in various cancers. We cover the classically known virus in infection and cancers, novel roles of other pathogens (e.g., bacteria and fungi) in cancer development, microbial biomarkers for diagnosis and therapy, and immune therapy in cancer. We focus on mechanistic concepts (e.g., inflammation, cell death, autophagy, and mitochondria) that underlie the complex relationships between host and microbes. We highlight the research tools, such as organoid models and germ-free animals in exploring the pathophysiology of infection and cancer. In addition to discussing the individual pathogen and its role in cancer, we also highlight emerging roles of microbiome as a microbial community in the pathogenesis of cancers and outcomes of therapy. We discuss approaches that can inhibit infection, suppress chronic inflammation, and reverse dysbiosis represented as reasonable strategies for restoring the balance between host and microbes, metabolites, mitochondrial functions, and immunity in cancer therapy. The integration of next-generation sequencing, "omics," and mega data has expanded the horizons of biomedical research, enabling the interrogation of complex systems. Furthermore, we have invited experts to discuss the application of machining learning and statistical analysis in microbiome research.

The main readers of this book will not only be cancer researchers, but also physiologists, pathologists, immunologists, microbiologists, and gastroenterologists working on the inflammatory diseases, infectious diseases, autoimmunity, and other human diseases. Students will benefit from this book by learning about the progress made in cancer research and gaps in the field of infection and microbiome.

During the journey of organizing this book project, I learned a lot from my peers. I would like to thank all the contributors/authors for their generous support and diligent work. Each chapter has been peer reviewed before we finalized the contents. I greatly appreciate the constructive suggestions and professional commitment of reviewers. Their expertise and support have helped us to improve the book. I would like to thank editors from *Nature/Springer* for their support and help. It is a great honor to publish my second APS e-book. I would like to thank APS for its recognition and Dr. Dee U. Silverthorn for her support. My ongoing research is supported by the NIH R01s, DOD breast cancer research awards, and a VA merit award.

I am in debt to my family who has helped me to handle the stresses of academic life. I would like to dedicate this book to my husband, sons, and parents. I thank them

for supporting me to develop my skills in independent judgment, critical thinking, and persistence in research. Since the beginning of 2020, the COVID-19 pandemic has been a major challenge for our medical practice and to our research community. We are in a world needing tolerance and facts. Hope my knowledge and training could eventually help and support our society.

> May the road rise up to meet you.
> May the wind be always at your back.
> May the sun shine warm upon your face;
> the rains fall soft upon your fields...

May you find inspiration from this book for the future research direction.

Chicago, IL, USA Jun Sun
August 8th, 2020

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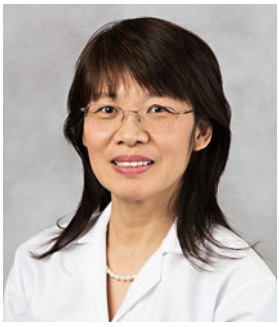
About the Editor



Jun Sun is a tenured Professor of Medicine at the University of Illinois at Chicago (UIC), USA. She is an elected fellow of the American Gastroenterological Association (AGA) and the American Physiological Society (APS). She chairs the AGA microbiome section. Her research interests are host–microbiome interactions in inflammation and cancer. Her key achievements include (1) characterization of vitamin D receptor regulation of intestinal barrier and microbiome in inflammation and cancer, (2) identification of dysbiosis and intestinal dysfunction in amyotrophic lateral sclerosis (ALS), (3) characterization of bacteria in regulating intestinal stem cells and leading to cancer, and (4) identification and characterization of the *Salmonella* protein AvrA in host–bacterial interactions. Dr. Sun has published over 190 scientific articles in peer-reviewed journals, including *Gut, Gastroenterology, Cell Stem Cells, Nature Genetics, JBC, Autophagy, American Journal of Pathology,* and *American Journal of Physiology-GI*. She is the leading editor of four books on microbiome, including a *Nature/Spring* book entitled "Mechanisms underlying host-microbiome interactions in pathophysiology of human diseases." This book has shown a novel theme and multiple disciplinary topics of microbiome research for a broad audience. She is the author of *Nature/Spring* book "Statistical Analysis of Microbiome Data with R**."** This timely book addresses the statistical modeling and analysis of microbiome data using cutting-edge R software. She serves on the editorial board of more than 10 peer-reviewed international scientific journals. She services study sections for the

NIH, American Cancer Society, and other national and international research foundations. She has been invited to chair meetings on microbiome, be a keynote speaker, and write reviews, editorials, and comments on microbiome in human diseases published for peer-reviewed journals. She is actively involved in advocating microbiome research at the international, national, and institutional levels. Her research is supported by the NIH, DOD, VA, and other research awards.

Dr. Sun is a believer in scientific art and artistic science. She enjoys writing her science papers in English and poems in Chinese. She teaches her medical fellows biomedical knowledge and also the way to translate the Chinese poems. In addition to her research papers and books, her poetry collection《让时间停留在这一刻》"*Let time stay still at this moment*," is published in 2018 by the Chinese Literature and History Press (中国文史出版社).

Chapter 1
Microbiome and the Hallmarks of Cancer

Rachel M. Bleich and Janelle C. Arthur

Abstract Microbes have long been linked to cancer: from studies on Epstein–Barr virus (EBV) and lymphoma to *Helicobacter pylori* and gastric cancer. Although single infectious agents were initially associated with carcinogenesis, technological advances have broadened our knowledge of microbial communities that may impact carcinogenesis, namely, the trillions of microorganisms that live in symbiosis with humans. Commensal microbes (microbiota) live in close association with their human hosts and impact host health, immunity, and homeostasis. Disruption to the composition of these microbial communities can dysregulate host cellular processes and promote the development of various diseases, including cancer. The "hallmarks of cancer" is an important framework for understanding the processes of how normal cells turn cancerous. This framework can also be applied to the mechanisms underlying how microbes and microbial communities influence carcinogenesis and cancer development in their human hosts. This chapter uses the hallmarks of cancer as a framework to discuss mechanisms for how microbiota promote tumorigenesis through crosstalk with the host, interactions between other microbes, and the role of microbial localization in relation to carcinogenesis.

Keywords *Escherichia coli* · *Fusobacterium nucleatum* · Enterotoxigenic *Bacteroides fragilis* · Colorectal cancer · Inflammation · DNA damage

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Abbreviations

AEEC	Attaching and effacing *E. coli*
AIEC	Adherent-invasive *E. coli*
AMP	Antimicrobial peptide
AOM	Azoxymethane
APC	Adenomatous polyposis coli
BFT	*B. fragilis* toxin
BGC	Biosynthetic gene cluster
CDT	Cytolethal distending toxin
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
CTLA4	Cytotoxic T lymphocyte-associated protein 4
DSS	Dextran sulfate sodium
EBV	Epstein–Barr virus
EMT	Epithelial to mesenchymal transition
EPEC	Enteropathogenic *E. coli*
ERK	Extracellular signal-regulated kinase
ETBF	Enterotoxigenic *Bacteroides fragilis*
HDAC	Histone deacetylase
HGF	Hepatocyte growth factor
HPV	Human papillomavirus
IBD	Inflammatory bowel disease
IgA	Immunoglobulin A
IL	Interleukin
KSHV	Kaposi sarcoma-associated herpesvirus
Lcn2	Lipocalin-2
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDSC	Myeloid-derived suppressor cell
c-MYC	Myelocytomatosis
MyD88	Myeloid differentiation primary response gene 88
NFAT	Nuclear factor of activated T cells
NK	Natural killer
NF-κB	Nuclear factor-κB
NLR	NOD-like receptor
NOC	*N*-nitroso compound
PCWBR2	Putative cell wall binding repeat 2
PD-L1	Programmed death-ligand 1
PI3K	Phosphoinositide 3-kinase
pks	Polyketide
ROS	Reactive oxygen species
SCFA	Short-chain fatty acid
SENP1	SUMO-specific peptidase 1

SFB	Segmented filamentous bacteria
SMO	Spermine oxidase
SPF	Specific pathogen free
Th17	T helper-17
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TLR	Toll-like receptor
TNF-a	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

1.1 Introduction

The close interaction between microbes and humans has evolved into an important symbiotic relationship impacting human health and survival. The presence of trillions of microbes in close contact with their human hosts is beneficial for host immunity and metabolism. However, shifts in microbial populations (dysbiosis) or infection by microbial pathogens negatively impact human health through inflammation, translocation to other body sites, and secretion of microbial products, resulting in a variety of disease conditions.

1.1.1 Oncomicrobes

Dysbiosis has been associated with cancer development and progression (Schwabe and Jobin 2013; Dzutsev et al. 2017); however the first links between microbes and cancer came through single infectious agents. Microbes have been directly linked to cancer over the past several decades. *Helicobacter pylori* (*H. pylori*), which causes gastric adenocarcinoma, was the initial bacterial link to cancer and is the classic example of an oncogenic bacteria (Vyshenska et al. 2017). Other oncogenic bacteria, such as *Fusobacterium* and colibactin-producing *Escherichia coli* (*E. coli*), have been emerging in more recent years. Even earlier, viruses have been directly linked to cancer development. Epstein–Barr virus (EBV) was discovered in 1964 as the first human-linked tumor virus and causes lymphoma (Moore and Chang 2010; Chen et al. 2017). Since then several other viruses have been directly linked to cancer including Hepatitis B and C viruses to liver cancer, human papillomavirus (HPV) to cervical cancer, and Kaposi sarcoma-associated herpesvirus (KSHV) to Kaposi sarcoma (Moore and Chang 2010; Chen et al. 2017). The fungus *Candida albicans* has recently been linked to an increased risk of carcinogenesis and metastasis in immunosuppressed patients (Ramirez-garcia et al. 2016). Additionally, several parasites can also directly cause cancer. The parasitic flatworm *Schistosoma haematobium* can cause urinary bladder cancer, and two other flukes (*Clonorchis sinensis* and *Opisthorchis viverrini*) can cause biliary tree cancer (Chen et al. 2017).

1.1.2 Hallmarks of Cancer

The "hallmarks of cancer" first proposed by Hanahan and Weinberg in 2000 and expanded in 2011 comprise key biological capabilities acquired by normal cells as they progress toward tumor development (Hanahan and Weinberg 2000, 2011). The initial six hallmarks included sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Two factors that enable these capabilities, genome instability and mutation and tumor-promoting inflammation, have been included among the hallmarks along with the more recently emerging traits of avoiding immune destruction and deregulating cellular energetics. These hallmarks provide a clear rationale for the multistep process of how neoplastic disease develops through acquisition of traits cancer cells need to become tumorigenic within the influence of the tumor microenvironment. At the end of their update to the hallmarks of cancer, Hanahan and Weinberg note that understanding the signaling circuitry and heterotypic interactions between the various cell types within the tumor microenvironment would be an important area of research over the next decade. Not only have interactions between cells within the tumor microenvironment become a critical area of research but also understanding the interactions between microbial cells and host cells within and without the tumor microenvironment.

1.1.3 Microbiota and Cancer

Commensal microbiota, or the microbial communities that live in close association with humans, play an important role in modulating host physiology and tissue homeostasis (Dzutsev et al. 2017). Microbiota impact host metabolism, inflammation, immunity, and cellular proliferation, which are all processes that when dysregulated become highly linked to tumorigenesis (Tibbs et al. 2019). Dysbiosis, or microbial imbalance of the resident microbiota, disrupts these processes and can promote the development of disease, including various cancers (Schwabe and Jobin 2013; Dzutsev et al. 2017). Ample evidence suggests that the microbiota can directly impact tumor formation. Fecal transplants from human patients with colorectal cancer (CRC) promote carcinogenesis in germ-free and conventional mice given the colon-specific carcinogen azoxymethane (AOM) (Wong et al. 2017). Transferring the microbiota of tumor-bearing mice vs. non-tumor-bearing mice accelerates the development and severity of tumorigenesis in the AOM/dextran sulfate sodium (DSS) mouse model (Zackular et al. 2013). As more research emerges on microbial mechanisms that directly impact carcinogenesis and tumor progression, our understanding of how our microbes and microbial communities influence most of the host factors described in the hallmarks of cancer is growing. In 2017, Fulbright et al. reviewed how specific members of the microbiota influence the hallmarks of cancer

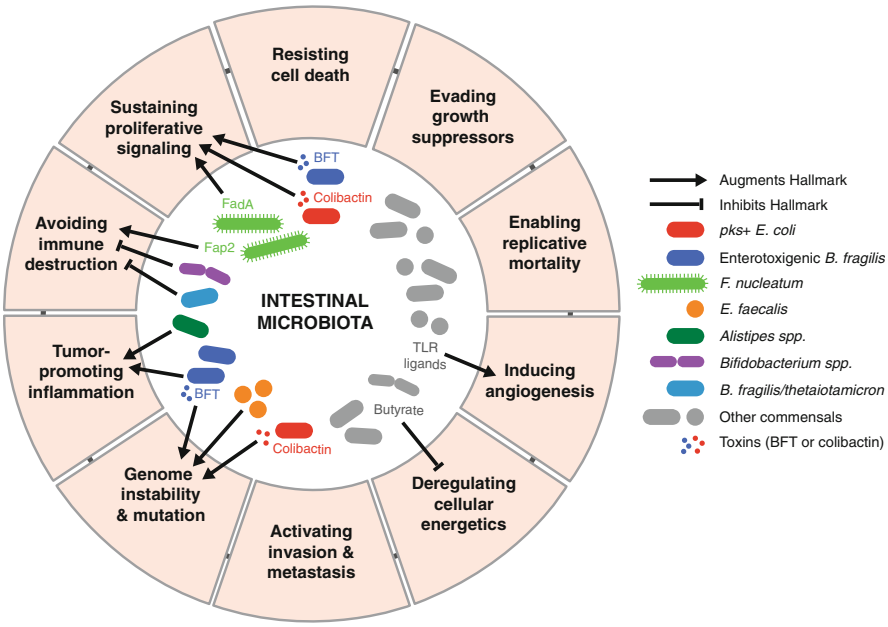

Fig. 1.1 Microbiota and microbial metabolites influence many hallmarks of cancer through diverse mechanisms and signaling. Fulbright LE, Ellermann M, Arthur JC (2017) The microbiome and the hallmarks of cancer. PLoS Pathog 13(9):e1006480. https://doi.org/10.1371/journal.ppat.1006480.g001

(Fulbright et al. 2017). Here we will review and expand on how the microbiota promote tumorigenesis through crosstalk with the host, interactions between microbes, and the role of microbial localization in relation to carcinogenesis (Fig. 1.1).

1.2 Mechanisms of Microbes and the Hallmarks of Cancer

1.2.1 Cellular Proliferation

Normal tissues carefully regulate and control the release of growth-promoting and death-inducing signals that guide progression through the cell cycle and maintain proper tissue architecture and function. By deregulating these signals, cancer cells are able to sustain cellular proliferation, which is one of the most fundamental hallmarks of cancer. The primary route microbiota impact cellular proliferation is through blocking cell-to-cell contact inhibition by targeting the adhesion molecule E-cadherin, which activates the Wnt/ß-catenin pathway. Mutations in the ß-catenin pathway are associated with numerous cancers; thus it is not surprising that this pathway is a major target of procarcinogenic microbes. Enterotoxigenic *Bacteroides*

fragilis (ETBF) secretes *B. fragilis* toxin (BFT), which is a zinc-dependent metalloprotease that cleaves E-cadherin, releasing ß-catenin, leading to activation of cell proliferation-regulating transcription factor c-MYC (myelocytomatosis) and proliferation of colonic epithelial cells (Wu et al. 1998, 2003). Similarly, *Fusobacterium nucleatum* (*F. nucleatum*) promotes colorectal cancer (CRC) proliferation by binding its adhesin FadA to E-cadherin on CRC cell surfaces (Rubinstein et al. 2019). The *Salmonella* effector AvrA activates Wnt/ß-catenin signaling intracellularly by deubiquitinating ß-catenin and blocking its degradation. This signaling upregulates c-MYC and cyclin D1 to promote proliferation, maintain the stem cell compartment, and promote tumorigenesis (Liu et al. 2010; Lu et al. 2014). AvrA can increase p53 acetylation in intestinal epithelial cells, which induces cell cycle arrest at G0/G1 and leads to ß-catenin activation (Wu et al. 2010). The *H. pylori* effector CagA promotes Wnt/ß-catenin signaling in gastric cancer by binding E-cadherin, disrupting its complex with ß-catenin and GSK-3ß, and disrupting degradation of cytosolic ß-catenin (Yong et al. 2015). CagA has also been shown to inactivate tumor suppressor pathways, including p53 (Yong et al. 2015).

Other microbes and microbial components can activate additional signaling pathways to promote cellular proliferation. *F. nucleatum* enrichment in CRC tumors is mediated by binding of the adhesin, Fap2, to a disaccharide motif (Gal-GalNAc) that is highly expressed on tumor cells (Abed et al. 2016; Brennan and Garrett 2019). Recognition of *F. nucleatum* lipopolysaccharide (LPS) by toll-like receptor 4 (TLR4) activates nuclear factor-κB (NF-κB), resulting in the production of microRNA-21 that then regulates the transcription of genes involved in proliferation and invasion (Yang et al. 2017). *Peptostreptococcus anaerobius* (*P. anaerobius*) also adheres preferentially to CRC cells via its cell surface protein PCWBR2 (putative cell wall binding repeat 2) by binding and activating α2/ß1 integrin, a cell surface molecule abundant on cancer cells. Blocking this interaction with a peptide, siRNA, or antibodies reduced *P. anaerobius* attachment and oncogenic effects in $Apc^{Min/+}$ mice (Long et al. 2019). Indeed, many members of the microbiota induce cellular proliferation through well-known pathways including NF-κB, ERK (extracellular signal-regulated kinase), and PI3K (phosphoinositide 3-kinase). Taken together, these studies highlight that a common mechanism among members of the microbial community is activating epithelial proliferation and initiating cancer development.

1.2.2 Deregulating Cellular Energetics

The increased levels of cellular proliferation associated with neoplastic disease also involve changes to energy metabolism to fuel the increased cell growth. Butyrate, a short-chain fatty acid (SCFA) produced by gut microbiota through fermentation of dietary fiber, has been linked to overall gut health and homeostasis. A decrease in butyrate-producing bacteria has been observed in CRC cases vs. controls (Perrin et al. 2001; Bultman 2014). While butyrate-producing bacteria can attenuate tumor

burden in CRC-susceptible mice, colonization with a mutant strain that produces reduced levels of butyrate fails to attenuate tumor burden to the extent of the wild-type butyrate-producing strain (Donohoe et al. 2014; Sebastián and Mostoslavsky 2014). Additional supplementation with dietary butyrate rescues this protective effect (Donohoe et al. 2014; Sebastián and Mostoslavsky 2014). Normal colonocytes rely on butyrate as an energy source, which undergoes ß-oxidation in the mitochondria, providing the energy needed for rapid proliferation of the colonic epithelium (Donohoe et al. 2011). CRC cells switch to glucose utilization as an energy source, allowing butyrate to accumulate and function as an HDAC (histone deacetylase) inhibitor, which regulates gene expression and reduces tumor burden (Vander Heiden et al. 2009; Donohoe et al. 2012, 2014). The full tumor-suppressive effects of butyrate are multifaceted and involve other hallmarks of cancer as well. Further studies are needed to understand the broader impact of the gut microbial metabolome on cellular energetics and the overall hallmarks of cancer

In response to aging and stressors, cells can stop dividing and enter cellular senescence to halt proliferation. However, senescent cells promote tumorigenesis by secreting growth factors that enable tumor growth. Senescent cells are metabolically active and secrete various growth factors in addition to reactive oxygen species (ROS), pro-inflammatory cytokines, and chemokines (Wang et al. 2017). In senescent cells, metabolism is upregulated but altered to include an increase in glycolysis and a reduction in oxidative phosphorylation and the TCA cycle, leading to a characteristic decrease in intracellular NAD+ (Sabbatinelli et al. 2019). Colibactin-producing *E. coli* help sustain tumor cell growth by inducing epithelial senescence and enhancing production of hepatocyte growth factor (HGF) (Cougnoux et al. 2014; Dalmasso et al. 2015). The underlying mechanisms are not fully clear but involve downregulating p53 SUMOylation through microRNA-20a-5p and SENP1 (SUMO-specific peptidase 1) (Secher et al. 2013; Cougnoux et al. 2014). Thus, by deregulating cellular energetics in cells of the tumor microenvironment, colibactin-induced senescent cells can promote tumor growth.

1.2.3 Avoiding Immune Destruction

The immune system keeps constant surveillance and is responsible for recognizing and eliminating cancer cells. Thus, for tumorigenesis to occur, neoplastic cells must avoid detection and destruction. Members of the microbiota can protect tumor cells from immune-mediated detection and killing and, in combination with immunotherapy and chemotherapy, may enhance antitumor immunity. Natural killer (NK) cells kill non-self cells (i.e., virus-infected and tumor cells) via coordination of activating and inhibitory receptors. *Fusobacterium nucleatum* inhibits NK cells by binding NK cell inhibitor receptor TIGIT (T cell immunoreceptor with Ig and ITIM domains) via the bacterial Fap2 adhesin, which allows tumor cells to evade immunosurveillance (Gur et al. 2015). Additionally, *Fusobacterium* induces immunosuppressive myeloid-derived suppressor cells (MDSCs), which can boost tumor formation by

interfering with immune surveillance (Montero et al. 2012; Gur et al. 2015; Goodman and Gardner 2018). *Helicobacter pylori* is able to subvert the adaptive immune system, allowing the bacteria to establish an infection and promote gastric carcinogenesis through several mechanisms. For example, it can induce T cell apoptosis by upregulating Fas ligand on Fas-expressing T cells (Wang et al. 2001). The *H. pylori* vacuolating toxin and virulence factor VacA inhibits T cell proliferation by blocking interleukin (IL)-2 secretion via nuclear factor of activated T cells (NFAT), an important transcription factor for T cell activation (Sundrud et al. 2004). *H. pylori* reduces the immune response in the gastric epithelium by inducing Tregs, which impair the response of memory T cells (Beswick et al. 2007). Finally, *H. pylori* induces programmed death-ligand 1 (PD-L1) expression on gastric epithelial cells, which regulates T cell programmed cell death and reduces T cell proliferation, causing a loss of immune surveillance (Beswick et al. 2007; Silva et al. 2016; Holokai et al. 2019).

A major area of current investigation relates to enhanced efficacy of immunotherapy and chemotherapy by members of the gut microbiota. Immune checkpoint inhibitors target and block inhibitory receptors on T lymphocytes, thus permitting robust T cell-mediated antitumor immunity. While these treatments have revolutionized cancer therapy, many patients simply do not respond (Agrawal 2019). Although the mechanisms are not yet well understood, several lines of evidence suggest that immunotherapy efficacy is driven by the functional characteristics of the microbiome, including "immunostimulatory" bacteria. It was first observed in 2013 that tumor-infiltrating myeloid cells were ineffective in germ-free and antibiotic-treated mice harboring subcutaneous xenograph tumors (Iida et al. 2013). Two years later, specific microbes were implicated. Administration of *Bifidobacterium* with an immunotherapeutic targeting PD-L1 almost completely stops tumor growth in mice (Sivan et al. 2015). *Bifidobacterium* promotes dendritic cell function and antitumor abilities of cytotoxic T cells, which leads to reduced growth of subcutaneous melanoma in a xenograft mouse model (Sivan et al. 2015). Likewise, *Bacteroides thetaiotaomicron* and nontoxigenic *B. fragilis* improve anti-cytotoxic T lymphocyte-associated protein 4 (CTLA4) immunotherapeutic efficacy in sarcoma, melanoma, and colorectal cancer xenograft mouse models (Vétizou et al. 2015). This is driven by microbe-specific T cell responses, as adoptive transfer of *B. fragilis*-specific T cells is also protective (Vétizou et al. 2015). More recent studies demonstrate protective effects of *Akkermansia muciniphila* (Routy et al. 2018), *Bifidobacterium longum* (Matson et al. 2018), and *Faecalibacterium prausnitzii* (Gopalakrishnan et al. 2018). In these studies, colonization of germ-free mice with "responder" patient stool vs. "non-responder" stool combined with immunotherapy enhances antigen presentation and activated T lymphocytes, leading to protective antitumor effects. Indeed, supplementing non-responder stool with *Akkermansia* induces similar protective effects (Routy et al. 2018). Although across these studies there is a lack of common protective microbial signature, meta-analyses suggest that functional attributes have more predictive power than taxonomy (Gharaibeh and Jobin 2019).

1.2.4 Tumor-Promoting Inflammation

Almost every neoplastic lesion contains immune cells, with some tumors densely packed. Once thought to be a solely antitumoral response, the inflammatory response can enhance tumor progression and help neoplasias acquire additional hallmarks. Inflammation can contribute to multiple hallmarks including cellular proliferation, angiogenesis, invasion and metastasis, and limiting cell death. In fact, treatment with anti-inflammatory drugs is effective in reducing colorectal cancer rates and death (Lasry et al. 2016). Patients with inflammatory bowel disease (IBD), a chronic immune-mediated inflammatory condition, are at a greater risk of developing colorectal cancer. The close proximity of the microbiota and mucosal immune system provides opportunity for resident microbes to elicit protumorigenic immune responses. In the AOM/*Il10*$^{-/-}$ model of colitis-associated cancer, microbes are required for inflammation and carcinogenesis (Uronis et al. 2009; Arthur et al. 2014). In specific pathogen-free (SPF) housed *Apc*$^{Min/+}$; *Il10*$^{-/-}$ mice, inflammation correlates with colon tumorigenesis (Tomkovich et al. 2017).

Intestinal microbiota impact mucosal barrier integrity, which alters immune responses (Bhatt et al. 2017). One layer of epithelial cells covered with a thick layer of mucus is all that separates the gut microbiota from the mucosal immune system. This physical barrier regulates interactions between the host and microbiota, along with secreted molecules like mucins (mucus), antimicrobial peptides (AMPs), and immunoglobulin A (IgA) (Yang and Jobin 2017). The microbiome of CRC patients is usually enriched for pro-inflammatory opportunistic pathogens and depleted of butyrate-producing bacteria that help maintain intestinal homeostasis (Marchesi et al. 2011; Gao et al. 2015). Butyrate-producing bacteria promote barrier function in part by upregulating the claudins and occludins involved in tight junctions (Kelly et al. 2015). Disruption of intestinal epithelial homeostasis leads to inflammation and tumorigenesis driven by bacterial translocation and the spread of bacterial metabolites (Yang and Jobin 2017). Inflammation increases epithelial oxygenation in the colon of mice, which can then drive expansion of *E. coli* through aerobic respiration (Cevallos et al. 2019). This aerobic expansion of colibactin-producing *E. coli* was required for the carcinogenic activity of this species in a mouse model of CRC (Cevallos et al. 2019).

Generally speaking, toll-like receptors (TLRs) detect bacterial antigens and signal through myeloid differentiation primary response gene 88 (MyD88) and NF-κB to release pro-inflammatory cytokines and trigger an immune response (Kawasaki and Kawai 2014). However, inflammation and tumorigenesis are modulated by cell type-specific responses. This is exemplified by a study demonstrating that genetic deletion of IL-1R1 (a cytokine receptor for the major pro-inflammatory cytokine IL-1) in epithelial cells or T cells decreases inflammation and cancer, whereas IL-1R1 deletion in neutrophils enhances inflammation and cancer in the APC (adenomatous polyposis coli) model (Dmitrieva-Posocco et al. 2019). Various aspects of NF-κB signaling and IL-6 sensing and production also mediate cell type-specific responses.

However, a common procarcinogenic immune signature involves IL-17 and T helper-17 immunity (Wang et al. 2014).

T helper-17 (Th17) immunity is associated with worse prognosis in CRC and is promoted by microbes and their metabolites (Grivennikov et al. 2012; Wang et al. 2014). Th17 immunity is trained as a protective host defense response under conditions of homeostasis by epithelial, adherent bacteria, including segmented filamentous bacteria (SFB) (Atarashi et al. 2015). However, these protective effects can turn pathogenic in the setting of inflammation and cancer. ETBF toxin activates Th17 T cell-mediated responses in $Apc^{Min/+}$ mice, and blocking IL-17 reduces inflammation and tumorigenesis in this model (Wu et al. 2009; Housseau et al. 2016). IL-17 is produced by immune cells and targets IL-17 receptor (IL-17RA) on epithelial cells, triggering a signaling cascade through MAPK (mitogen-activated protein kinases) and NF-κB pathways that promote proliferation particularly in neoplastic cells (Wang et al. 2014). *Alistipes* are also implicated in inflammation-related carcinogenesis through human metagenomic studies of CRC (Feng et al. 2015). Mouse modeling in $Il10^{-/-}$ $Lcn2^{-/-}$ (lipocalin-2) mice revealed that *Alistipes* induces tumorigenesis associated with enhanced pro-inflammatory cytokine production, STAT3 activation, and epithelial hyperplasia (Feng et al. 2015; Moschen et al. 2016; Tilg et al. 2018). Although IL-17 RNA and protein were elevated in the epithelium, the dependence of IL-17 was not tested in this study. Instead, inflammation and tumorigenesis were significantly reduced through genetic deletion of IL-6, a cytokine that stimulates IL-17 production from Th17 cells (Moschen et al. 2016).

1.2.5 Genome Instability and Mutation

Many of the hallmarks of cancer are acquired through changes and mutations to the genomes of neoplastic cells that confer growth and survival advantages. *E. coli* expressing the genotoxin colibactin enhance tumorigenesis in mouse models and are enriched in human CRC tissues (Arthur et al. 2012; Buc et al. 2013). Colibactins are hybrid polyketide-nonribosomal peptides produced by *Enterobacteriaceae* harboring the 54 kilobase genomic polyketide (*pks*+) island (Nougayrède et al. 2006; Homburg et al. 2007). Epithelial cells that encounter colibactin have DNA double-strand breaks and are characterized by γ-H2AX foci, G2/M cell cycle arrest, megalocytosis, and activation of ATM/CHK/CDC25/CDK1 DNA damage signaling cascades (Nougayrède et al. 2006; Arthur et al. 2012). Colibactin works, at least in part, by alkylating DNA to form adducts that cause DNA damage in colonic epithelial cells in cellulo and in mice (Bleich and Arthur 2019; Wilson et al. 2019). *Pks* + *E. coli* have been shown to cause DNA cross-links directly on purified DNA (Bossuet-Greif et al. 2018). *E. coli* lacking *pks* induce similar levels of inflammation in $Il10^{-/-}$ mice, but fewer tumors and less invasion than mice colonized with *pks* + *E. coli* (Arthur et al. 2012, 2014). Attaching and effacing *E. coli* (AEEC; *pks*-negative) can also reduce expression of DNA mismatch repair proteins

MSH2 and MLH1, which are mutated in hereditary nonpolyposis colorectal cancer (Kim et al. 2002; Maddocks et al. 2009). Enteropathogenic *E. coli* (EPEC) inhibits DNA repair in a pks-independent manner via NleE, a secretory cysteine methyltransferase, that blocks DNA annealing helicase and endonuclease ZRANB3 (Yao et al. 2014). However, AEEC and EPEC are generally acute pathogens, and their role in cancer development is not known.

Another bacterial toxin that damages DNA and is therefore thought to be related to cancer development is cytolethal distending toxin (CDT). CDT is produced by various *Proteobacteria* and induces DNA damage and activation of DNA damage response in cells (Hassane et al. 2003; Thelestam and Frisan 2004). CDTs are heterotrimeric toxins with three subunits (CdtA, CdtB, and CdtC) with different functions (Song et al. 2013). CdtB can activate DNAseI, while CdtA and CdtC help with binding of the toxin to the plasma membrane of host cells (Scuron et al. 2016). In *Salmonella*, CdtB is also part of the typhoid toxin complex with pertussis-like toxin A (pltA) and pertussis-like toxin B (pltB) (Rosadi et al. 2016). CdtB induces DNA damage and cell cycle arrest, which is potentially linked to carcinogenesis in the gallbladder upon chronic infection (Iyer et al. 2016; Di Domenico et al. 2017). In $Rag2^{-/-}$ mice, CDT-producing *Helicobacter hepaticus* (*H. hepaticus*) downregulates genes involved in DNA repair pathways (Mangerich et al. 2012). The cancer-related activity of CDT is linked to chronic exposure at low doses as a possible side effect of infection (Guidi et al. 2013; Rosadi et al. 2016).

In addition to interactions with bacterial products, gut microbes can cause DNA damage through the formation of host-derived reactive oxygen species (ROS). This can be mediated by inflammation caused by infection. For example, *H. pylori* can induce a chronic inflammatory state with increased production of ROS, leading to DNA strand breaks and genomic instability (Wong et al. 2019). Bacterial proteins can also induce ROS formation. *B. fragilis* enterotoxin upregulates expression of spermine oxidase (SMO), a polyamine catabolic enzyme that is induced by inflammatory stimuli (Goodwin et al. 2011). SMO induces ROS formation and DNA damage, which is evidenced by increased ɣ-H2AX foci, indicative of double-strand DNA breaks, in human colon cancer cells exposed to purified enterotoxin (Goodwin et al. 2011). *Enterococcus faecalis* (*E. faecalis)* can induce ROS in epithelial cells through generation of superoxide through interaction with macrophages (Huycke et al. 2002; Wang et al. 2015). In vitro and in vivo studies have shown that *E. faecalis* induces macrophages to generate superoxide and hydrogen peroxide that damage epithelial cell DNA by forming DNA-protein cross-links, DNA breaks, and DNA point mutations (Huycke et al. 1996, 2001, 2002; Huycke and Moore 2002; Ley et al. 2006; Wang et al. 2008). Gene knockout experiments identified membrane-associated quinones as the source for superoxide (Ramsey et al. 2014; Wang et al. 2017). Further studies have shown that when *E. faecalis* activates macrophages and polarizes them to an M1-like phenotype, those macrophages can then cause mutations and chromosomal instability in primary epithelial cells through a bystander effect (Wang et al. 2015; Wang and Huycke 2015). This is at least partially mediated by trans-4-hydroxy-2-nonenal (4-HNE), a highly reactive aldehyde produced by lipid peroxidation in macrophages that induces cyclooxygenase-2

(COX-2) and inflammatory cytokines like tumor necrosis factor alpha (TNF-a) that enhance procarcinogenic effects (Yang et al. 2012; Emerit 2007; Wang et al. 2012).

Additional byproducts of bacterial metabolism are implicated in tumorigenesis. N-nitroso compounds (NOCs) can either be ingested through consumption of processed meats or produced as products of bacterial transformation of nitrate into nitrite, which reacts with other nitrogenous compounds in the body (Gill and Rowland 2002; Dubrow et al. 2010). Many of these NOCs are DNA alkylating agents that have been associated with gastrointestinal cancer (Loh et al. 2011). Sulfate-reducing bacteria are anaerobic organisms that produce hydrogen sulfide (H_2S) by reducing sulfate and oxidizing organic compounds or molecular hydrogen (Mandal 2018). Various species like *Bilophila wadsworthia* and *Alistipes* spp. are abundant in some CRC patients and produce H_2S that is toxic to epithelial cells and causes DNA damage (Attene-Ramos et al. 2007; Yazici et al. 2017). Hydrogen sulfide can also modulate gene expression, cell cycle progression, and DNA repair (Wang et al. 2017). Elevated H_2S is a feature of the microbiome of Crohn's disease patients, with *Atopobium parvulum* acting as a central hub of H_2S-producing microbes. Administering *Il10*$^{-/-}$ mice the H_2S scavenger bismuth reduces *A. parvulum*-induced colitis, but the impact on colorectal cancer is currently unknown (Mottawea et al. 2016).

Eukaryotes and gut bacteria produce polyamines, small cationic molecules that can damage DNA and induce oxidative stress (Gobert and Wilson 2017). Certain bacterial strains, including ETBF and *H. pylori*, can upregulate polyamine production in host cells (Pegg 2013). Polyamine oxidation by spermine oxidase, such as upon *H. pylori* infection, causes hydrogen peroxide release, DNA damage in gastric epithelial cells, and apoptosis of macrophages, leading to an increased risk of gastric carcinogenesis (Hardbower et al. 2013). Polyamines can also target bacteria: a recent study found that the polyamine spermidine is required for full genotoxic activity of colibactin-producing *E. coli* (*pks*+) (Chagneau et al. 2019). Spermidine is required for direct damage of DNA and may be involved in the regulation of the synthesis of colibactin (Chagneau et al. 2019). Another polyamine, N(1), N(12)-diacetylspermine, that regulates cellular proliferation is detected in metabolomic analyses of biofilms from colon cancer patients (Hiramatsu et al. 2005; Johnson et al. 2015). The levels of this polyamine metabolite are higher in the proximal colon, corresponding to a higher incidence of biofilms detected on CRCs in the proximal colon (Allgayer et al. 2007; Dejea et al. 2014). This indicates the biofilm microbial community may differentially produce cancer-associated metabolites (Dejea and Sears 2016). Overall, bacterially produced or induced metabolites are influential in promoting DNA damage and mutations that can help promote carcinogenesis and fuel other hallmarks of cancer. Analysis of Human Microbiome Project data has revealed several thousand biosynthetic gene clusters (BGCs) within human-associated bacterial genomes (Donia et al. 2014). With such a widespread distribution of small-molecule metabolite biosynthesis systems, exploring their impact on the process of carcinogenesis is an area we expect will grow rapidly in the near future.

1.2.6 Remaining Hallmarks

In addition to the previously discussed hallmarks of cancer, five more remain. Although evidence exists these are impacted by the microbiome, it is an open area of investigation into microbial mechanisms driving these hallmarks. These hallmarks include evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting cell death. These hallmarks are employed by cancer cells to avoid suppression and destruction by the body while promoting their own spread and survival.

Epithelial to mesenchymal transition (EMT) is associated with invasion and metastasis in CRC (Vu and Datta 2017). During EMT, cells lose epithelial traits including cell-cell contact and gain mesenchymal traits like increased motility (Vu and Datta 2017). Gut microbes can induce an epithelial-to-mesenchymal transition through various signaling pathways that lead to the suppression of tumor-suppressor E-cadherin and thus increased tumor invasion and metastasis (Thiery et al. 2009; Chandrakesan et al. 2014). The *Salmonella* effector, SopB, is linked to EMT by increasing expression of EMT transcriptional activators and downregulating E-cadherin (Knodler et al. 2005; Zavadil and Böttinger 2005; Clevers 2006). SopB has been shown to do this through activation of the serine and threonine kinase Akt (protein kinase B), which increases the transcriptional activity of ß-catenin through phosphorylation, and activates the Wnt/ß-catenin pathway (Knodler et al. 2005; Zavadil and Böttinger 2005; Clevers 2006). This promotes proliferation and resistance to apoptosis, allowing transformed cells to spread beyond the initial tumor (Knodler et al. 2005).

During normal development and under conditions of homeostasis, the gut microbiota promotes angiogenesis responses through toll-like receptors (TLRs) and NOD-like receptors (NLRs) (Schirbel et al. 2013). An early observation was that germ-free mice developed fewer capillaries in their small intestinal villi (Stappenbeck et al. 2002). Lipopolysaccharide (LPS), part of the cell wall of Gram-negative bacteria that is recognized by TLR4, can increase angiogenesis and metastasis by stimulating endothelial cells to produce vascular endothelial growth factor (VEGF) and increase vasculature permeability (Harmey and Bouchier-Hayes 2002; Pollet et al. 2003). Cell wall extracts from *Streptococcus gallolyticus* can induce IL-8 expression that promotes angiogenesis (Biarc et al. 2004). While we have some mechanistic information linking the microbiota and these remaining hallmarks of cancer, future studies will hopefully reveal further connections. Although we have described these hallmarks individually, it is important to note that many of these hallmarks of cancer work together and are influenced by one another. Additionally, the microbial populations shift and respond to these hallmarks, thereby driving other hallmarks. For example, increased inflammation leads to changes in the microbial community, like increasing levels of *pks* + *E. coli* (Arthur et al. 2012). Developing cancer impacts the transcriptome of intestinal *pks* + *E. coli*, including genes of the *pks* island (Arthur et al. 2014) that may increase the likelihood of developing mutations that promote tumorigenesis.

1.3 Additional Microbial Factors that Influence Cancer

1.3.1 Establishing a Chronic Infection

In order to influence cancer development, some microbes need close proximity to the host for extended periods of time. Several microbes can subvert the immune system by hiding and surviving with host epithelial or immune cells. This allows the bacteria to establish a chronic infection, increasing the ability to influence tumorigenesis. The *Salmonella* effector AvrA helps the bacteria survive within macrophages and epithelial cells by preventing apoptosis and promoting bacterial propagation (Wu et al. 2012). To further evade the immune system, *Fusobacterium* can live within cells, as FadA binding to E-cadherin on epithelial cells enables cellular uptake. This may also drive production of inflammatory cytokines (Rubinstein et al. 2013; Goodman and Gardner 2018). Adherent-invasive *E. coli* (AIEC) can invade intestinal epithelial cells and survive and replicate inside macrophages, helping them subvert the immune system (Prorok-Hamon et al. 2014). *E. faecalis* is effective at evading immune responses and is resistant to macrophage killing (Gentry-weeks et al. 1999). *E. faecalis* can survive intracellularly by preventing formation of vacuoles needed for acidification of phagolysosomes, and it interferes with transport of vacuoles to lysosomes (Zou and Shankar 2016). Microbes have evolved mechanisms for their survival with the host, and chronic contact with the host cells can impact possible carcinogenesis and cancer development.

1.3.2 Microbial Interactions

Although there has been progress in elucidating mechanisms of the role of individual microbes in cancer development and progression, no single species is universally present among cancer or CRC patients. There is also variation in microbial communities between individual patients, suggesting that different combinations of microbes work together to drive or protect from tumorigenesis (Sears and Garrett 2014). Not only are host-microbe interactions important for understanding cancer development, but microbe-microbe interactions influence host disease state and the overall function of the microbial community.

In the gut, microbes are in close proximity and their interactions are important for gut health and the development of cancer. One model of bacterial interactions in CRC pathogenesis includes "driver" microbes that initiate CRC development that are followed by "passenger" microbes that have a growth advantage in the tumor microenvironment established by the drivers (Sears and Pardoll 2011; Tjalsma et al. 2012). This model suggests that disease progression changes the microenvironment, which in turn, changes the microbial community to one that can perpetuate tumor progression (Saus et al. 2019). Proteobacteria like *E. coli*, *Shigella*, *Citrobacter*, and *Salmonella* are enriched in early stages of CRC and may function more as drivers,

while *Fusobacterium* and other passengers are enriched in later stages (DuPont 2009; Lazarovitch et al. 2013). It is possible, although not proven, that drivers may function primarily in the initiation stage of cancer and passengers may be essential contributors to the promotion stage.

Furthermore, ample evidence exists that bacteria and viruses interact both directly and indirectly via the host to impact oncovirus-driven cancers. Very little is known about interactions between the virome—comprising all viruses including bacteriophages and eukaryotic viruses that are resident in and on a host—and these cancers. Surely bacteriophages influence microbiome composition, and this may impact the bacterial microbiome's effect on cancer. There is some evidence of compositional differences in the viromes of healthy vs. CRC individuals, with the cancer-associated virome consisting of mainly temperate bacteriophages (Hannigan et al. 2018). The impact of these viromes on cancer has not been experimentally investigated.

Additional inter-kingdom interactions have only recently been explored, such as between bacterial microbiome and fungal mycobiome. A study exploring squamous cell carcinoma of the tongue found alterations to the mycobiome, the consequences of which are unknown (Mukherjee et al. 2017). Another study reports an increased ratio of *Basidiomycota-Ascomycota*, increased *Malasseziomycetes*, and decreased *Saccharomycetes* and *Pneumocystidomycetes* in CRC patients compared to health controls (Coker et al. 2019). Principal component analysis reveals that these mycobiome populations cluster according to stage of CRC, suggesting mycobiome profiles are stage-specific (Coker et al. 2019). There is also evidence for synergistic intrafungal and antagonistic bacterial-fungal correlations (Coker et al. 2019). It is intriguing to consider that these inter-kingdom interactions and stage-specific clustering of the mycobiome may be a cause or biomarker for the cancer.

1.3.3 Location and Tumorigenesis

We have microbiomes specific to all niches in and on our bodies (Huttenhower et al. 2012). Even within the gut microbiome, there exist functionally and compositionally distinct communities. An example of these are communities residing in the lumen/stool, mucus layer, adherent to the epithelial cells (mucosa), and intratumoral. In future studies, rather than simply evaluating bulk stool samples, acquiring location-specific information could be more helpful in making meaningful connections. This is especially informative when combining multi-omics analyses such as microbiome, host transcriptome, and metabolome. Yet proximity to a tumor may not be the only way a microbe can influence tumor progression, as bacterial products and specialized metabolites can act as signaling molecules from more distal locations.

Tumors harbor their own microbial populations that thrive in the hypoxic environment. Leaky vasculature can help bacteria enter the tumor and evade immunosurveillance (Syed Khaja et al. 2017). Intratumoral bacteria can have immunomodulatory functions, impacting immune responses to tumors and cancer immunotherapy (Kim et al. 2017; Zheng et al. 2017). In fact, intratumoral

Gammaproteobacteria can produce an enzyme that inactivates gemcitabine in pancreatic cancer (Geller et al. 2017). Due to their proximity to the gut microbiome, CRC tumors have been studied most extensively. Not only are changes to the microbial population restricted to cancerous tissue, but often times non-cancerous adjacent tissue is impacted as well (Flemer et al. 2017). In CRC, there are differences in microbial communities between proximal (right-sided, ascending colon) and distal (left-sided, descending colon) tumors (Flemer et al. 2017; Gao et al. 2017). There are also differences in the physiological state of the bacterial communities, with biofilm-positive communities dominating proximal cancers and biofilm-negative communities in distal cancers (Dejea et al. 2014, 2018; Drewes et al. 2017). In mouse models, biofilm communities from both CRC and healthy individuals induce more tumorigenesis than non-biofilm communities (Tomkovich et al. 2019). Thus, the interactions and functions of the mucosal community have a greater impact on carcinogenesis than donor host health status.

Differences in the microbiota have been observed between mucosal tissue from tumors and adjacent "normal" tissue (Picardo et al. 2019). However, adjacent "normal" mucosal microbiomes may still differ from that patient's former healthy state. The potential functional attributes of tumor-associated microbes may also be altered. One study observed a decrease in *Firmicutes* and *Actinobacteria* and increase in *Fusobacterium*, with LPS biosynthesis-associated microbial genes enriched in tumor tissues (Gao et al. 2017). *Fusobacterium* itself is more highly abundant in CRC patient tissue vs. healthy controls (Kostic et al. 2012, 2013; Castellarin et al. 2012) and is most abundant in rectal tissue biopsies from patients with adenocarcinoma vs. adenoma vs. healthy controls (McCoy et al. 2013). *Fusobacterium*-containing tumors harbored microbiomes that were highly similar to their *Fusobacterium*-positive distant metastases (versus *Fusobacterium*-negative tumors) (Bullman et al. 2017), suggesting that *Fusobacterium* may be a hub for multi-species procarcinogenic activities. Another study that found *Fusobacterium* and *ε-Proteobacteria* enriched on tumors also found differences in metabolites, including increased taurine, isoglutamine, choline, lactate, phenylalanine, and tyrosine and decreased lipids and triglycerides in tumor vs. adjacent tissue (Kinross et al. 2017). Taken together, the tumor microbiome and associated metabolites are very likely to contribute to the hallmarks of cancer, as we have discussed in relation to mainly the luminal microbiome. Greater precision in sampling and lower cost, higher-throughput sequencing and analyses pipelines will surely accelerate our understanding of how tumor-associated communities contribute to the hallmarks of cancer.

1.4 Conclusion

The hallmarks of cancer provide an important framework to help understand the complexities of cancer biology and the broad phenotypes discovered in cancer research. We can also use the hallmarks as a framework to classify specific

mechanisms by which microbes, microbial communities, and microbial metabolites may impact cancer development. Individual microbes have been associated with cancer for decades from studies with *H. pylori* to various viruses like HPV and EBV. However, we now have a better understanding of not only host-microbe interactions but also the microbe-microbe interactions that influence host physiology and microbial community dynamics. Microbes act as a community and influence one another both through physical (proximity) and chemical (metabolite) interactions and signaling. There is also constant communication and feedback between members of this community and the host. Dysbiosis occurs when this communication goes from a nice conversation (homeostasis) to a heated argument (dysbiosis and disease). Understanding this dialogue will improve our ability to prevent, diagnose, and treat various microbially linked cancers in the future.

Initial studies primarily examined correlative data to define members of the microbial community who were present or associated with specific disease states. Continuing to define specific mechanisms by which microbial factors target these hallmarks is crucial to further understanding cancer development. This includes both host and microbial targets and how they are induced. For example, is it an insult from the microbial community? Is it linked to the environment external to the host (stress, diet, etc.) or internal to the host? When thinking about microbial targets, we need to move beyond just bacteria and embrace emerging technology to detect and define the mechanisms of other microbes: virus, fungus, and archaea. As we uncover the roles of these less-characterized microbes, we need to consider the interspecies interactions between these different microbial types. Finally, as we define microbial factors, we need to examine evidence that link the specific microbial "lesions" (i.e., the DNA alkylation due to colibactin) to human cancers. For example, we find viral genes inserted into the genome that drive oncogenes, so we should be able to detect molecular signatures that indicate a bacterially induced carcinogenic effect has occurred. This would truly demonstrate that the lesion is linked to the disease and could potentially be used in the near future to identify precursor lesions that direct prognosis and treatment. In summary, treatment for cancers will become more personalized and target specific components of the microbiota as we uncover specific mechanisms by which microbes influence carcinogenesis and cancer development.

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Chapter 2
Microbiome in Human Gastrointestinal Cancers

Olabisi Oluwabukola Coker and Jun Yu

Abstract Human gastrointestinal tract houses several millions of microbes, with which they form complex symbiotic and mutualistic relationships. The resident microbes encode unique genes that are important in several host beneficial processes. As such, alteration of the optimal composition and ecology of human gastrointestinal microbes can be detrimental to the host. Indeed, evidences of the association of altered gut microbiome with gastrointestinal carcinogenesis including esophageal, gastric, pancreas, and colorectal cancers are emerging. This chapter details the essential roles of gut microbes including bacteria, fungi, viruses, and archaea in the gut, shift of gut microbes in gastrointestinal cancers, and the potential manipulation of the gut microbes in the prevention and treatment of human gastrointestinal cancers.

Keywords Gastric cancer · Colorectal cancer · Pancreatic cancer · Esophageal cancer · Microbiome · Bacteria · Fungi · Archaea · Virus

2.1 Introduction

Human gut microbiome refers to the trillions of microorganisms, including their genetic material, that reside within the gastrointestinal tract. These microorganisms comprise of a dynamic community of species from the bacteria, fungi, virus, archaea, and protozoa kingdoms (Bengmark 1998). Compared with other body sites, the human gut hosts the largest numbers of microbial species whose composition is acquired within 2 years after birth, followed by the establishment of mutualistic relationships with the host (Dieterich et al. 2018). Site-specific microbiome have also been determined for sections of the gastrointestinal tract subject to conditions

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such as oxygen concentration, pH, nutrient availability, and surface area available for colonization (Donaldson et al. 2016). Majority of studies have focused on the microbial composition of the intestine, due to the relative ease with which fecal samples can be obtained. However, recent studies have revealed that several microbes also form the normal microflora of the esophagus, stomach, and pancreas of healthy human. The gastrointestinal microbes are alive, actively metabolizing and engaging in dynamic ecological interactions among themselves. In a homeostatic gut microbiome state, the products of the metabolic processes of the gut microbes or direct host interactions impact host health positively (Jandhyala et al. 2015). However, when the composition of the normal flora is disturbed, the derivable positive impacts of the gut microbes may be lost, while colonization of the gut by opportunistic pathogens may be enhanced. This chapter details the essential roles of bacteria, fungi, virus, and archaea in the gut, shift of gut microbes in gastrointestinal cancers, and the potential manipulation of the gut microbes in the prevention and treatment of human gastrointestinal cancers.

2.2 Microbiome in Gastrointestinal Health

The composition of the gut microbiome is largely unique to individuals (Franzosa et al. 2015). However, gut microbes perform essentially similar direct and indirect physiological functions which impact the gastrointestinal health of the host. Gut bacteria, fungi, virus, and archaea reportedly play important roles in the gut. These functions, among others, include food digestion, production of essential vitamins, absorption of nutrients, immune modulation, and resistance of colonization by pathogenic microbes (Fig. 2.1).

2.2.1 Functions of Bacteria in the Gastrointestinal Tract

Bacteria are the most abundant component of the gastrointestinal microbiome. The distribution of bacteria varies along the gastrointestinal tract ranging from 10^1 colony-forming unit (CFU) per gram of contents in the esophagus and stomach to 10^{12} CFU per gram of contents in the colon and distal gut (O'Hara and Shanahan 2006). Human gut bacteria can be aerobic, facultative anaerobic, or strictly anaerobic, depending on the level of oxygen along the gastrointestinal tract. They mostly belong to the *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* phyla. Members of *Actinobacteria*, *Verrucomicrobia*, *Acidobacteria*, and *Fusobacteria* phyla are also present, although usually less than 1% of the bacterial population. Esophageal bacteria have been demonstrated by many studies to be dominated by *Streptococcus* (Corning et al. 2018), while gastric bacteria are dominated by *Prevotella*, *Streptococcus*, *Veillonella, Rothia*, and *Haemophilus* (Nardone and Compare 2015). Mucosa-associated bacteria from the colon are dominated by members of the

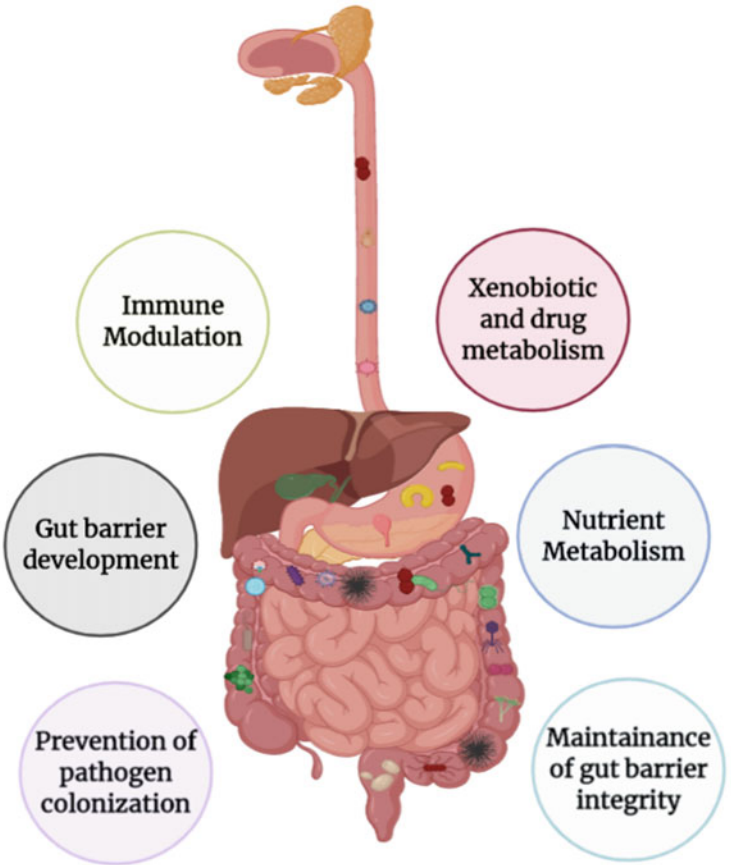

Fig. 2.1 Roles of gut microbes in human gastrointestinal health: functions of gut microbiota include food digestion, production of essential vitamins, absorption of nutrients, immune modulation, and resistance of colonization by pathogenic microbes

Lachnospiraceae and *Bacteroidetes* (Sekirov et al. 2010; Dieterich et al. 2018). The commensal bacteria in the gastrointestinal tract perform several essential functions including nutrient metabolism, xenobiotic and drug metabolism, host immune modulation, gut barrier development, and prevention of pathogen colonization (Fig. 2.1).

Nutrient Metabolism Gut bacteria make essential contribution to the metabolism of nutrients in the host through specific enzymes that are not encoded by the human genome. They particularly aid in breaking down indigestible polysaccharides and polyphenols. Bacteria aid in the digestion of carbohydrates into short-chain fatty acids (SCFA) such as acetate, butyrate, succinate, and propionate which in turn are beneficial to the host. Carbohydrates are digested into butyrate and propionate mainly by *Lachnospiraceae*, *Negativicutes*, *Clostridium*, and *Bacteroides* species and *Faecalibacterium prausnitzii*, *Bacteroides thetaiotaomicron*, *Ruminococcus*

obeum, and *Roseburia inulinivorans* (Macfarlane and Macfarlane 2003; Jandhyala et al. 2015). *Bacteroides* species can express glycoside hydrolase, polysaccharide lyases, and glycosyl transferases necessary for host carbohydrate metabolism (Cantarel et al. 2012). Gut bacteria are also involved in lipid metabolism by enhancing lipase activity (Jandhyala et al. 2015). For example, *Bacteroides thetaiotaomicron* reportedly expresses a colipase, necessary for efficient lipid digestion by pancreatic lipase (Hooper et al. 2001). Furthermore, gut bacteria can aid in the synthesis of vitamins, including vitamin K and group B vitamins such as pyridoxine, thiamin, riboflavin, biotin, cobalamin, and folates, which are essential for human health. It was reported that human subjects on low vitamin K diets coupled with gut bacteria depletion, by antibiotics treatment, showed significantly suppressed prothrombin levels compared with subjects on low vitamin K diets alone (Frick et al. 1967). The predominant producer of riboflavin and biotin are the *Bacteroidetes*, *Fusobacteria*, and *Proteobacteria*, while the major producers of vitamin B12 are members of the *Fusobacteria* phylum (Magnusdottir et al. 2015). Moreover, gut bacteria, namely, *B. intestinalis*, *B. fragilis*, and *Escherichia coli*, can deconjugate and dehydrate primary bile acids and convert them into secondary bile acids such as deoxycholic and lithocholic acids in the human colon (Fukiya et al. 2009). Polyphenols present in diet are also metabolized into active molecules by the gut bacteria. Flavanols in onions and grapes are metabolized by *B. diastonis*, *B. uniformis*, and *Eubacterium ramulus* (Winter et al. 1989; Rechner et al. 2004; Schneider et al. 2000), while chlorogenic acids in peach, plums, and coffee are metabolized by *E. coli*, *Bifidobacterium* sp., and *Lactobacillus gasseri* (Couteau et al. 2001).

Xenobiotic and Drug Metabolism Humans are continuously exposed to xenobiotics from dietary components, pharmaceuticals, and environmental chemicals. Several studies have presented evidences for the gut bacteria in metabolizing xenobiotics, which are unmetabolizable by human enzymes. In a global study, 850 bacteria genera with xenobiotic-metabolizing potential were identified. Notably, lower bacteria diversity was associated with high rate of xenobiotic drug consumption (Das et al. 2016). *Comamonadaceae* and *Burkholderiaceae* which are abundant in the aerial gut environment possessed larger xenobiotic-metabolizing repertoire (Das et al. 2016), an observation which can aid in issues related to drug bioavailability, drug overdose, and side effects. Moreover, the gut bacteria contribute broader range of glycosidases, sulfatases, lyases, and proteases which are important in altering the physical properties and activities of xenobiotic compounds. For example, gut bacterial C-S B-lyases could cleave polychlorinated biphenyls to produce thiol metabolites for further methylation in host tissues (Claus et al. 2016).

Immune Modulation The gut bacteria can modulate both innate and adaptive immunity in the gastrointestinal tract. Colonization of germ-free mice with a community of eight bacterial species comprising two *Lactobacilli*, one *Bacteroides*, one *Flexispira*, and four *Fusobacterium* species, selected for their dominance in mice microflora, resulted in compartmentalized expansion, activation, and de novo generation of mucosal Treg cells in the colon lamina propria. The induced Treg cells

were important for homeostasis of CD4 T cells, reflected by the absence of mucosal Th17 or Th1 cell responses (Geuking et al. 2011). *Bacillus fragilis* have also been characterized to induce Treg cells via TLR2 signaling cascade. Also, germinal centers and Peyer's patches, which are major sites of gut immunoglobulin A immune response generation, are induced by gut bacteria. The direct stimulation of Peyer's patches with bacterial products and retinoic acid enhanced the expression of chemokine CXCL13 (Suzuki et al. 2010). Metabolite indole-3-aldehyde, synthesized by *Lactobacillus*, can stimulate innate lymphoid cells via the aryl hydrocarbon receptor to induce the expression of interleukin 22, which in turn contribute to the maintenance of gut homeostasis (Liu et al. 2016).

Gut Development and Barrier Integrity There are evidences that the gut bacteria are important for gut permeability integrity. Gut bacteria can induce the expression of angiogenin, a transcription factor important in the development of intestinal microvasculature (Stappenbeck et al. 2002). Modulation of mucosal glycosylation by gut bacteria is another means through which gut bacteria can protect the gut barrier (Cayuela 2000). Germ-free mice generally have thin villi (Banasaz et al. 2002), increased cell cycle time (Alam et al. 1994), impaired peristalsis (Husebye et al. 1994), and reduced villus capillary network (Jandhyala et al. 2015). For example, *B. thetaiotaomicron* can stimulate the expression of fucose on epithelial cell surface glycoconjugates (Hooper and Gordon 2001) and induce the expression of small proline-rich protein 2A (sprr2A), required for maintaining desmosomes of the epithelial villus (Lutgendorff et al. 2008). Moreover, peptidoglycan, the bacterial cell wall component, can maintain tight junctions through TLR2-mediated signaling (Cario et al. 2007). Furthermore, soluble proteins, namely, p40 and p75 produced by *Lactobacillus rhamnosus*, can protect the gut by preventing cytokine-induced epithelial cell apoptosis in a manner dependent on epithelial growth factor receptor (EGFR) and protein kinase C (Shen et al. 2018).

Prevention of Colonization by Pathogens Enteric bacteria can also confer protection against colonization by pathogens. Certain commensal bacteria can secrete molecules with bactericidal or bacteriostatic properties (Zipperer et al. 2016). Examples include bacteriocins by Gram-positive *Enterobacteriaceae* and microcins by Gram-negative *Enterobacteriaceae* (Sassone-Corsi et al. 2016). *Enterococcus faecalis* was demonstrated to produce plasmid-encoded bacteriocin to prevent infection of mice gastrointestinal tract by vancomycin-resistant *Enterococcus* (Kommineni et al. 2015). *R. obeum* can prevent mice colonization by *Vibrio cholerae* through the production of quorum-sensing signal AI-2, which disrupts the expression of *V. cholerae* pilus operon needed for intestinal colonization (Hsiao et al. 2014). Moreover, bacteria can metabolize host-derived molecules into secondary metabolites that can confer protection against pathogens. *Clostridium scidens* encodes 7a-hydroxysteroid hydrogenase enzyme, which converts primary bile acids to secondary bile acids. *C. scidens* is associated with resistance to *Clostridium difficile* infection (Buffie et al. 2015). Colonization of antibiotics-treated mice with *C. scidens* increased intestinal secondary bile acids and improved the survival of *C. difficile*-infected mice (Buffie et al. 2015). Furthermore, gut bacteria

can prevent infection of the gastrointestinal tract by pathogens through nutrient deprivation. It was demonstrated that the infection of mice gut with pathogenic *E. coli* O157:H7 was severely impaired in mice pre-colonized with two commensal *E. coli* HS and Nissle 1917 strains. This was reportedly due to competition for important sugars needed for the growth of *E. coli* O157:H7 in the gut (Maltby et al. 2013).

2.2.2 Functions of Virus in the Gastrointestinal Tract

Human gastrointestinal virome comprises of nucleic acids including single-stranded (ss) RNA, double-stranded (ds) RNA, ssDNA, and dsDNA that belong to viral-like particles and resident in healthy human gut. Human endogenous retroviruses, eukaryotic viruses, and bacteriophages have been described as part of the human virome (Shkoporov et al. 2019). Although present in comparatively low proportions, the adult colon-associated virome is stable and correlates with the bacteriome and with varied diversities among individuals (Beller and Matthijnssens 2019; Shkoporov and Hill 2019). Most bacteriophages from human gut belong to order *Caudovirales* and families *Myoviridae*, *Podoviridae*, *Siphoviridae*, and *Microviridae* (Beller and Matthijnssens 2019). Viral populations have also been described in human esophagus (Deshpande et al. 2018b) and gastric compartment (Hu et al. 2018b). Although viruses can infect the pancreas and the liver, commensal virome has not been described in them. Gut viruses can be involved in host beneficial functions with evidences from colon-associated virome.

Control of Bacteria Population The lytic and lysogenic life cycles of certain phages can be linked with their potential to control the population of host bacteria. Studies involving marine phages showed the ability of some phages to induce bacterial reduction in response to temperature and radiation (Weinbauer and Suttle 1996). In human, the proportion and identity of strictly lytic and lysogenic enteric phages have been shown to vary according to health status. It was therefore speculated that maintenance of optimal bacterial population in the gut by bacteriophages is possible and may contribute to gut health (Santiago-Rodriguez and Hollister 2019). Phages can lyse invading pathogenic bacteria and thus contribute to innate immunity against infections (Barr et al. 2013). Additionally, gut phages in healthy individuals were shown to correlate with the bacteriome (Shkoporov and Hill 2019), further supporting their role in bacteria population control. Some phages can change the fitness and phenotype of host bacteria by genetic transfer, resulting in evolutionary advantages. Lysogenic conversion in the human gut may also promote niche-specific bacterial colonization along the gastrointestinal tract, which is characterized by varied competitive ecosystems (Santiago-Rodriguez and Hollister 2019).

2.2.3 Functions of Fungi in the Gastrointestinal Tract

Enteric fungi are key components of the gut microbiome. Although the composition of gut fungi has been shown to be lower and less stable compared to bacteria and largely influenced by environmental factors, majorly diet (Hallen-Adams and Suhr 2017), enteric fungi do contribute to gastrointestinal and systemic health of the host. Fungi in a healthy gut are usually dominated by the *Ascomycota* and *Basidiomycota* phyla. The mostly widely reported fungi genera in the gastrointestinal tract include *Candida*, *Saccharomyces*, *Malassezia*, *Cladosporium*, *Cryptococcus*, *Fusarium*, *Penicillium*, *Galactomyces*, *Pichia*, *Trichosporon*, and *Aspergillus* (Hallen-Adams and Suhr 2017; Coker et al. 2019). Enteric fungi have been described with the capacity to calibrate host immunological responses. A recent study revealed that gut mycobiota could recapitulate the protective effect of commensal bacteria in mice (Jiang et al. 2017). Mice treated with broad-spectrum antibiotics developed more severe dextran sodium sulfate (DSS)-induced colitis and generated reduced levels of protective $CD8^+$ T cells when infected with influenza A virus. However, gavage of antibiotics-treated mice with *Saccharomyces cerevisiae* and *Candida albicans* reversed the effect of bacterial depletion. The protective impact of the two fungal species was demonstrated to be due to mannans, an abundant component of fungal cell walls (Jiang et al. 2017). Innate immune receptors such as Dectin-1, a C-type lectin receptor, specialize in the recognition of fungal B-1,3 glucan (Iliev et al. 2012). Dectin-1 activates intracellular caspase recruitment domain protein 9 (CARD9) which leads to induction of T helper 17 (TH17) immune responses (Cheng et al. 2011; Gringhuis et al. 2012; LeibundGut-Landmann et al. 2007). Increased disease susceptibility had been demonstrated in mice lacking Dectin-1 (Taylor et al. 2007). Human monocytes stimulated in vitro with β-glucan or chitin, another fungal cell wall component, showed changes in their ability to secrete pro-inflammatory cytokines (Rizzetto et al. 2016). However, variations have been reported for the capacity of fungi and its components to modulate immunocytes (Rizzetto et al. 2010, 2013; Wagener et al. 2017). The diverse enteric symbiotic fungal species may, therefore, modulate homeostatic immune responses through different mechanisms, subject to the composition of the mycobiota in each individual (Rizzetto et al. 2014). Notably, fungi were described as key drivers of secondary lymphoid organ (Koslowski, #3) maturation in mice (Zhang et al. 2016). Antifungal, but not antibiotics, treatment of mice dampened the migration of dendritic cells (DCs) expressing retinol dehydrogenase enzyme ($RALDH^+$ DCs) into SLOs, while inoculation of neonates with a single species of murine indigenous mycobiota, *Candida tropicalis*, augmented the numbers of $RALDH^+$ DCs in lymph nodes (Zhang et al. 2016). Enteric fungi can also promote T cell responses. Antibiotics-treated mice colonized with *C. albicans* developed strong Th17 responses without any obvious signs of intestinal inflammation (Atarashi et al. 2015; Leonardi et al. 2018). Moreover, antibiotics-treated mice inoculated with *C. albicans* produced effector and memory T cells in the gut (Xin et al. 2014). In human, *C. albicans*-specific T cells can secrete mixed Th1-Th17 phenotype IL-17A, IL-22, and IFN-γ

(Zielinski et al. 2012) cytokines. As such, *C. albicans* may drive diverse and functional T cell responses in the gut. It is possible that mycobiota-driven T cell subsets can confer cross-protection against infections, since microbiota-induced T cell subsets have been described to mediate heterologous protection against pathogens at the mucosal surface (Ivanov et al. 2009; Wang et al. 2019).

Moreover, fungi can secrete a variety of metabolites that can potentially modulate host tissue function. One of the most frequently identified fungi in the gastrointestinal tract is *Malassezia*. On cutaneous surfaces, members of *Malassezia* produce metabolites that serve as potent ligands for the aryl hydrocarbon receptor (Ahr), such as malassezin, pityriacitrin, and indolo[3,2-*b*]carbazole (Gaitanis et al. 2008; Mexia et al. 2015). These metabolites can promote epithelial repair, melanogenesis, and barrier homeostasis (Esser and Rannug 2015; Furue et al. 2014). Skin *Malassezia* species also secrete lipases and phospholipases to convert skin triglycerides into short-chain fatty acids (Velegraki et al. 2015; White et al. 2014). It is postulated that the many undefined metabolites derived from the mycobiota may play similar role in the gut. In addition to the colon, stable fungi populations have been found in the esophagus (Deshpande et al. 2018b), the stomach (von Rosenvinge et al. 2013; Sam et al. 2017), and the pancreas (Aykut et al. 2019b).

2.2.4 Functions of Archaea in the Gastrointestinal Tract

Archaea are a group of single-celled prokaryotes with unique molecular characteristics such as lack of peptidoglycan and D-glycerol esters or fatty acids, distinguishing them from bacteria and eukaryotes (Kandler and Konig 1998). Most archaea are found in extreme acidophilic, alkaliphilic, halophilic, and thermophilic ecosystem (Eme et al. 2018). However, some species are mesophilic (Brochier-Armanet et al. 2008) and have been isolated from human skin, nose, lungs, oral cavity, and vagina (Lurie-Weinberger and Gophna 2015). They are also reportedly stable commensals of the gastrointestinal tract where they participate in host beneficial biological processes. The most widely studied is the role of archaeal methanogens such as *Methanobrevibacter smithii*, *Methanosphaera stadtmaniae*, and *Methanomassiliicoccus luminyensis* in methanogenesis. Methanogenic archaea are responsible for reducing carbon dioxide, produced during bacterial fermentative nutrient digestion, into methane in the presence of hydrogen (Roccarina et al. 2010). This process is essential to facilitate excess hydrogen removal from the gut, because accumulated hydrogen can inhibit digestive processes and impair energy derivation from food (Gaci et al. 2014). Archaea undergoes active metabolism and optimizes the fermentation and metabolic pathways of fermentative bacteria in the human gut (Nakamura et al. 2010). Archaea can also aid in the removal of trimethylamine (Tanji, #17) from the gut. TMA is produced from bacteria digestion of dietary choline, betaine, and carnitine. TMA is transferred to the liver and oxidized through the action of flavin monooxygenase into trimethylamine-*N*-oxide (TMAO), a molecule that has been mechanistically linked to cardiovascular disease, atherogenesis,

and fish odor syndrome (Koeth et al. 2013; Wang et al. 2011, 2015). It was reported that *Methanobrevibacter smithii*, *Methanosarcina mazei*, and *Methanomicrococcus blatticola* which could use TMA as growth substrates essentially reduced plasma concentration of TMAO, with a atherosclerosis reduction tendency in mice (Ramezani et al. 2018) and immune modulation (Brugere et al. 2014; Blais Lecours et al. 2014). Moreover, just like commensal bacteria, gut archaea has also been shown to be capable of activating antigen-specific adaptive immune responses and may be important for maintaining immune homeostasis in human health (Bang and Schmitz 2015).

2.3 Microbiome in Gastrointestinal Cancers

Cancers in the gastrointestinal tract account for more than 34% of cancer-related deaths (Bray et al. 2018) and remain a serious global concern. Carcinogenesis is a multifactorial process that involves both genetic and environmental factors. An evolving prominent environmental factor in the pathogenesis of gastrointestinal cancers is the gut microbiome. This is especially so because the entire length of the gastrointestinal tract has direct contact with microorganisms whose genetic capacities are largely unique and have been studied to influence host metabolic processes as described above. Compositional and ecological alterations of the gut microbiome are associated with esophageal, gastric, liver, pancreatic, and colon cancers. Majority of studies on gut microbiota dysbiosis and gastrointestinal cancers have been carried out on bacteria, apparently due to its high abundance and availability of comparatively more defined reference databases. With the advancement of sequencing technologies, studies on the role of enteric fungi, virus, and archaea in gastrointestinal cancers are emerging and are described below.

2.3.1 Microbiome Alteration in Esophageal Cancer

Esophageal cancer is multifactorial, developing from a complex interplay of host genetic and epigenetic factors, host immune response, as well as environmental factors, of which the microbiome is increasingly identified to be important (Yang et al. 2009; Blackett et al. 2013; Macfarlane et al. 2007). Alteration of esophageal-associated microbiome has been reported in esophageal adenocarcinoma (EAC) patients as well as in patients with Barrett's esophagus (BE), a high-risk group for EAC development (Lv et al. 2019). Reduced bacterial diversity in esophageal microbiome is associated with EAC. In particular, Gram-negative bacteria such as *Veillonella*, *Neisseria*, *Leptotrichia*, *Fusobacterium*, *Campylobacter*, and *Capnocytophaga* are most often enriched in EAC patients (Yang et al. 2009, 2014; Macfarlane et al. 2007). The shift from Gram-positive aerobic microbiota to Gram-negative anaerobic microbiota may enhance the production of inflammatory

cytokines and chemokines. Additionally, increased lipopolysaccharide (LPS) from Gram-negative bacteria may stimulate toll-like receptors (TLRs) on host cell surface. For example, the esophageal epithelial expression of TLR4, which could activate nuclear factor kappa B (NF-κB) signaling cascade, was increased in both of BE and EAC patients compared to control subjects (Yang et al. 2012). Gram-positive *Granulicatella*, *Rothia*, and *Lactobacillus* are also associated with EAC (Yang et al. 2012). Dysregulated lactate metabolism is one of the distinctive feature of carcinogenesis (San-Millan and Brooks 2017; Flemer et al. 2018). Bacterial lactic acid production pathways such as homolactic and heterolactic fermentation were found increased in EAC subjects. In particular, *Lactobacillus fermentum* was found enriched in EAC patients compared to control subjects.

Non-bacterial microbes including virus, fungi, and archaea have been reported in healthy human esophagus, signifying commensal relationships with the host (Deshpande et al. 2018a). However, changes in esophageal mycobiome and virome in esophageal cancer have not been described. Infections with *C. albicans* were described in esophageal squamous cell carcinoma (ESCC) patients (Rautemaa et al. 2007), while colonization with oral fungi, including *Cladosporium cladosporioides*, increased the formation of esophageal cancer in mice model (Zhu et al. 2017). The importance of fungi to the development of esophageal cancer was further demonstrated by the prevention of ESCC in mice following antifungal treatment (Zhu et al. 2017). Moreover infections with human papillomavirus (HPV) and Epstein-Barr virus (EBV) are associated with the development of ESCC (Xu et al. 2015).

2.3.2 Microbiome Alteration in Gastric Cancer

Helicobacter pylori is a well-established risk factor for gastric cancer (Kumar et al. 2019). *H. pylori* infects more than 50% of global population and induces gastric inflammation, thereby increasing the risk of gastric diseases including cancer (Ruggiero 2010). The chance of gastric cancer development is determined by the pathogenic potential of the infecting *H. pylori*. The presence of cag pathogenicity island (cag PAI), which comprise of genes encoding bacterial type IV secretion systems, is a well-characterized virulence determinant of *H. pylori*. Strains of *H. pylori* with cag PAI possess increased potential to promote severe gastritis, atrophic gastritis, and gastric cancer compared to strains without cag PAI. *H. pylori* virulence determinants also include its ability to express vacuolating VacA toxin, adhesins, and virulence-associated outer membrane proteins (Sgouras et al. 2015). Moreover, host factors such as genetic polymorphism that favor the high expression of pro-inflammatory cytokines such as interleukin-1B (IL-1B) and tumor necrosis factor alpha (TNF-alpha) or low expression of anti-inflammatory cytokines including interleukin-10 (IL-10) are associated with the enhanced risk of gastric cancer development in *H. pylori*-infected individual (Shanks and El-Omar 2009). In addition to the virulence potential of infecting *H. pylori* strain and host genetic

polymorphisms, high salt intake, helminth infection, and smoking can enhance the chance of developing gastric cancer in infected individuals (Wroblewski et al. 2010).

Modern sequencing technologies have enabled the discovery of other commensal bacteria that can thrive at the low pH condition of the stomach, in addition to *H. pylori*. *H. pylori* dominate the gastric microbiota of infected individuals, revealing its ability to modulate the gastric microbiota (Gantuya et al. 2019; Noto and Peek 2017). Whether the suppression of other gastric microbes by *H. pylori* represents a means to support its gastric cancer-promoting ability is yet to be defined. There are evidences that *non-H. pylori* bacteria may be involved in promoting gastric cancer. *H. pylori* infection concomitant with colonization by intestinal flora accelerated the development of gastrointestinal intraepithelial neoplasia using insulin promoter regulating the overexpression of gastrin (INS-GAS) mouse model, signifying the importance of *non-H. pylori* gastric microbes in gastric cancer development (Lertpiriyapong et al. 2014). Alterations of gastric microbiota along the stages of human gastric carcinogenesis have been described, although there are inconsistencies on the direction of change in bacterial diversity (Wang et al. 2016; Eun et al. 2014; Ferreira et al. 2018; Aviles-Jimenez et al. 2014; Coker et al. 2018; Hu et al. 2018a). Bacteria genera *Streptococcus*, *Prevotella*, *Veillonella*, and *Lactobacillus* are recurrently identified to be more abundant in gastric cancer patients compared to patients with superficial gastritis, atrophic gastritis, or precancerous intestinal metaplasia (Coker et al. 2018; Jo et al. 2016; Wang et al. 2016; Aviles-Jimenez et al. 2014; Ferreira et al. 2018). Moreover, an overgrowth of microbes of potential oral origin was positively associated with gastric cancer (Castano-Rodriguez et al. 2017; Coker et al. 2018). Oral microbes *Peptostreptococcus stomatis*, *Slackia exigua*, *Parvimonas micra*, *Streptococcus anginosus*, and *Dialister pneumosintes* were indicated to be significantly important in gastric carcinogenesis, from their significant contribution to gastric microbial ecology of gastric cancer patients (Coker et al. 2018). The involvement of non-*H. pylori* oral microbes in the development of gastric cancer was further demonstrated by the positive association of *Peptostreptococcus*, *Streptococcus*, *Parvimonas*, *Prevotella*, and *Granulicatella* with the emergence and persistence of gastric atrophy and intestinal metaplasia, 1 year after *H. pylori* eradication therapy (Sung et al. 2020). How the gastric cancer-associated microbes function to promote carcinogenesis remains unclear. A widely supported notion is that nitrosating bacteria may convert nitrogen compounds in gastric fluid to potentially carcinogenic *N*-nitroso compounds (NOCs) (Mowat et al. 2000; Weng et al. 2019). Consistent with this view, nitrosating bacteria including *Veillonella*, *Neisseria*, and *Clostridium* species were reportedly twofold enriched in gastric cancer patients without *H. pylori* infection (Jo et al. 2016). *Nitrospirae* bacteria were also found present in all 103 gastric cancer patients but absent in 212 chronic gastritis patients with chronic gastritis in a separate study (Wang et al. 2016), supporting a role for NOCs in gastric carcinogenesis.

Fungi such as *Candida* and *Phialemonium* are able to survive acidic ecosystem and are present in gastric fluids (von Rosenvinge et al. 2013; Schulze and Sonnenborn 2009). Compared to bacteria, the potential role of fungi in gastric carcinogenesis is largely unexplored. However, gastric growth of yeast and

pseudohyphae of *Candida* were observed in a patient with intestinal gastric adenocarcinoma (Subramanian et al. 2015), while fungal infection, worsened with increased age, was associated with delayed gastric ulcer healing (Minoli et al. 1982). Given that impaired host immunity contributes to susceptibility to fungal infections (Kumar et al. 2018), it is plausible that opportunistic gastric fungi may play a role in gastric carcinogenesis under immunocompromised conditions.

2.3.3 Microbiome Alteration in Pancreatic Cancer

Pancreatic cancer is a lethal and devastating disease. Pancreatic ductal adenocarcinoma (PDAC) patients have been characterized with distinct microbiome at oral, gut, and pancreatic tissues, compared with healthy subjects (Wei et al. 2019). Due to its high alkalinity and presence of several proteases, the pancreas was considered a sterile organ. However, using shotgun metagenomics analysis, pancreatic cancer-associated microbiome has been described, including about 1000-fold increase of bacteria in pancreatic tissues of PDAC patients (Pushalkar et al. 2018; Dickson 2018). Direct comparison of the gut and pancreas microbiomes in PDAC patients revealed increased translocation of Gram-negative *Proteobacteria* to the pancreas. *Prevotella* and *Bacteroides* were more abundant in the gut of PDAC patients (Pushalkar et al. 2018). Investigation of intratumoral microbiota in PDAC progression and immunotherapy response in mice revealed the enrichment of *Bifidobacterium pseudolongum*, underscoring the significance of the intratumoral microbiota in PDAC (Pushalkar et al. 2018). Mechanistically, PDAC-associated microbiome was shown to drive suppressive monocytic cellular differentiation in pancreatic cancer through selective TLR ligation leading to T-cell anergy. Bacterial ablation protected against oncogenesis and reversed intratumoral immune tolerance in mice (Pushalkar et al. 2018).

Moreover, the fungi composition and diversities in pancreatic tumor tissues of PDAC patients were found to be distinct and about 3000-fold more than in healthy control subjects (Aykut et al. 2019a). Gut fungi, enriched in *Malassezia*, infiltrated the pancreas, both in human and mouse PDAC. Repopulation with *Malassezia* species accelerated PDAC in mice, following antifungal treatment, signifying that particular species of fungi may sufficiently promote the progression of PDAC and that the mycobiome may be a new therapeutic target (Aykut et al. 2019b) in pancreatic cancer therapy.

2.3.4 Microbiome Alteration in Liver Cancer

The liver does not contain its own microbiome; however, there are mounting evidences that the gut microbiota plays important roles in the development of liver diseases and the pathogenesis of hepatocellular carcinoma (HCC) (Weng et al.

2019). The gut microbiota is crucial for the maintenance of gut barrier function and host immunity. Therefore, alteration of the gut microbes affecting these functions may be detrimental to distal organs including the liver, which connects to the gut through the portal vein. Compromised gut permeability exposes the liver to microbial metabolites, toxins, and microbiota-associated molecular patterns (Csak et al. 2011). These patterns are recognized by immune receptors on liver cells such as Kupffer cells and hepatic stellate cells, which initiate and maintain inflammatory cascades that can lead to liver damage, including HCC (Anand et al. 2016). Patients with chronic liver diseases reportedly manifested shifts in the composition of the gut microbiota when compared to healthy subjects (Anand et al. 2016). In a cross-sectional study, fecal microbial diversity was observed to increase from cirrhosis to HCC, while phylum *Actinobacteria* was increased in early HCC versus cirrhosis (Gupta et al. 2019). Most commonly, healthy bacteria that promotes healthy gut are reduced, while potentially pathogenic bacteria are increased. *Ruminococcaceae* and *Bacteroides* were enriched, while *Bifidobacterium* was found to be depleted in HCC patients compared to healthy subjects. Increased fecal counts of *E. coli* were also observed in cirrhotic patients with HCC (Ponziani et al. 2019).

Hepatic chronic infection with hepatitis B or C virus (HBC or HCV) increases the risk of HCC development (Zamor et al. 2017), with about 56% and 20% of liver cancer attributed to HBC and HCV, respectively (Maucort-Boulch et al. 2018). Studies have shown that the HBV and HCV proteins enhance the population of hepatic cancer stem cells and modulate the epigenetic modification and cancer-associated molecular pathways in the liver (Mani and Andrisani 2018; Sasaki et al. 2017). In addition to the direct role of HBV in inducing cancer formation in the liver, the gut microbiota of HBV-infected HCC (HBV-HCC) patients were reportedly distinct from non-HBV and non-HCV HCC patients in bacterial compositional and functional profiles (Liu et al. 2019a). HBV-HCC patients exhibited increased bacteria richness, reduced pro-inflammatory *Escherichia-Shigella* and *Enterococcus*, and increased *Faecalibacterium, Ruminoclostridium*, and Ruminococcus compared with non-HBV HCC and non-HCV HCC patients and healthy subjects (Liu et al. 2019a). Moreover, compared with healthy subjects, HCV-HCC patients had reduced bacteria diversity, with increased *Streptococcus* and *Lactobacillus* compared to healthy subjects. *Streptococci*-encoded urease genes was found enriched in the predicted metagenome of HCV-HCC patients during disease progression (Inoue et al. 2018). These suggest that the gut microbiota could promote virus-induced HCC in the liver.

Moreover, the involvement of the gut microbiota in the promotion and progression of HCC has been demonstrated in animal studies, in the absence of HCV and HBV. An increase in plasma LPS levels was concomitant with increased tumor size and number in diethylnitrosamine (DEN)-treated rats (Yu et al. 2010). Another study demonstrated that the intestinal mucosa was damaged, leading to increased abundance of Gram-negative bacteria, *E. coli*, *Atopobium*, *Collinsella*, *Eggerthella*, and *Coriobacterium*, and decreased *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* after DEN treatment (Zhang et al. 2012). Translocation of bacteria and bacteria products due to impaired gut barrier can trigger inflammatory response by activating

liver TLRs. LPS from Gram-negative bacteria can stimulate TLR-4, while TLR-2 and TLR-5 can be activated by peptidoglycan and lipoteichoic acid from Gram-positive bacteria (Kawai and Akira 2009, 2010). Chronic activation of TLRs can lead to the production of inflammatory TNF-alpha, IL-1-beta, and IL-6, through NF-κB pathway (Luedde and Schwabe 2011; Pikarsky et al. 2004). Activation by IL-6 through the Janus kinase (JAK) or the STAT3 pathway can induce proliferation and immortalization of hepatic cells, leading to HCC (Jung et al. 2015; Hatziapostolou et al. 2011).

2.3.5 Microbiome Alteration in Colorectal Cancer

Compared to other gastrointestinal sites, a more complicated microbial community covers the colon epithelial surface, participating in many host metabolic processes. The most widely demonstrated index described in the gut microbiome of CRC patients is the reduction in bacteria diversity (Yu et al. 2017; Ahn et al. 2013), although certain studies reported the opposite (Hibberd et al. 2017). Dysbiotic gut microbes play an active role in colon tumorigenesis. The causal relationship between gut bacteria and CRC was demonstrated by a study which showed that transplantation of stools from CRC patients could promote colon tumorigenesis in both conventional and germ-free mice (Wong et al. 2017). Normalization of the gut microbiota close to healthy microbiota, after treatment of CRC patients, has also been reported (Sze et al. 2017). Moreover, a multi-cohort study based on 526 metagenomic samples from China, Austria, the United States, Germany, and France identified seven CRC-enriched bacteria, namely, *Bacteroides fragilis*, *Fusobacterium nucleatum*, *Porphyromonas asaccharolytica*, *Parvimonas micra*, *Prevotella intermedia*, *Alistipes finegoldii*, and *Thermanaerovibrio acidaminovorans*, with a demonstrated area under the receiver operating characteristics curve (AUC) of 0.80 across the different populations (Dai et al. 2018). Moreover, 62 bacteria species, mainly probiotic bacteria including *Streptococcus thermophilus*, *Lactobacillus gallinarum*, and *S. salivarius*, were found to be consistently depleted across all cohorts (Dai et al. 2018). As in gastric cancer, microbes of potential oral origin were highlighted to be consistently enriched in CRC patients (Nakatsu et al. 2015; Flemer et al. 2018).

The mechanisms through which CRC-enriched bacteria including *F. nucleatum*, *P. anaerobius*, and *Bacteroides fragilis* promote colon tumorigenesis have been elucidated. *F. nucleatum* activates the E-cadherin/β-catenin signaling pathway through its FadA adhesin protein, leading to cancerous transformation of colon epithelial cells (Xu et al. 2007; Rubinstein et al. 2013). *F. nucleatum* could also alter the tumor microenvironment (TME) by enriching myeloid-derived suppressor cells (MDSCs) to support colon tumorigenesis (Rubinstein et al. 2013). Enterotoxigenic *B. fragilis* (ETBF), which possesses *bft* gene, encoding *Bacteroides fragilis* toxin (BFT) is associated with CRC. BFT targets the epithelial cell tight junctions, resulting in E-cadherin cleavage, impaired gut barrier, followed by Wnt/β-catenin

and NF-κB signaling (Peloquin and Nguyen 2013), to promote colon cell proliferation. *P. anaerobius* could promote CRC through the binding of its surface protein, putative cell wall binding repeat 2 (PCWBR2) to host cell receptor integrin α2/β1, thereby activating pro-carcinogenic PI3K-Akt-NF-κB signaling cascade (Long et al. 2019).

Evidences of association between fungi and CRC are also emerging as with other intestinal diseases such as IBD (Sokol et al. 2017). Although no significant change in the fungal alpha diversity in CRC patients compared to control subjects, *Basidiomycota* to *Ascomycota* ratio, a measure of fungi ecological dysbiosis was higher in Chinese CRC patients than control subjects. Moreover, distinct fungal composition characterized by increased *Malasseziomycetes* and decreased *Saccharomycetes* was observed in CRC patients. Abundances of 14 fungal biomarkers including species of *Aspergillus*, *Malassezia*, *Rhodotorula*, *Pseudogymnoascus*, *Kwoniella*, *Talaromyces*, *Debaryomyces*, *Moniliophthora*, *Pneumocystis*, and *Nosema* distinguished CRC patients from controls with an AUC of 0.93 and validated AUCs of 0.82 and 0.74 in independent Chinese cohort and European cohort, respectively (Coker et al. 2019). Moreover, abnormal immune responses to fungi are frequently reported in IBD (Qiu et al. 2015) and ulcerative colitis (Sokol et al. 2017), which are CRC risk factors. Such dysregulated immunity may thus be present in CRC patients. Commensal gut fungi reportedly promoted inflammasome activation during AOM-DSS-induced colitis, while antifungal treatment exacerbated colitis and CRC in mice (Malik et al. 2018).

Changes in human virome have been reported in metabolic diseases. Many studies have also found higher presence of viral DNA in CRC tumor tissues compared to normal tissues in human. Individual virus infections with human papillomaviruses (HPV) (Liu et al. 2011; Damin et al. 2007; Bodaghi et al. 2005), human polyomaviruses (Mou et al. 2012; Lin et al. 2008), human herpesviruses (Dimberg et al. 2013; Tafvizi and Fard 2014), human bocavirus (Schildgen et al. 2013), and Inoue–Melnick virus (Ito et al. 1992; Nishibe et al. 1990) were reportedly associated with CRC. Moreover, untargeted metagenomic analysis revealed that the taxonomic composition of gut virome was consistently associated with CRC in multiple cohorts, including Chinese, Austrian, German, and French cohorts. Relative increases in bacteriophage richness and diversity were observed in CRC-associated gut metagenomes compared with those of control subjects (Nakatsu et al. 2018). Differences in the colorectal cancer virome could have been driven by eukaryotic viruses or by bacteriophages (Hannigan et al. 2018). A combination of four CRC-enriched enteric viruses *Betabaculus virus*, *Epsilon15likevirus*, *Mulikevirus*, and *Punalikevirus* was associated with high risk of mortality (Nakatsu et al. 2018).

2.4 Gut Microbe Interactions in Gastrointestinal Health and Cancer

The gut microbiome is a complex ecosystem that needs to be maintained in a homeostatic state for a healthy gut. In the healthy stomach, complex networks of bacteria interactions were reportedly formed among members of the *Proteobacteria* and *Firmicutes* phyla, with *Helicobacter* being negatively correlated with *Prevotella*, *Bacteroides*, *Faecalibacterium*, *Phascolarctobacterium*, and *Roseburia* (Liu et al. 2019b). The positive interactions observed among gastric bacteria in a normal stomach were reportedly absent in peritumoral and tumoral microbes in gastric cancer patients (Liu et al. 2019b). Moreover, the correlation strengths among gastric cancer-associated microbes were found to increase from superficial gastritis to atrophic gastritis, intestinal metaplasia, and gastric cancer (Coker et al. 2018). These suggest that alteration of microbial ecology may promote gastric carcinogenesis.

Compared to control subjects, polymicrobial ecological interactions were found to be disturbed in CRC patients. Bacterial community ecology was observed with lower strength of associations across stages, from normal to adenoma and to CRC at the mucosal surface (Nakatsu et al. 2015). Intra-phylum interactions within the *Firmicutes* phylum were higher in healthy subjects than in adenoma and CRC patients (Nakatsu et al. 2015). Synergistic relationships between gut bacteria and fungi have been indicated in healthy subjects, while antagonistic bacteria-fungi associations were observed in CRC patients. Within the fungi kingdom, co-occurring intra-kingdom relationships were found enhanced in CRC patients compared with control healthy subjects (Coker et al. 2019). Alteration of gut bacteria by antibiotic treatment has been shown to lead to fungi overgrowth and increased colonization by *Candida albicans*, an opportunistic fungal pathogen associated with gastrointestinal and systemic candidiasis (Kobayashi-Sakamoto et al. 2018).

Moreover, negative association determined between bacterial and viral community diversities in control subjects was lost in CRC patients (Nakatsu et al. 2018), supported by the role of bacteriophages in bacteria population control in a healthy gut (Santiago-Rodriguez and Hollister 2019). The diversities of bacteria and archaea in the colon were observed to be positive in healthy guts (unpublished data). This may be explained by the role of archaea as electron acceptors for substrates originating from anaerobic digestive processes of gut bacteria (Gaci et al. 2014). Although interactions among virus, fungi, and archaea in the colon are yet unexplored, it is admissible that balanced fungal, bacterial, and viral ecological interactions are important in a healthy gut but are disrupted in CRC (Fig. 2.2).

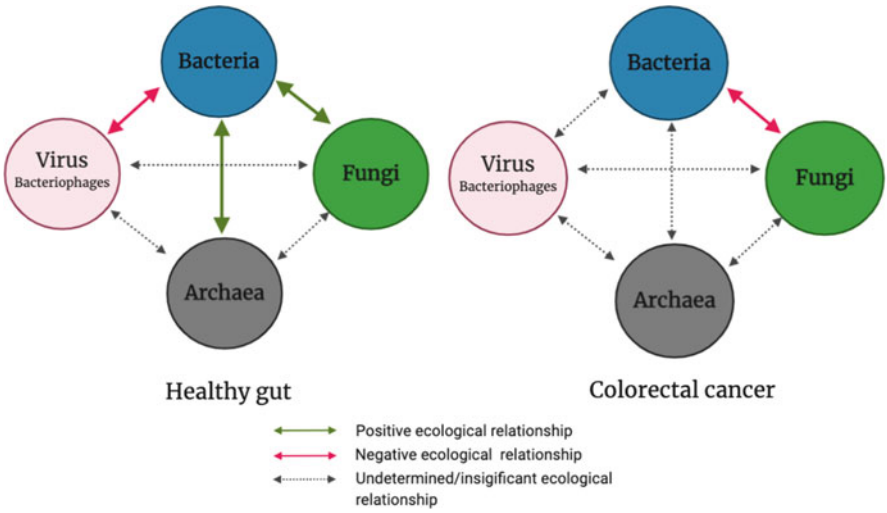

Fig. 2.2 Ecological network depicting interactions among bacteria, fungi, virus, and archaea in healthy gut and in colorectal cancer. Synergistic ecological relationship between bacteria and fungi and between bacteria and archaea in healthy subjects is reversed or lost, respectively, in colorectal cancer. Antagonistic relationship between bacteria and bacteriophages is lost in colorectal cancer

2.5 Therapeutic Manipulation of the Gut Microbiome for Prevention and Treatment of Gastrointestinal Cancers

It is agreeable that gut microbiota imbalance plays significant role in the pathogenesis of gastrointestinal cancers. Thus, modulation of the gut microbiota toward enhancing its gut protective functions is widely recommended as a promising strategy in gastrointestinal cancer prevention and treatment (Wong and Yu 2019).

2.5.1 Fecal Microbiome Transplantation

Fecal microbiota transplantation (FMT) is a process of transplanting stools from healthy donors into the gastrointestinal tract of recipients with the aim of gut microbiota restoration. Successful application of FMT has been recorded in gastrointestinal diseases such as recurrent *Clostridium difficile* infection, irritable bowel syndrome, constipation, and colitis through restoration of bacterial diversity, metabolites, and bile acid metabolism (Kelly et al. 2015; Konturek et al. 2015). The concept behind FMT is the competitive niche exclusion of dysbiotic microbiome by "normal" and beneficial microbiome. The active involvement of gut microbiota in gastrointestinal carcinogenesis has been described for gastric cancer, pancreatic

cancer, liver cancer, and colorectal cancer. It is possible that the replacement of the pro-carcinogenic microbial ecosystem with a healthy one may be important in prevention and treatment of gastrointestinal cancers. The potential usefulness of FMT in the treatment of pancreatic cancer has been demonstrated. FMT from patients with long-term survival of pancreatic cancer, characterized by high bacterial diversity, modulated the tumor microbiome and reduced tumorigenesis via immunosuppression in mice (Riquelme et al. 2019). As a potential application in liver cancer therapy, FMT alleviated precancerous steatohepatitis by inducing decreased intrahepatic accumulation of lipid, triglyceride, and cholesterol in mice fed with high-fat diet. This was described as a result of increased bacterial diversity and abundance of beneficial *Lactobacillus*, *Christensenellaceae*, and butyrate concentration facilitated by FMT (Zhou et al. 2017). A human pilot study also showed that FMT increased survival and resolved ascites in patients with HCC-predisposing severe alcoholic hepatitis. Moreover, the benefit of gut microbiota modulation by FMT in the treatment of chronic hepatitis B, hepatic encephalopathy, and liver cirrhosis in human has been reported (Ren et al. 2017; Bajaj et al. 2017). Mechanistic studies have determined that the gut microbiota from CRC patients play direct role in colon tumorigenesis by activating Wnt signaling pathway which play essential role in tumor development (Wong et al. 2017; Li et al. 2019). The potential therapeutic ability of healthy microbiome against colon tumorigenesis was demonstrated in the improved fitness and resistance to CRC shown by laboratory mice transplanted with stools from wild mice which had higher relative abundance of *Bacteroides* and *Proteobacteria* and lower abundance of *Firmicutes*, *Tenericutes*, and *Verrucomicrobia* (Rosshart et al. 2017).

Some undesirable outcomes ranging from abdominal tenderness, fatigue, nausea, and potential transfer of diseases to recipients can occur following FMT (Russell et al. 2018). A consortium of known beneficial gut bacteria can alternatively be transferred to patients for potential modulation of the gut microbiome. It was demonstrated that the transplantation of a bacterial consortium of 11 bacteria species from *Actinobacteria*, *Proteobacteria*, *Firmicutes*, *Fusobacteria*, and *Bacteroides* phyla, purified from mouse stools, proffered comparable effects in the restoration of barrier integrity in mice with microbiota dysbiosis (Li et al. 2015). With standardization of fecal material preparation, these evidences support the future application of FMT in treatment of gastrointestinal diseases including cancer.

2.5.2 Phage Therapy

An alternative to antimicrobials in targeting known pathogenic microbes is phage therapy. Bacteriophages inject their genome into bacterial cells through specific recognition of protein receptors and replace the bacteria genome, thereby preventing bacterial replication and infection. Bacteriophages are highly specific against strains of bacteria. As such, risks of side effects and development of bacterial resistance are reduced. Phage therapy has been used to reduce pathogenic bacteria. Patients can be

administered multiple lytic phages or a combination of phages and antibiotics (Paule et al. 2018; McCallin et al. 2013, 2018). Human studies have shown that oral bacteria are important in esophageal, gastric, pancreatic, and colon cancers (Zhang et al. 2019). The ecological analysis between oral bacteria and bacteriophages was found to be altered in CRC patients compared to healthy control subjects. *Streptococcus phage K13*, *Streptococcus phage pH10*, *Streptococcus phage SpSL1*, and *Erwinia phage phiEaH2* exhibited stronger positive correlation with oral bacteria in healthy subjects, but not in late CRC patients (Nakatsu et al. 2018). Owed to the bacterial population control ability of bacteriophages, restoration of depleted bacteriophages may be employed in gastrointestinal cancer therapy. Moreover, efforts have been put to increase the specificity of certain phages in patients undergoing phage therapy (Schooley et al. 2017). Bacteriophages may be engineered to carry genes encoding metabolites such as SCFA that can positively modify commensal bacteria toward prevention or treatment of gastrointestinal cancers (Paule et al. 2018).

2.5.3 Use of Antimicrobials

Antimicrobials against known pathogenic microbes may be effective in preventing the initiation and progression of diseases. Antibiotics regimen targeting *H. pylori* can reduce the incidence of gastric cancer (Kumar et al. 2020). The use of antimicrobials can alter gut microbiota composition. Increased gastric bacterial diversities concomitant with reduced inflammation were observed in subjects after *H. pylori* eradication with 1 week regimen of clarithromycin omeprazole and amoxicillin (Sung et al. 2020). Long-term administration of antibiotics cocktail comprising ampicillin, metronidazole, and vancomycin attenuated colon tumorigenesis in mice. Moreover, antifungal treatment reduced tumor by up to 40% in mouse model of pancreatic cancer (Aykut et al. 2019b). The use of antimicrobials for gut microbiota modulation is, however, not recommended due to their nonspecificity and the fact that many gastrointestinal microbes are unculturable and unknown. As such potential detrimental effects of antibiotics on unknown "beneficial" microbes are obscure. Moreover, uncontrolled used of antimicrobials can lead to the emergence of resistance. For example, increased prevalence of *H. pylori* resistance to clarithromycin-based therapy has been reported. Adoption of more effective empirical treatment such as bismuth quadruple and levofloxacin and use of probiotics as adjuncts in *H. pylori*-targeted eradication therapy are being recommended.

2.5.4 Probiotics and Prebiotics

Ingestion of probiotics or prebiotics promotes gut health and has the capacity to prevent gastrointestinal diseases including cancers (Gorska et al. 2019). Many

studies have revealed the beneficial impact of probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* in the modulation of the gut microbiota by inhibiting inflammation and suppression of pathogenic bacteria, thereby abrogating their pro-carcinogenic potentials (Azad et al. 2018). The potential usefulness of probiotics in esophageal cancer prevention has been reported. Co-culture of two Barrett's esophageal cell lines with *B. longum* and *Lactobacillus acidophilus* resulted in decreased expression of cancer-associated biomarkers including TNFα, IL-18, and cyclooxygenase 2 (COX2) (Mozaffari namin et al. 2015). Probiotics use also holds promise in gastric cancer prevention and treatment. Oral administration of *Lactobacillus* reportedly accelerated gastric ulcer healing. *Lactobacillus johnsonii* No. 1088, a highly acid-resistant strain of *L. johnsonii*, inhibited the growth of *H. pylori* in vitro and suppressed gastric acid secretion in mice by reducing the gastrin-positive cells of the stomach. Moreover, probiotics diminished *H. pylori*-induced Th1 response, dampened *H. pylori*-associated hypochlorhydria, and secreted bacteriocins in vitro. In human, probiotic monotherapy with *S. thermophilus*, *L. acidophilus*, *B. longum*, and *L. plantarum* for 10 days effectively reduced *H. pylori* load by up to 32.5% (Rosania et al. 2012). However, the probability of subsequent recurrence was reportedly high as *H. pylori* antigen test was no longer negative after 4 weeks (Rosania et al. 2012). Probiotics can be used as adjunct in *H. pylori* eradication therapy to increase the rate of successful elimination of *H. pylori* (Bhandari and Crowe 2012) (55). As a complement to *H. pylori* eradication antibiotics regimen, probiotics *B. bifidum*, *L. acidophilus*, *L. rhamnosus*, and *L. salivarius* were shown to proffer inhibitory effects on *H. pylori* infection in many animal models (Zhu and Liu 2017). Moreover, probiotics supplement including *L. gasseri*, *L. reuteri*, *L. acidophilus*, *Saccharomyces boulardii*, *Streptococcus faecalis*, *Bacillus subtilis*, and *Bifidobacterium* sp. improved *H. pylori* eradication rate and reduced total adverse effects in Asian patients (Zhu et al. 2014). A potential mechanism proposed for the effect of probiotics of *H. pylori* eradication is that probiotics can colonize the stomach temporarily, improve host immune response, and dampen the effect of *H. pylori*-induced inflammation on the gastric mucosa (Du et al. 2012). The use of antibiotics alone in the eradication of *H. pylori* toward prevention of gastric cancer is often associated with emergence of antibiotics resistance (Savoldi et al. 2018) with possibility of horizontal transfer to other gastric microbes (Pot et al. 2001). While probiotics help replenish beneficial bacteria, probiotics alone may not proffer sustained suppression of infection, given that *H. pylori* can modulate the gastric microbiota and can successfully outcompete other gastric microbes (Gantuya et al. 2019). The use of probiotics as adjuncts in antibiotics therapy will be more beneficial than either antibiotics or probiotics alone in *H. pylori*-targeted control of gastric cancer.

The potential use of probiotics in prevention and treatment of pancreatic cancer has also been indicated by studies showing that probiotics can prevent pancreatitis, obesity, and pancreatic necrosis. The use of *L. plantarum* for 7 days reduced pancreatic sepsis and the number of surgical interventions of severe acute

pancreatitis (SAP) patients (Olah et al. 2002). Also, ingestion of *B. longum*, *L. bulgaricus*, and *S. thermophilus* for 7 days alleviated abdominal pain, restored serum amylase, and reduced the incidence rate of complications and hospitalization of SAP patients (Javanmard et al. 2018). Moreover, *B. longum*, *L. bulgaricus*, and *E. faecalis* for 14 days resulted in significant lowering of pro-inflammatory cytokines, early restoration of gastrointestinal function, and decreased SAP complications (Cui et al. 2013). Additionally, probiotics use was demonstrated to inhibit hepatocellular carcinoma (HCC) progression in mice. Feeding a probiotics mixture to tumor-injected mice shifted the gut microbiota, reduced liver tumors, and downregulated angiogenic factors (Li et al. 2016).

Reduction in CRC risks with probiotics intake was observed in several studies including randomized placebo-controlled trials. Intake of *L. rhamnosus* and *Propionibacterium freudenreichii* by healthy human subjects resulted in decreased activity of beta-glucosidase which has potential colon carcinogenic activity (Hatakka et al. 2008). In addition to colon cancer prevention, probiotics can alleviate the symptoms and complications of CRC patients undergoing surgery and treatment. CRC patients treated with a cocktail of *Bifidobacterium lactis* and *Lactobacillus acidophilus* manifested increased gut bacterial diversity concomitant with increased butyrate-producing *Faecalibacterium* and *Clostridiales* spp. in the tumor, non-tumor mucosa, and fecal microbiota (Hibberd et al. 2017). Treatment of CRC patients undergoing surgery with *L. plantarum*, *L. acidophilus*, and *B. longum* for 16 days improved gut barrier integrity and decreased infection complications (Taremi et al. 2005). A separate study reported decreased postoperative major complication rate, decreased TNF expression, and circulating IL-6 in CRC patients treated with four probiotics regimen comprising *L. acidophilus*, *L. plantarum*, *B. lactis*, and *Saccharomyces boulardii* (Kotzampassi et al. 2015). Moreover, less diarrhea, abdominal pain, and hospital care and radiation therapy-related toxicity were reported in radiation-/chemotherapy-treated CRC patients that received probiotic *L. rhamnosus* GG for 24 weeks (Osterlund et al. 2007).

The mechanisms employed by probiotics in conferring gastrointestinal health and potential prevention of gastrointestinal cancers include production of SCFA, production of antimicrobial products, modulation of microbiota, reduction of inflammation, alteration of tumor gene expressions, modification of differentiation process in tumor cells, inhibition of the pro-carcinogens, and activation of the host's immune system (Javanmard et al. 2018; Sanders et al. 2019; Rowland et al. 2018) (Fig. 2.3). Overall, the results of clinical trials showed that probiotics can manipulate the composition of gut microbiota, improve intestinal barrier integrity, inhibit pathogen growth, and reduce metabolism of pro-carcinogenic substances (Javanmard et al. 2018).

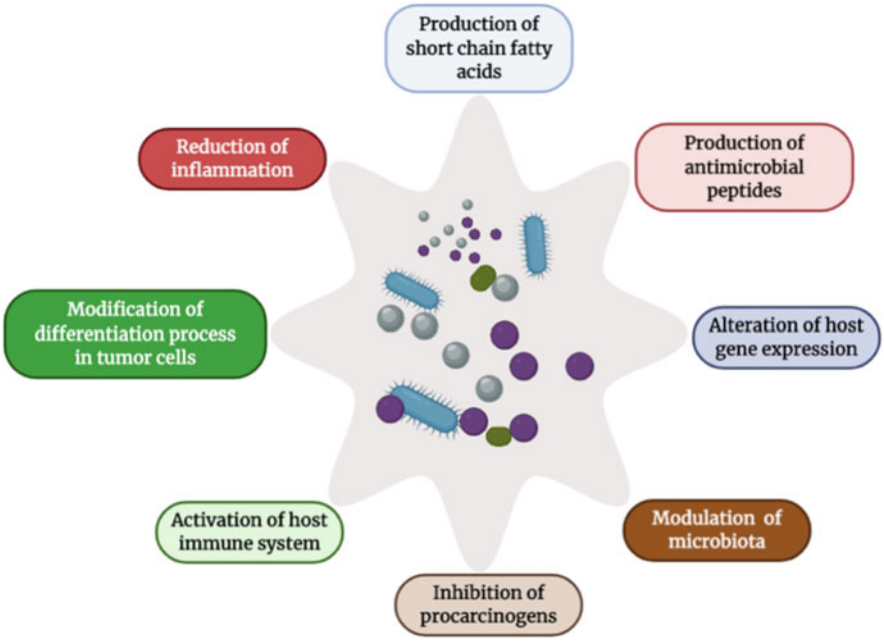

Fig. 2.3 Mechanisms of prebiotics and probiotics in preventing gastrointestinal cancers: the mechanisms employed by prebiotics and probiotics include production of SCFA, production of antimicrobial products, modulation of microbiota, reduction of inflammation, alteration of tumor gene expressions, modification of differentiation process in tumor cells, inhibition of the pro-carcinogens, and activation of the host's immune system

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Chapter 3
The Gut Microbiome and Colorectal Cancer

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Abstract The gut microbiome, the consortium of bacteria, viruses, and fungi, that reside in the gastrointestinal tract, has been linked to disease in recent years. Specifically, the gut microbiome can contribute to colon cancer development and severity. Specific bacteria have been identified as promoters of colon tumorigenesis through a variety of mechanisms, including promoting mutagenesis of tumor-related genes and modulating immune responses. Additionally, metabolites produced by the gut microbiome are also implicated in colon cancer development where microbial metabolites have both pro- and anti-tumor effects. Here, we discuss, in depth, the significance of the gut microbiome, and in particular, gut bacteria, in colon cancer pathogenesis.

Keyword Microbiota · Colorectal · Cancer

3.1 Introduction

Colorectal cancer (CRC) is the third most common type of cancer and fourth leading cause of cancer-related deaths worldwide (Arnold et al. 2017). While incidence and mortality rates are declining in the USA, Western Europe, and Australia, likely due to increased and improved screening and therapies, CRC incidence rates are rising in Asia, Eastern Europe, and South America, some of which may be attributable to westernization and economic transitioning of countries as a higher incidence of CRC has typically been associated with economically developed countries (Keum and Giovannucci 2019; Arnold et al. 2017). In addition, there has been a disturbing

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increase in incidence of young onset colorectal cancer and, in particular, rectal cancer in individuals aged 50 years and younger. This rise in incidence cannot be entirely explained by genetic predisposition and points to lifestyle and environment factors potentially playing a role (Stoffel and Murphy 2019). Besides genetic predisposition and inflammatory bowel disease (IBD), which account for a small percentage of CRC, risk factors related to dietary and lifestyle habits include smoking, obesity, increased alcoholic consumption, and a diet rich in red meat and reduced whole grains and dairy (World Cancer Research Fund International/American Institute for Cancer Research. Continuous Update Project Report 2018).

Many of these factors—diet, nutrition, physical activity, and colorectal cancer—can influence or be influenced by the composition of the microbiota (Li et al. 2019a; Capurso and Lahner 2017; Turnbaugh et al. 2006, 2009; Song et al. 2019; Makki et al. 2018). In addition, just as different parts of the colon are associated with specific subtypes of CRC, the composition of the gut microbiota also changes with anatomic location, further suggesting a potential link between the microbiota and CRC pathogenesis (Keum and Giovannucci 2019; Flynn et al. 2018; Missiaglia et al. 2014; Kim et al. 2018). Thus, there has been increasing interest in understanding the role of the gut microbiome in dictating CRC risk. Indeed, there is now significant evidence that the gut microbiome composition is altered in patients with CRC and that these perturbations from a healthy state, often referred to as dysbiosis, may contribute to the development and/or progression of CRC.

The gut microbiome consists of trillions of bacteria comprising at least 1000 different species and plays an important role in promoting health and intestinal homeostasis (Rajilic-Stojanovic and de Vos 2014). For example, the gut microbiota is required for the digestion and provision of certain nutrients including resistant starches and vitamins, the development and education of the host immune system, and promotion of resistance against colonization by harmful pathogens (Rajilic-Stojanovic and de Vos 2014; Roy and Trinchieri 2017; Savage 1977). The gut microbiota is dominated by two phyla, Bacteroidetes and Firmicutes, but also includes Actinobacteria, Fusobacteria, Proteobacteria, and Verrucomicrobia (Arumugam et al. 2011; Eckburg et al. 2005). Although there is significant interindividual heterogeneity in the composition of the gut microbiota such that a "core" microbiome has not been identified, the human microbiome may be stratified into specific enterotypes defined by the relative abundance of certain phylotypes, such as *Bacteroides*, *Prevotella*, and *Ruminococcus* genera, as well as co-occurring bacterial species found in the healthy human gut (Turnbaugh et al. 2009; Arumugam et al. 2011; Costea et al. 2018; Ding and Schloss 2014). On the other hand, there is much more similarity in the functional gene content of the gut microbiome and the metabolic pathways they represent between individuals (Turnbaugh et al. 2009). These observations suggest the possibility of using the composition of the microbiota and the metabolites they produce as potential biomarkers or therapeutic targets for disease. Indeed, when the microbiome is perturbed, the resultant dysbiotic or imbalanced microbiome can promote colon inflammation and intestinal pathology including infectious colitis, IBD, and CRC (Carding et al. 2015). There is now significant evidence that CRC patients also have dysbiosis that may be associated

with potential metabolic signatures (Thomas et al. 2019; Wirbel et al. 2019). In this review, evidence supporting a role for dysbiosis, and in particular, gut bacteria, which have been the best-studied, in promoting CRC and potential mechanisms by which gut bacteria may modulate CRC risk will be presented.

3.2 Dysbiosis and CRC

There are a growing number of studies that show differences in the composition of the gut microbiome between individuals that are healthy or have CRC (Table 3.1). However, identifying specific bacteria that are found in all cases of CRC that may be used as potential biomarkers of disease development, has been more of a challenge and may reflect the preexisting interindividual heterogeneity as well as the diversity of molecular subtypes of CRC. Furthermore, the relatively small numbers of patients examined to date, limitations in taxonomic resolution to the species level, differences in the source material used for sequencing analysis (e.g., tissue versus stool), and differences in sequencing method have also made it difficult to identify universal microbial signatures of CRC. Most studies evaluating the presence of dysbiosis in CRC patients have been small case-control studies, typically less than 100 subjects per group, comparing either fecal or tissue samples from CRC patients and normal, healthy controls, some of which were performed with the goal of identifying potential microbial biomarkers of disease. These studies have not shown significant concordance on specific species that are predictive of or are associated with CRC; however, CRC patients generally have altered gut microbiomes, characterized by decreased *Firmicutes* and increased *Bacteroidetes* bacteria compared to healthy controls (Wang et al. 2012; Ahn et al. 2013; Baxter et al. 2014; Zackular et al. 2014).

Despite the lack of significant concordance between studies on microbiome differences between CRC and non-CRC control subjects to date, multiple studies have reported an enrichment of *Fusobacterium nucleatum* as well as other oral commensal and pathogenic bacteria in either the tissues or stool of CRC patients. Although the reason behind this association is poorly understood, it has been posited that the ability of oral bacteria to produce and reside in biofilms enables them to colonize and adhere to the colon epithelium under predisposing conditions (e.g., during inflammation) that result in CRC development (Rajilic-Stojanovic and de Vos 2014; Koliarakis et al. 2019). Consistently, the presence of biofilms is associated with the development of CRC particularly in the proximal colon, and consistently, increased colonization of *Fusobacterium* has been observed in right-sided colon cancers (Mima et al. 2016a). Despite the fact that *Fusobacterium* and other oral microbes are repeatedly identified in colon tumor tissue, one study suggests that the presence of these bacteria is still not predictive of CRC (Baxter et al. 2016). Rather, the depletion of typically beneficial bacteria such as those capable of producing butyrate and other short-chain fatty acids (e.g., *Ruminococcaceae*, *Lachnospiraceae*, and *Eubacterium* spp.) was more strongly predictive of CRC (Baxter et al. 2016). A meta-analysis identified eight taxa whose fecal abundance

Table 3.1 Microbiome studies involving stool samples of individuals with colorectal cancer

Study	Year	Country	No. of patients	Sequencing	Major findings
Sobhani et al.	2011	France	6 CRC patients, 6 healthy controls	V3/V4 region, pyrosequencing	• *Bacteroides*/*Prevotella* group bacteria are increased in CRC patients compared to healthy controls
Wang et al.	2012	China	46 CRC patients, 56 healthy controls	V3 region, pyrosequencing	• *Bacteroidetes* phylum bacteria significantly increased in healthy controls and *Proteobacteria* phylum bacteria significantly increased in CRC patients • *Bacteroides*, *Roseburia*, *Alistipes*, *Eubacterium*, and *Parasutterella* genera increased in healthy controls • *Porphyromonas*, *Escherichia*/*Shigella*, *Enterococcus*, *Streptococcus*, and *Peptostreptococcus* genera increased in CRC patients • Healthy controls were enriched in OTUs of *Alistipes*, *Phascolarctobacterium*, *Oscillibacter*, unclassified genera of the order *Clostridiales*, as well as butyrate-producing *Roseburia* and the *Lachnospiraceae* family • CRC patients were enriched in OTUs from *Escherichia*/*Shigella*, *Klebsiella*, *Streptococcus*, *Enterococcus*, *Peptostreptococcus*, *Eggerthella*, *Fusobacterium*, and *Gemella* genera, as well as *Citrobacter* from the *Enterobacteriaceae* family

(continued)

Table 3.1 (continued)

Study	Year	Country	No. of patients	Sequencing	Major findings
Chen et al.	2012	China	Stool, 21 CRC patients, 22 healthy controls; lumen swabs, 32 CRC patients, 34 healthy controls	V1-V3, pyrosequencing	• *Erysipelotrichaceae*, *Prevotellaceae*, *Coriobacteriaceae*, and *Peptostreptococcaceae* family bacteria are enriched in CRC patient intestinal lumens compared to healthy controls • *Peptostreptococcus*, *Porphyromonas*, *Mogibacterium*, *Anaerococcus*, *Slackia*, *Anaerotruncus*, *Collinsella*, *Desulfovibrio*, *Eubacterium*, and *Paraprevotella* genera bacteria are enriched in CRC patient stool compared to healthy controls
Ahn et al.	2013	USA	47 CRC patients, 94 healthy controls	V3-V4 region, pyrosequencing	• CRC patients have decreased community diversity but no difference in community evenness compared to healthy controls • CRC patients have increased *Bacteroidetes* and decreased *Firmicutes* bacteria • In the *Firmicutes*, the *Clostridia* family, which include butyrate producers, are particularly reduced in CRC patients • *Fusobacterium*, *Atopobium*, and *Porphyromonas* genera are increased in CRC patients
Chen et al.	2013	China	47 CRC (advanced colorectal adenoma) patients, 47 healthy controls	V1-V3 region, pyrosequencing	• *Clostridium*, *Roseburia*, and *Eubacterium* genera (butyrate producers) are reduced in CRC patients • *Enterococcus*, *Streptococcus*, and *Bacteroidetes* genera are enriched in CRC patients

(continued)

Table 3.1 (continued)

Study	Year	Country	No. of patients	Sequencing	Major findings
Weir et al.	2013	USA	11 CRC, 10 healthy controls	V4 region, pyrosequencing	• Several *Bacteroides* and *Prevotella* spp. as well as *Dialister* and *Megamonas* spp. enriched in healthy controls • Increased representation of *Akkermansia* in colorectal cancer patients
Zackular et al.	2014	USA and Canada	30 CRC (carcinoma) patients, 30 adenoma patients, 30 healthy controls	V4 region, Illumina MiSeq (16S rRNA sequencing)	• CRC patients are enriched for *Fusobacterium*, *Porphyromonas*, *Lachnospiraceae*, and *Enterobacteriaceae* bacteria and reduced *Bacteroides*, *Lachnospiraceae*, and *Clostridiales* bacteria
Zeller et al.	2014	France	53 CRC (carcinoma) patients, 42 adenoma patients, 61 healthy controls	V4, Illumina MiSeq (16S rRNA sequencing); Illumina HiSeq (metagenome)	• *Bacteroidetes*, *Fusobacteria*, and *Proteobacteria* are enriched in CRC patients; *Firmicutes* and *Actinobacteria* bacteria are reduced in CRC patients • Healthy control metagenomes are enriched for fiber-degrading enzymes and fiber-binding domains • CRC patient metagenomes suggest an increase in degradation of host glycans and amino acid uptake
Feng et al.	2015	Austria	41 CRC patients, 42 adenoma patients, 55 healthy controls	Illumina HiSeq (metagenome)	• *Bacteroides* and *Parabacteroides* spp. as well as *Alistipes putredinis*, *Bilophila wadsworthia*, *Lachnospiraceae* bacterium, and *E. coli* enriched in CRC compared with healthy and advanced adenomas. Fusobacterium, *Parvimonas micra*,

(continued)

Table 3.1 (continued)

Study	Year	Country	No. of patients	Sequencing	Major findings
					Gemella morbillorum, and *Peptostreptococcus stomatis* elevated in carcinomas and adenomas compared with control • *Bifidobacterium animalis* and *Streptococcus thermophilus* decreased in feces from adenoma or CRC patients
Baxter et al.	2016	USA and Canada	120 CRC patients, 198 adenoma patients, 172 no colonic lesions	V4, Illumina MiSeq (16S rRNA sequencing)	• Depletion of *Lachnospiraceae* and *Ruminococcaceae* families (butyrate producers) in CRC • Higher levels of *Fusobacterium*, *Porphyromonas*, *Parvimonas*, *Peptostreptococcus*, and *Prevotella* in CRC
Flemer et al.	2017	Ireland	59 CRC patients, 21 individuals with polyps, 56 healthy controls	V3/V4 region, Illumina MiSeq (16S rRNA sequencing)	• CRC patients are enriched for *Bacteroides*, *Roseburia*, *Ruminococcus*, and *Oscillibacter* genera and certain genera containing known oral pathogens such as *Fusobacterium* and *Porphyromonas* • CRC patients enriched for bacteria in the *Prevotella* and pathogen bacteria clusters and positively correlated with CXCL1, SERPINE1, and IL-17a and IL-23 genes • Microbiota composition differed between proximal and distal colon tumors
Yu et al.	2017	China	74 CRC patients, 54 healthy controls	Illumina HiSeq (metagenome)	• Strong association between *Parvimonas*, *Fusobacterium*, *Solobacterium*, and *Peptotreptococcus* with CRC

was significantly associated with carcinomas, namely, *Fusobacterium*, *Parvimonas*, *Porphyromonas*, and *Peptostreptococcus*, which are commonly found in the oral cavity, and *Clostridium*, *Enterobacteriaceae*, *Escherichia*, and *Ruminococcus* with decreases rather than increases in *Clostridium* and *Ruminococcus* associated with CRC (Sze and Schloss 2018). Interestingly, no individual taxa were sufficient to predict for the presence of cancer, suggesting that multiple bacterial populations contribute to cancer susceptibility (Sze and Schloss 2018).

Whether the altered microbiomes observed in CRC patients cause the development of CRC or occur as a result of carcinogenesis and cancer progression remains unclear. In a longitudinal study of patients who developed CRC, the reversion of the gut microbiome to that associated with a normal colon in patients after treatment of their colorectal cancer suggests that a CRC-associated microbiome may in fact contribute to disease (Sze et al. 2017). In addition, studies using germ-free (GF) mice strongly suggest that dysbiosis directly contributes to colon tumorigenesis (Zackular et al. 2013). A commonly used mouse model to study the effects of the microbiota on colon carcinogenesis is the azoxymethane-dextran sulfate sodium (AOM/DSS) model of inflammation-associated colon cancer in which mice are treated with the experimental carcinogen azoxymethane followed by multiple rounds of water containing dextran sulfate sodium which causes bacteria-driven inflammation in the colon by disrupting the epithelial barrier (Tanaka et al. 2003). This results in the generation of adenomatous polyps, which, although are premalignant, can eventually progress into adenocarcinomas and, therefore, have been used as a surrogate marker for cancer (Tanaka et al. 2003). AOM/DSS treatment also causes microbiome alterations similar to that observed in human CRC patients, including reduced species richness and alpha diversity and significant shifts in beta diversity (Zackular et al. 2013). Using this model, it was shown that the colonization of GF C57BL/6 (B6) mice with the microbiome of tumor-bearing AOM/DSS-treated mice resulted in significantly more and larger tumors compared to GF mice colonized with the microbiota of healthy, untreated mice (Zackular et al. 2013). Similarly, conventionalization of GF B6 mice with the fecal microbiome of five CRC patients also resulted in increased tumors, intestinal dysplasia, and inflammation after injection of a single dose of AOM compared to GF mice that were gavaged with stool from healthy controls (Wong et al. 2017). $Apc^{Min/+}$ mice, which spontaneously develop intestinal tumors due to a mutation in the tumor suppressor gene Apc that occurs in the majority of human CRC, developed more tumors after gavage of fecal contents from CRC patients than from healthy controls (Li et al. 2019b). The potential carcinogenicity of biofilm-associated microbiota was demonstrated in a study in which GF Apc mutant mice developed significantly more tumors when gavaged with the homogenates of biofilm-positive colon mucosa compared to biofilm-negative colon mucosa (Tomkovich et al. 2019). However, in one study, GF B6 mice that were gavaged with the stool from either three CRC or three healthy individuals had different susceptibilities to colon tumorigenesis after AOM/DSS treatment that did not correlate with donor cancer status, which may in part be due to incomplete reconstitution of GF mice with human donor microbiota (Baxter et al. 2014). As human-derived microbiota may not interact with the mouse immune system in the same way as an indigenous mouse microbiome, studies involving "humanized" GF

mice should still be interpreted with caution although remain one of the few methods to evaluate causality between the microbiome and disease (Nguyen et al. 2015; Chung et al. 2012).

3.3 CRC-Associated Microbiota

Although a microbial signature defining colorectal cancer has not yet been identified, there are several commensal bacteria that have been repeatedly shown to be enriched in CRC tissue compared to normal adjacent, which also have pro-tumorigenic activities in mouse models of colon cancer. The most well-studied are *Fusobacterium nucleatum*, enterotoxigenic *Bacteroides fragilis*, and *Escherichia coli*. In addition, certain pathogenic species have also been associated with increased incidence of CRC after infection, such as *Streptococcus gallolyticus* and *Salmonella enterica*. It is important to note that none of these bacteria are universally present in all CRCs, and therefore, it remains possible that these CRC-associated bacteria represent opportunistic organisms that can thrive in the tumor microenvironment or in preneoplastic lesions, but are capable of accelerating tumor growth. Evidence supporting an association with these bacterial species and CRC will be presented here.

3.3.1 Fusobacterium nucleatum

Fusobacterium spp. were first noted to be enriched in tumor compared to normal adjacent tissue in a small study of six CRC patients (Marchesi et al. 2011). This was further confirmed in a larger study of 95 paired tumor and normal tissues from CRC patients in which there was overrepresentation of the *Fusobacterium* taxon, including *Fusobacterium nucleatum* (Kostic et al. 2012). *F. nucleatum* is a Gram-negative, anaerobic bacteria typically found in the oral cavity and was initially recognized to promote gingivitis and periodontitis (Han 2015; Signat et al. 2011). Transcriptomic analysis of tumor tissue from 11 matched pairs of CRC and adjacent normal tissue revealed that *Fusobacterium nucleatum* was disproportionately increased in most tumor tissue samples, although it was not more abundant in all cases (Castellarin et al. 2012). Increased *Fusobacterium* spp. was also found to be increased in premalignant adenomatous tissue compared to adjacent tissues, suggesting that *Fusobacterium* may be involved in tumor progression. Indeed, oral gavage of *F. nucleatum* into $Apc^{Min/+}$ mice increased the growth and number of tumors that developed (Kostic et al. 2013). Examination of over 1069 cases of CRC from 2 large US prospective cohort studies, the Nurses' Health Study and the Health Professionals Follow-up Study, revealed detectable *F. nucleatum* DNA in only 13% of cases, although tissue fixation may have affected the detection of *F. nucleatum* in this particular study (Lee et al. 2018). Interestingly, *F. nucleatum* DNA levels

directly correlated with CRC-specific mortality, proximal tumor location, and poor tumor differentiation (Mima et al. 2016a, b). Furthermore, higher levels of *F. nucleatum* DNA correlated with microsatellite instability (MSI-high) and the CpG island methylator phenotype (CIMP-high), which was also observed in a second retrospective analysis of 246 Asian patients (Lee et al. 2018). *F. nucleatum* was also more abundant in the tumor tissue of patients who had recurrences, suggesting *F. nucleatum* can promote chemotherapy resistance (Yu et al. 2017). Altogether, these studies suggest that the presence of *F. nucleatum* in CRC tissue correlates with poorer prognosis. The link between *F. nucleatum* with microsatellite instability is interesting given that *F. nucleatum* levels are otherwise associated with poor prognostic features since patients with MSI-H tumors tend to have better prognosis. This may reflect the fact that MSI-H tumors tend to be located in the proximal colon which coincides with the preferential location of *F. nucleatum* in biofilms that are dominant on the right side in both tumors and normal adjacent tissue and not on the left (Dejea et al. 2014). Similarly, CIMP-high tumors also tend to be locally proximally, which may also partially explain the association with F. nucleatum, but unlike MSI-H CRC, CIMP-high tumors, can be associated with worse prognosis, which may be dependent on the presence of certain mutations (Kim et al. 2017; Chen et al. 2019). Regardless, it remains unclear why Fusobacterium preferentially colonizes proximal tumors. How it affects tumor biology and whether it contributes to the molecular phenotype of CRC is also not fully understood; however, as it has been shown to preferentially bind to CRC cells rather than precancerous adenoma cells and induces their cellular proliferation, it has been suggested that it acts as a cancer promoter rather than as an initiator (Rubinstein et al. 2019).

3.3.2 *Enterotoxigenic* Bacteroides fragilis

Enterotoxigenic *B. fragilis* (ETBF) is a strain of *B. fragilis* characterized by the expression of the zinc-dependent metalloprotease toxin *B. fragilis* toxin (BFT). ETBF causes inflammatory diarrhea in children and asymptomatically colonizes 20–35% of adults (Sears et al. 2008). However, a potential link between ETBF and colon carcinogenesis was suggested by a study in which inoculation of $Apc^{Min/+}$ mice with ETBF, but not non-toxigenic *B. fragilis* (NTBF), increased tumor numbers (Wu et al. 2009). Subsequently, at least two studies were able to identify enhanced levels of ETBF and the BFT gene (bft) in the stool of CRC patients by PCR although the numbers of patients evaluated were small (<100) (Toprak et al. 2006; Haghi et al. 2019). Interestingly, like *F. nucleatum*, *B. fragilis* and the bft gene can be found in biofilms that largely occur in the proximal colon and are also prevalent in patients with familial adenomatous polyposis (FAP), a hereditary condition in which the Apc gene is mutated and universally leads to the development of CRC, suggesting the interesting possibility that biofilm formation and aggregation of cancer-associated microbiota may be a precursor to and predictive of malignant

transformation (Dejea et al. 2014, 2018). In addition, like *F. nucleatum*, *B. fragilis* was increased in biofilms associated with right-sided CRC (Drewes et al. 2017). Also consistent with the enrichment of *B. fragilis* in right-sided colon tumors, *B. fragilis* is more abundant in MSI-H tumors; however, in a study of 83 individuals with CRC, there was no significant difference in the presence of the bft gene between MSI-H and microsatellite stable (MSS) CRC (Hale et al. 2018).

3.3.3 Escherichia coli

Several studies suggest a possible role for *E. coli* in promoting CRC. In one study, 90–92% of CRC patients had tumor-associated bacteria compared to 3% of healthy controls where *E. coli* was enriched in 62–77% of CRC patients (Swidsinski et al. 1998). Another study found 71% of CRC patients to have mucosa-associated bacteria where the majority of the Gram-negative mucosa-associated bacteria were *E. coli* (Martin et al. 2004). Finally, in a third study, mucosa-associated *E. coli* were found in 50% of adenocarcinoma samples (Maddocks et al. 2009). *E. coli* in the B2 phylogenic group are enriched for the polyketide synthase (*pks*) genomic island which encodes the genotoxin called colibactin (Nougayrede et al. 2006). A role for *pks*+ *E. coli*, specifically, in modulating susceptibility for colon tumorigenesis was discovered in a sentinel study by Arthur et al., in which increased pks+ *E. coli* was observed in 21 CRC tissue specimens compared to 24 non-CRC controls (66.7% vs. 20.8%) (Arthur et al. 2012). The accumulation of this bacteria in tumors may, in part, be due to the presence of chronic inflammation as *pks*+ *E. coli* were also enriched in patients with IBD, a major risk factor for the development of colitis-associated CRC. In mice, Arthur et al. found increased colonization of *E. coli* in conventionalized GF *Il10*$^{-/-}$ mice, which developed spontaneous colitis in SPF conditions, compared to that of conventionalized GF WT mice that do not develop colitis (Arthur et al. 2012). This is consistent with the association of *Enterobacteriaceae* and *E. coli* in IBD and suggests that inflammation promotes the bloom of *E. coli* that occurs prior to frank carcinogenesis (Arthur et al. 2012; Kuhn et al. 1993). More importantly, mono-association of GF *Il10*$^{-/-}$ mice treated with the carcinogen AOM, which results in the development of inflammation-associated colon tumors, with *pks*+ *E. coli*, but not *pks*-deficient *E. coli* resulted in increased numbers of invasive adenocarcinomas. The ability of *pks*+ *E. coli* to promote colon tumorigenesis was not necessarily due to an effect on inflammation as GF *Il10*$^{-/-}$ mice mono-associated with *pks*-deficient *E. coli* or *E. faecalis*, both of which induced similar levels of colitis as *pks*+ *E. coli*, did not result in any tumors (Arthur et al. 2012). However, in the context of IL-10 deficiency, inflammation is required for the tumor-promoting effects of *pks*+ *E. coli*, as *Il10*$^{-/-}$ mice that were deficient in T cells and are non-colitic (*Il10*$^{-/-}$;*Rag2*$^{-/-}$) did not develop tumors after AOM treatment despite the presence of similar levels of *pks*+ *E. coli* (Arthur et al. 2014). Subsequent studies, using other mouse models, namely, the AOM/DSS, *Apc*$^{Min/+}$, and human xenograft models of colon tumorigenesis, also demonstrated

a tumor-enhancing effect for *E. coli* in specific pathogen-free (SPF) mice (Bonnet et al. 2014; Cougnoux et al. 2014).

E. coli of the B2 phylotype is also capable of forming biofilms, which may contribute to their ability to adhere to intestinal epithelium and colonize the gut (Raisch et al. 2014). Consistently, *pks+ E. coli* was identified in the biofilms of FAP patients in addition to *B. fragilis* (Dejea et al. 2018). Regardless, it is important to note that *pks+ E coli* has not been consistently observed to be significantly elevated in all CRC patient cohorts compared to non-CRC controls, and therefore other bacteria likely contribute or are required for full malignant transformation (Dejea et al. 2018; Raisch et al. 2014). Indeed, in GF genetically engineered mouse models of CRC, colonization of mice with both *B. fragilis* and *pks+ E coli* resulted in significant greater tumor induction including adenocarcinoma formation compared to either strain alone (Dejea et al. 2018).

3.3.4 Streptococcus gallolyticus *(Previously Known as* Streptococcus bovis *Biotype I and II/2)*

Streptococcus gallolyticus are Gram-positive, opportunistic pathogens that often reside in the gut and have been linked to sepsis and endocarditis (Leport et al. 1987; Pasquereau-Kotula et al. 2018). The *S. gallolyticus* subspecies gallolyticus (SGG) was previously known as *S. bovis* biotype I, while the *S. gallolyticus* subspecies *pasteurianus* and *macedonicus* were identified as *S. bovis* biotype II/2 (Schlegel et al. 2003). Epidemiological studies since the 1970s have suggested a correlation between SGG infection and CRC, and it has been reported that 25–80% of patients with SGG bacteremia have colorectal tumors (Abdulamir et al. 2011; Pasquereau-Kotula et al. 2018; Corredoira-Sanchez et al. 2012; Klein et al. 1977; Kwong and Dove 2009; Boleij et al. 2011b; Ellmerich et al. 2000b). Patients with higher levels of SGG in the blood were at higher risk for CRC compared to healthy controls with no SGG in the blood (Kwong et al. 2018), and in a large case-controlled study of 4210 CRC cases and 4210 matched controls, antibody responses to a specific SGG protein was significantly associated with a 40% increase in CRC risk in patients diagnosed within 10 years of testing, although the percentage of CRC cases with seropositivity was low (Butt et al. 2018). Thus, patients who present with SGG bacteremia are often referred for a colonoscopy. All three subspecies of *S. gallolyticus* have been found to be increased in CRC tumor tissues compared to non-tumor tissues (Abdulamir et al. 2010). Furthermore, an analysis of 148 tumor and 128 normal adjacent tissues from CRC patients also demonstrated enrichment of SGG in tumor tissues, which can be directly visualized by immunofluorescence (Kumar et al. 2017). In a mouse model of CRC in which mice are injected with two doses of the carcinogen AOM, mice orally gavaged with SGG developed significantly more tumors compared to mice inoculated with a *Lactobacillus* strain or saline (Kumar et al. 2017). In a separate study, it was determined that rats that were treated

with two doses of AOM followed by administration of SGG also developed more adenomas compared to rats treated with AOM alone (Ellmerich et al. 2000b).

How SGG becomes enriched in CRC remains to be fully elucidated. However, increased colonization efficiency was observed in the tumors of mice that are genetically predisposed to developing colon cancer via over-activation of Notch signaling in the intestinal epithelium (Notch/APC mice) after oral administration of SGG without any pretreatment of antibiotics (Aymeric et al. 2018). Specifically, SGG was able to outcompete enterococci through the production of the antimicrobial gallocin, enabling it to create a niche for colonization. The bactericidal activity of gallocin was enhanced in vitro in the presence of secondary bile acids, which is more abundant in CRC patients (Aymeric et al. 2018; Jia et al. 2018; Hill et al. 1975), suggesting that the presence of colon neoplasia may produce a suitable environment for SGG colonization. In addition, SGG is capable of forming biofilms particularly on collagen-rich surfaces, which can be found at adenomatous tissue (Boleij et al. 2011a). Taken together, as with other CRC-associated bacteria, it has been proposed that SGG colonizes the colon under preneoplastic conditions and acts to promote rather than initiate the development of CRC (Pasquereau-Kotula et al. 2018).

3.3.5 Salmonella

Salmonella are Gram-negative bacterial pathogens that can cause gastroenteritis, which is linked to an increased risk for developing IBD (Zha et al. 2019; Gradel et al. 2009; Axelrad et al. 2019). A nationwide registry-based study of Dutch residents that were diagnosed with severe *Salmonella* infection ($n = 14{,}264$) determined that patients infected with *Salmonella*, especially with *Salmonella enterica*, had greater risk for developing CRC and, in particular, cancers of the ascending and transverse colon than that of the general population (Mughini-Gras et al. 2018). In particular, the *Salmonella* effector protein AvrA that is injected by *Salmonella* into host cells through a type III secretion system is detectable in the stool of CRC patients as well as in CRC-adjacent tissue (Hardt and Galán 1997; Lu et al. 2017). Additional evidence that *Salmonella* may be involved in colorectal carcinogenesis comes from mouse studies. In the AOM/DSS model of colitis-associated CRC, all WT mice infected with AvrA+ *S. typhimurium* developed colon tumors, whereas only 56% of WT mice infected with AvrA-*S. typhimurium* developed tumors (Lu et al. 2014b). In addition, infection of $Apc^{Min/+}$ mice with *S. typhimurium* resulted in more mice with malignant transformation compared to uninfected mice. Interestingly, $Apc^{Min/+}$ mice infected with mutant *Salmonella* that lacked a functional type III secretion system and therefore unable to inject effector proteins, such as AvrA, developed fewer adenocarcinomas similar to that of uninfected mice (Scanu et al. 2015).

3.4 Mechanisms by Which the Gut Microbiome Contribute to CRC

As discussed above, there are a number of well-studied bacteria that are linked to CRC. Although the mechanisms by which CRC-associated bacteria and dysbiosis in general promote CRC development remain to be fully elucidated, studies, largely in mice, suggest a mechanism related to an effect on host immune responses, tumor suppressor activity, epithelial transformation, and cellular proliferation via the production of bacterial immunostimulatory molecules, microbial metabolites, and genotoxins (Fig. 3.1). Pro-tumorigenic processes that are promoted by dysbiosis will be reviewed in this section.

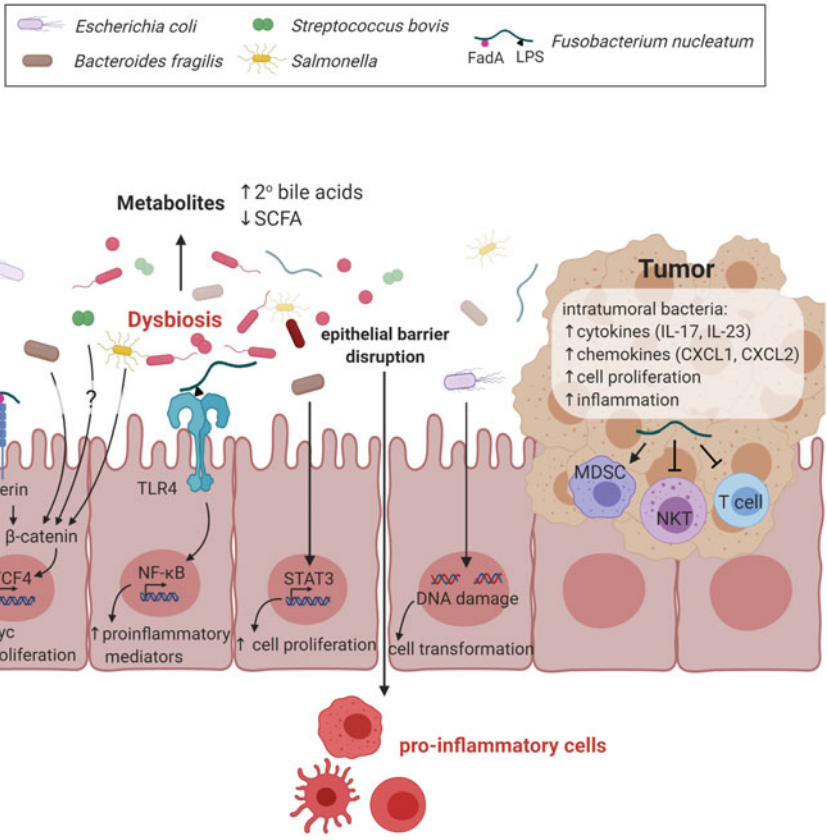

Fig. 3.1 Potential mechanisms by which the gut microbiome contribute to CRC. Dysbiosis can lead to the accumulation of bacteria that have pro-inflammatory, DNA-damaging, and/or cellular growth-promoting properties. For example, *F. nucleatum, B. fragilis, S. bovis*, and *Salmonella* are capable of activating β-catenin to promote cellular proliferation. Recognition of pathobionts such as *F. nucleatum* can activate NFκB to upregulate pro-tumorigenic, pro-inflammatory responses via innate immune receptors. Activation of STAT3 by *B. fragilis* can also promote cellular proliferation, and DNA-damage induced by genotoxic bacteria such as *pks+ E. coli* can lead to cellular transformation. Finally, dysbiosis may alter the production of specific metabolites, resulting in, for example, an imbalance in SCFA and secondary bile acid levels, which can further contribute to tumorigenesis

3.4.1 Modulation of Host Immune Responses

Chronic inflammation is a major risk factor for the development of CRC. An inflammatory response arises when tissue homeostasis is disrupted and is characterized by the recruitment of inflammatory cells to damaged tissue and production of soluble factors to promote tissue repair. These soluble factors promote cellular survival and proliferation, angiogenesis, and matrix remodeling that are required for effective tissue repair and regeneration (Coussens and Werb 2002). However, with chronic inflammation that is not self-limited, these same factors can result in the generation of DNA-damaging reactive oxygen species that can initiate tumorigenesis as well as promote a microenvironment that is conducive to tumor growth and survival (Coussens and Werb 2002). Thus, patients with IBD, for example, have an increased risk for developing CRC (Lutgens et al. 2013). Inflammation also plays a role in the pathogenesis of sporadic CRCs as elevations in cytokines such as IL-8, IL-8, IL-17, and IL-23 have been observed in the serum and tissue of CRC patients (Johdi et al. 2017; Lu et al. 2014a; Yan et al. 2018; Grivennikov et al. 2012). In the context of a breached epithelial barrier, the gut microbiome can further induce inflammation by stimulating innate immune receptors, such as the Toll-like receptors (TLRs) (Grivennikov et al. 2012). Indeed, in mice that harbor a mutation in the *Apc* tumor suppressor gene, colon tumors exhibit increased intestinal permeability indicative of a disrupted epithelial barrier, and the presence of intratumoral bacteria is associated with upregulation of IL-17 and IL-23 expression that is TLR-dependent (Grivennikov et al. 2012). Deficiency in either IL-17 or IL-23 ameliorated tumorigenesis (Grivennikov et al. 2012). Thus, shifts in the microbial community that result in the accumulation of bacteria that are highly immunostimulatory and pro-inflammatory would be a potential mechanism by which dysbiosis and CRC-associated microbiota can potentiate tumorigenesis. Consistently, treatment of mice with antibiotics prior to the development of dysbiosis results in reduction in tumor numbers in the AOM/DSS model of colon tumorigenesis (Zackular et al. 2013).

F. nucleatum also interacts with the immune system and stimulates cytokine production through TLR4 and can be sensed by retinoic acid-inducible gene I (RIG-I) (Liu et al. 2007; Lee and Tan 2014). In periodontal disease, NK cells can recognize and bind to *F. nucleatum* via the NK killer receptor, NKp46 in humans, or NCR1 in mice, which promotes a TNF-α inflammatory response that promotes disease (Chaushu et al. 2012). Although *F. nucleatum* does not instigate or worsen intestinal inflammation in mice, daily gavage of *F. nucleatum* into $Apc^{Min/+}$ mice resulted in expansion of tumor-infiltrating myeloid cells, which are capable of promoting tumorigenesis, as well as upregulation of pro-inflammatory genes in tumor tissue similar to what is observed in human CRC tissue (Kostic et al. 2013).

The induction of chronic inflammation is also a potential mechanism by which ETBF promotes tumorigenesis as colonization of WT GF mice with ETBF results in colitis (Rhee et al. 2009). ETBF-colonized $Apc^{Min/+}$ also developed inflammation in the colon, resulting in epithelial hyperplasia and neoplasia. Moreover, ETBF-

colonized *Apc*$^{Min/+}$ mice had increased colonic IL-17 expression, which, in turn, activates NF-κB signaling in colon epithelial cells to promote the production of the chemokines CXCL1, CXCL2, and CXCL5, which are neutrophil chemoattractants, in the distal colon (Wu et al. 2009; Chung et al. 2018). Consistently, ETBF-colonized mice have increased colon and intratumoral neutrophils that are dependent on IL-17 and CXCL2 signaling (Chung et al. 2018; Thiele Orberg et al. 2017). Loss of IL-17 signaling either by antibody blockade or by genetic deletion in *Apc*$^{Min/+}$ mice resulted in suppression of tumors after ETBF colonization (Wu et al. 2009; Chung et al. 2018). Additionally, in several human intestinal epithelial cell lines, BFT treatment induces IL-8 chemokine expression, which is increased in IBD patient colons (Sanfilippo et al. 2000; Hwang et al. 2013; McCormack et al. 2001; Kim et al. 2001). IL-8 secretion by BFT-treated HT29/C1 cells is NF-κB-dependent via activation of ERK and p38 MAPK pathways (Kim et al. 2001; Wu et al. 2004).

The mechanism by which SGG promotes CRC development is not well-understood; however, increased expression of NF-κB as well as COX-2 and c-myc, all of which can promote colon tumorigenesis, was found in the tissue of CRC patients that were seropositive for *S. gallolyticus* compared to seronegative groups (Abdulamir et al. 2009, 2010; Greten et al. 2004). SGG also upregulates the production of pro-inflammatory mediators such as IL-8 via stimulation of macrophages and epithelial cells, for example, which can lead to chronic inflammation with persistent colonization (Ellmerich et al. 2000a; Boleij et al. 2011a).

Although chronically dysregulated inflammation can lead to tumorigenesis, an effective immune response is also important for protecting against cancer development as exemplified by the increased incidence of cancer in immunocompromised patients. Immune responses can lead to the recruitment of immune cells, such as cytotoxic lymphocytes, that can eliminate nascent transformed cells in a process known as tumor immunoediting, or immune surveillance (Bui and Schreiber 2007). Indeed, studies of biopsy samples from colon cancer patients suggest that a robust immune response as measured by increased infiltration of activated T cells at the site of the cancer is associated with better prognosis and less aggressive behavior of the cancer (Galon et al. 2006; Pages et al. 2005). *F. nucleatum* has been shown to promote an immunosuppressive environment. For example, *F. nucleatum*, when measured in colorectal carcinoma tissue, was associated with lower density of CD3+ T cells, which may reflect reduced anti-tumor immunity resulting in worse prognosis although the association between F. nucleatum and the density of CD8+, CD45RO+, and FOXP3+ intratumoral T cells was not statistically different (Mima et al. 2015; Hamada et al. 2018). Additionally, NK cells are less cytotoxic to *F. nucleatum*-incubated tumor cells compared to tumor cells not incubated with *F. nucleatum* (Gur et al. 2015). In addition, the *F. nucleatum* protein Fap2 interacts with the receptor T cell immunoreceptor with Ig and ITIM domains (TIGIT) on intratumoral NK and T cells to inhibit tumor cell killing (Gur et al. 2015). Finally, *F. nucleatum*-colonized *Apc*$^{Min/+}$ mice had increased intratumoral myeloid-derived suppressor cells (MDSCs), which are capable of suppressing CD4 T cells (Kostic et al. 2013). In ETBF-colonized *Apc*$^{Min/+}$ mice, tumor-infiltrating neutrophils have a transcriptional signature similar to MDSCs, including upregulation of iNOS, and were able

suppress CD8 T cell proliferation in vitro, suggesting that *B. fragilis* may also affect anti-tumor immunity (Thiele Orberg et al. 2017). Thus, CRC-associated microbiota such as *F. nucleatum* and ETBF may not only accelerate tumorigenesis via upregulating tumor-promoting pro-inflammatory mediators, but, also promote a tumor environment that is deficient in anti-tumor activity. On the other hand, the gut microbiota have also been implicated in activating immune responses that may not only promote anti-tumor immunity, but also enhance the anti-tumor effects of both chemotherapy and immunotherapy. For example, a mix of 11 strains of bacteria isolated from human microbiota was shown to increase colon CD8+ IFNγ+ T cells in the lamina propria (Tanoue et al. 2019). Importantly, this 11-strain mix was capable of suppressing the growth of subcutaneously implanted MC38 cells as well as synergize with anti-PD-1 immunotherapy, which did not occur with antibody depletion of CD8+ T cells (Tanoue et al. 2019). MC38 cells harbor defects in DNA mismatch repair proteins, a hallmark of MSI-H tumors that are responsive to immunotherapy and is the only type of metastatic CRC for which immunotherapy has been FDA approved (Efremova et al. 2018). The mechanism by which the gut microbiota can upregulate CD8 T cell responses remains to be fully understood, but may be related to intestinal epithelial-derived chemokine induction by specific bacteria followed by the expansion and bacterial antigen-mediated differentiation of CD8 T cells (Tanoue et al. 2019). Gut bacteria can also act on other cells to enhance T cell-mediated tumor immunity. For example, oral gavage of Bifidobacterium, A. muciniphila, or E. hirae into GF or antibiotic-treated mice resulted in increased activation of dendritic cells, which, in turn, can prime T cells to mount tumor-specific responses and augment the effects of immunotherapy (Sivan et al. 2015; Routy et al. 2018). Besides immunotherapy, the gut microbiota also enhanced the effects of oxaliplatin, a commonly used chemotherapy drug in the treatment of locally advanced and metastatic CRC, via the induction of inflammation and production of myeloid-specific reactive oxygen species that can have anti-tumor effects (Iida et al. 2013). However, whether specific bacterial populations in CRC patients actually influence therapeutic responses to either chemotherapy or immunotherapy remains to be determined.

3.4.2 Stimulation of Cellular Proliferation

CRC-associated bacteria can also act directly on the intestinal epithelium to activate pathways involved in cellular proliferation. *F. nucleatum* is known to have adherent and invasive properties via its FadA adhesin (Han et al. 2000; Xu et al. 2007), which is highly expressed in human adenoma and adenocarcinoma tissues compared to tissue from healthy patients (Rubinstein et al. 2013). FadA binds to E-cadherin (CDH1), which is expressed by epithelial cells, enabling *F. nucleatum*'s invasion into the host cell (Rubinstein et al. 2013). Furthermore, binding of FadA to E-cadherin promotes E-cadherin's phosphorylation and internalization into the host cell, resulting in increased β-catenin translocation into the nucleus and increased

transcription of Wnt signaling genes, including the oncogenes *c-myc* and *cyclin D1* (Rubinstein et al. 2013), which are directly involved in cellular proliferation and stem cell activity. Consistently, FadA increases the proliferative activity of multiple human CRC cells in vitro and in vivo in a FadA-dependent manner (Rubinstein et al. 2013). FadA also upregulates the expression of annexin A1 (ANXA1), a phospholipid-binding protein, in CRC cells via E-cadherin, and ANXA1 can engage β-catenin to activate cyclin D1 to promote cellular proliferation (Rubinstein et al. 2019).

Other CRC-associated microbiota such as *B. fragilis* and *Salmonella* can affect Wnt signaling to facilitate neoplastic transformation. In the case of *B. fragilis*, treatment of the CRC cell line HT29/C1 with BFT resulted in the cleavage of E-cadherin and activation of β-catenin, resulting in increased c-myc transcription and cellular proliferation (Wu et al. 1998, 2003). AvrA+ *S. typhimurium* infection is also associated with activated β-catenin signaling in colon epithelial cells (Lu et al. 2012). Furthermore, AvrA displays deubiquitinase activity and was able to block the degradation of IκBα and β-catenin, resulting in increased c-myc protein expression (Ye et al. 2007). Consistently, tumors from AOM/DSS-treated AvrA+ *S. typhimurium*-infected mice displayed increased phosphorylated c-myc expression compared to AvrA- *S. typhimurium* tumors (Lu et al. 2014b). Infection with *Streptococcus gallolyticus* also increased cellular proliferation in vitro and tumor growth in vivo of certain, but not all, CRC cell lines, which was associated with increased nuclear β-catenin, c-myc, and cyclin D1 expression in responsive cells; knockdown of β-catenin abrogated this effect (Kumar et al. 2017). The responsiveness of CRC cells to SGG was not related to the presence of preexisting mutations affecting the β-catenin/Wnt pathway or to the ability to bind cells, but may reflect other downstream events that remain to be identified (Kumar et al. 2017).

Bacteria can also stimulate cellular proliferation via mechanisms that do not necessarily involve Wnt signaling. For example, *B. fragilis* colonization of $Apc^{Min/+}$ mice results in upregulated STAT3 signaling, which not only promotes Th17 differentiation, but can also drive epithelial proliferation such that loss of STAT3 signaling in epithelial cells resulted in significantly fewer tumors (Chung et al. 2018; Grivennikov et al. 2009). *Salmonella* was capable of inducing cellular transformation of mouse embryonic fibroblasts (MEFs) harboring pre-transforming mutations that lead to the inactivation of the tumor suppressor protein p53 inactivation or overexpression of c-myc since infection resulted in anchorage-independent soft agar growth and permitted tumor growth in immunocompromised mice (Scanu et al. 2015). This process was dependent on intact MAPK and Akt signaling (Scanu et al. 2015).

3.4.3 Promotion of DNA Damage

Another potential mechanism by which CRC-associated bacteria may facilitate the development of CRC is the promotion of DNA damage as in the case with pks+

E. coli (Arthur et al. 2012; Nougayrede et al. 2006), which produces colibactin that is genotoxic. HeLa cells infected with pks+ *E. coli* exhibited signs of DNA double-stranded breaks and cell cycle arrest (Nougayrede et al. 2006). This colibactin-associated DNA damage induced DNA repair responses resulting in cells with chromosomal instability, including chromosomal alterations (e.g., translocations, ring chromosomes, etc.) and abnormal chromosome number (i.e., aneuploidy and tetraploidy) (Cuevas-Ramos et al. 2010). *Pks+ E. coli* were able to induce carcinogenic mutations in infected HCT116 (human colon carcinoma), IEC-6 (rat intestinal epithelial), and CHO (hamster ovarian epithelial) cells, revealing the mutagenic and pro-tumorigenic ability of *pks+ E. coli* (Cuevas-Ramos et al. 2010). Rat epithelial cells infected with *pks+ E. coli* also exhibited increased DNA damage compared to cells infected with *E. coli* without the *pks* pathogenicity island (*E. coli* Δpks) (Arthur et al. 2012). In vivo, AOM-treated GF *Il10*$^{-/-}$ mice monocolonized with *pks+ E. coli* developed more tumors and had increased DNA damage and cell cycle arrest compared to *E. coli* Δ*pks*-monocolonized mice despite no difference in inflammation, strongly suggesting that the tumor-promoting effects of *E. coli* were related to its genotoxic rather than inflammation-promoting effects (Arthur et al. 2012).

3.4.4 Production of Metabolites

There has been growing recognition that microbial-derived metabolites can affect both health and disease including CRC. Specific metabolites, such as short-chain fatty acids (SCFAs) and secondary bile acids, have received the most attention for their role in modulating immune responses, epithelial homeostasis, and cell signaling that can affect tumor susceptibility and will be briefly reviewed here.

3.4.4.1 Short-Chain Fatty Acids

Consistent with a potential significant role for SCFAs in CRC pathogenesis, patients with CRC can have significant reduction in butyrate-producing bacteria (Wang et al. 2012; Baxter et al. 2016). Furthermore, risk for CRC is inversely associated with intake of dietary fiber, which is a source of SCFAs via microbial metabolism of resistant starches (Aune et al. 2011). SCFAs, which include butyrate, propionate, and acetate, are generated from the digestion of dietary fibers, such as polysaccharides from plant cell wells, by the gut microbiota, and are then absorbed by host cells (Gill et al. 2006). In particular, butyrate is produced by *Firmicutes* bacteria and is an energy source of epithelial cells and helps maintain colon epithelial integrity (Peng et al. 2009). Notably, butyrate can inhibit histone deacetylaces (HDACs) in colon epithelial cells and immune cells, which can have anti-tumorigenic effects, including the downregulation of pro-inflammatory cytokines such as IL-6 (Candido et al. 1978; Davie 2003; Chang et al. 2014; Bolden et al. 2006). Increased histone

acetylation at the *Foxp3* locus results in the differentiation of regulatory T cells (Treg) (Furusawa et al. 2013). Consistent with members of *Clostridia* being relatively high producers of butyrate, colonization of GF WT mice with either a cocktail of 46 strains of *Clostridium* (clusters IV and XIVa) isolated from mice or 17 Clostridia strains isolated from a healthy human donor promoted colon Treg differentiation in GF WT mice (Atarashi et al. 2011, 2013). The impact of SCFAs on Treg differentiation may have implications on anti-tumor immunity and response to therapy given its immunosuppressive effect although it remains to be determined whether regulatory T cells play a significant role in colon carcinogenesis. SCFAs, however, can promote intestinal homeostasis as the administration of SCFAs to GF mice made them more resistant to the epithelial damaging effects of DSS (Maslowski et al. 2009). Similarly, butyrate- or *Clostridium*-treated mice developed less severe colitis compared to their control counterparts (Atarashi et al. 2011; Furusawa et al. 2013). Deficiency in the receptor for butyrate that is expressed on epithelial cells, Gpr109a, resulted in reduced numbers of Tregs and the anti-inflammatory cytokine IL-10 as well as decreased susceptibility to DSS-induced colitis (Chen et al. 2011; Zaki et al. 2010; Elinav et al. 2011; Singh et al. 2014). GPR109A signaling was also required for butyrate-mediated epithelial expression of IL-18, which is important for promoting epithelial repair and resistance to epithelial injury-induced inflammation (Chen et al. 2011; Zaki et al. 2010; Elinav et al. 2011; Singh et al. 2014).

Butyrate and SCFAs in general also have anti-tumor effects. Mice deficient in GPR109a, for example, have increased tumor development in both AOM/DSS and $Apc^{Min/+}$ models (Singh et al. 2014). One mechanism, besides their anti-inflammatory activity, is by sensitizing cancer cells to apoptosis. Cancer cells often express Fas, the receptor for Fas ligand (FasL), but are able to evade apoptotic cell death induced by Fas-FasL interactions by effector CD8 T cells and NK cells (Bonnotte et al. 1998; Owen-Schaub et al. 1994; Owen-Schaub et al. 1995). Interestingly, in several human CRC cell lines, the addition of soluble FasL and sodium butyrate to the culture promoted increased CRC cell apoptosis compared to cells that were incubated with sodium butyrate alone (Bonnotte et al. 1998). Similarly, many cancer cells, including CRC cells, are able to evade apoptotic death induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and its receptors, including the death receptors DR4 and DR5 (Zhang and Fang 2004; Hernandez et al. 2001b; Zhang et al. 2000). TRAIL-resistant human colon cancer cell lines KM12C, KML4A, and KM20 were incubated with TRAIL and with or without sodium butyrate. Cells that received sodium butyrate displayed increased TRAIL-mediated cell death (Hernandez et al. 2001a). Other mechanisms have been proposed including downregulation of pathways involved in cellular proliferation, induction of antioxidant pathways, and effects on microbiome composition (Ohara and Mori 2019; Sivaprakasam et al. 2016). However, microbial regulation of SCFA levels is unlikely to be the main contributor to colon cancer risk as fecal SCFA levels were not found to be predictive of either adenomas or carcinomas (Sze et al. 2019).

3.4.4.2 Secondary Bile Acids

Primary bile acids are secreted by the liver into the gastrointestinal tract where they aid in lipid digestion (Begley et al. 2005). Bile acids have antimicrobial properties and can modify the gut microbiome composition (Begley et al. 2005; Islam et al. 2011). Primary bile acids pass through and are mostly reabsorbed by the small intestine without microbial alterations (Ridlon et al. 2006). However, about 5% of total bile acids are not reabsorbed and enter the large intestine and undergo modification by the gut microbiome via bile acid hydrolases to generate secondary bile acids (Begley et al. 2005; Ridlon et al. 2006). High levels of secondary bile acids have been measured in CRC patients (Ou et al. 2012; Louis et al. 2014; Reddy et al. 1980). Interestingly, a meta-analysis of eight fecal metagenomic studies of CRC encompassing 386 cancer cases and 392 tumor-free controls demonstrated a significant enrichment of the bai operon, which encodes bile acid-converting enzymes involved in secondary bile acid production, in the stool of CRC patients (Wirbel et al. 2019).

Two well-studied secondary bile acids that have been linked to CRC are deoxycholic acid (DCA) and lithocholic acid (LCA). They can induce reactive oxygen and nitrogen species (ROS and RNS) production by human colon tissue and human adenocarcinoma cells (Payne et al. 2007; Venturi et al. 1997; Casellas et al. 1996; Bernstein et al. 2009). ROS and RNS induce DNA damage, and DCA and LCA have been shown to induce DNA breaks in human adenocarcinoma cell lines and human colon tissue (Venturi et al. 1997; Pool-Zobel and Leucht 1997; Bernstein et al. 2009). Secondary and conjugated secondary bile acids can activate multiple pathways, including Wnt, EGFR, MAPK, and NF-κB, which, in turn, can stimulate the proliferation of CRC cells as well as induce tumor formation in mice (Reddy et al. 1976; Cook et al. 1940; Magnuson et al. 1993; Cao et al. 2017; Cheng and Raufman 2005; Pai et al. 2004; Liu et al. 2018; Dong et al. 2018; Cao et al. 2014). Administration of either primary (e.g., cholic acid, CA) or secondary bile acids have also been associated with changes in the gut microbiome, which may contribute to tumor promotion as gavage of feces from CA- or DCA-treated mice into $Apc^{Min/+}$ mice resulted in inflammation and tumorigenesis, respectively (Cao et al. 2017; Wang et al. 2019). Specifically, DCA ingestion resulted in a reduction in Firmicutes, including *Lactobacillus* and *Roseburia*, while Bacteroidetes, and certain members of Proteobacteria, were increased in abundance (Cao et al. 2017).

3.5 Conclusion

Since the advent of 16S rRNA and metagenomic sequencing technologies and the establishment of germ-free mouse modes, significant advances have been made in our understanding of the role of the gut microbiome in the pathogenesis of colorectal cancer. It has now become apparent that the presence of CRC is associated with significant shifts in the microbial community compared to healthy individuals. The exact nature and timing of these changes and whether these changes directly cause

colorectal cancer in humans remain active areas of intense research and would require a concerted effort by the scientific community to embark in large population studies that are prospective in nature and involve the longitudinal analysis of stool or mucosal tissue samples. Moreover, much research has focused on bacteria; however, the gut microbiome also consists of fungi and viruses that have yet to be fully studied for their role in colon carcinogenesis although early studies suggest this to be the case (Anandakumar et al. 2019; Hannigan et al. 2018; Nakatsu et al. 2018). Regardless, it has now become generally accepted that colon carcinogenesis is a multistep process that requires the accumulation of genomic mutations that precipitate cellular transformation and that the gut microbiota can act either as tumor promoters or tumor suppressors by modulating inflammation. Inflammation, in turn, can also allow the bloom of harmful pathobionts that outcompete and deplete potential beneficial bacteria to further facilitate tumor progression. Thus, it has become tantalizing to speculate that identification of specific bacteria associated with CRC and/or preneoplastic lesions (e.g., adenomas) would allow the establishment of microbial biomarkers to assess colorectal cancer risk or identify strategies to manipulate the microbiome or target specific microbes and their products for cancer chemoprevention. As we acquire more information by applying multiomics approaches to microbiome research and continue to improve models of colorectal cancer, efforts toward microbial therapeutics and biomarker development will translate into reality.

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Chapter 4
The Impacts of *Salmonell*a Infection on Human Cancer

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Abstract Non-typhoidal *Salmonella enterica* is the leading cause of foodborne illnesses resulting over 153 million of incidence per year worldwide, while typhoidal *Salmonella* infection disproportionally affects low- to middle-income countries. Sufficient epidemiological data support causal association between typhoidal *Salmonella* infection and gallbladder cancer. The accumulated evidence suggests that the risk associated with this infection disproportionally affects individuals who are also susceptible to cholelithiasis. On the other hand, clinical and epidemiological evidence to support a causal association between non-typhoidal *Salmonella* infection and colorectal cancer has been modest. However, there have been several recent intriguing findings indicative of carcinogenicity in humans, along with rather strong biological data from experimental studies to support mechanistic pathways to colorectal cancer. Due to greater burden of non-typhoidal *Salmonella* infection, further studies are urgently needed to identify molecular signatures of potentially oncogenic bacterial proteins in the carcinogenic pathway in human tissues as well as to develop physiologically relevant experimental animal models and 3D in vitro cultures, which can reflect the changes of chronic infection and bacterial-host interactions in vivo.

Keywords Bacteria · Beta-catenin · Colon cancer · Dysbiosis · Inflammation · Infection · Microbiome · p53 · Wnt beta-catenin

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4.1 Introduction

The bacterial genus *Salmonella* (S) consists of two species, *S. enterica* and *S. bongori.* We focus on *S. enterica* in the remaining section as it represents 99.5% of all *Salmonella* strains (Gossner et al. 2016), while *S. bongori* is largely associated with reptiles (Fookes et al. 2011).

S. enterica is traditionally classified by serotype based on combinations of two surface proteins, flagellar (H) and somatic (O) antigens. To date, more than 2600 serotypes have been reported (Gossner et al. 2016; Jajere 2019). These serovars are also often grouped according to their clinical presentations in humans, typhoidal vs. non-typhoidal. The former causes typhoid/enteric fever, a serious systemic condition that is often life-threatening, and includes *S. serovars* Typhi and Paratyphi A,B,C. The remaining serovars are considered non-typhoidal and represent the major cause of foodborne illness (gastroenteritis/diarrhea disease) (Jajere 2019). Many *Salmonella* serovars have a broad host range, infecting a wide variety of animals, including mammals, birds, reptiles, amphibians, fish, and insects, while others are very limited in their host range (Silva et al. 2014; Jajere 2019). *Salmonella* can also grow in plants and can survive in protozoa, soil, and water, extending its transmission routes (Silva et al. 2014). Chronic asymptomatic carriage of either type of *Salmonella* develops in some patients after initial infection, which is more often documented for typhoidal *Salmonella* (Gal-Mor 2018).

Broad-host-range, ubiquitous/generalist, *Salmonella* pathogens are generally non-typhoidal, but typhoidal serovars take only humans as the host (Silva et al. 2014; Jajere 2019). In the literature to date, two types of human cancer have been under vigorous study in relation to *Salmonella* infection. These include colorectal cancer by non-typhoidal *Salmonella* and biliary (gallbladder) tract cancer by typhoidal *Salmonella*. In this chapter, we first update exposure data of each *Salmonella* group worldwide, then present new findings on each cancer, which were published in the past decade, and finally address gaps in the current research and potential future developments in the field.

4.2 Human Exposure Data

4.2.1 Non-typhoidal Salmonella

Global and regional burden of various disease and health conditions has been estimated by the World Health Organization (WHO) since the 1990s (Stein et al. 2007), and the WHO established the Foodborne Disease Burden Epidemiology Reference Group (FERG) in 2007. Assembling an assortment of data, including systematic reviews, cohort studies, surveillance studies, and other burden of disease assessments, the group estimated the burden of 22 diseases around year 2010 (Kirk et al. 2015). Illnesses due to non-typhoidal *S. enterica* infection resulted in the

largest disease burden globally, reflecting the ubiquitous nature of non-typhoidal *Salmonella* (regardless of geographical regions), causing over 153 million of incidence worldwide (Kirk et al. 2015). This is reflected to the median incidence rate of approximately 1% per year, implying that cumulative incidence over lifetime per individual is substantial. Overall, a half was estimated to be foodborne, though this fraction varies with the regions of the world, the rest from human to human, water, and animal contacts (Hald et al. 2016). Poultry, pork, and eggs were major food sources consistently found throughout the world, while other sources, such as dairy, beef, and vegetables, were reported less frequently (Hoffmann et al. 2017). It is important to note that these estimates concern symptomatic cases only. Serosurveillance is an alternative to laboratory-based passive surveillance, which has been used in many developed countries but is known to be limited in sensitivity. Using almost 10,000 serum antibody measurements against non-typhoidal *Salmonella*, Mølbak et al. reported seroincidence around 2010 in 13 European countries, which was lowest in Sweden (0.06 infections per person-year), Finland (0.07), and Denmark (0.08) and highest in Spain (0.61), followed by Poland (0.55) (Mølbak et al. 2014). These numbers were not correlated with the reported national passive surveillance of *Salmonella* infection data, but were well correlated with prevalence of *Salmonella* in laying hens, broilers, and slaughter pigs (Mølbak et al. 2014). These numbers were also substantially higher than the incidence rate estimated by the WHO (Kirk et al. 2015), suggesting much larger burden of non-typhoidal *Salmonella* exposure in human populations.

4.2.2 Typhoidal Salmonella

In contrast to ubiquitous presence of non-typhoidal *Salmonella* worldwide, typhoidal *Salmonella* infection has been essentially eliminated in high-income countries over the past century, but it disproportionally affects low- to middle-income countries with the highest incidence of typhoid fever in South and Southeast Asia and sub-Saharan Africa (Gibani et al. 2018; Bhutta et al. 2018). The aforementioned WHO reports also covered typhoidal *Salmonella* infection, although the majority was considered not foodborne but predominantly waterborne (Kirk et al. 2015; Hald et al. 2016). The estimated burden in 2010 was 25.8 million of incidence worldwide (Kirk et al. 2015), which was in a close range of the estimates made by a few others for the same time frame, using different methods (Mogasale et al. 2014).

4.3 Association with Human Cancer

4.3.1 Colorectal Cancer and Its Precursor Lesions

In the past decades, several important studies have been published concerning the association of non-typhoidal intestinal *Salmonella* infection with colorectal cancer or its well-established precursor lesions, specifically inflammatory bowel disease (IBD), patients with which are known to be at increased risk of colorectal cancer (Lutgens et al. 2013; Jess et al. 2012). Evidence for IBD have been provided by cohort linkage studies conducted in Scandinavian countries for extended follow-up periods (>10 years) after infection (Gradel et al. 2009; Jess et al. 2011; Axelrad et al. 2019). Two of these studies from Denmark were based on laboratory-confirmed infection with stool specimens (Kirk et al. 2015; Jess et al. 2011) and investigated *Salmonella* as well as *Campylobacter* infections. These studies (Kirk et al. 2015; Jess et al. 2011) pointed out detection bias within 1 year from the infection, demonstrated by substantially heightened incidence of IBD in this period, which was consistent with an earlier report (Helms et al. 2006). Excluding this period, the standardized incidence ratio (SIR) was approximately 2 for individuals who were positive to non-typhoidal *Salmonella*, compared to general population negative to both types of infection (Kirk et al. 2015; Helms et al. 2006). In the newest study from Sweden, *Salmonella* infection was identified via diagnostic codes from nationwide inpatient and outpatient databases (Axelrad et al. 2019). Compared with the general population without diagnosis of any infectious gastroenteritis, a history of non-typhoidal *Salmonella* enteritis led to 70% increase in risk of IBD (Axelrad et al. 2019). Although all estimates from these record linkages suggest a modestly increased risk of IBD associated with non-typhoidal *Salmonella* infection, underestimation of the risk may be possible, given low sensitivity of laboratory-based public heath surveillance of these infections, as discussed above. The second caveat is the limited specificity of *Salmonella* as an causative agent, as these studies equally reported similarly increased risk of IBD in the patients who had other types of gastrointestinal infection, i.e., *Campylobacter* (Kirk et al. 2015; Helms et al. 2006) or any other types of bacterial, viral, or parasitic gastroenteritis (Axelrad et al. 2019). Thus, besides bacterial species-specific virulence factors, common pathological pathways through persistent inflammatory reactions may be involved.

For colorectal cancer, a pioneer study in human subjects was a case-control study, involving two countries, the USA and the Netherlands, using archived blood samples (Kato et al. 2013). They found that subjects with colorectal tumor (cancer and polyps) had higher antibody titers against *Salmonella enterica* flagellin than controls, showing a two- to threefold increase in risk of colorectal neoplasm in subjects who had titers higher than the median level (Kato et al. 2013). Important points of this study were observations made in the two independent samples as well as the association with premalignant stage (e.g., polyps) of colorectal cancer, suggesting potential etiological involvement. In addition, this study also suggested a possibility of interactions between this bacterial infection and other colorectal cancer risk

factors, such as smoking and red meat intake. However, the study had a major weakness as case-control studies have an inherent limitation in addressing a temporal relationship between exposures and outcomes.

The same group also studied fecal carriage of *Salmonella* using a molecular method, specially PCR to amplify *Salmonella* 16S–23S internal transcribed spacer, demonstrating that *Salmonella* is commonly detected as a low-abundance bacterium in healthy human feces (Lu et al. 2017). Most importantly, this study was the first to report detection of a *Salmonella*-specific effector protein AvrA in actual human tissue specimens (Lu et al. 2017). AvrA has been shown to modulate multiple key oncogenic pathways, namely, Wnt/β-catenin (Wang et al. 2018; Sun et al. 2004; Sun 2009; Lu et al. 2012, 2014; Duan et al. 2007) and P53 (Wu et al. 2010b), in post-transcriptional manners. Analyzing 155 tissue microarray cores ranging from normal colorectal mucosa to metastasized cancer, they found that cancer adjacent mucosa had a statistically significantly higher mean staining ($P = 0.018$) than normal mucosa without any colorectal pathology, while primary tumors themselves exhibited a lower staining score ($P = 0.013$). Benign lesions and lymph node metastases showed equivalent staining to normal mucosa (Lu et al. 2017). Staining of separate clinical samples clearly depicted dense red staining of AvrA in colorectal cancer tissue, including nuclei, but no staining in the controls. Although the results from this study provide strong evidence to support oncogenic potential of *Salmonella* infection in humans, the retrospective nature of the study does not allow inferring a causal association. Moreover, being a low-abundance pathogen, *Salmonella* has not been reported in studies for colorectal cancer, using global metagenomic sequencing of stool or tissue samples to date.

Further supporting evidence is derived from a recent prospective study based on laboratory-confirmed *Salmonella* infection, which was linked to colon cancer incidence data from the Netherlands Cancer Registry (Mughini-Gras et al. 2018). After excluding cases diagnosed within 1 year from infection, the SIR compared with general population was 1.54 (95% confidence interval: 1.09–2.10) for individuals infected with *Salmonella* before age 60. In addition, there was a declining trend in the SIRs with increasing age at infection, implying a long incubation time may be required for development of colon cancer. The association was more pronounced for right-sided colon cancer, infection with serovar Enteritidis, and enteric rather than systemic infection (Mughini-Gras et al. 2018). It is noteworthy that the stronger association with enteric infection is consistent with expression patterns of *S. enterica* potential oncoprotein, AvrA, which is predominantly expressed in enteric infection, but not often in systemic disease (Streckel et al. 2004).

4.3.2 Biliary Tract Cancer and Its Precursor Lesions

The association between typhoidal *Salmonella* infection and biliary tract (specifically gallbladder) cancer has been reported by a larger number of clinic o-epidemiological studies. To date, there have been more than 20 studies and 2 recent

meta-analyses have estimated the summary risk (Koshiol et al. 2016; Nagaraja and Eslick 2014). These two studies have produced very similar results, i.e., the overall summary risk ratio of 4~5, although the summary risk estimates for some subgroups varied considerably between these two analyses, due to different inclusion/exclusion criteria (Koshiol et al. 2016; Nagaraja and Eslick 2014). Most, except three cohort studies, were case-control studies, the majority were from Asian countries where enteric/typhoid fever is/was endemic, and various methods were used for exposure assessment, including bile/gallstones/blood/stool culture, serum antibody assays for specific typhoidal *Salmonella* antigens, PCR, and medical history of enteric fever. The association was confirmed not only by case-control studies but also by a limited number of cohort studies, regardless of geographic locations (despite apparent over-representation of studies from India), and the association was stronger for the studies based on objective laboratory measurements rather than those based on medical history (Koshiol et al. 2016; Nagaraja and Eslick 2014). Moreover, types of control subjects had pronounced effects on the risk estimates for case-control studies. To obtain culture specimens, many studies have used patients with gallstones or other hepatobiliary conditions as controls. However, the comparison of gallbladder cancer cases to these types of controls led to attenuated associations (Koshiol et al. 2016; Nagaraja and Eslick 2014), which was credible given well-known associations of gallstones with the risk of gallbladder cancer (Di Domenico et al. 2017). A more recent case-control study not included in the meta-analyses replicated these associations using PCR to detect *S*. Typhi *fliC* and *staA* genes, reporting the odds ratios of four compared with individuals with benign gallbladder disease and 51 compared with those deceased without gallbladder disease (Scanu et al. 2015).

Interestingly, several recent studies have revealed that typhoidal *Salmonella* produces biofilms on the surfaces of cholesterol gallstones and thus *Salmonella* can survive and grow, encased within a macromolecular matrix of biofilms and protected from antimicrobial properties of bile (Crawford et al. 2010; Gonzalez-Escobedo et al. 2013; Marshall et al. 2014). This facilitates development of chronic gallbladder carriage of typhoidal *Salmonella*, and approximately 90% of chronic carriers in endemic areas have been reported to have gallstones (Di Domenico et al. 2017). Thus, the association of gallstones with gallbladder cancer is likely to be mediated through protracted exposure to oncogenic virulence factors from typhoidal *Salmonella*, such as cytolethal distending toxins (CDT) (Di Domenico et al. 2017), and other *Salmonella* effectors, SopB, SopE, SopE2, and SptP, have been demonstrated to activate MAPK and AKT pathways in the presence of cMYC overexpression and pretransforming p53 mutations, which is crucial for sustained transformation (Scanu et al. 2015). There was a report of detection of non-typhoidal *Salmonella* DNA in a small number of gallbladder cancer tissues, but its etiological involvement was uncertain (Iyer et al. 2016).

4.4 Summary and Future Direction

There is sufficient epidemiological evidence to support causal association between typhoidal *Salmonella* infection and gallbladder cancer. There have been consistent robust associations across the studies. The accumulated data suggest that the risk associated with this infection disproportionally affects individuals who are also susceptible to cholelithiasis. In addition, typhoid toxin belongs to CDT toxins that induce DNA damage and cell cycle alternations (Di Domenico et al. 2017) and bile enhances virulence of typhoidal *Salmonella*, but not that of non-typhoidal *Salmonella* (Johnson et al. 2018).

There is insufficient evidence in clinic-epidemiological studies to permit a conclusion as to a causal association between non-typhoidal *Salmonella* infection and colorectal cancer. Compared with the strength of the associations observed for gallbladder cancer, the magnitude of the association for colorectal cancer reported in a limited number of human studies is lower.

However, there have been several recent intriguing findings indicative of carcinogenicity in humans, along with rather strong biological data from experimental studies to support mechanistic pathways to colorectal cancer.

Despite the weaker association with colorectal cancer, due to highly ubiquitous exposure in human populations, the percent population attributable risk to non-typhoidal *Salmonella* infection for colorectal cancer is in fact larger than that of typhoidal *Salmonella* infection for gallbladder cancer, if the association is determined to be causal. This is especially the case for developed countries, e.g., the USA and European Union. Besides, due to much higher global incidence of colorectal cancer than that of gallbladder cancer (Ferlay et al. 2019), the number of cases attributable to non-typhoidal *Salmonella* infection becomes much larger than that attributable to typhoidal S*almonella* infection. Thus, future research priority should be given to the effort to clarify carcinogenicity of non-typhoidal *Salmonella* in humans.

There are several gaps in the current knowledge concerning *Salmonella*-induced carcinogenesis in humans. First, little has been known about natural history of non-typhoidal *Salmonella* infection in human intestine. The traditional method to determine carriage of non-typhoidal *Salmonella* after acute episodes has been stool culture, which has limited sensitivity, and not all carriers shed bacteria in the stool constantly. Others and we have demonstrated that use of culture-independent, DNA-based methods evidently increases *Salmonella* detection rates (Lu et al. 2017; Tack et al. 2019). In addition, most information about non-typhoidal *Salmonella* infection has been derived from stains that caused conditions requiring medical attention, and there are almost no data concerning long-term carriage following to mild self-limiting non-typhoidal *Salmonella* infection. Certainly, long-term follow-up studies with repeat molecular and/or serological monitoring in general population are warranted.

Equally unknown are if there are specific histopathological changes associated with sustained infection beyond inflammation, e.g., atrophy, meta/dysplasia, and

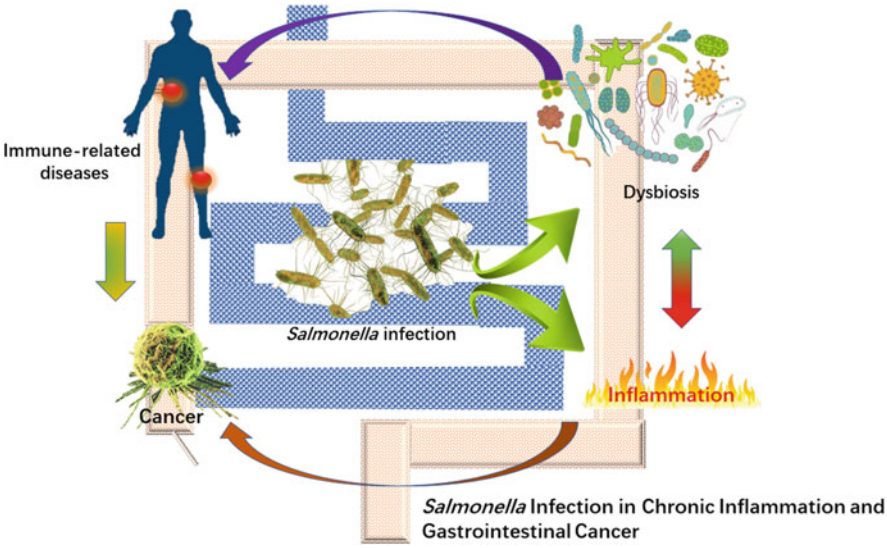

Fig. 4.1 The working model *of Salmonella* infection and its progression and contribution to inflammation and cancer. *Salmonella* can cause chronic infection, dysbiosis, and chronic inflammation resulting in DNA damage and genome instability, which can be exasperated by external factors such as diet, obesity, and inactivity. Ultimately, *Salmonella* can activate the host ontogenetic signaling pathways, thus leading to cancer

aberrant crypt foci in human intestine, and if there are genetic and behavioral changes in *Salmonella* to adopt the host during long-term colonization. Specifically, we do not know whether non-typhoidal *Salmonella* forms biofilm on the intestinal mucosa as typhoidal *Salmonella* does. While antibiotic use is a known risk factor for non-typhoidal *Salmonella* infection (Crum-Cianflone 2008; Gal-Mor 2018), there is little information about its social interactions with human intestinal commensals, i.e., whether certain types of gut microbiome or prebiotic diet prevent *Salmonella* long-term colonization and whether *Salmonella* colonization alters gut microbiome structure. Thus far, evidence is limited to mouse models (Martz et al. 2015; Deatherage Kaiser et al. 2013).

Based on the current progress of *Salmonella* infection and its contribution to inflammation and cancer, we believe that *Salmonella* can cause chronic infection, gut dysbiosis, and chronic inflammation resulting in DNA damage and genome instability, which can be exasperated by external factors such as diet, obesity, and inactivity. Ultimately, *Salmonella* can manipulate the host signaling, e.g., the Wnt/β-catenin signaling pathway and P53 through AvrA and MAPK and AKT pathways through SopB, SopE, SopE2, and SptP, thus leading to cell transformation and development of cancer (Zha et al. 2019; Scanu et al. 2015) (Fig. 4.1). These transformations are more likely to occur in the presence of pretransforming mutations in tumor suppressors and oncogenes, such as KRAS, P53, and APC PAC. In fact, mutations in these genes are linked to exposure to other environmental risk

factors, such as diet, alcohol, and cigarette smoking (Kato et al. 2014). However, the exact mechanisms of interplays between bacterial virulence factors and other environmental risk factors in driving definitive transformation are unknown and warrant further investigation.

To establish a causal association, detection and quantitation of actual *Salmonella* proteins with oncogenic potential in well-characterized human tissue samples is crucial. It is equally important to identify molecular signatures of such potentially oncogenic bacterial proteins in the carcinogenic pathway in human tissues. The majority of laboratory studies still focus on *Salmonella* Typhimurium. However, *Salmonella* Enteritidis has emerged as one of the most important foodborne pathogens for humans, and it is mainly associated with the consumption of contaminated poultry meat and egg (Patrick et al. 2004; Wright et al. 2016). Infection caused by *Salmonella* Enteritidis is the second most common cause of bacterial gastroenteritis in the developed world and results in significant economic loss to the poultry industry and places a substantial burden on the healthcare system (Wright et al. 2016; Scallan et al. 2011; Majowicz et al. 2010). Thus, more studies are needed to understand *Salmonella* Enteritidis, an important pathogen with a public health concern (Lin et al. 2016). We need work on physiologically relevant experimental models, which can reflect the changes of chronic infection in vivo (Lu et al. 2010; Wu et al. 2010a). In vitro, we need to use the 3D or polarized epithelial cells (Zhang et al. 2014; Sun 2017). We need to consider human organoids derived from intestinal stem cells for studying *Salmonella*-host interactions (Zhang et al. 2019). Finally, *Salmonella* possesses a myriad of virulence factors derived from multiple pathogenicity islands (Hayward et al. 2014; Sabbagh et al. 2010; Hensel 2004; Kaur and Jain 2012; Kuhle and Hensel 2004; Phoebe Lostroh and Lee 2001). It is highly plausible that there are synergistic and antagonistic interplays among these virulence factors in modulating carcinogenic risk, which remains to be further investigated.

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Chapter 5
Biomarkers of Esophageal Cancers and Precancerous Lesions

Manisha Bajpai and Zhongren (David) Zhou

Abstract Esophageal cancer is one of most deadly cancers worldwide although the two subtypes differ in their geographical distribution and natural history. The challenge is to intercept the disease in its premalignant stages to improve the curative effect and survival rate. To this end, most efforts have been focused on finding biomarkers for early diagnosis and early treatment of esophageal cancer. In this chapter, we reviewed biomarkers of esophageal cancer and precancerous lesions that have already been in clinical application as well as those that are in different stages of discovery and validation. In addition, we briefly introduce the microbiome and other less conventional biomarkers of esophageal cancers.

Keywords Esophageal cancer · Esophageal squamous cell carcinoma · Esophageal adenocarcinoma · Barrett's epithelium · Gastroesophageal junction · Biomarker · Microbiome

Abbreviations

AOL	*Aspergillus oryzae* lectin
ATP	Adenosine triphosphate
BE	Barrett's epithelium
CA	Carcinoma
cfDNA	Circulating cell-free DNA
CISH	Chromogenic in situ hybridization
CTC	Circulating tumor cells
EAC	Esophageal adenocarcinoma

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EC	Esophageal cancer
EGFR	Epidermal growth factor receptor
ESCC	Esophageal squamous cell carcinoma
FDA	Food and Drug Administration
GCA	Gastric cardia adenocarcinoma
GEJ	Gastroesophageal junction
GERD	Gastroesophageal reflux disease
GI	Gastrointestinal
HER2	Human epidermal growth factor receptor 2
HGD	High-grade dysplasia
IHC	Immunohistochemistry
KEGG	Kyoto Encyclopedia of Genes and Genomes
LGD	Low-grade dysplasia
LncRNA	Long non-coding ribonucleic acid
LOH	Loss of heterozygosity
MSI	Microsatellite instability
NE	Normal esophagus
OCCAMS	Oesophageal Cancer Clinical and Molecular Stratification
ORR	Objective response rate
OS	Overall survival
PD-1	Programmed cell death receptor 1
PD-L1/PD-L2	Programmed cell death receptor ligand 1 and 2
SCNA	Somatic copy number alterations
TCGA	The Cancer Genome Atlas
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VOC	Volatile organic compounds

5.1 Introduction

Every year, approximately 570,000 patients are diagnosed with esophageal cancers (EC), and 500,000 patients die from this fatal disease worldwide. Although esophageal squamous cell carcinoma (ESCC) is the most common subtype of esophageal cancer, the incidence of esophageal adenocarcinoma (EAC), the other subtype, has increased 600–800% in the last three decades and continues to rise in the western world (Pohl et al. 2010). The esophageal cancer has become the sixth leading cause of cancer deaths in the United States (Njei et al. 2016). The esophageal cancer researchers have been studying the biology of esophageal cancer development to devise the best methods to prevent, monitor, and treat this disease for many years. However, the answer remains pessimistic, and the 5-year survival rate is still between 15% and 20% although many treatments including surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy are available for EC (Njei et al.

2016). Cancer is an extremely heterogeneous disease with various genomic changes including chromosomal aberrations, somatic mutations, and other genetic and epigenetic changes that remain to be discovered. Esophageal cancers present differently in each patient; even the genomic changes from different areas of the same tumor are heterogeneous (Junker and van Oudenaarden 2014; Li et al. 2018; Pectasides et al. 2018).

Both ESCC and EAC are known to arise from a background of chronic inflammation triggered by underlying health conditions like gastroesophageal reflux disease or obesity in EAC and smoking/alcohol consumption in ESCC. Both subtypes are more common in men and have overlapping risk factors (Table 5.1) compounded by genetics, ethnicity, gender, and dietary preferences. The ESCC is more common in Southeastern Africa, Asia, and South America, whereas EAC is prevalent in the developed nations like Western Europe, North America, and Australia. Squamous dysplasia is the precursor lesion of esophageal squamous cell carcinoma, and Barrett's esophagus is the precursor for EAC; these premalignant lesions provide a window of opportunity to intercept the progression of the deadly disease. Effective screening tools in the form of noninterventional methods and biomarkers with high specificity and sensitivity are necessary to achieve success with early detection and timely intervention. Thousands of cancer-related genomic changes have been identified in esophageal cancers (Dulak et al. 2013; Bandla et al. 2012; Kaz et al. 2015), and the diversity of such observations has baffled the investigators and impeded the development of universally acceptable methods for risk stratification.

In 1998, the National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Goossens et al. 2015). In this chapter, we provide a succinct review of the literature related to the biomarkers in esophageal cancers and precancerous lesions. The review is organized into sections based on the nature of the biomarkers and their stage of clinical development. The first section discusses the biomarkers already in use in clinical applications; the second section focuses on potential molecular biomarkers related to Barrett's esophagus, dysplasia, and esophageal cancers in the discovery stagesand the third section introduces the microbiome and other less conventional biomarkers of EC. Our goal is to systematically discuss the limitations of the currently available biomarkers and the methods used to identify and evaluate newer, more sensitive and specific molecular biomarkers. We hope this article will help clinicians, clinical and translational researchers, as well as people with interest in the development and validation of EC biomarkers understand the methods and challenges of early stage biomarker discovery and the significance of the need for sensitive and specific biomarkers.

Table 5.1 Studies identifying gene signatures for esophageal cancer and premalignant conditions

Study (year)	Samples in discovery set (*n*)	Histological tumor type	Gene signature	Samples in signature validation set (*n*)	Risk assessed
Hammoud (2009)	89	AC	9 genes	Not conducted	Survival
Kim (2010)	64	AC	10 genes	52	Disease-free survival
Peters (2010)	75	AC	4 genes	371	Disease-free survival
Goh (2011)	56	AC	4 genes	371	Disease-free survival
Rao (2011)	35	AC	165 genes	165 genes	Disease-free survival
Rao (2011)	35	AC	113 genes	113 genes	Response to CT (epirubicin/cisplatin/capecitabine) + surgery
Wen (2014)	28	AC	3	32	Response to CRT (cisplatin/vinorelbine/40Gy) + surgery
Luthra	18	16 EAC 2 ESCC	3 genes	Not conducted	CRT (docetaxel/5-FU/irinotecan/50.4Gy) + surgery
Lu (2014)	10	ESCC	1 gene	198	Disease-free survival
Motoori (2010)	25	ESCC	199 genes	10	CT (cisplatin/5-FU/doxorubicin) + surgery (17)
Schauer	47	EAC	1 gene Ephrin B3	Not validated	Response to CT (cisplatin/5-FU/leucovorin) + surgery
Lagarde (2008)	61	EAC	5 genes		Lymph node metastasis
Gao et al. 2014a, b	113	ESCC	70 genes	Not validated	Survival and therapeutic response
Maher (2009)	13	10 EAC 3 ESCC	12 genes	27	Response to CRT (cisplatin/5-FU/40.5-44Gy) + surgery
Varghese	150	28 non-dysplastic BE 10 low-grade dysplasia 13 high-grade dysplasia 8 EAC	90 genes	169	Risk for disease progression

n number, *AC* adenocarcinoma, *SCC* squamous cell carcinoma, *FU* follow-up, *OS* overall survival, *DFS* disease-free survival

5.2 Biomarkers of Esophageal Cancer and Precancerous Lesions in Clinical Application

5.2.1 Human Epidermal Growth Factor Receptor 2 or HER2

The human epidermal growth factor receptor 2 or HER2 (also known as ERBB2 or HER2/neu) is a member of the epidermal growth factor receptor (EGFR) family and encodes a 185-kDa transmembrane tyrosine kinase receptor (Iqbal and Iqbal 2014). Functionally, the HER2 promotes cell proliferation, controls differentiation, or suppresses apoptosis and is expressed in several tissues such as the nervous system, epithelial cells, or the mammary gland (Iqbal and Iqbal 2014). HER2 overexpression/gene amplification results in excessive cell growth, angiogenesis, and tumorigenesis. Aberrant HER2 levels are detected in breast cancer, lung cancer, glioblastoma, head and neck cancer, pancreatic cancer, colorectal cancer, gastric cancer, and EAC (Roskoski 2019; Gaibar et al. 2020). A humanized monoclonal antibody trastuzumab that selectively targets the extracellular domain of the HER2 receptor has been used extensively in these cancers to attack the tumor cells via antibody-mediated cellular cytotoxicity (Hudis 2007).

5.2.1.1 HER2 Amplification and Overexpression in Esophageal Cancer

In 2010, the clinical trial ToGA showed that the gastric cancer treatment by trastuzumab, combined with chemotherapy improved the overall survival by 2 months compared to chemotherapy alone (Bang et al. 2010). Based on this trial, the team at University of Rochester set out to investigate the status of HER2 in EC. A tissue microarray containing 116 cases of esophageal adenocarcinoma, 34 cases of BE, 18 cases of low-grade dysplasia (LGD), and 15 cases of high-grade dysplasia (HGD) found *HER2* amplification and overexpression in (18.10%; 21/116) EAC tumor cells by immunohistochemistry (IHC) and chromogenic in situ hybridization (CISH) methods (Hu et al. 2011). The amplification frequency was validated in an independent set of 116 esophageal adenocarcinoma samples using Affymetrix SNP 6.0 microarrays (16.4%, 19/116). HER2 protein overexpression was observed in 12.1% (14/116) of esophageal adenocarcinoma and 6.67% (1/15) of HGD. It was confirmed that *HER2* amplification does not associate with poor prognosis in total 232 esophageal adenocarcinoma patients by CISH and high-density microarrays (Hu et al. 2011). Since then multiple studies found HER2 gene amplification in 15–28% EC (Reichelt et al. 2007; Yoon et al. 2012, 2014; Subasinghe et al. 2018; Plum et al. 2019; Brien et al. 2000; Phillips et al. 2013; Van Cutsem et al. 2015). However, the association of HER2 amplification or overexpression with patients' prognosis is controversial. While Brien et al. found that patients with *HER2* amplification ($n = 11$) had shorter survival durations compared to patients without amplification of this gene ($n = 43$) (Brien et al. 2000), some studies found that HER2 amplification significantly associated with better prognosis (Plum et al. 2019)

and improved overall survival ($n = 713$); 35% of HER2-positive patients lived for 5 years as compared to 26% of patients who were HER2 negative (Yoon et al. 2012, 2014). On the other hand, some studies found no difference (Reichelt et al. 2007) or very modest 2-month (23 months vs. 25 months) (Hu et al. 2011) survival difference between the *HER2* amplification and no *HER2* amplification groups. In 2019, Plum et al. confirmed that HER2 amplification is associated with better prognosis (Plum et al. 2019).

5.2.1.2 HER2 Clinical Application in EAC

With the evidence of HER2 amplification or overexpression in EAC, multiple clinical trials have been conducted to study the effect of treatment of EAC patients with HER2 monoclonal antibody. ToGA clinical trials in patients with gastric adenocarcinoma (trial vs. control: 236 vs. 243 patients) and gastroesophageal junction adenocarcinoma (trial vs. control: 58 vs. 48 patients) have shown a significant survival benefit for patients treated with a combination of trastuzumab and standard chemotherapy (Bang et al. 2010; Press et al. 2017). In the TRIO-013/LOGiC trial, that accrued 545 patients (gastric, 87.3%; GEJ, 8.3%, and esophageal cancer, 4.4%) and 487 patients (89%) were centrally confirmed as having *HER2*-amplified disease; the lapatinib-treated Asian participants less than 60 years of age showed significant improvement in progression-free survival (PFS), particularly the subgroup that had 5.01–10.0 and >10.0-fold amplification of *HER2 in their tumors* (Press et al. 2017). A recent meta-analysis, of four cohort studies and one randomized controlled trial (RCT) with 200 patients who received second-line trastuzumab plus chemotherapy and 183 who received chemotherapy alone (Zaanan et al. 2018) showed that trastuzumab plus chemotherapy did not prolong overall survival [HR = 0.72, 95% confidence interval (95% CI) = 0.47–1.08, $p = 0.11$). Progression-free survival was longer with trastuzumab plus chemotherapy compared to chemotherapy alone (HR = 0.64, 95% CI = 0.45–0.91, $p < 0.05$). The treatment outcomes of targeting HER2 in EAC seems different from the outcomes observed in breast cancer. More clinical trials are probably needed to get a more definitive conclusion for treating EAC patients with HER2 inhibitors (Palle et al. 2020).

5.2.2 *Programmed Cell Death 1 or PD-L1: Immunotherapy and Expression in Esophageal Cancer*

The checkpoint programmed cell death 1 (PD-1) protein is expressed in tumor-infiltrating T lymphocytes, B lymphocytes, natural killer cells, monocytes, and dendritic cells. The immune cells are engaged by the tumor cells that express the ligands PD-L1 and PD-L2. PD-L1 increases the apoptosis of activated tumor-reactive T cells and promotes the growth of tumor cells in vivo (Dong et al. 2002).

The advent of immunotherapy, especially immune checkpoint inhibitors, opened a new therapeutic venue for several human cancers, including melanoma (Hua et al. 2016; Ribas et al. 2016) and lung (Gettinger et al. 2016; Rizvi et al. 2016; Garon et al. 2015; Fehrenbacher et al. 2016; Herbst et al. 2016; Hellmann et al. 2017), bladder, (Bardoli et al. 2016; Sidaway 2016), and renal cancers (Wallin et al. 2016). In Japan, PD-L1 antibody was also used to treat hepatocellular carcinoma and esophageal squamous cell carcinoma (ESCC) in clinical trials (Kudo 2017; Kato et al. 2019; Shah et al. 2019).

5.2.2.1 PD-L1 Immunotherapy in Clinical Application

Numerous studies have demonstrated association of PD-L1 tumor expression with disease prognosis in patients with ESCC (Derks et al. 2015; Fan and Mao 2017; Akutsu et al. 2018; Doi et al. 2018; Salem et al. 2018; Fassan et al. 2019; Kato et al. 2019; Kelly 2019; Konno-Kumagai et al. 2019; Shah et al. 2019; Yan et al. 2019; Tamura et al. 2020). Based on these findings, the Food and Drug Administration (FDA) approved the immunotherapy drug pembrolizumab (KEYTRUDA) to treat patients with locally advanced or metastatic squamous cell carcinoma of the esophagus (ESCC). The patients were selected for this drug treatment if they had certain levels of the protein PD-L1 in their tumors and had failed to respond to one or more lines of standard therapy (Doi et al. 2018; Kato et al. 2019; Shah et al. 2019). A companion diagnostic test PD-L1 IHC 22C3 pharmDx was approved by FDA measuring PD-L1 levels in the tumors (Shah et al. 2019).

The FDA approval was based primarily on two clinical trials, both sponsored by the drug's manufacturer, Merck. The first was KEYNOTE-180, a phase 2 clinical trial that enrolled 121 patients with advanced, metastatic esophageal cancer that had progressed even after two or more lines of standard therapy (Shah et al. 2019). In this trial, the objective response rate was 14.3% (95% CI, 6.7–25.4%) among patients with ESCC (9 of 63) and 5.2% (95% CI, 1.1–14.4%) among patients with adenocarcinoma (3 of 58). Among patients with PD-L1-positive tumors, the objective response was 13.8% (95% CI, 6.1–25.4%) (8 of 58), while patients with PD-L1-negative tumors have a response rate of 6.3% (4 of 63). In the KEYNOTE-180 trial, 35 patients with ESCC had a PD-L1 expression of 10 or greater with combined positive score (CPS), and their overall response rate was 20%, ranging from approximately 4 months to more than 25 months. The second clinical trial was KEYNOTE-181, a phase 3 trial comprising 628 patients with advanced esophageal cancer that had progressed on or after one line of treatment. In this study, the patients were randomized to receive either pembrolizumab or the treating clinician's choice of three different chemotherapy regimens including paclitaxel and docetaxel (Metges et al. 2019). The median overall survival in patients with PD-L1 expression of tenfold or greater was 10.3 months with pembrolizumab versus 6.3 months for patients receiving standard chemotherapy. The overall response rate in patients who received pembrolizumab was 22%, compared with 7% in patients who received alternative chemotherapy regimen (Metges et al. 2019).

KEYTRUDA® (pembrolizumab) was also approved for the treatment of recurrent, locally advanced or metastatic gastric or gastroesophageal junction (GEJ) adenocarcinoma in patients whose tumors express PD-L1 [CPS≥1] (as determined by the PD-L1 IHC 22C3 pharmDx test). These patients were treated with pembrolizumab as they were either nonresponsive to or their disease progressed on or after two or more prior lines of therapy (including fluoropyrimidine- and platinum-containing chemotherapy and if appropriate, HER2/neu-targeted therapy) (Fashoyin-Aje et al. 2019; Shah et al. 2019). Promising data from a global, multicenter, non-randomized, open-label multi-cohort trial, KEYNOTE-059, accelerated approval for KEYTRUDA. Observations of the study was based on 259 patients with gastric or GEJ adenocarcinoma and whose disease progressed on at least two prior systemic treatments for advanced GEJ adenocarcinoma. Fifty-five percent (143/259) of the patients in this cohort had tumors that expressed PD-L1 with a CPS $\geq$ 1 and microsatellite stable (MSS) tumor status or undetermined microsatellite instability (MSI) or mismatch repair (MMR) status. The objective response rate (ORR) in these 143 patients was 13.3% (Fuchs, Doi et al. 2018).

Several clinical trials have studied the efficacy of PD-1/PD-L1 blockade by drugs other than KEYTRUDA in advanced gastroesophageal cancers. CheckMate 032, a phase I/II trial, studied the clinical impact of nivolumab (N), an anti-PD-1 monoclonal antibody, along with ipilimumab (I), a CTLA-4 inhibitor in 160 patients with advanced gastroesophageal malignancy (Janjigian et al. 2018). The ORRs were 19% with nivolumab alone, 40% with N1mg+I3mg combination, and 23% with N3mg +I1mg combination. These ORRS were greater in cancers with PD-L1 expression; however a modest response was noted in PD-L1-deficient malignancies as well (12% nivolumab alone, 22% N1mg+I3mg, 0% N3mg+I1mg). ATTRACTION-02, a randomized, double-blind, placebo-controlled phase III trial conducted in Japan, South Korea, and Taiwan, studied 493 patients with refractory gastroesophageal cancer. Patients were randomized (2:1) to receive either nivolumab 3 mg/kg or placebo, and the primary endpoint was overall survival (OS). Nivolumab improved the median OS to 5.26 months (95% CI, 4.60–6.37) compared to 4.14 months (95% CI, 3.42–4.86) in the placebo cohort. In addition, 12-month OS rates were 26.2% (95% CI, 20.7–32.0) in the nivolumab arm compared with 10.9% (95% CI, 6.2–17.0) in the placebo cohort. In this study PD-L1 tumor status did not appear to significantly impact OS of the 26 (14%) PD-L1-positive patients after an exploratory analysis. The median OS in tumors with PD-L1 positivity in experimental vs. placebo arms was 5.22 months (95% CI, 2.79–9.36) vs. 3.83 (95% CI, 0.79–9.36). This was not significantly different from the PD-L1-negative tumors that had a median OS of 6.05 months (95% CI, 4.83–8.54) vs. 4.19 months (95% CI, 3.02–6.93) in experimental vs. placebo arms (Kang et al. 2017). Although it is difficult to conclude how PD-L1 status may impact the choice of therapy, given the small sample size, this study led to the approval of nivolumab in Japan for use as third-line therapy in advanced gastroesophageal cancer.

5.2.2.2 PD-L1 Expression in Esophageal Cancer

The PD-L1 expression in ESCC has been extensively studied in China and other Asian countries (Ito et al. 2016; Leng et al. 2016; Qu et al. 2016; Chen et al. 2017; Jesinghaus et al. 2017; Jiang et al. 2017a, b; Lam et al. 2017; Zhang et al. 2017, 2019; Guo et al. 2018a, b, 2019; Hsieh et al. 2018; Ng et al. 2018; Wang et al. 2018; Fukuoka et al. 2019; Jiang et al. 2019; Rong et al. 2019). PD-L1-positive expression ranging from 18.9 to 45% has been reported in ESCC tumor cells (Chen et al. 2016; Ito et al. 2016; Lim and Soo 2016; Rong et al. 2019). These differences might be due to several factors including type of neoadjuvant therapy, cutoff points, commercial antibodies for different epitopes of PD-L1, and IHC methodology. For example, Chen and his colleagues reported positive PD-L1 immunoreactivity in 45% of ESCC tissues including neoadjuvant chemoradiotherapy-treated patients. Lim et al. reported PD-L1 (5H1) expression increased in ESCC patients who received neoadjuvant therapy (Lim and Soo 2016). The study by Rong et al. excluded the patients who received neoadjuvant chemoradiotherapy and found that PD-L1 was expressed on 29.9% (113/378) ESCC tumor cells and 40.2% (152/378) tumor-infiltrating immune cells. Similarly, the method of scoring for PD-L1 expression may have introduced variability. Ito S et al. found that 18.9% of ESCC tissues had positive PD-L1 (LS-B480) expression (Ito et al. 2016). However, their study used the scoring for PD-L1 expression based on adding both the proportion score and the intensity score with cutoff as $\geq$7%, which is different from the current PD-L1 evaluation guideline from clinical application. Recently, pembrolizumab was approved for the treatment of patients with recurrent locally advanced or metastatic squamous cell carcinoma of the esophagus whose tumors express PD-L1 with combined positive score [CPS] $\geq$10, as determined by a US Food and Drug Administration (FDA)-approved test, with disease progression after one or more prior lines of systemic therapy based on findings from the open-label phase III KEYNOTE-181 trial (ClinicalTrials.gov identifier NCT02564263) and the phase II KEYNOTE-180 trial (NCT02559687) (Shitara et al. 2018; Metges et al. 2019; Shah et al. 2019). The FDA also approved a new use for the PD-L1 IHC 22C3 pharmDx kit as a companion diagnostic device for selecting patients for this indication.

The related data for PD-L1 in EAC and ESCC in the United States is limited, with only a few studies examining its expression in esophageal adenocarcinoma (Ohigashi et al. 2005; Loos et al. 2011; Derks et al. 2015; Dislich et al. 2017). In a study, of 109 EAC cases, 14 (13%) were positive for PD-L1 immunostain; of the 34 ESCC cases, 6 (18%) were positive for PD-L1 immunostain. The PD-L1 expression in EAC was significantly associated with age, T stage, and stroma/inflammatory cell PD-L1 expression (Abu-Farsakh et al. 2017). PD-L1 expression in EAC showed worse survival but was not statistically significant. Recently, Derks et al. reported their PD-L1, PD-L2, and PD-1 immunohistochemistry study on EAC tissue microarray (Derks et al. 2015). They found that PD-L1 was expressed on only 2% of EAC and on 18% inflammatory cells. However, we found that PD-L1 expression on tumor

cells was about 11%, which is higher than the PD-L1 expression rate in their study (Abu-Farsakh et al. 2017). Dislich et al. also found that PD-L1 expression was detected in 8% of EAC and 51.8% of tumor-associated inflammatory cells. The rate of PD-L1 expression (43%) in tumor-associated inflammatory cells in our study is close to Distich's results (51.8%), but it is higher than that in Derks' data. Derks et al. used the monoclonal antibody (PD-L1, clone 405.9A11) from Dr. Gordon Freeman's laboratory, Dana-Farber Cancer Institute (Derks et al. 2015), but Dislich et al. used clones SP142 (Spring Bioscience, Pleasanton, CA) and E1L3N (Cell Signaling Technology, Danvers, MA) antibodies. In our study, we used a commercially available 22C3 PharmDx IHC kit from Dako with their Autostainer and manufacturer protocols, which includes the antibody approved by FDA for evaluating lung non-small cell carcinoma for pembrolizumab (KEYTRUDA) in clinic. The antibodies and protocols may contribute to the discrepancies.

The association between PD-L1 expression and clinicopathological features like lymph node metastasis and tumor stages was reported in several studies (Chen et al. 2016; Ito et al. 2016; Lim and Soo 2016). PD-L1 expression was also found to be associated with age and tumor differentiation (Rong et al. 2019). Older patients (35%) had higher expression of PD-L1 than young patients (25%). Poorly differentiated ESCC tumors had higher PD-L1 expression (42%) compared to well- (25%) and moderately (27%) differentiated tumor groups (Chen et al. 2016; Yu and Guo 2018). Few meta-analysis reports convene to a similar conclusion that PD-L1 overexpression is associated with unfavorable outcomes and lower OS in patients with ESCC, notably in Eastern Asian countries such as China, Japan, and South Korea (Qu et al. 2016; Guo et al. 2018a, b). However, a limited number of studies reported that increased PD-L1 expression is associated with improved disease-free survival and OS (Jesinghaus et al. 2017, Jiang et al. 2019). This controversy may be attributed to numerous factors, including different methodological approaches and different assessment criteria to define high PD-L1 expression and heterogeneity of PD-L1 expression. These factors may result in differing detection of infiltrating lymphocytes in tumor from the biopsy or the postoperative pathological specimens. However, staining cutoff values tumor proportion score (TPS) of 1 or 5% are frequently used to define the positive rate of PD-L1 expression. Various studies have defined the cutoff values differently. Most of clinical trials for KEYTRUDA defined TPS $\geq$1% as a positive tumor PD-L1 protein expression as a cutoff (Katsuya et al. 2016; Bodor et al. 2020), whereas other trials for atezolizumab or durvalumab used TPS $\geq$5% as the threshold (Eckstein et al. 2019; Gennen et al. 2020).

5.2.3 Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is a secreted cytokine that plays a central role in angiogenesis, the process of new blood vessel formation, and is essential for numerous physiological processes such as embryonic development and wound healing. VEGF receptor-2 (VEGFR-2) is a 200–230-kDa receptor for VEGF-A,

VEGF-C, VEGF-D, and VEGF-E. VEGFR-2 is expressed by vascular and lymphatic endothelial cells and by other cell types such as megakaryocytes and hematopoietic stem cells (Katoh et al. 1995). VEGF and VEGFR-2-mediated signaling and angiogenesis seem to have an important role in the pathogenesis of gastric cancer. Ramucirumab is a fully human IgG1 monoclonal antibody VEGFR-2 antagonist that prevents ligand binding and receptor-mediated pathway activation in endothelial cells (Fuchs et al. 2014).

5.2.3.1 VEGF Clinical Application in EAC

In 2014, ramucirumab, an angiogenesis inhibitor, was approved by the US FDA in the second-line setting by itself or in combination with paclitaxel based on phase 3 REGARD clinical trials (Fuchs et al. 2014). Three hundred fifty-five patients with advanced gastric or gastro-esophageal junctional adenocarcinoma were assigned to receive ramucirumab ($n = 238$) or placebo ($n = 117$). Median overall survival was 5.2 months (IQR 2.3–9.9) in patients in the ramucirumab group and 3.8 months (1.7–7.1) in those in the placebo group (hazard ratio [HR] 0.776). The survival benefit with ramucirumab remained unchanged after multivariable adjustment for other prognostic factors (multivariable HR 0.774). Ramucirumab by itself has only a marginal effect, but the combination of ramucirumab with paclitaxel has a decent efficacy profile (Fuchs et al. 2018). In the RAINBOW experimental arm, the overall survival (OS) was significantly longer with the combination ramucirumab with paclitaxel (median 9.6 months compared to paclitaxel alone at 7.4 months) (Wilke et al. 2014). The combination of ramucirumab with paclitaxel significantly increases overall survival compared with placebo plus paclitaxel and could be regarded as a new standard second-line treatment for patients with advanced gastric cancer (Wilke et al. 2014). The RAINBOW regimen is further recommended in the third-line setting when patients fall out of the first-line therapy due to considerable neuropathy.

5.2.3.2 VEGF Expression in Esophageal Carcinoma

Vascular endothelial growth factors C (VEGF-C) and D (VEGF-D) are important lymphangiogenic factors in several human cancers. High expression of VEGF-C and VEGF-D were observed in 54.7% (40/73) and 65.7% (48/73) of resected esophageal cancer specimens after immunohistochemistry. The higher expression correlated positively with the histological grade of the tumors ($p = 0.038$) and lymph node metastasis ($p = 0.018$). Both VEGF-C and VEGF-D high expression correlated with decreased overall survival, disease-free survival, and cancer-specific survival in this patient cohort. This study confirmed that overexpression of VEGF-C and VEGF-D in locally advanced disease may be useful in identifying patients who are more likely to have a poor prognosis even after curative resection (Kozlowski et al. 2011). However, an earlier study on 46 EAC patients concluded that clinicopathological factors did not show any significant correlation with VEGF expression in the

22 patients (47.8%) to be positive for VEGF, in that cohort. There was no significant association between VEGF expression and long-term survival, reported in this study (Cavazzola et al. 2009). Later, a systematic review of 31 studies ($n = 2387$ patients) and a meta-analysis of 30 studies ($n = 2345$ patients) by Chen et al. investigated the prognostic importance of elevated VEGF expression on overall survival among patients with esophageal cancer (Chen et al. 2012). They reported that high VEGF expression was associated with poor survival in esophageal squamous cell carcinoma (HR, 1.81) and that there was no significant heterogeneity between the studies published in literature ($P = 0.185$). However, the data collected were not sufficient to determine the prognostic value of VEGF in patients with esophageal adenocarcinoma.

5.2.4 *Other Biomarkers in Clinical Application for Diagnosis of EAC and Precancerous Lesions*

Due to interobserver variability in diagnosing LGD, the frequency of progression from low-grade dysplasia (LGD) to high-grade dysplasia/carcinoma (HGD/CA) in Barrett's esophagus (BE) varies among studies. Skacel and colleagues analyzed the immunohistochemical staining for p53 in patients diagnosed with LGD with known clinical outcome and interobserver agreement data (Skacel et al. 2002). They correlated p53 immunoreactivity with clinical progression and with the interobserver agreement among three GI pathologists. In a 2–28-months' follow-up of total 16 LGD cases, 8 patients progressed to HGD/CA. Of the eight patients progressed to HGD/CA, seven cases stained positively for p53, but of the other eight patients without progressing to HGD/CA, only two patients stained positively for p53. With these observations, Skacel et al, claimed that p53 positivity resulted in improved sensitivity (100%) with no change in specificity (75%) in predicting the progression of LGD to HGD/CA when combined with complete interobserver agreement on LGD among three experienced GI pathologists. Therefore, immunohistochemical staining for p53 can be used as an adjunctive test, as it correlated with progression to HGD/CA in this series (Skacel et al. 2002). Late multiple researchers confirmed their finding and agreed that addition of p53 IHC significantly improves the histological assessment of Barrett's esophagus biopsies (Kaye et al. 2016; van der Wel et al. 2018). In one of the confirmatory studies, 10 GI pathologists assessed 60 referral BE cases-single hematoxylin and eosin (HE) slide per case including 20 low-grade dysplasia (LGD); 20 high-grade dysplasia (HGD); and 20 non-dysplastic BE reference cases. After a "washout" period, the same cases were reassessed with the addition of a corresponding p53 IHC slide. It was concluded that addition of p53 IHC decreased the mean proportion of indefinite dysplasia diagnoses from 10 of 60 to 8 of 60 ($P = 0.071$). The mean interobserver agreement, between the pathologists' assessments, increased significantly from 0.45 to 0.57 ($P = 0.0021$), and the mean diagnostic accuracy increased significantly from 72% to 82% ($P = 0.0072$)

after p53 IHC addition (van der Wel et al. 2018). In another study 10 pathologists from 4 other institutions were provided a brief training session in p53 staining interpretation and then asked to review 72 cases encompassing the full spectrum of BE. Each pathologist classified cases on hematoxylin and eosin alone using the Vienna classification and assessed the p53 staining using a qualitative system. Using the three recognized patterns, for p53 staining the unweighted kappa (interobserver agreement) was 0.6 (confidence interval 0.58–0.63), while the weighted kappa values varied from 0.27 to 0.69 with an average of 0.47. When cases were evaluated with both H&E and p53 IHC, the average kappa was 0.61 for definite dysplasia versus no definite dysplasia. Based on these observations, it was agreed that p53 immunohistochemistry interpretation is more reliable than dysplasia diagnosis, even with limited training. Due to the fact that p53 IHC was predictive of prognosis and improved diagnostic reproducibility, it is considered suitable for routine use by pathologists as an adjunct to dysplasia diagnosis. The use of ancillary markers like p53 IHC may help to prevent overdiagnosis of dysplasia in Barrett's and inform appropriate treatment for the patients based on their disease stage (Kaye et al. 2016).

Recently, two panels of biomarkers in BE were found to predict future risk of progression and prevalent dysplasia, respectively (Bird-Lieberman et al. 2012; di Pietro et al. 2015). The first panel includes a consensus diagnosis of LGD by experts, presence of aneuploidy, and aspergillus oryzae lectin (AOL) immunohistochemistry (IHC), which was applied to a retrospective nested case-control study (Northern Ireland Barrett's Registry) (Bird-Lieberman et al. 2012). Based on a risk score created for individuals positive for one or more of these abnormalities, a reduced biomarker panel was constructed. With each additional positive biomarker in the reduced model, the odds for progression to EAC increased by fourfold (OR, 3.90; 95% CI, 2.39–6.37) in BE patients with LGD and by threefold (OR, 3.31; 95% CI, 1.81–6.05) in BE patients without LGD. The second panel, which comprises aneuploidy, p53 (IHC), and cyclin A (IHC), was tested in a multicenter prospective study (di Pietro et al. 2015). This panel predicts inconspicuous prevalent HGD/EAC with a sensitivity of 100% and a specificity of 85%. Duits et al. combined two panels together to investigate their powers for identifying high-risk BE patients. Their nested case-control cohort comprised BE patients who progressed to high-grade dysplasia (HGD)/EAC ($n = 130$) and BE patients who never progressed ($n = 130$), in a 2-year follow-up, matched on age, sex, length of the BE segment, and duration of endoscopic surveillance. This study confirmed that expert consensus LGD diagnosis, abnormal expression of p53, and abnormal expression of AOL all independently predicted the risk of progression to HGD/EAC and this biomarker panel was able to discriminate well (73%) between progressors and nonprogressors as predicted by the ROC curve. Based on this study, a combination of these three markers could help select patients for prophylactic ablation therapy or intensified endoscopic surveillance (Duits et al. 2019).

Apart from molecular markers, Parasa and colleagues developed a scoring system based on demographic data and endoscopic and histologic findings at the time of index endoscopy (Parasa et al. 2018). This longitudinal study involved patients with BE from five centers in the United States and one center in the Netherlands enrolled

in Barrett's Esophagus Study database from 1985 to 2014. Of the 4584 patients in the database, 2697 were included in their analysis (84.1% men; 87.6% Caucasian; mean age, 55.4 ± 20.1 years; mean body mass index, 27.9 ± 5.5 kg/m^2; mean length of BE, 3.7 ± 3.2 cm). During the follow-up period, 154 patients (5.7%) developed HGD or EAC, with an annual rate of progression of 0.95%. Male sex, smoking, length of BE, and baseline low-grade dysplasia (changes in the appearance of the cells in the esophageal epithelium) were found to be significantly associated with BE progression. Using a scoring system, patients with BE at low, intermediate, and high risk for HGD or EAC could be identified with 76% certainty as predicted by the ROC curve (95% confidence interval, 0.72–0.80; $P < 0.001$). This scoring method was validated in an independent cohort, and the calibration slope was 0.9966 ($P = 0.99$), confirming the utility of this scoring system (Progression in Barrett's Esophagus score) based on the biopsy diagnosis, sex, smoking, and length of BE as biomarkers for predicting the risk of patients to develop EAC (Parasa et al. 2018).

5.3 Molecular Markers in Development for Esophageal Cancer and Precancerous Lesions

Due to limited success of existing therapeutic regimens in the management of esophageal cancers, more comprehensive knowledge of the biology of the esophageal cancers is necessary to design effective treatment plans. Efforts have been directed to find molecular biomarkers that could not only diagnose early stages of esophageal cancer (diagnostic markers) but also predict the patient's risk of progression to cancer (progression marker), response to therapy (predictive marker), and survival or prognosis (prognostic marker). Observations on genetic and epigenetic changes in the esophageal cells from in vitro cell culture models (Bus et al. 2012) including the BE carcinogenesis model (Das et al. 2011; Minacapelli et al. 2017), 3D culture models (Whelan et al. 2018), and animal models (Kapoor et al. 2015; Nair and Reddy 2016; Jiang et al. 2017a, b) have been instrumental in understanding the molecular mechanisms of Barrett's esophagus and esophageal carcinogenesis. However, all existing disease models have been criticized for not being physiologically relevant to human esophageal pathobiology. Therefore, in this article we mostly focus on the findings from primary human tissues.

Aberrant transcript levels resulting from epigenetic changes or mutations of several genes were early events observed in the carcinogenesis process, even before the appearance of malignant histological changes (Kalatskaya 2016). Several such events were deemed to be potential biomarkers for early diagnosis (surveillance) and prevention as well as therapeutic management in esophageal squamous cell carcinoma (Gao et al. 2014a, b) and esophageal adenocarcinoma (Dulak et al. 2013). However, data on genomic profiling and potential genetic biomarkers in esophageal cancer are disparate between investigations from different centers. There is a critical need for rigorous clinical validation and replication in independent cohorts before

the molecular biomarkers can make the transition from bench to bedside. The following sections describe in detail some of the outstanding efforts led by esophageal research consortia in the United States and European nations and other innovative independent investigators in the discovery of molecular markers for both ESCC and EAC.

5.3.1 Gene Mutations and Aberrant Expression in Esophageal Cancer and Precancerous Lesions

5.3.1.1 Esophageal Adenocarcinoma

More than 8331 genes were found to be mutated in the 165 EACs after examining exome sequencing datasets curated by our TCGA team (Dulak et al. 2013). However, 3639 genes were found to be mutated in two or more samples, and only 26 genes were significantly mutated (FDR $q < 0.1$) in this cohort. TP53 (72%) had the highest mutation frequency followed by CDKN2A (12%). Twenty-six cancer-related genes with mutation are present in more than 10% tumor. This led the investigators to suggest that point mutations in specific genes may not provide robust discrimination as predictors of progression to EA (Dulak et al. 2013). Around the same time, our group found that genomic mutation load was a potential biomarker to differentiate EAC from BE and columnar metaplasia without goblet cells (Bandla et al. 2014). In this study, we also highlighted for the first time that there was significant difference in genomic changes between columnar cell metaplasia with and without goblet cells. It indicated that goblet cells are the essential criteria for the diagnosis of BE (Bandla et al. 2014).

The Oesophageal Cancer Clinical and Molecular Stratification (OCCAMS) Consortium took a different approach: they identified inherent differences in the mutational profiles from whole-genome sequencing analysis of 129 EAC cases and classified them into three distinct subtypes of EACs. They are (1) enrichment for BRCA signature and defects in the homologous recombination pathway; (2) dominant T>G mutational pattern associated with a high mutational load and neoantigen burden; and (3) C>A/T mutational pattern representative of aging. These signatures when independently verified in another cohort of 87 patients and were suggested to be clinically relevant for therapeutic decision (Secrier et al. 2016). In another recent study, whole genome sequencing (WGS) was performed on 61 junctional adenocarcinomas across all three Siewert types (GEJ1: 26, GEJ2: 22, GEJ3: 13). Based on transcriptome profiling and biological function (based on key gene networks identified on the basis of gene expression), the GEJ adenocarcinomas were classified into three groups. Group 1 was enriched for pathways involved in cell turnover, Group 2 for metabolic processes, and Group 3 for immune-response pathways. Patients in group 1 showed the worst overall survival ($p = 0.019$). The transcriptomic, mutational, and protein expression signatures used to classify the subgroups were successfully verified in independent transcriptomic data with clinical outcomes from

four independent European and Asian datasets. The pooled analysis confirmed the prognostic effect of the new subtypes (Bornschein et al. 2019). A comprehensive integrative analysis combining clinical data with methylation, transcriptome, and genome profiles of more than 400 BE and EAC tissues classified BE and EAC tissues into four subtypes (described subsequently). The tissues classified as subtype 1 showed gain in methylation in CpG islands and enrichment of genes like GATA4, CCND1, and others involved in DNA repair. Subtype 2 also had gained methylation like the subtype 1 but with a unique pattern of unmethylation and enrichment for genes associated with ATP synthesis, fatty acid metabolism, and oxidation processes. Subtype 3 had increased presence of both myeloid and lymphoid cell lineages in the tumor tissue, and subtype 4 was characterized by hypomethylation and a high degree of genome stability from copy number alterations and structural variants. Stratification into these subtypes informed potential therapeutic options ideal for the characteristics of the particular tumor type, e.g., subtype 1 representative of CIMP could possibly be sensitive to DNA methyltransferase and topoisomerase I inhibitors and subtype 4 with hypermethylation to CDK2 inhibitors. Subtype 3 was found to associate with poor prognosis due to immune cell involvement (Jammula et al. 2020).

Genomic changes were also extensively studied as the predicating biomarkers for the chemotherapy and prognosis. EACs were divided by two molecular subtypes with disparate response to chemotherapy and characterized their somatic mutation patterns as well as differential gene expression. They identified a subtype I, with 24 distinctive genes including SMAD4 gene. This subtype was less sensitive to frontline chemotherapy compared to the subtype II EACs that presented a different mutation profile comprising set of 30 different genes including *ARID1A*. Compared to the subtype II, the gene expression in subtype I EACs was enriched for biological processes including epithelial cell differentiation, keratinocyte differentiation, and KEGG pathways including basal cell carcinoma (Guo et al. 2018a, b). Visser et al. systematically compiled 22 peer-reviewed studies from literature that used RNA next-generation sequencing to analyze transcriptional profiles of esophageal tumors (Visser et al. 2017). Only four (three on EACs and one on ESCC discussed later) studies that investigated gene expression profiles in relation to survival actually validated their findings in independent cohorts (see Table 5.1). The first study led by Peters et.al. used gene expression data derived from tumor tissue specimens of 75 patients and stringently selected 10 genes strongly associated with survival and with the number of involved lymph nodes (a prognostic feature) using statistical modeling. In the external validation dataset that consisted of 371 cases from 5 OCCAMS centers, patients with none of the 4 genes dysregulated (5-year survival, 58%) had better survival compared to those with 1–2 of 4 genes dysregulated (5-year survival, 26%), who in turn did better than those with 3–4 of 4 genes dysregulated (5-year survival, 14%) (Peters et al. 2010). The second validated study characterized gene expression profiles in 75 EAC and 28 NE tissue samples from 64 patients. Unsupervised hierarchical clustering analysis based on Pearson correlation coefficients identified three subclasses of EAC, each with a remarkable difference in the clinical outcomes of these patients. Comparison of the differential gene expression

patterns between the EAC subgroups revealed ten genes *AKR1B10*, *CD93*, *CSPG2*, *DKK3*, *LUM*, *MMP1*, *SOX21*, *SPP1*, *SPARC*, and *TWIST 1* that were more than fourfold different between the poor prognosis and better prognosis groups. Among these, *AKR1B10* and *SOX21* were protective genes associated with better survival and the others are risk genes. Another two gene signatures comprising *SPP1* and *SPARC* had prognostic relevance and potential clinical utility (Kim et al. 2010). The third study, by Goh et al., identified a cluster (32% of cohort) with differential log2 ratios of 16 CGH probes ($p < 4 \times 10^{-7}$) using K-means clustering on a CGH array of 56 EAC resection samples and found that the cluster was associated with worse prognosis (median survival = 1.37 years; $p = 0.015$) (Goh et al. 2011). The fourth study identified signatures in ESCC, discussed later in the text (Table 5.2).

Better response to chemotherapy in EAC was associated with overexpression of Ephrin B3 (Schauer et al. 2010). A 165-gene signature in combination with endoscopic ultrasound and traditional staging reliably predicted overall survival (OS; $P < 0.01$) and outcomes of resection after perioperative chemotherapy, with a poor outcome group ($N = 17$) (1 year OS 46.2%) and a good outcome group ($N = 18$) (1 year OS 1005). This set of genes is associated with the regulation of the TOLL receptor-signaling pathway (Rao et al. 2011). Lower expression of gene sets associated with arginine metabolism pathways and lipid metabolism pathways in general and, particularly, argininosuccinate synthetase expression were reported to be correlated with ($P = 0.048$) lymph node metastasis in EAC (Lagarde et al. 2008). Another study found a set of 21 genes that were overexpressed in T1-2 compared with T3-4 tumors (false discovery rate of 0). One of those genes could discriminate between N+ and N0 tumors (false discovery rate of 0), and subset of nine correlated with longer survival (Hammoud et al. 2009). The most interesting fact that emerges from these studies is the heterogeneity of the EACs that could probably explain why all the studies arrived at a different subset of prognostic genes. Only eight genes ALDH1A3, BIN1, CSPG2, DOK1, IFIT1, IFIT3, PHB, SPP1 have been mentioned in other two studies. Smaller sample sizes used in the studies, lack of external validation, dissimilar endpoints (progression, response, survival, metastasis, etc.), and methods used for arriving at gene signatures are other variables that complicate the discovery of efficient biomarkers. In a systematic evaluation of published literature on BE and EAC genome sequencing and genes implicated in BE progression, 77 genes names were extracted. Using an integrated text mining approach, six genes, TP53, CDKN2A, β-catenin or CTNNB1, CDH1, GPX3, and NOX5, were identified as the most frequently altered during BE carcinogenesis, because their name appeared in two or more publications. All six genes prioritized by the text-mining approach accumulate genomic, transcriptomic, and/or proteomic alterations in the large subpopulation of EAC patients, and this subpopulation correlates well with progression stage. It denotes that each of the six genes plays a certain role in BE progression. In addition, all six genes are functionally interrelated, which might indicate that they can serve as an essential core for BE progression (Kalatskaya 2016).

Table 5.2 List of studies investigating epigenetic signatures for esophageal cancer and premalignant conditions

Study	Tumor	Gene signature	Risk assessed
Methylation			
Jin (2009)	145 nonprogressors and 50 progressors	p16, RUNX3, HPP1, NELL1, TAC1, SST, AKAP12, and CDH13	Neoplastic progression
Alvi (2013)	22 Barrett's esophagus and 24 esophageal adenocarcinoma Validated in 98 samples; 60 non-dysplastic BE, 36 dysplastic Barrett's, and 90 early EAC	*SLC22A18*, *PIGR*, *GJA12*, and *RIN2*,	Three risk groups based on the number of genes methylated (low risk, <2 genes; intermediate, 2; and high, >2)
Dilworth (2019)	67 20 progressors 47 nonprogressors Validation set 32 patients (progressors 18, nonprogressors 14)	OR3A4	Risk of progression
Chettouh (2018)	Pilot cohort ($n = 20$ cases, $n = 10$ controls) and a validation cohort ($n = 149$ cases, $n = 129$ controls)	TFPI2*, TWIST1, ZNF345 and ZNF569,	Risk of progression
Wang (2019)	80 patients (52 in the training set; 28 in the test set)	p16, HPP1, NELL1, TAC1, and AKAP12	Risk of progression
Lu (2019)		ABCD1, SLC5A10, SPIN3, ZNF69, ZNF608	Risk of progression
Howarth		15 genes	Progression to neoplasia
miRNA			
Mallick (2016)		miR-192, miR-194, miR-203, miR-205, and miR-215	Diagnosis and monitoring of BE
Clark (2018)		Regulatory miRNA controlling BMP, NOTCH, NF-κB, MAPK signaling pathways, and CDX2 expression	BE progression to EAC
Li (2018)		MIR7, MIR30a, MIR181a, MIR192, MIR196a, and MIR199a or MIR192, MIR196a, MIR199a, and trefoil factor 3 (TFF3)	BE diagnosis

(continued)

Table 5.2 (continued)

Study	Tumor	Gene signature	Risk assessed
Craig (2020)		11 miRNA signatures including miR-29c-3p and miR-193b-5p,	Risk prediction for EAC development
Zhang (2010)		miR-10a, miR-22, miR-100, miR-148b, miR-223, miR-133a, and miR-127-3p miRNAs,	Discriminate between stage I/II ESCC patients
Xie (2012)		miR-10b*, miR-144, and miR-451 detectable in whole saliva and miR-10b*, miR-144, miR-21, and miR-451	Diagnosis of ESCC
Hirajima (2013)		miR-18	Presence of tumor
Skinner (2014)		mir-505*, mir-99b, mir-451, and mir-145*	Response to neoadjuvant therapy
LncRNAs			
Fanelli (2018)	EAC	AFAP1-AS1 (actin filament-associated protein 1-antisense RNA 1) and LncRNA HNF1A-AS1 (hepatocyte nuclear factor 1 alpha-antisense RNA 1)	Progression
Dong (2015)	GCA	UCA1 (urothelial cancer-associated 1), LSINCT-5, and PTENP1	Survival in GC tumors
Wang (2015)	ESCC	MALAT1 (metastasis-associated lung adenocarcinoma transcript 1)	Lymph node metastasis and poor overall survival
Gupta	ESCC	HOTAIR (HOX transcript antisense RNA)	Lymph node metastasis and poor overall survival
Chen (2014)	ESCC	ANRIL (antisense lncRNA in the INK4 locus)	Prognostic marker
Kang (2018)	ESCC	PART1 (prostate androgen-regulated transcript 1)	Drug resistance
Fanelli (2018) Table 5.1	ESCC tumors and cell lines	UCA1 (urothelial cancer-associated 1); CCAT1, 2, and 3 (colon cancer-associated transcript 1, 2, and 3); PCAT-1 (prostate cancer-associated ncRNA transcript 1); H19; POU3F3 (lnc-POU class 3 transcription factor 3); TUG1 (taurine-upregulated lncRNA); SOX2-OT (SOX2	Disease progression, poor clinical outcome disease progression, poor clinical outcomes and poor overall survival

(continued)

Table 5.2 (continued)

Study	Tumor	Gene signature	Risk assessed
		overlapping transcript); CBR3-AS1 (carbonyl reductase 3 antisense RNA 1; also known as PlncRNA-1: prostate cancer-upregulated long non-coding RNA 1); FOXCUT (LncRNA Fork head box C1 Upstream transcript); SPRY4-IT1 (sprouty 4 intronic transcript 1); CASC9 (cancer susceptibility candidate 9); and PEG10 (lcnRNA paternally expressed gene 10)	

5.3.1.2 Esophageal Squamous Cell Carcinoma

ESCC is the dominant histological type of esophageal cancer worldwide, and cigarette smoking and alcohol consumption are the major population-attributable risks in the United States (Cook et al. 2010); additionally genetic factors are also known to contribute to ESCC etiology (Wu et al. 2012). The largest exome sequencing performed on 113 ESCC tumors and paired normal samples by Gao et al. in the Chinese population identified a total of 9197 non-silent mutations and 2825 silent mutations, and 70 genes were mutated in at least 5% of the samples including TP53, CDKN2A, NFE2L2, KDM6A, PIK3CA, FBXW7, PTCH1, BRCA2, AJUBA, RB1, NOTCH1, and NFE2L2 (Gao et al. 2014a, b). Other less frequently mutated genes include KMT2D, FBXW7, PTCH1, KDM6A, PIK3CA, CREBBP, EP300, and FAT1 (in $\geq$5% of cases) (Gao et al. 2014a, b). Cell cycle, apoptosis, and DNA damage control pathways are unequivocally disrupted in 95% of ESCC tumors due to TP53, CCND1, CDKN2A, NFE2L2, and RB1 mutations. The tumor suppressor Hippo pathway with mutations on AJUBA and FAT1-4 genes and recurrent mutations in histone modifier genes such as EP300 and KMT2D is another most frequently affected pathway in ESCCs. Collectively these mutations have prognostic and potentially therapeutic implications (Gao et al. 2014a, b). The TCGA sequenced 90 ESCC tumors and found novel focal deletions at 3p25.2 in ESCC, encompassing the negative regulator of the Hippo pathway (VGLL4 and autophagy factor ATG7 (Cancer Genome Atlas Research et al. 2017)) and other recurring focal somatic copy number alterations (SCNAs) including amplifications of SOX2, TERT, FGFR1, MDM2, and NKX2-1 and deletion of RB1. Amplification or mutation of EGFR and alterations of PIK3CA, PTEN, or PIK3R1 that lead to activation of the PI3K pathway were found in 24% of ESCC tumors, thus making them targetable by kinase inhibitors (Cancer Genome Atlas Research et al. 2017). Overexpression of cortactin

(CTTN) was observed in 126/198 (63.6%) of ESCC cases and was significantly associated with lymph node metastasis, pathologic stage, and poor survival ($P < 0.001$) of ESCC patients (Lu et al. 2014).

Due to the poor 5-year survival, and lack of markers to guide the best course of therapeutic management, molecular properties of ESCCs have been studied elaborately to predict their therapeutic sensitivity. Luthra et al. identified a three-gene (PERP, S100A2, and SPRR3) differential expression pattern between pretreatment cancer biopsies and patients with complete response to neo-CRT with 85% specificity (Luthra et al. 2006). Responders to chemo-radiotherapy in both ESCC and EAC could be identified from nonresponders by five-gene signature (low expression of EPB41L3, NMES1, RNPC1, STAT5B and overexpression of RTKN) with 95% accuracy in a subset of patients (Maher et al. 2009). With the help of gene expression profiling on 50 pretreatment biopsy specimens from 11 patients who responded and 14 patients who did not respond to first-line FAP chemotherapy (cisplatin combined with doxorubicin and 5-fluorouracil), Motoori et al. developed a 199-gene predictive diagnostic system that was able to predict response in the validation cohort with 82% accuracy. They also reported that nonresponders to chemotherapy had reduced expression of PERP combined with overexpression of four genes (PRDX6, DAD1, SELPINB6, and SRF) (Motoori et al. 2010). A combination of reduced expression of ClOrf226 and LIMCHI1 and overexpression of MMP1 was found to be a signature predictive of responders to neo-CRT (radiotherapy with cisplatin and vinorelbine concurrently) and was able to successfully predict the responders in another independent cohort of ESCCs receiving the same regimen with 81% accuracy (Wen et al. 2014). Panels of genes to predict outcomes to therapy are still experimental and need further confirmation for clinical application.

5.3.1.3 Molecular Gene Mutation: ESCC Versus EAC

The ESCC and EACs involve the same organ and share similar risk factors but have very different histology and pathophysiology. The most common substitution mutations in both EACs (46%) and ESCCs (35%) were C:G>T:A transitions but with distinct spectra. Overall, A:T>C:G substitutions were more common in EACs, whereas C:G>G:C transversions and indels were more frequent in ESCCs ($P < 0.0001$) (Agrawal et al. 2012). Some known oncogenes like *MCL1*, *EGFR*, *CDK6*, *SMURF1*, *KRAS*, *ERBB2*, *CCNE1*, *VEGFA*, *MET*, and *IGF1R* are amplified in both ESCC and EAC at similar frequencies, while others like *SOX2*, *CCND1*, *MYC*, and *PIK3CA* have a higher amplification frequency in ESCC, and *GATA4* and *GATA6* are higher in EACs (Cancer Genome Atlas Research et al. 2017). In addition, larger genomic changes like deletion of 13q12.2 was found in 20% of ESCC samples, but the same region was amplified in 17% of the EAC tumors. Similarly, chromosome 14 amplification was observed in 35% of ESCC cases but only 4% of EAC (Bandla et al. 2012). The ESCC also shows frequent genomic amplifications of TP63, the master regulator of squamous epithelial cell differentiation, and/or CCND1 and SOX2 similar to squamous carcinomas of other organs (Cancer

Genome Atlas Research et al. 2017). These molecular signatures were suggestive of the distinct pathophysiology of the tumors and the need for different therapeutic approaches, despite their anatomical overlap and similar risk factors.

5.3.2 Epigenetic Markers: Methylation, miRNA, and lncRNA

Epigenetic changes of the DNA are heritable changes in gene activity or function that are not associated with any change of the DNA sequence itself (Table 5.2).

5.3.2.1 DNA Methylation

DNA methylation involves the transfer of a methyl group onto the C5 position of the cytosine to form 5-methylcytosine. DNA methylation mediates the diversified gene expression profiles in a variety of cells and tissues in multicellular organisms. Both hypermethylation and hypomethylation can cause dysregulation of gene expression by altering the binding of transcription factor(s) to DNA binding sites (Robertson 2005). Aberrant DNA methylation has been extensively studied as a tool for stratifying Barrett's esophagus patients' risk of developing esophageal adenocarcinoma. Selective hypermethylation of promoter regions of multiple genes such as CDKN2A and APC has been reported as part of the neoplastic progression from Barret's esophagus to EAC (Kaz et al. 2011). Some exploratory studies also reported global hypomethylation at the genomic level in the BE and EAC tissues compared to normal counterparts (Agarwal et al. 2012). Gastric reflux-induced toxic acidic environment was implicated as a cause of the aberrant methylation patterns that in turn affected the gene expression levels in favor of carcinogenesis (Bajpai et al. 2013). Obesity and tobacco smoking, the other major risk factors for the development of BE and EAC, were also found to have a statistically significant correlation with aberrant methylation patterns observed in BE and EAC (Kaz et al. 2016). When differentially methylated genes between Barrett's esophagus and normal squamous esophageal biopsies were identified from whole methylome data, a combination of 8-marker tissue methylation biomarkers panel (i.e., p16, RUNX3, HPP1, NELL1, TAC1, SST, AKAP12, and CDH13) was found to accurately predict risk in approximately half of HGDs and EACs (Jin et al. 2009). Such a methylation biomarker-based panel had potential clinical value in improving both the efficiency of surveillance endoscopy and the early detection of BE neoplasia. The methylation of AKAP12 in particular seemed to be a specific biomarker for the early detection of BE associated with EAC. Using an array-based approach, Alvi et al. discovered a novel panel of genes that appear to be hypermethylated in HGD/EAC compared with non-dysplastic BE (Alvi et al. 2013). Interestingly, this study did not find the genes from the previously described eight-gene panel as being differentially methylated or other previous candidates such as APC. Instead, they not only developed a new four-gene panel that not only allowed diagnosis of BE and EAC but also facilitated risk

stratification into three groups depending on the number of methylated genes in the panel: <2 = low risk, 2 = intermediate risk, and >2 = high risk. This panel consisted of four markers, *SLC22A18*, *PIGR*, *GJA12*, and *RIN2*, and generated a high AUC score (0.988), with 97% specificity and 94 % sensitivity to separate BE and EAC. It was validated using pyrosequencing in a retrospective cohort that spanned the BE, dysplasia, and EAC (60 non-dysplastic BE, 36 dysplastic Barrett's, and 90 early EACs). In a prospective multicenter study ($n = 98$), the statistical separation between the groups was maintained (Alvi et al. 2013). A more recent study identified 44 methylation markers that may be able to discriminate non-dysplastic Barrett esophagus that either progress to adenocarcinoma or remain as nonprogressive disease (Dilworth et al. 2019). Hypomethylation of the tumor suppressor OR3A4 (probe cg09890332) ranked at the top of the list among the 44 markers and was validated in a separate cohort of samples. Multivariable reverse stepwise logistic regression analysis showed that OR3A4 probe cg09890332 can predict progression to invasive carcinoma (with 70.8% sensitivity and 86% specificity). The positive predictive value being 85% and negative predictive value being 72.5%. However, in another validation test using bisulfite sequencing and a threshold of 58%, the hypomethylation of OR3A4 demonstrated reduced sensitivity of only 33.3% and specificity of 78.6%. The positive predictive value dropped to 10.5% with a negative predictive value of 94%, making this method not sensitive enough for clinical application (Dilworth et al. 2019).

The methylation marker panels are being studied to reduce the need for upper GI-endoscopic procedures performed during BE surveillance and to increase the specificity of EA detection at earlier stages. A panel of four candidate genes, TFPI2, TWIST1, ZNF345, and ZNF569, and a simple and cost-effective non-endoscopic Cytosponge cell collection device showed promise as noninvasive diagnostic biomarkers for Barrett's esophagus (Chettouh et al. 2018). Another noninvasive method of obtaining mucosal cells from the esophagus is the EsophaCap. Investigators were able to differentiate BE samples from non-BE controls using a set of five methylation biomarkers (p16, HPP1, NELL1, TAC1, and AKAP12) with 78.6% sensitivity (95% C+I 48.8% ~94.3%) and 92.8% (95% CI 64.1% ~99.6%) specificity. The markers were different from Chettouh et al. but were found to be significantly higher in BE patients (Wang et al. 2019). Abnormal methylation patterns have also been studied in ESCC with five other candidate genes (ABCD1, SLC5A10, SPIN3, ZNF69, ZNF608) that were claimed to serve as independent prognostic biomarker for ESCC (Lu et al. 2019).

5.3.2.2 MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are small non-coding RNAs, about 18–25 nucleotides in length that regulate gene expression by binding to the 3′-untranslated regions (3′-UTR) of target mRNAs. Such binding may lead to inhibition of mRNA translation or facilitate their degradation. MiRNAs have been shown to regulate cell growth, differentiation, and migration and are frequently dysregulated in cancer

(Bartel 2004). Therefore, miRNAs have potential diagnostic and prognostic value as disease biomarkers, and there is considerable interest in identifying miRNA signatures characteristic of disease stage or therapeutic responsiveness. Serum miRNA biomarkers have been of particular interest in diseases like EAC that are associated with extended monitoring periods, poor early detection, and costly or invasive methodologies. Distinct miRNA signatures associated with GERD and BE were first described in 2008, and subsequent studies have expanded the list of miRNAs dysregulated in BE to at least 105 miRNAs potentially associated with BE pathophysiology. miR143, miR-145, miR-191, miR-192, miR-22, miR-25, miR-661, and let-7 are upregulated in BE and are validated to target p53. miR-149, miR-210, miR-32, and miR-378 are downregulated by p53, which is associated with progression to advanced neoplasia (Horvath et al. 2016). In addition, miR-192, miR-194, miR-203, miR-205, and miR-215 have been identified as promising tissue biomarkers for the diagnosis and monitoring of BE (Mallick et al. 2016).

The regulatory role of the miRNAs, such as mir-125b and mir-378 on Hedgehog signaling and mir-200c, mir-130, and let-7c on BMP signaling, has been associated with the activation of these pathways during the squamous to columnar transition. The mir-21, mir-200c, mir-122-5p, and mir-146a target the NOTCH pathway and promote the squamous to columnar transition. The mir-21 is also known to promote intestinal metaplasia of the columnar epithelium by altering the CDX2 and MAPK signaling. The mir-125b, miR-130b, miR-181b, and miR-501-5p are known to drive inflammation through NF-κB by targeting the ubiquitin-specific processing protease (CYLD), a known NF-κB suppressor during the development of EAC (Clark et al. 2018). However, longitudinal studies are necessary to better understand the role of miRNAs in separating BE from HGD/EAC epithelia.

Ongoing efforts to reduce the need for endoscopic intervention for BE surveillance have led to investigations on finding biomarker signatures in biospecimens derived using minimally invasive methods. A panel of six miRNAs (MIR7, MIR30a, MIR181a, MIR192, MIR196a, and MIR199a) identified BE patients from non-BE with a sensitivity of 86.2% and specificity of 91.6%, and the results were not affected by the method used to obtain the esophageal cells (conventional upper endoscopy vs. Cytosponge). However, the investigators noted that the efficiency of differentiating between the same set of BE and normal samples was improved (93.1% sensitivity and 93.7% specificity) when three specific miRNA (MIR192, MIR196a, MIR199a) expression levels were combined with that of trefoil factor 3 (TFF3) (Li et al. 2018). In another study, miRNA profiles were obtained after high-throughput sequencing of miRNAs extracted from serum and tissue biopsies of normal, GERD, BE, LGD, or EAC. Logistic regression modeling of the whole miRNA profiles identified 11 miRNA signatures including miR-29c-3p and miR-193b-5p that could differentiate between normal, GERD/BE, or LGD/EAC and help stratify patients at risk of progressing to EAC (Craig et al. 2020).

After the first report of presence of miRNA in various bodily fluids (Weber et al. 2010), the possibility of testing serum and saliva samples has also been explored to measure dysregulated miRNAs to diagnose ESCC patients. Markedly increased levels of 7 miRNAs, miR-10a, miR-22, miR-100, miR-148b, miR-223, miR-133a,

and miR-127-3p miRNAs were identified in serum samples of 25 ESCC patients. The investigators claimed that this panel of miRNAs had a higher sensitivity (78.5%) and sensitivity (87.0%) in distinguishing stage I/II ESCC patients samples from healthy controls compared to the standard clinical marker carcinoembryonic antigen (3.05 μg/L as the cutoff value) (13.4% sensitivity and 100% specificity) (Zhang et al. 2010). A panel of six other miRNAs, miR-10b*, miR-144, and miR-451 detectable in whole saliva and miR-10b*, miR-144, miR-21, and miR-451 in saliva supernatant, were also reported to be significantly increased in ESCC patients and had the ability to discriminate between healthy and ESCC patients (Xie et al. 2012, 2013). The miR-18a known to be highly expressed in the ESCC tumors was also detected in the serum of the patients. The levels of miR-18a in the serum were reduced significantly after surgical removal of the tumors ($P = 0.0076$) (Hirajima et al. 2013). This association between tumor and serum levels of miR-18a established that the miRNA levels of serum and tissue are comparable in the ESCC as also observed in oral squamous cancer (Wiklund et al. 2011). Although inconsistent at this time, the methods for measuring biomarkers from body fluids could potentially revolutionize noninvasive esophageal cancer surveillance.

A predictive role of miRNAs in treatment response has been studied by Skinner et al. They developed a miRNA expression profile (MEP) score derived from four miRNAs (mir-505*, mir-99b, mir-451, and mir-145*) to predict complete response to neoadjuvant chemoradiotherapy in patients with esophageal adenocarcinoma (Skinner et al. 2014). Although available data are not conclusive, the possible clinical application of miRNAs as biomarkers or as a potential target of treatment in esophageal cancer deserves further investigation.

5.3.2.3 Long Non-coding RNAs (lncRNAs)

Long non-coding RNAs (lncRNAs) are becoming one of the next frontiers of cancer research as the role of these non-coding RNAs in carcinogenesis and metastasis are beginning to unfold. LncRNAs are RNA transcripts longer than 200 nucleotides with almost no protein-coding capacity. These transcripts are known to regulate gene expression at multiple levels through epigenetic regulation of DNA modification, regulation of transcriptional factor binding, and post-transcriptional steps like splicing and protein modifications. The lncRNAs are promising candidates as early-stage diagnostic markers. They are known to have tissue-specific expression and thus a high specificity. Just like the miRNAs, they are also detectable in body fluids, including plasma or serum, gastric juice, saliva, cerebrospinal/peritoneal/pleural fluid, and urine, making repeated noninvasive assessment feasible (Fanelli et al. 2018). The role of lncRNAs is not well studied in the EACs. Differential expression of an lncRNA AFAP1-AS1 (actin filament-associated protein 1-antisense RNA 1) and an LncRNA HNF1A-AS1 (hepatocyte nuclear factor 1 alpha-antisense RNA 1) has been reported between BE and EAC, but the significance of this finding remains to be elaborated (Fanelli et al. 2018). A microarray-based screening of gastric cardia adenocarcinoma (GCA) that closely relates to EAC revealed that 1598 lncRNAs

were significantly upregulated and 327 were significantly downregulated (fold change $\geq$2.0) in the GCA compared to paired noncancerous tissue. These molecules were associated with biological pathways involved in development, invasion, and metastasis of GCA tumors, thus supporting a strong rational for their potential role as biomarkers for the clinical diagnosis of and targets for further therapy in these malignancies (Wang et al. 2014). Dong et al. studied the sera from 110 patients with GC and 106 age- and sex-matched healthy subjects and identified three lncRNAs UCA1, LSINCT-5, and PTENP1 to be significantly downregulated in GC patients compared with the control group. The set of three lncRNAs correlated with worse survival rates in the GC patients (Dong et al. 2015).

Several lncRNAs are known to be differentially expressed in the ESCC compared to normal esophageal tissue. High levels of MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) RNA expression was negatively correlated with miR-101 or miR-217 expression in 42 ESCC samples compared to paired normal controls and regulated the proliferation, migration, and invasion abilities of ESCC cells. MALAT1 expression increases EZH2 enhancer of zeste homolog 2 (EZH2) expression significantly in ESCC tissue and activation of the Wnt/β-catenin pathway to increase lymph node metastasis and poor overall survival (Wang et al. 2015). HOTAIR (HOX transcript antisense RNA) plays a role in chromatin remodeling of the promoter region of the Wnt inhibitory factor-1 (*WIF-1*), thus shutting down its expression and facilitating upregulation of the Wnt/β-catenin pathway. Due to its epigenetic role, high HOTAIR expression is associated with an elevated risk of mortality in ESCC and other cancers (Gupta et al. 2010; Ge et al. 2013). Several other lncRNAs UCA1 (urothelial cancer-associated 1); CCAT1, 2, and 3 (colon cancer-associated transcript 1, 2, and 3); PCAT-1 (prostate cancer-associated ncRNA transcript 1); H19; POU3F3 (lnc-POU class 3 transcription factor 3); TUG1 (taurine-upregulated lncRNA); SOX2-OT (SOX2 overlapping transcript); CBR3-AS1 (carbonyl reductase 3 antisense RNA 1; also known as PlncRNA-1: prostate cancer upregulated long non-coding RNA 1); FOXCUT (LncRNA Fork head box C1 Upstream Transcript); SPRY4-IT1 (sprouty 4 intronic transcript 1); CASC9 (cancer susceptibility candidate 9); and PEG10 (lcnRNA paternally expressed gene 10) have been summarized from studies on ESCC cell lines and human samples and are found to be associated with ANRIL (antisense ncRNA in the INK4 locus) that acts as an oncogene, and blocking it prevents cancer cell proliferation which could be a good candidate prognostic biomarker and target for new target therapy in ESCC (Chen et al. 2014). High levels of serum lncRNA PART1 in ESCC patients were found to promote gefitinib resistance by regulating miR-129/Bcl-2 pathway and be associated with poor response in these individuals (Kang et al. 2018).

The knowledge on the biological roles of the lncRNAs is constantly evolving. Although levels of these molecules are discriminatory between disease and normal, which makes them ideal for diagnostic purposes, several questions remain about their stability and interaction with other molecules like the miRNAs, mRNAs, DNA, and proteins. Since they are known to have complex regulatory and interactive

networks, just like the miRNAs, using lncRNAs as therapeutic targets is still debatable.

5.4 Microbiome Application in Esophageal Cancer and Precancerous Lesion

The gastrointestinal (GI) microbiome comprises the complex symbiotic community of some 1014 bacteria colonizing in the human GI tract (Abreu and Peek 2014). More recent research has focused on characterizing these bacterial communities in the gut and identifying global differences in healthy gut and disease conditions like esophageal, gastric, and colorectal cancers (Schwabe and Jobin 2013; Fraher et al. 2012). Data on esophageal microbiome and dysbiosis during EAC and ESCC is still very limited and controversial (Snider et al. 2016). Both oral and esophageal microbiomes are influenced by diet and oral hygiene and have been associated with the risk for development of EAC (Lagergren et al. 2013) and ESCC (Yamamura et al. 2016). Using bacterial 16S ribosomal RNA gene sequencing, ~100 unique taxa were identified in the esophageal microbiome. These bacteria belong to six major phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and TM7. Majority of the bacteria (39%) were *Streptococcus*; other major genera were *Prevotella* (17 %) and *Veillonella* (14 %), among others (Pei et al. 2004). A more recent study used mucosal brushings instead of traditional biopsy and found a greater number of taxa in the esophageal microbiome (Gall et al. 2015). The use of antibiotics and decrease in the incidence of *H. pylori*, an organism strongly implicated in gastric cancer, have been linked with the increase in incidence of EAC (Polk and Peek 2010); a study actually found protective effect of *H. pylori* eradication to be protective in BE (Lagergren and Lagergren 2013). The acidic environment in BE and reflux esophagitis was reported to alter the microbial balance of the esophagus, by reducing the relative abundance of the Gram-positive *Streptococcus* and increasing the abundance of Gram-negative bacteria, including *Fusobacterium*, *Neisseria*, *Campylobacter*, *Bacteroides*, *Proteobacteria*, and *Veillonella* taxa (Yu et al. 2014; Gall et al. 2015). The altered abundance of several genera, including *Bifidobacteria*, *Bacteroides*, *Fusobacteria*, *Veillonella*, *Staphylococcus*, and *Lactobacilli*, observed in GERD and BE, reversed with progression to EAC and comparable to levels seen in normal esophagus. Some species like *Campylobacter* had a reversed trend, with low abundance in healthy and cancer patients but higher prevalence in GERD and BE (Blackett et al. 2013). High relative abundance of Gram-negative bacterial antigens like the lipopolysaccharide (LPS), in the reflux-associated esophagus, promotes tissue inflammation via increased expression of NF-κβ that is implicated in the progression from BE to EAC (Yang et al. 2012).

Studies have identified a decrease in alpha diversity (Yu et al. 2014) and a greater abundance of *Clostridiales* and *Erysipelotrichales* and a specific bacteria called *F. nucleatum* in esophageal cancer and dysplasia tissue specimens compared to

normal esophageal mucosa (Yamamura et al. 2016). The *F. nucleatum* is known to participate in colorectal cancer carcinogenesis via activation of the beta-catenin pathway and epigenetic alteration of CpG methylation (Ito et al. 2015). Another upper digestive tract study found microbial richness and β-diversity (pairwise difference in microbiota among samples), and serum PGI/II ratio in the Cytology Sampling Study 2 (CSS2), a cancer screening study in Linxian, China, a region with very high rates of ESCC and gastric cancer. Microbial richness (number of bacterial genera per sample) was significantly associated with lower PGI/II ratio ($P = 0.034$) and the presence of ESD ($P = 0.018$). Collectively, these studies suggest that microbiome alterations occur in ESCC, yet it is not clear if this is the cause or an effect of the carcinogenesis process. Future studies are warranted to better understand the role of the microbiome in esophageal carcinogenesis and to identify potential risk biomarkers and therapeutic targets.

5.5 Other Promising Biomarkers for Esophageal Cancer

5.5.1 Circulating Tumor Cells

Circulating tumor cells (CTCs) are cancer cells that shed or break away from the tumor into the blood vessels and are carried around the body via circulation. Liquid biopsy is a technological advancement that will enable detection of CTCs from the blood. However, this method is only investigational and is not in clinical use. The number of CTCs in the blood of metastatic breast cancer patients after therapy is strongly correlated with progression-free survival (Cristofanilli et al. 2004). Meta-analysis of pooled data from 16 trials and 1260 patients showed that the presence of CTCs was significantly associated with poor overall survival (HR = 1.71, 95% CI [1.30, 2.12], $P < 0.001$) and progression-free survival (HR = 1.67, 95% CI [1.19, 2.15], $P < 0.001$) in EC patients especially if they were Asian and had a diagnosis of ESCC (Qiao et al. 2016). The esophageal CTCs could also be examined for cytokeratin markers and epithelial as well as mesenchymal markers like CK7, CK8, and EpCAM, to evaluate their malignant potential and inform the staging of tumor, without the need for invasive biopsy (Woestemeier et al. 2020). Further well-designed prospective studies are needed to explore the clinical applications of CTCs in patients with esophageal carcinoma.

5.5.2 Circulating Cell-Free DNA

Circulating cell-free DNA (cfDNA) detection of circulating cell-free tumor DNA from blood samples has great potential as noninvasive "liquid biopsy" cancer biomarker (Duffy 2019). In a small group of patients, it was found that the frequency of BE-specific LOH and MSI markers was statistically higher in the dysplastic

tissues compared to that of metaplastic BE ($P = 0.005$). Among the examined markers, those that map nearby *TP53* gene were the most discriminant between metaplastic and dysplastic BE. The frequency of LOH and MSI in the cfDNA dropped after endoscopic treatment, suggesting potential use monitoring curative effects (Rumiato et al. 2017). In a recent longitudinal study conducted on patients with stage 1-4 EAC, the levels of cfDNA detected in the plasma of EAC patients and the allele frequencies of the mutations correlated with disease burden and could be used to predict recurrence or response to therapy (Egyud et al. 2019). Although detection of circulating tumor DNA in early-stage EAC is challenging and may limit diagnostic applications, the cfDNA has great promise as a dynamic biomarker for monitoring real-time treatment efficacy in patients with EAC undergoing neo-adjuvant drug therapy and immunotherapy (Kosovec et al. 2018).

5.5.3 Breath Volatile Organic Compounds

Breath volatile organic compounds (VOCs) have shown clinical utility as possible biomarkers for lung, breast, prostate, colorectal, gastric, and, recently, esophageal cancer (Yazbeck et al. 2016). Twelve VOCs, pentanoic acid, hexanoic acid, phenol, methyl phenol, ethyl phenol, butanal, pentanal, hexanal, heptanal, octanal, nonanal, and decanal, were present at significantly higher concentrations ($P < 0.05$) in exhaled breath samples from patients in the esophageal cancer groups than in the noncancer controls. These significant VOCs could discriminate esophageal and gastric adenocarcinoma from those with normal upper GI tracts (Kumar et al. 2015).

5.6 Conclusion and Future Directions

Multiple cancer-related genomic changes are listed in this review including genomic mutation, methylation, amplification, deletion, aberrant expression, miRNA, mRNA, and lncRNA, which could be potential biomarkers for early stages of esophageal cancer (diagnostic markers), predicting the patient's risk of progression to cancer (progression marker), responding to therapy (predictive marker for treatment), and survival or prognosis (prognostic marker). As we know, the prognosis of esophageal cancer has been not improved significantly in many years although the various therapies are developed including surgery, chemotherapy, radiotherapy, target therapy, and immunotherapy. Probably, it is time to change the direction to focus on the prevention. The early detection of patients with high risk progressing to EC could shed a light on the eradication of EC instead of focusing on treatment of later stage of EC. Recently Fitzgerald and colleagues used Cytosponge with TFF3 immunohistochemistry and molecular biomarker tests to screen Barrett's esophagus which showed a high sensitivity and specificity in Europe (Chettouh et al. 2018; Katzka and Fitzgerald 2020). Similarly, combining EsophaCap cytological method

with MUC2 immunohistochemistry showed a reasonable sensitivity and higher specificity to screen Barrett's esophagus, dysplasia, and EAC in the United States (Zhou et al. 2019). Innovative combinatorial approaches are in the horizon that will provide cheaper, convenient nonendoscopic methods with objective biomarkers that will help us find the high-risk patient populations with progressive disease. Such improvements in surveillance with early intervention procedures including endoscopic mucosal resection and radiofrequency ablation for the high-risk patients before the EC happens will make the esophageal cancer history.

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Manisha Bajpai, PhD, a translational scientist, trained with Dr. Kiron M. Das, an internationally prominent gastroenterologist and clinician scientist on methods of biomarker discovery and validation in premalignant conditions of the gut. She has led the development of in vitro models of disease to study mechanisms of Barrett's development and malignant progression and discovery of innovative tools for early detection of EAC using fluorescent in situ hybridization. Currently she leads multi-omics GI research programs for early stage biomarkers discovery with Dr. Steven Brant, a gastroenterologist and pioneer in IBD genomics and Dr. Zhongren Zhou, a GI pathologist and established clinical investigator.

Dr. Zhongren (David) Zhou, who is a pathologist and basic researcher, first started his investigations on the biomarkers of esophageal cancer 12 years ago at the University of Rochester with Dr. Tony Godfrey and Dr. Jeff Peter, Chairman of Department of Surgery and well-known esophageal cancer surgeon. His interest and passion for GI pathology and research have led to imminent contributions in the field of biomarker for the early diagnosis of esophageal cancer with combined cytology and genomic methods and predicting Barrett's esophagus patients with high risk to progress to esophageal adenocarcinoma. In addition, he and colleagues developed a new gene therapy for gastroesophageal reflux disease.

Chapter 6
Epithelial and Immune Cell Responses to *Helicobacter pylori* That Shape the Gastric Tumor Microenvironment

Meaghan Torvund, Jayati Chakrabarti, and Yana Zavros

Abstract Gastric cancer is the third most common cause of cancer-related death worldwide with a 5-year survival rate of only 29%. The incidence of gastric cancer in the United States is relatively low due to the diagnosis and treatment of the major risk factor *Helicobacter pylori* (*H. pylori*). Even after *H. pylori* infection has been eradicated, there is still a risk of developing gastric cancer. Gastric cancer is the final clinical outcome that is often initiated by a sustained inflammatory response to *H. pylori* infection and immune cell-epithelial crosstalk. The review focuses on reporting the mechanisms by which the bacterium modulates the host's innate and adaptive immune response and the gastric epithelium as part of a strategy to create an immunosuppressive microenvironment that ultimately leads to gastric cancer.

Keywords *Helicobacter pylori* · SPEM · CD44V9 · PD-L1 · HIF-1α · Hypoxia · Inflammation

List of Abbreviations

ALPK1	Alpha-protein kinase 1
ATP	Adenosine triphosphate
CAF	Cancer-associated fibroblast
CagA	Cytotoxin-associated gene A
CD44	Cluster of differentiation 44
CD8	Cluster of differentiation 8
c-MET	Mesenchymal-epithelial transition factor
COX-1/2	Cyclooxygenase-1/2
CSC	Cancer stem cell
CSF	Colony-stimulating factor

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CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DC	Dendritic cell
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
ENO1	Enolase 1
FAP	Fibroblast activation protein
F-FDG	Fluorodeoxyglucose (^{18}F), fluorodeoxyglucose
FOXP3	Forkhead box protein 3
GCSF	Granulocyte colony-stimulating factor
GLUT1	Glucose transporter 1
G-MDSCs	Granulocytic MDSCs
GOF	Gain-of-function
H. pylori	*Helicobacter pylori*
HA	Hyaluronic acid
HIF	Hypoxia-inducible factor
HKII	Hexokinase II
HRE	Hypoxia response element
IL-1/6/8	Interleukin 1/6/8
IM	Intestinal metaplasia
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
LDH	Lactic acid dehydrogenase
MDSCs	Myeloid-derived suppressor cells
MHC-1	Major histocompatibility complex −1
MIP2	Macrophage inflammatory protein 2
M-MDSCs	Monocytic MDSCs
MMP-2/9	Matrix metalloproteinase 2/9
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor-kappa B
NKCs	Natural killer cells
NOD1	Nucleotide-binding oligomerization domain-containing protein 1
NSAID	Nonsteroidal anti-inflammatory drug
PD-1	Programmed death-1
PDK	Pyruvate dehydrogenase kinase
PD-L1	Programmed death-ligand 1
PGE2	Prostaglandin E2
PHD	Prolyl hydroxylase domain-containing enzyme
PKM2	Pyruvate kinase m2
PMN	Polymorphonuclear leukocytes
PPRs	Pattern recognition receptors
PTEN	Phosphatase and tensin homolog
ROS	Reactive oxygen species
Shh	Sonic Hedgehog
SLFN 4/12	Schlafen 4/12

SMA	Smooth muscle actin
SPEM	Spasmolytic polypeptide/trefoil Factor (TFF)2-expressing metaplasia
STAT	Signal transducer and activator of transcription
T4SS	Type 4 secretion system
TAM	Tumor-associated macrophages
TCA	Tricarboxylic acid cycle
TFF2	Trefoil factor 2
TGF-β	Transforming growth factor -β
Th1/2	T helper cell 1/2
TLRs	Toll-like receptors
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor-α
Tregs	Regulatory T cells
vacA	Virulence factor A
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau protein
xCT	Cystine-glutamate transporter

6.1 Introduction: *Helicobacter pylori* and the Attributes of Virulence

In the January 1983 issue of the British medical journal *The Lancet*, Australian physicians Drs. Barry Marshall and Robin Warren, in a report titled "Unidentified Curved Bacilli on Gastric Epithelium in Active Chronic Gastritis," documented that gastric ulcers were caused by a bacterial infection and not by excessive acidity (Warren and Marshall 1983). The link between bacterial infection and ulcers was initially considered a foolish idea, but given the tenacity of these physician scientists, Dr. Marshall ingested the bacteria and documented that indeed the infection induced gastric ulcers that were cured following treatment with a combination of antibiotics and acid neutralization. In 1994, *H. pylori* was recognized as a type I carcinogen by the World Health Organization and is recognized as the leading risk factor for infection-related gastric cancers representing the third most common cause of cancer-related death worldwide with a 5-year survival rate of only 29% (Ferlay et al. 2015). In 2005 Drs. Warren and Marshall were awarded the Nobel Prize of Medicine for their pioneering discovery linking bacterial infection and its role in peptic ulcer disease and demonstrated the importance of perseverance in science.

The major cause of chronic inflammation in the normal, acid-secreting stomach is Gram-negative bacterial pathogen *Helicobacter pylori* (*H. pylori*) (Correa et al. 1975). It is widely accepted that inflammation that is caused by *H. pylori* infection is a trigger for the development of gastric cancer (Correa et al. 1975). An explanation for the causal role of *H. pylori* infection in the pathogenesis of gastric cancer has been described by disruption of differentiation of epithelia as a consequence of elevated pro-inflammatory cytokines such as IFNγ, TNFα, and IL-1β (Moss et al.

1994; Padol IT 2004; Sawai et al. 1999; Smythies et al. 2000; Zavros et al. 2003). The cellular adhesion molecule CD44 is involved in multiple important physiological functions including cell proliferation, adhesion, migration, hematopoiesis, and lymphocyte activation. Our laboratory has shown that Cluster of differentiation 44 (CD44) mediates epithelial cell proliferation associated with *H. pylori* infection (Bertaux-Skeirik et al. 2015). Eventually within the inflammatory environment of the tumor, there is the presence of tumor-associated macrophages (TAMs), neutrophils, cancer associated fibroblasts, T and B cells, and myeloid-derived suppressor cells (Chen et al. 2014a, b, c; Ding et al. 2016, 2020; Grivennikov et al. 2010; Quante et al. 2013) (Fig. 6.1). These cells have tumorigenic properties via their production of cytokines, growth factors, enzymes, and angiogenic mediators. The immune cells promote not only proliferation but also an antitumor immune response (Galon et al. 2006; Halama et al. 2011). While our knowledge of the gastrointestinal tumor microenvironment is predominantly based on studies of colon cancer (Galon et al. 2006; Halama et al. 2011), there is limited investigation of the gastric tumor microenvironment (Houghton et al. 2004; Quante et al. 2011). The current review focusses on the epithelial and immune cell responses to *H. pylori* infection that are likely to initiate the progression from chronic inflammation to neoplasia. In addition, we explore the early epithelial and immune responses immediately after the bacterial infection that are likely to persist within the tumor microenvironment.

6.2 Early Epithelial and Immune Cell Responses to Helicobacter Infection

The correlation between *H. pylori* infection and gastrointestinal diseases is facilitated by the complex interplay between bacterial virulence factors, host, and environmental influence. Studies have demonstrated that the persistence of chronic inflammation of gastric mucosa is directly associated with virulence factors (vacA), cytotoxin-associated gene A (CagA), and its type 4 secretion system (T4SS). The development of atrophic gastritis and intestinal metaplasia abruptly increases the risk of developing gastric adenocarcinoma (Moyat and Velin 2014). The Hedgehog family of proteins, mainly Sonic Hedgehog (Shh), plays an important role in the development of multiple organ systems, including neuronal and gastrointestinal systems, and also has been found in a variety of solid tumors including stomach cancer. The progression from inflammation to cancer includes the disruption of normal epithelial cell differentiation and the development of atrophic gastritis and metaplasia. The loss of Shh during inflammation correlates with the atrophic gastritis, which has recently been shown in vivo using a unique mouse model of targeted gastric Shh deletion (Xiao et al. 2010). Based on earlier studies, it has been shown that CagA-induced Sonic Hedgehog (Shh) signaling within parietal cells is facilitated by the NF-kB signaling pathway (Schumacher et al. 2012, 2015). Importantly, *H. pylori* induces the secretion of Shh and activates its signaling pathway,

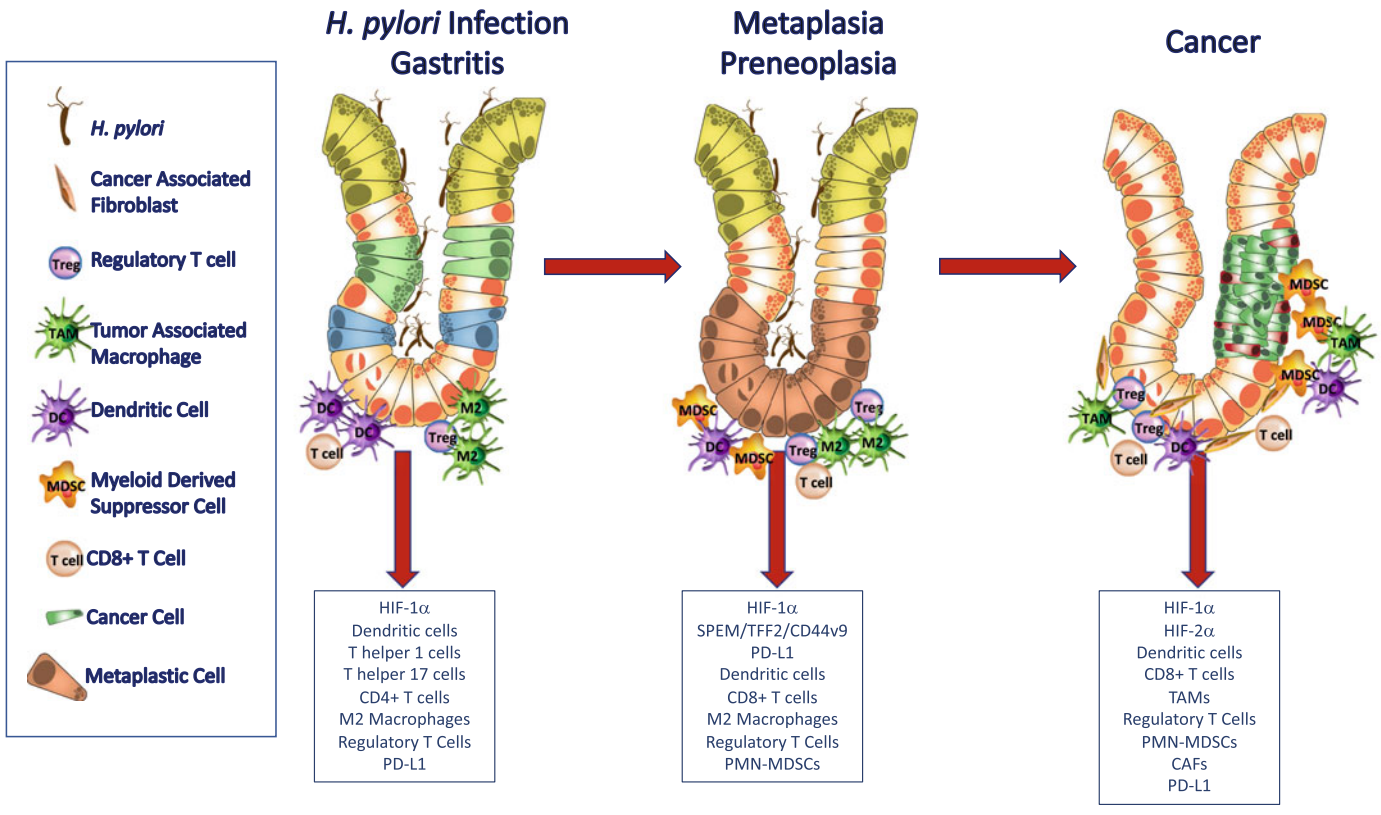

Fig. 6.1 Epithelial and immune changes in response to *H. pylori* infection during disease progression. Early epithelial changes including induction of CD44v9 and PD-L1 may act to evade early immune surveillance. Infiltration of PMN-MDSCs, TAMs, and Tregs during chronic gastritis act as immunosuppressive cells within the gastric tumor microenvironment

which is crucial for initiation of gastritis (Schumacher et al. 2012). Chronic *H. pylori*-induced inflammation ultimately leads to loss of the normal gastric mucosal architecture, destruction of gastric glands, and development of intestinal metaplasia. Atrophy of the acid-secreting parietal cells leads to the development of spasmolytic polypeptide/trefoil factor (TFF)2-expressing metaplasia (SPEM) (Nomura et al. 2004, 2005), which initiates neoplastic changes in the gastric epithelium before the onset of gastric cancer (Petersen et al. 2014) (Fig. 6.1). In a cohort of 47 gastric cancer patients, it has been observed that SPEM was present in 82% of the biopsies obtained prior to the diagnosis of cancer whereas intestinal metaplasia was found adjacent to the tumor in 76% of cases. Immunostaining for spasmolytic polypeptide suggested that SPEM is highly present in fundic biopsies of patients who subsequently developed gastric adenocarcinoma, indicating an increased risk for developing gastric adenocarcinoma (Halldórsdóttir et al. 2003).

The genetic heterogeneity of *H. pylori* arises upon adaptation to host's gastric environment, as well as to the distinct patterns of the host-mediated immune response to *H. pylori* infection reviewed in Kuipers et al. (2000). The factors regulating different immune cells against inflammatory responses due to *H. pylori* infection still need further investigation. The persistence of *H. pylori* within the stomach epithelium initiates pro-inflammatory signaling cascades via the production of neutrophil-activating protein (NAP) (Chesney et al. 2017). HP-NAP, a major virulence factor, plays important role in the gastric inflammatory response to *H. pylori* infection. It also functions as a protective antigen by attracting neutrophils and other immune cells to the infection site. The nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and alpha-protein kinase 1 (ALPK1) negatively regulate expression of nuclear factor-kappa B (NF-κB) activation to prevent intestinal metaplasia and gastric cancer (Evans et al. 1995; Viala et al. 2004). In a review article, Lehours and Ferrero stated that *H. pylori* controls host immune responses by altering cytokine signaling in epithelial and myeloid cells, increasing proliferation of regulatory T cells (Tregs) and downregulating the effector T-cell functions (Chesney et al. 2017; Lehours and Ferrero 2019). But this commensal bacterium has developed extensive adaptations to sustain itself in the host stomach and escape innate and adaptive immune response by reprogramming the immune system toward tolerance. The rapid infiltration of macrophages post-infection is essential for the innate immune response to *H. pylori*-induced signals and is crucial to the development of gastritis (Kaparakis and Price 2008). During *H. pylori* infection, Shh also plays an immunoregulatory role during epithelial immune response by acting as a macrophage chemoattractant (Schumacher et al. 2012). Following a sustained increase in Shh secretion and signaling, macrophages are recruited to the infection site (Schumacher et al. 2012). These macrophages secrete IL-1β, which inhibits acid secretion causing atrophic gastritis and the atrophy of parietal cells (Schumacher et al. 2012; Waghray et al. 2010). Programmed death-ligand 1 (PD-L1/B7-H1) represents an adaptive immune resistance mechanism by binding with PD-1 and shutting down T-cell effector function (Reissfelder et al. 2015; Sun et al. 2007). Our earlier studies demonstrate that Shh signaling induces PD-L1 expression as an early epithelial response to *H. pylori* infection and a mechanism to evade the

immune response (Holokai et al. 2019). We have shown the mechanism by which PD-L1 is specifically localized to SPEM cells to survive chronic inflammation, for the persistence of infection, and progression of the disease to cancer (Holokai et al. 2019; Wu et al. 2010) (Fig. 6.1).

H. pylori induces a strong immune response with infiltration of neutrophils and B and T cells into the gastric mucosa that fails to clear the infection. Immune cells predominantly express specialized receptors called pattern recognition receptors (PPRs) and warn the body about the presence of potentially harmful pathogens. *H. pylori* possess several mechanisms that prevent their recognition via Toll-like receptors (TLRs), and by rearranging LPS and flagellin can prevent recognition of the pathogen by immune cells (Cadamuro et al. 2015). It has been shown that the *H. pylori* flagellin is not recognizable by the immune cell receptors, PRRs, and, as a result, diminishes the primary host immune response mechanisms, such as phagocytosis and natural killer (NK) cell activity (Allen et al. 2000; Bäckhed et al. 2003). It has been shown that TLR4 is expressed on antigen-presenting cells such as monocytes and dendritic cells. Bacterial infection leads these monocytes to secrete pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-8 which, in turn, act as local chemoattractants for granulocytic infiltration (Crabtree 1996). Adaptive immunity is also impaired during *H. pylori* infection which induces macrophage apoptosis and diminishes dendritic cell (DC) and macrophage maturation, leading to decreased T-cell activation (Mnich et al. 2015). The function of PD-L1 on gastric epithelial cells is to inhibit proliferation and differentiation of naïve T lymphocytes and stimulate DCs to increase secretion of the anti-inflammatory cytokine IL-10. Monocytes and macrophages are important controllers of innate immune responses to pathogens. During *H. pylori* infection, they activate adaptive immunity along with DCs, by producing factors such as IL-12 which stimulate Th1 cells, resulting in production of cytokines such as IFN-γ (Haeberle et al. 1997; Meyer et al. 2003; Peek et al. 2010).

In recent publications, our group has demonstrated that the host's immune response is an important driver of *H. pylori* pathogenesis. Data indicates that *H. pylori*-induced PD-L1 expression within the gastric epithelium is mediated by the Shh signaling pathway as an early response to infection. Using a patient tissue-derived organoid model, we incorporated the patient's immune cells with the gastric epithelium in the absence and presence of *H. pylori* infection (Holokai et al. 2019). Organoids infected with *H. pylori* were co-cultured with autologous CTLs and treated with a PD-1 inhibitor. The data suggest that while bacterial infection results in decreased CTL proliferation, inhibition of PD-L1/PD-1 interactions induces proliferation of CTLs within the co-culture in the presence of *H. pylori* infection. These results support the fact that once a patient progresses to a (Chen et al. 2016) metaplastic state, the eradication of *H. pylori* does not decrease the risk of developing gastric cancer. Therefore, this organoid-immune cell co-culture model can be used to develop a diagnosis for patients that have progressed to metaplasia or even to discover new therapies for gastric cancer (Chakrabarti et al. 2018a, b; Holokai et al. 2019).

6.2.1 Induction of Protective Responses

The immune response toward bacterial pathogens stems from either the innate or adaptive immune system. Adaptive immune responses, which can be both protective and damaging to the host, follow the failure of the innate immune response to eliminate the pathogen. Chronic gastritis is linked to an increased CD4+/CD8+ T-cell ratio and accumulation of CD4+ T-helper lymphocytes in the gastric mucosa, where *H. pylori* infection results in a Th1-predominant host immune response and induction of IFN-γ-related genes. A Th1-predominant immune response is associated with elevated levels of the pro-inflammatory cytokines IL-12, IL-18, and TNF-α (Tummala et al. 2004). Th17 cells, CD4+ T cells associated with infections and inflammation in the gastric mucosa, are induced during both *H. pylori* infection and gastric cancer and may be an important link between inflammation and carcinogenesis (Pinchuk et al. 2013).

DCs embody a critical bridge between the innate and adaptive immune responses and have been identified as antigen-presenting cells as well as primary responders to *H. pylori* infection (Chieppa et al. 2006). In a review article, Banchereau et al. demonstrated that after activation of their TLRs, DCs may activate T cells in different ways, by inducing either a Th1 or Th2/regulatory T cell (Treg) response and by generating IL-12 or IL-10, respectively (Banchereau et al. 2000). Studies have reported that in human blood monocyte-derived DCs, the activation and maturation of DCs occur independently of the presence of the CagA and vacA genotype and may be partially lipopolysaccharide dependent (Kranzer et al. 2005). DCs also specialize in priming different types of effector T cells, CD4+ versus CD8+ T cells, and uniquely respond to distinct stimuli (Eisenbarth 2019). It has been shown that DCs pulsed with *H. pylori* for 48 h show significantly attenuated ability of IFN-ɣ production upon co-culture with naive T cells compared to 8 h activation, and this suggests that continuous exposure of DCs to *H. pylori* results in a loss of induction of the Th1 response that could contribute to the persistence of the infection (Mitchell et al. 2007).

In the immune response to *H. pylori*, CD4+T cells are the key effector cells of adaptive immunity, while the role of CD8+T cells has not been fully explored, besides human. Clinical studies in humans have shown a positive correlation between *H. pylori* colonization and increased CD8+ T-cell infiltration during the development of gastric ulcers (Helmin-Basa et al. 2011; Kronsteiner et al. 2014). *H. pylori*-induced immune response was originally considered as a Th1 response, but other CD4+ T-cell subsets, including Th17 and Tregs, also play a major role during infection (Bagheri et al. 2018). Earlier studies established that gastric lymphocytes from *H. pylori*-infected patients have increased IFN-ɣ-producing T cells, consistent with a Th1 cytokine response (Bamford et al. 1998). Mucosal T cells during *H. pylori* infection produce ample levels of Th1 cytokines, IFN-ɣ and IL-2, and low levels of Th2 cytokines, IL-4, and IL-5 (Bamford et al. 1998). IFN-ɣ−/− mice have shown impaired gastritis, and other *H. pylori*-infected mice lacking T and B cells require an adoptive transfer of CD4+T cells to attenuate *H. pylori*

colonization and repair gastritis (Akhiani et al. 2002). Studies have demonstrated that *H. pylori* infection induces $CD4^{+}CD25^{high}FOXP3^{+}$ Tregs, suppresses circulating memory T-cell responses, and stimulates high levels of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) protein, thus contributing to the persistence of the infection (Lundgren et al. 2003) (Fig. 6.1).

6.2.2 Recruitment and Polarization of Macrophages

Macrophages and dendritic cells are the first responders to *H. pylori*-induced signals from epithelial cells on the surface of the gastric mucosa that modulate inflammatory responses. Monocytes and macrophages, along with DCs, control the immune response to pathogens by producing factors like IL-12 that stimulate Th1 cells, resulting in the production of cytokines such as IFN-ɣ (Meyer et al. 2003). Macrophages are also involved in the amplification of the inflammatory response by producing various cytokines such as IL-1, TNFα, and IL-6, which is linked to activation of TLR4, MAP kinase, and NF-κB signaling events (Pathak et al. 2006; Schumacher et al. 2015).

During *H. pylori* infection, release of IL-8, macrophage inflammatory protein (MIP2), and CXCL1/KC is necessary for lymphocyte and neutrophil recruitment (Algood et al. 2007; Ferrero et al. 2008). The Sonic Hedgehog (Shh) signaling pathway is activated during infection and regulates gastric epithelial differentiation and function. Previous publications from our group demonstrated that the targeted removal of Hedgehog signaling within macrophages resulted in a failure of *H. pylori*-induced macrophage recruitment to the site of infection using bone marrow chimera experiments on LysMCre/SmoKO mice (Schumacher et al. 2012).

Increased levels of reactive oxygen and nitrogen species that are released by macrophages in the gastric mucosa are strongly associated with the pro-inflammatory response during *H. pylori* pathogenesis (Pignatelli et al. 2001), which may promote cancer development. Murine studies have suggested that mice that are deficient for the enzyme inducible nitric oxide synthase (iNOS) have a reduced incidence of gastric cancer after *H. pylori* infection compared to normal mice (Nam et al. 2004). While iNOS contributes to the development of gastric cancer, a high level of the chemokine CCL18 in gastric tumors is associated with prolonged survival of gastric cancer patients (Leung et al. 2004). Interestingly, Martinez et al. stated in a review article that iNOS is produced by naturally activated/M1 macrophages, whereas CCL18 production is associated with alternatively activated/M2 macrophages (Martinez et al. 2009). These findings suggest that macrophage polarization may have an important role in the development of *H. pylori*-associated gastric cancer. Wilson's group has demonstrated that during *H. pylori* infection, the l-arginine metabolic enzymes get induced in gastric tissue. They have also stated that, *H. pylori* upregulates polyamine metabolism as a method of survival to impair M1 macrophage responses and polarizes to an M2 phenotype by increasing the rate of ROS-induced macrophage apoptosis, which enhances the

risk of disease progression. This supports innate immunity by inducing polarization of macrophages to a pro-inflammatory response (Latour et al. 2020).

The polarization of macrophages is controlled by the microenvironment. M1 macrophages usually play a role in the initial immune response for attacking microorganisms and promote T helper (Th) 1 immunity, whereas $CD163^+$ M2 macrophages are induced during tissue remodeling and promote Th2 immunity. M1 macrophages are induced by IFN-γ and LPS, while M2 macrophages are induced by Th2- or anti-inflammatory cytokines and growth factors, including IL-4, IL-10, and TGF-β. During *H. pylori* infection, macrophages are recruited to the gastric mucosa, where they contribute to the production of pro-inflammatory cytokines and chemokines (Bergin et al. 2004; Dzierzanowska-Fangrat et al. 2008; Fehlings et al. 2012) (Fig. 6.1).

One of the most common myeloid infiltrates in infection sites is composed of polarized M2 macrophages which are recruited by chemo-attractants, such as colony-stimulating factor (CSF), CC ligand 2 (CCL2), vascular endothelial growth factor A (VEGFA), or CXCL12 (Cortez-Retamozo et al. 2012; Franklin et al. 2014; Noy and Pollard 2014; Qian et al. 2011). In different review articles, the Mantovani group discusses how most TAMs present M2 phenotypes and promote the suppression of effective antitumor activity via IL-10, TGF-β, and VEGFA signaling (Mantovani and Marchesi 2014; Mantovani et al. 2002). These cells promote tumor growth by several mechanisms, including their characteristic immunosuppressive activity, the involvement of epithelial-mesenchymal transition, and alteration of cellular metabolism during infection.

6.2.3 *Recruitment and Polarization of Myeloid-Derived Suppressor Cells*

H. pylori-induced gastritis shows increased infiltration of inflammatory cells, which play a crucial role in protecting *H. pylori* from immune attack by damaging gastric mucosa and inducing immune evasion and oncogenesis. Myeloid-derived suppressor cells (MDSCs) have appeared as a new cell type which plays a crucial role in controlling T-cell activity. In addition, *H. pylori* adheres to the cells of gastric mucosa and secretes different molecules that can change gastric epithelial cell function (Avilés-Jiménez et al. 2012) (Fig. 6.1).

MDSCs are precursors of granulocytes, monocytes, macrophages, and dendritic cells. The reviews from Shipp et al. and Szebeni et al. demonstrated that murine MDSCs are classified as Ly6C+ monocytic (M-MDSC) and Ly6G+ granulocytic (G-MDSC) subpopulations. Human MDSCs consist of a phenotypically heterogeneous population of myeloid cell precursors, M-MDSC (CD11b+, HLA-DR+/low, CD33+, CD14+, CD15-), and PMN-MDSC (CD11b+, HLA-DR+/low, CD33+, CD15+, or CD66b+) (Movahedi et al. 2008; Shipp et al. 2016; Szebeni et al. 2016). PMN or granulocytic MDSC-derived ROS disrupts T-cell receptor (TCR),

IL-2 receptor signaling, and MHC-TCR interactions (Nagaraj et al. 2007). The depletion of L-arginine by *H. pylori* arginase decreases the expression of CD3 zeta chains associated with the T-cell receptor, thus inhibiting T-cell activation and proliferation (Zabaleta et al. 2004). MDSC-derived IL-10 and VEGF-A inhibit dendritic cell maturation and promote the expansion and recruitment of Tregs, which further skew the tumor-specific immune response into tolerance.

The Merchant research group has discovered that during chronic *H. pylori* infection, the Shh-derived myeloid cells become polarized to MDSCs in response to tissue IFNα. Using stomach tissue-derived organoids and a patient-derived xenograft model, they showed that this subset of MDSCs expresses the protein Schlafen4 (SLFN), a transcriptional target of GLI1. MiR130b, the endogenous small, non-coding RNA produced by $SLFN^+$ MDSCs, enhances T-cell suppression, induces epithelial cell proliferation, and promotes metaplastic changes. $SLFN4^+$ MDSCs, as well as their human orthologs $SLFN12L^+$ PMN-MDSCs, express VEGF, IL-1β, and TNF-α, known factors associated with MDSC regulation, and create immunosuppression in the tumor microenvironment (TME) of gastric cancer patients (Ding et al. 2016, 2020).

H. pylori infection is one of the known risk factors for gastric cancer, which is also the second leading cause of cancer-related deaths worldwide. Tu et al. have shown a link between IL-1β and gastric cancer, which implicates the importance of MDSCs in the early stages of gastric carcinogenesis. Their data also shows a strong correlation between MDSC infiltration and tumor progression and MDSC activation in response to tumor-derived signals (Tu et al. 2008). Proliferation and activation mechanisms of MDSCs can be triggered through distinct pathways (reviewed in (Condamine et al. 2015)), mainly directed by STAT3, a transcription factor activated by GM-CSF, G-CSF, and VEGF, as well as IL-6 (Song et al. 2005). STAT3, once activated, induces expression of S100A8 and A9 (reviewed in (Foell et al. 2007)), which block the differentiation of immature myeloid cells and cause MDSC proliferation (Cheng et al. 2008). In vivo inhibition of STAT3 via receptor tyrosine kinase inhibitors resulted in a decrease in MDSCs (Movahedi et al. 2008; Munera et al. 2010). Downstream, MDSC activation is mainly mediated by NF-κB, which is induced by IL-1β and TNF-α or TLR signaling (Tu et al. 2008).

6.2.4 Induction of CD44V9 and Metaplasia

Helicobacter pylori colonization of human gastric mucosa damages gastric epithelial cells and promotes epithelial progression to neoplasia. In mice with chronic *H. pylori* infection, parietal and chief cell atrophy in the gastric mucosa can occur, leading to spasmolytic polypeptide/trefoil factor (TFF)2-expressing metaplasia (SPEM) and subsequently increasing the risk for progression to cancer (Nam et al. 2010; Nomura et al. 2004, 2005) (Fig. 6.1). Studies have shown how bacterial toxins and their effector proteins can provide insights into parietal cell physiology and the mechanisms by which pathogens gain control of cellular activities and the immune

response. Data from our laboratory has revealed the emergence of SPEM/TFF2 glands at the base of the ulcer margin in the stomach mucosa as the major reparative lineage for healing after severe gastric injury (Bertaux-Skeirik et al. 2017; Wright et al. 1990). We have also shown that to protect the tissue from further injury and reinstate epithelial continuity from bacterial infection, epithelial cells migrate from the ulcer margins via a process known as re-epithelialization (Bertaux-Skeirik et al. 2017).

The cell-surface glycoprotein CD44 is involved in various important physiological functions including cell proliferation, adhesion, migration, and hematopoiesis. The diverse physiological activity of CD44 embodies a number of diseases including cancer, arthritis, bacterial and viral infections, interstitial lung disease, vascular disease, and wound healing. During chronic *H. pylori* infection, the CD44 cell surface receptor and its variable isoforms (CD44v9) have been implicated as key players in malignant transformation (Engevik et al. 2016; Wada et al. 2013). The emergence of SPEM along with CD44v9 expression significantly contributes to defense against reactive oxygen species (ROS) (Ishimoto et al. 2011). CD44v9 interacts with the glutamate-cysteine transporter xCT, stabilizing the protein and inducing defense against ROS by increasing glutathione (GSH), promoting tumor growth (Bertaux-Skeirik et al. 2015, 2017; Ishimoto et al. 2011).

Using a gastric organoid orthotopic transplantation model, Bertaux-Skeirik et al. demonstrated that CD44v9 emerges at the ulcer margin in response to injury and contributes to the regeneration of the gastric epithelium (Bertaux-Skeirik et al. 2017). During *H. pylori* infection, SPEM expands into proliferative metaplasia, and the infection leads to the development of intestinal metaplasia (IM), with loss of parietal cells and inflammation throughout the mucosa (Bertaux-Skeirik et al. 2017). Infection with *H. pylori* is the greatest influencing factor for developing gastric cancer; however, it has been found that IM occurs at an equal frequency in patients with dysplasia and gastric cancer regardless of their *H. pylori* status. Importantly, in the stomach of both mouse and human metaplastic tissues, M2 macrophages promote the advancement of SPEM, associated with the emergence of CD44v9 in the presence of inflammation and parietal cell atrophy (Petersen et al. 2014) during gastric repair in response to infection and injury. It has been shown that upon *H. pylori* infection, Mongolian gerbils progressively develop chronic gastritis, followed by loss of parietal cells and metaplasia (Shimizu et al. 2016). Expression of SPEM and intestinal metaplasia (IM) was observed on the gastric glands of those gerbils after 1 year of infection. In humans there is evidence that suggests SPEM can either progress directly to dysplasia or become IM in the presence of continuous chronic inflammation (Goldenring et al. 2010). Single-cell transcriptional analyses have shown that SPEM/Tff2 cells that lack mature chief cells remain highly preserved and not found in the normal mucosa. It has been found also that the immunoregulatory phenotypes that emerge during chronic inflammation may play a role in regulating downstream events such as intestinalization, dysplasia, and carcinogenesis (Bockerstett et al. 2020).

6.2.5 Induction of Programmed Death-Ligand 1 (PD-L1)

Gastric cancer is often preceded by a precancerous process, frequently initiated by *H. pylori* infection, via series of events like chronic atrophic gastritis, intestinal metaplasia, dysplasia, and eventually carcinoma. Checkpoint proteins, such as programmed cell death-ligand 1 (PD-L1; B7-H1 or CD274), are a group of molecules involved in immune evasion mechanisms and, consequently, are thought to play an important role in the persistence of chronic infection. With the onset of infection, *H. pylori* injects virulence factors, mainly CagA, directly into the gastric epithelium. This event is highly associated with the development of gastric cancer. One of the major signaling pathways induced by CagA upon entering a gastric epithelial cell is the Sonic Hedgehog (Shh) signaling pathway which functions during *H. pylori* infection to stimulate secretion of IL-1β, inhibit acid secretion from parietal cells, induce atrophic gastritis and SPEM followed by intestinal metaplasia and dysplasia, and finally boost the development of gastric cancer. Unfortunately, clearance of *H. pylori* does not decrease the risk of developing gastric cancer once a patient has progressed to a metaplastic state. *H. pylori* infection upregulates programmed death-ligand 1 (PD-L1) expression on the surface of gastric epithelial cells. PD-L1 interacts with programmed death-1 (PD-1) on the surface of cytotoxic T lymphocytes, diminishing their ability to induce apoptosis in infected or cancer cells. Therefore, it is crucial to determine the Shh-mediated signaling pathway responsible for PD-L1 expression to discover treatment modalities that prevent expression, thereby improving patient outcome (Engevik et al. 2016; Holokai et al. 2019; Schumacher et al. 2012, 2015).

Various studies have shown that the gastric epithelium has been the target of damage induced by infection (Jones et al. 1999). Specifically, gastric T cells contribute to apoptosis of the epithelium by a Fas/Fas ligand and receptor expression on T cells (Gobert et al. 2002; Wang et al. 2000) enabling persistence in the host. Gastric biopsies have discerned that epithelial cells (GEC) infected with *H. pylori* show higher PD-L1 expression compared with uninfected samples. T cells co-cultured with these GEC showed decreased proliferation, IL-2 secretion, and CD69 expression. However, the neutralization of PD-L1 with a specific antibody reinstated the responses to levels close to those of T cells cultured alone. Synergistic expression of IFN-ɣ by *H. pylori* happens as a result of the increased PD-L1 expression. An interesting possibility is that this mechanism could also downregulate immune surveillance mechanisms needed to clear transformed cells that arise within the site of infection. Interestingly, the apoptotic effect of B7-H1 was suggested to be mediated by receptors other than PD-1. Although the *H. pylori* virulence factor responsible for inducing PD-L1 expression is not clear, this may open up new therapeutic targets against diseases associated with *H. pylori* infection (Das et al. 2006; Freeman et al. 2000).

6.3 Inflammation and Hypoxia

In response to bacterial or viral infection, the host immune system activates programs to facilitate wound healing, tissue homeostasis, and clearance of foreign pathogens. These programs trigger the release of cytokines and chemokines to activate and recruit lymphocytes to the site of infection (Korniluk et al. 2017). However, persistence of these inflammatory conditions has been associated with cancer development, as these actions can result in damage to normal tissues over time. A number of pro-inflammatory molecules, such as TNF-α, IL-1, IL-6, and IL-8, have been implicated in tumor progression (Aggarwal and Gehlot 2009; Balkwill and Mantovani 2001; Pierce et al. 2009). An acute inflammatory response is beneficial to the host, resulting in elimination of the inappropriate agent. However, when the inflammatory response fails to resolve after clearance of an insult, as is most often the case with *H. pylori* infection, prolonged activation can lead to a chronic condition and present a predisposition to disease, such as cancer (Korniluk et al. 2017; Niemela et al. 1995).

The prolonged availability of growth factors, cytokines, and chemokines associated with chronic inflammation may result from the dysregulation of a number of pro-inflammatory and pro-growth signaling pathways. For example, aberrant activation of nuclear factor-kappa B (NF-KB), Janus kinase (JAK)/STAT signaling, and hypoxia-inducible factor-1α (HIF-1α) have all been implicated in tumor growth, proliferation, and angiogenesis, among others (Semenza 2002; Taniguchi and Karin 2018; Thomas et al. 2015). Many of the cells and molecules involved in chronic inflammation are the same as those that respond acutely to tissue insult. How these factors turn a beneficial function into a pathological one has been an active area of study. Ongoing research using known instigators of inflammation, such as *H. pylori* infection in the stomach, continues to provide insight into how the host inflammatory response can be weaponized against the affected epithelial barrier.

NF-κBs are a family of transcription factors that regulate genes involved in innate immunity, inflammation, and apoptosis (Ghosh et al. 1998). NF-κB signaling can be activated by cytokines, infectious agents, and oncogenes (Ghosh and Karin 2002). In the stomach, constitutively active NF-κB has been linked to gastritis, an inflammatory condition that predisposes patients to gastric cancer (Isomoto et al. 2000; Keates et al. 1997). Targets of NF-κB signaling include anti-apoptotic proteins, proliferative proteins, and angiogenic factors. NF-κB activation, as well as many of its associated target genes, has been intimately associated with multiple cancers, especially those developing in inflammatory environments (Taniguchi and Karin 2018).

Prior to activation, NF-κB is bound to a set of IKB proteins that inhibit its activity. Activity from cytokines, such as TNF-α and IL1, cause phosphorylation of IKB, leading to its degradation and releasing NF-KB for nuclear translocation where it can promote the expression of additional pro-inflammatory cytokines, such as iNOS, and COX-2, TNF-a, and IL-1 (Ben-Neriah 2002; D'Ignazio et al. 2016; Hayden and Ghosh 2004; Xie et al. 1994). Regular NSAID use is known to decrease GI cancer risk partly through its modulation of the inflammatory response (Thorat and Cuzick

2013). NSAIDs target COX-1 and COX-2, enzymes responsible for the rate-limiting step in prostaglandin E2 (PGE2) synthesis. COX-2 has been implicated in tumorigenesis in gastrointestinal tissues and measurements show elevated COX-2 expression in gastric adenocarcinomas, more significantly so in those that are *H. pylori* positive (Gupta and Dubois 2001; Oshima et al. 2006; Ristimäki et al. 1997; Zhao et al. 2017).

The recruitment of immune cells to sites of inflammation creates metabolic stress that leads to hypoxia, as the demands of increased cell metabolism and proliferation deplete available oxygen (DeBerardinis et al. 2008). Endothelial cells are critical to the integrity of vascular architecture. *H. pylori* infection of the gastric epithelium induces the expression of chemoattractants that attract neutrophils, often leading to tissue damage (Fu 2014; Takemura et al. 1996; Wallace 1991). Neutrophils activated by *H. pylori* have been shown to disrupt endothelial cell function in vitro, and increased *H. pylori* infection is associated with greater neutrophil infiltration, initially operating in a positive feedback loop with the secretion of additional pro-inflammatory signals (Fu 2014; Takemura et al. 1996). Epithelial tissue damage resulting from *H. pylori* infection can allow the bacterium to spread to endothelial cells themselves, leading to increased IL-8 secretion and microvascular leakage (Aspholm et al. 2006; Kurose et al. 1994; Necchi et al. 2007; Tafreshi et al. 2018). Furthermore, advances in endoscopic technology have developed methods to identify gastritis and potential *H. pylori* infection through changes in the mucosal surface and in microvasculature patterns, suggesting broader scale vascular dysfunction in infected individuals (Anagnostopoulos et al. 2007; Ji and Li 2014; Yagi et al. 2002).

Vasculature is often compromised in inflammation, leading to diminishing oxygen supply, and hypoxic areas where oxygen is less than or equal to 1% (Bhandari and Nizet 2014; Eltzschig and Carmeliet 2011; Palazon et al. 2014). While this is problematic in normal cells and may lead to cell death, tumor cells often adapt and thrive under hypoxic conditions. The occurrence of hypoxia in tumor cells has been repeatedly demonstrated with different methodologies and more recently correlated with metastasis and survival in several cancers (Höckel and Vaupel 2001; Vaupel et al. 2002, 2007; Vaupel and Harrison 2004). Hypoxia can in turn regulate and exacerbate inflammation. When gastric cancer cells are cultured with 1% oxygen, expression of the cytokine IL-1α is upregulated, and tissue collected from gastric cancer tumors also shows increased IL-1α expression. IL-1α serves as a prognostic marker, correlating with poor survival in gastric cancer patients. Further study has shown that IL-1α is a potentiator of proliferation, migration, and metastasis in gastric cancers (Xuan and Wang 2017). Furthermore, the authors demonstrated that IL-1α expression was regulated by the hypoxia-inducible factors, HIFs, whose activity was directly due to the environmental effects of hypoxia.

6.3.1 Regulation of Inflammation by Hypoxia-Inducible Factors (HIFS)

As discussed previously, sites of inflammation, as well as solid tumors, often acquire regions of hypoxia (Vaupel and Mayer 2007). HIFs are transcription factors that are stabilized and activated under these hypoxic conditions (Huang et al. 1998b; Wang et al. 1995). HIFs are basic helix-loop-helix transcription factors that regulate the cell's response to hypoxia by signaling as heterodimers consisting of an alpha and beta subunit. HIF-1β is constitutively expressed and can associate with any of the three alpha subunits, HIF-1α, HIF-2α, and HIF-3α. Of these subunits, HIF-1α seems to be expressed ubiquitously in different tissue types, whereas the expression of HIF-2α and 3α is more restricted (Wiesener et al. 2003; Wood et al. 1996).

Under normoxic conditions, key residues of HIF-1α are hydroxylated by an oxygen-dependent, prolyl hydroxylase domain-containing enzyme (PHD), leading to its ubiquitination by an E3 ubiquitin ligase, the von Hippel-Lindau (VHL) tumor suppressor protein, and resultant proteasomal degradation (Bonicalzi et al. 2001; Cockman et al. 2000; Huang et al. 1998b; Jaakkola et al. 2001; Kamura et al. 2000; Ohh et al. 2000). As PHD protein activity uses O_2 as a cofactor, hypoxia prevents hydroxylation by PHD, stabilizing the alpha subunit and resulting in a functional HIF heterodimer and nuclear translocation of the complex. In this way HIFs are typically regulated not by expression levels but by protein stability. Dozens of HIF target genes have been identified containing the requisite hypoxia response element (HRE) (Semenza et al. 1994; Wang and Semenza 1993).

Experiments using gain-of-function (GOF) of HIF-1α in epithelial cells suggest a reciprocal role in inflammation. Mouse keratinocytes altered to express GOF HIF-1α show elevated chemokine production, resulting in elevated inflammatory cell accumulation in affected epidermis (Scortegagna et al. 2008). HIF-1α GOF keratinocytes also show elevated basal levels of NF-κB transcription, an important consideration since elevated NF-κB signaling can lead to a hypersensitive reaction to inflammatory stimuli.

In the intestinal epithelium, HIF-1α has been described to be both protective and pro-inflammatory. Mice lacking HIF-1α in intestinal epithelial cells subjected to epithelial lesion by sulindac sulfide developed milder inflammation in the colon than their littermate controls (Mladenova et al. 2015). However, Karhausen et al. have also demonstrated that the severity of colitis symptoms in a mouse model varied in accordance with HIF-1α levels in deficient and overexpressing mice (Karhausen et al. 2004). In a mouse model of chronic *H. pylori* infection, deletion of HIF-1α from myeloid cells has been shown to exacerbate *H. pylori*-induced gastritis. These myeloid cells were also less effective at killing *H. pylori*. While decreased IL-1β, NOS2, and IL-6 levels were observed in gastric mucosa from mice with HIF-1-α-deficient myeloid cells, tissue biopsies revealed increased proliferation which correlated with the severity of the corresponding gastritis and pathological abnormalities (Matak et al. 2015). The importance of HIF signaling in myeloid cell populations adds a degree of complexity to the HIF-1α signaling environment,

further illustrating that each cell type in an environment needs to be considered to fully understand the signaling implications.

Macrophages are found at higher frequencies in areas of hypoxia, such as infection sites and tumors (Cramer et al. 2003; Murdoch and Lewis 2005). In this hypoxic environment, macrophages upregulate HIF-1α and HIF-2α, switch to a glycolysis-heavy metabolic program, and exhibit changes in surface receptor expression. Macrophages express both HIF-1α and HIF-2α in response to hypoxia, and both play a role in upregulating transcriptional programs that promote inflammation and angiogenesis, inducing expression of IL-1b and VEGF, both relevant factors in tumor progression (Fang et al. 2009). Myeloid cell-specific HIF-1α depletion in mice results in altered macrophage metabolism and impaired inflammatory responses in response to acute skin challenge by TPA, a compound commonly used to initiate skin inflammation (Cramer et al. 2003). Hypoxia-treated macrophage cell isolates increase production of a number of pro-inflammatory cytokines and chemokines, such as IL-1B, and angiogenic factor and VEGF. These effects are ameliorated when either HIF-1α or HIF-2α is decreased.

6.3.2 HIFs and Cancer

While HIFs are normally regulated by oxygen-dependent degradation, HIF signaling can be induced by a number of other mechanisms in a normoxic state (Semenza 2010). HIF-1α is a direct transcriptional target of NF-κB, allowing inflammatory signaling to increase HIF-1α transcription and override its degradation kinetics, resulting in downstream signaling (Frede et al. 2006; Rius et al. 2008). HIF activity is targeted by several other inflammatory signaling components, such as TNF-α and IL-1 (Albina et al. 2001; Jung et al. 2003). HIF-1α activity is increased with the loss of function of certain tumor suppressors, such as PTEN and VHL (Li et al. 2005; Pugh and Ratcliffe 2003; Zundel et al. 2000), and has also been shown to be stabilized by multiple oncogenes in normoxia (Lee et al. 2008). Aberrant mTOR activity can also lead to increased HIF-1a translation (Lang et al. 2007). ROS generation in the gastric epithelia has been strongly implicated in gastric carcinogenesis. In gastric cancer cells, nonmitochondrial ROS production induced by *H. pylori* infection can lead to HIF-1α stabilization in normoxic conditions, demonstrating a plausible mechanism by which HIF-1α could be elevated in vivo (Park et al. 2003). More recently, Bhattacharya et al. have demonstrated that *H. pylori*-induced ROS drives increased HIF-1α expression through apurinic/apyrimidinic endonuclease 1 (APE1) and the transcriptional coactivator, p300 (Bhattacharyya et al. 2010). HIF-1α induction in response to *H. pylori* has also been measured in patient-derived gastric epithelia, further validating this relationship (Fig. 6.1) (Griffiths et al. 2007). This is important, as HIFs aberrantly activated under normoxia can still promote adaptive measures that are useful for tumor proliferation.

HIF-1α and HIF-2α are similar in structure and share some downstream targets. Despite these similarities, multiple reports have also demonstrated that each has

unique targets and sometimes even opposing functions (Holmquist-Mengelbier et al. 2006; Imamura et al. 2009; Rasheed et al. 2009; Song et al. 2009; Hu et al. 2003; Raval et al. 2005). HIF-1α and HIF-2α also differ in tissue- and cell-type-specific expression, complicating global inferences about the function of these proteins in all tissue types (Talks et al. 2000). As such, an interpretation of the role of either of these proteins in cancer depends heavily on the tissue of origin and context being discussed. Initial reports identifying HIF-2α observed high expression in endothelial cells, where its expression regulates vascularization (Tian et al. 1997). Its expression has since been reported to be a prognostic factor in multiple cancers (Bangoura et al. 2007; Giatromanolaki et al. 2001; Koukourakis et al. 2006; Onita et al. 2002). HIF-2α is also found in macrophages and has been identified in the parenchyma of multiple organs (Wiesener et al. 2003).

Immunohistochemical analysis has shown that increased HIF-1α expression is found in multiple cancers and neoplasms, including lung, breast, colon, and gastric cancers (Zhong et al. 1999). HIF-1α protein expression was also found to significantly correlate with Ki67 in multiple tumor types, suggesting that proliferation is associated with HIF-1α activity. Given its role in the regulation of diverse, proliferative processes, such as angiogenesis, metabolism, invasion, and metastasis, HIF-1α is well-placed to provide an adaptive advantage to cancer cells.

HIF-1α overexpression has been repeatedly linked to gastric cancer (Chen et al. 2014a, b, c; Sumiyoshi et al. 2006; Urano et al. 2006; Wang et al. 2010). Increases in HIF-1α have been linked to tumor metastasis, and its expression is correlated with other markers associated with aggressive tumor phenotypes, including VEGF and TGF-β (Chen et al. 2014a, b, c). HIF-1α expression has repeatedly been correlated with the development and severity of gastric cancer and has been shown to be detrimentally associated with 5-year survival, invasion depth, and metastasis in gastric cancer (Isobe et al. 2013; Kim et al. 2009; Rohwer et al. 2009; Sumiyoshi et al. 2006). As such, it is considered a prognostic marker for gastric cancer and is currently being investigated as a potential therapeutic target. This has particular importance in gastric cancer, where it has been shown to be induced by the carcinogen, *H. pylori* (Fig. 6.1) (Griffiths et al. 2007). Multiple studies have associated HIF-1α overexpression with decreased survival in patients with various cancers, including gastric. In an immunohistochemical study, HIF-1α was not detected in normal gastric tissue but was induced with *H. pylori* infection and often appeared in gastric tissues with various neoplastic pathologies (Griffiths et al. 2007). Observations of HIF-1α expression suggest that it occurs in the early stages of carcinogenesis, prior to apparent evidence of angiogenesis (Zhong et al. 1999).

HIF-2α expression has also been implicated in gastric cancer (Wang et al. 2010). A recent report has shown that while normal gastric mucosa does not express HIF-2α, tumor expression is linked to tumor stage, differentiation, metastasis, and poor prognosis in gastric cancer. Follow-up experiments using knockdown and overexpression of HIF-2a suggest that HIF-2α's role in gastric cancer progression occurs via regulation of survivin, cyclin D1, MMP2, and MMP9. Through these

effectors, HIF-2α could potentially impact cell survival, proliferation, and motility (Tong et al. 2015).

6.3.3 HIF Signaling Targets

To adapt to hypoxic environments, increased angiogenic signaling and changes in metabolic programming are employed to ensure cell survival. HIFs regulate multiple programs that stimulate adaptive functions to hypoxia, providing a crucial advantage to tumor cells in hypoxic environments. Many of these changes are associated with aggressiveness in tumors and therefore predict a poor prognosis in patients (Muz et al. 2015; Rankin and Giaccia 2016). Dozens of transcriptional targets of HIF-1α have been identified, many of which are important for tumor survival and proliferation. Snail is a transcription factor involved in inducing epithelial-mesenchymal transition (EMT), a process by which epithelial cells acquire mesenchymal characteristics and become more likely to metastasize. HIF-1α induction in gastric cancer stem cells has been linked to increased Snail expression (Yang et al. 2017). In addition, the cytoskeletal regulator RhoE, which has also been implicated in EMT, has been identified as a direct target of HIF-1α (Zhou et al. 2011). Angiogenesis is a key process for tumor proliferation, and VEGF is a critical factor for promoting the proliferation of endothelial cells. VEGF overexpression has been linked to metastasis and tumor progression, is frequently associated with poor prognosis, and is regulated by HIF-1α (Macedo et al. 2017; Song et al. 2009). Taken together, HIF-1a modulates the activity of a host of tumor-associated genes.

PD-L1

In recent years, much attention has been given to immune checkpoint inhibitors as treatments for certain types of cancer (Darvin et al. 2018; Pico de Coaña et al. 2015). Programmed death-1 (PD-1) is one such drug target, with treatment currently approved for multiple cancers, including gastric adenocarcinoma (Smyth and Thuss-Patience 2018). Generally, overexpression of PD-L1 is associated with decreased overall survival in multiple cancers (Wu et al. 2015). A recent meta-analysis of PD-L1 and gastric cancer has associated PD-L1 expression with tumor infiltration depth, lymph node metastasis, and overall patient survival (Gu et al. 2017).

Like other cells in the TME, MSDCs (see Sect. 6.2.2) are regulated by hypoxia, which promotes their differentiation into macrophages (Corzo et al. 2010). Basal levels of PD-L1 in MDSCs are upregulated under hypoxia. The PD-L1 promoter has an HRE, accounting for its upregulation during hypoxia in a HIF-1α-dependent manner (Noman et al. 2014). HIF-1α is associated with decreased effectiveness of multiple therapeutics. In fact, co-expression of PDL1 and HIF-1α in hepatocellular carcinoma is linked to decreased survival (Dai et al. 2018).

As previously discussed, infection with *H. pylori* significantly increases PD-L1 expression in normal primary gastric epithelial cells (Wu et al. 2010). Furthermore,

studies conducted using both iPSC-derived gastric organoids and human-derived gastric monolayers have shown that PDL1 expression on the gastric epithelium is induced by *H. pylori* infection in a Shh-dependent manner (Holokai et al. 2019).

While direct control of PD-L1 by HIF-1α has not yet been explicitly demonstrated in gastric tissue, a recent study using a radioisotope-labeled glucose analog, F-FDG, has correlated F-FDG accumulation with PDL1 expression in gastric cancer tissues. Previous studies have linked F-FDG accumulation to HIF-1α activity, likely through its relationship to glucose metabolism. Since the PD-L1 promoter has an HRE and is regulated by HIF-1α in other cell types, it is possible that this report demonstrates an indirect link of HIF-1α to PD-L1 gastric cancer (Chen et al. 2019).

CD44

CD44 has multiple functions in physiological processes, such as lymphocyte homing, growth signaling, and ECM organization (Ponta et al. 2003). Expressed on cells with stem-like properties, CD44 labels cancer stem cells (CSC) in the tumor niche through which tumor proliferation and differentiation is believed to occur (Batlle and Clevers 2017). CD44 expression is increased by cytokine signaling in gastric cancer cells, and its expression is enhanced in tissues from patients with gastritis (Fan et al. 1996; Mayer et al. 1993).

CD44 proteins are expressed in various isoforms due to insertions or alternatively spliced exons in the extracellular domain, resulting in the expression of different variants of the receptor (Screaton et al. 1992). The standard isoform, dubbed CD44s, consists of the base set of 10 of the 19 exons that make up the CD44 gene. This can be augmented by the presence of variable additional exons, dubbed "CD44v." Expression of these variant isoforms is linked to proliferation, as they are typically found in tissues that are actively proliferating (Rudzki and Jothy 1997). The expression of multiple variants have been implicated in aggressive tumor phenotypes or associated with poor prognosis in cancer patients (Kobayashi et al. 2016; Ozawa et al. 2014; Yamakawa et al. 2017). Indeed, when antibodies are used to inhibit CD44v binding in animal models, metastasis decreases (Guo et al. 1994; Seiter et al. 1993). CD44vs have been shown to convey different binding affinities for members of the extracellular matrix ligands that CD44 interacts with, an alteration likely to have downstream consequences for cancer progression (Dougherty et al. 1994).

In the gastric mucosa, CD44 marks a population of self-renewing cells that can produce differentiated cell populations (Takaishi et al. 2009). In normal gastric tissue, CD44s is the predominantly expressed CD44 transcript, with little to no detectable levels of most CD44v. When CD44 levels are examined in gastric cancer cell lines, CD44s expression is decreased, while CD44v expression appears to be elevated. In gastric cancer, both CD44 and CD44v6 are linked to disease progression and poor patient prognosis (Fang et al. 2016). While CD44v6 is expressed in normal gastric mucosa, in gastric cancer tissue, increased CD44v6 is linked to gastric dysplasia and has been connected to c-Met signaling, whose amplification promotes invasion and metastasis in many cancers (Fan et al. 1996). The role of CD44v6 as a co-receptor in c-Met signaling provides a potential mechanistic link between increases in CD44v6 levels and aggressive phenotypes in gastric cancer.

Generally, CD44 expression is increased in the gastric mucosa of individuals infected with *H. pylori* (Fan et al. 1996). More particularly, CD44v9 has been identified as factor emerging early following *H. pylori* infection in the gastric epithelium (Bertaux-Skeirik et al. 2017; Fan et al. 1996). CD44v9 is of particular importance for aberrant cell survival, as it has been shown to stabilize xCT, which plays an important role in protecting the cell from ROS (Ishimoto et al. 2011). Greater xCT expression can disrupt the redox balance and protect cancer cells, which are often exposed to oxidative stress, from death (Trachootham et al. 2009).

In gastric cancer cells, CD44 mRNA and protein expression are increased in hypoxia, and knockdown of HIF-1α mitigates these changes (Liu et al. 2016). HIF-1α has been shown to regulate CD44 expression in breast cancer cells, with hypoxia increasing the number of cells expressing CD44. Specifically, HIF-1α induces expression of CD44v6 and CD44v8. This activity is unique to HIF-1α, but not HIF-2α, making these CD44 variants unique targets of HIF-1α signaling (Oliveira-Costa et al. 2011). Initial studies in *H. pylori* infected epithelia have shown that CD44v6 is increased with infection (Fan et al. 1996). Given the relationship between CD44v6 and c-Met activation, this provides a potential mechanistic link between hypoxia and tumor aggressiveness (Orian-Rousseau et al. 2002).

6.3.4 HIF-1a and Increased Glycolysis in Tumor Cells

In the event of hypoxia, cells shift their metabolism from oxidative phosphorylation to glycolysis (Rempel et al. 1996; Younes et al. 1996). In glycolysis, glucose is metabolized into lactic acid, which is secreted from the cell. Glucose is an abundant extracellular nutrient, and while the metabolism of a single molecule does not yield as much ATP as oxidative phosphorylation, it produces ATP more quickly. This means that cells predominantly using glucose metabolism can produce more ATP over a short period of time than with oxidative phosphorylation. In addition to this increased efficiency, metabolizing glucose while truncating the use of the TCA cycle allows the cell to use many of the intermediates for alternative biosynthetic pathways (Liberti and Locasale 2016). In this manner, glycolytic metabolism supports the need for the production of nucleotides, lipids, and other materials for cell proliferation.

Typically, cells only engage in glycolysis in the absence of oxygen (DeBerardinis et al. 2008). It was first noted by Otto Warburg that proliferating tumor cells depend largely on glucose consumption, despite available oxygen (Warburg et al. 1927). This characteristic, now recognized as a hallmark of cancer, has been defined as the "Warburg effect." Since HIF-1α can be stabilized in normoxia, much emerging research has focused on its role in affecting the metabolic changes seen in the Warburg effect (Stubbs and Griffiths 2010). Many glycolytic proteins are targets of HIF-1α, but not HIF-2α (Song et al. 2009). Hypoxia response elements have been found in the promoters of GLUT1, ENO1, PKM2, and LDH, among others (Firth et al. 1995; Luo et al. 2011; Semenza et al. 1994, 1996).

In gastric cancer, studies have reported increases in the expression of glucose transporters and glycolytic enzymes. Specifically, gastric cancers have been shown to have increased expression of GLUT1, LDHA, hexokinase II (HKII), pyruvate kinase M2 (PKM2), pyruvate dehydrogenase (PDK), and enolase 1 (ENO1) (Kim et al. 2004). GLUT1 is shown to have little to no expression in normal gastric tissue yet is reported in gastric cancer, with some reports correlating its presence with poor overall survival (Chen et al. 2017; Noguchi et al. 1999; Yu et al. 2017). HKII catalyzes a rate-limiting step in glycolysis. Reports indicate that HKII is overexpressed in gastric cancer and has been linked to poor patient prognosis (Wu et al. 2017). It has been reported that *H. pylori* infection leads to increased ENO1 expression, while ENO1 knockdown has been shown to increase potential efficacy of cisplatin in gastric cells (Chen et al. 2014a, b, c). Both PKM2 and PDK have been linked to infection with CagA-positive *H. pylori*, showing increased expression in gastric cancer tissues (Shiroki et al. 2017). Furthermore, PKM2 knockdown in gastric cancer cells decreased proliferation and tumorigenicity in vitro. This isoform of PKM2, the M2 isoform of pyruvate kinase, is associated with cell proliferation and is known to be less active than PKM1, allowing for the accumulation of glycolytic intermediates that may be important for adjacent anabolic pathways (Christofk et al. 2008). Lactate dehydrogenase (LDH) catalyzes the conversion of pyruvate to lactate. There are five isoforms of LDH, with LDH-5 expression found to be elevated in cancer cells (Koukourakis et al. 2003). LDH-5 is frequently expressed at high levels in gastric cancer, and its expression correlates with HIF-1α and VEGF, two proteins linked to aggressive cancer phenotypes (Kolev et al. 2008). In addition to the metabolic changes observed in the Warburg effect, gastric cancer cells show differences from normal cells in lipid and amino acid metabolism. Overall, the metabolic profile of a gastric cancer cell differs significantly from a healthy one and is in part altered by HIF-1α activity (Liu et al. 2019). Since gastric cancer cells exhibit significant metabolic differences from their normal counterparts, several drugs have been developed to target the activity of crucial metabolic enzymes. Indeed, targeting the activity of glycolytic enzymes has shown promise as a potential cancer therapeutic (Martinez-Outschoorn et al. 2017).

6.4 Impact of Early Epithelial and Immune Cell Responses on the Gastric Tumor Microenvironment

6.4.1 Defining the Gastric Tumor Microenvironment

In addition to malignant cells, tumor tissues contain a stromal compartment consisting of fibroblasts, endothelial cells, lymphocytes, and macrophages. A complex network of associated extracellular matrix (ECM) and vascularization is also found with these cell populations (Hanahan and Coussens 2012). Infection with *H. pylori* can impact each of these components, creating an inflamed environment

and paving the way for gastritis and neoplastic changes. *H. pylori*'s effect on endothelial and immune cells resulting in increased cytokine secretion and compromised vasculature was discussed in earlier sections (see Sects. 6.2 and 6.3.1). The relationship between the tumor and stromal cells is bidirectional, with each releasing factors that impact the fates and behaviors of the other. Soluble factors signal between stromal cells and the TME, resulting in local concentrations of active molecules such as cytokines and growth factors. These interactions are often crucial in determining the aggressiveness of the resident tumor, influencing processes such as proliferation, inflammation, metastasis, angiogenesis, and epithelial-to-mesenchymal transition (EMT) (Bhowmick et al. 2004; Brivio et al. 2017; Comito et al. 2012; Olumi et al. 1999).

Fibroblasts in the tumor environment can be activated by growth factors or cytokines to dynamically participate in tumor progression (Kalluri 2016). These cancer-associated fibroblasts (CAFs) are typically characterized by the expression of alpha-smooth muscle actin (SMA) or fibroblast activation protein (FAP). In addition to activation by intrinsic factors, fibroblast activation can be induced by *H. pylori* in a CagA-dependent manner (Krzysiek-Maczka et al. 2018; Zhang et al. 2013). These activated CAFS can increase the expression of factors associated with gastric carcinogenesis and EMT, such as IL-6 and TGF-β (Kinoshita et al. 2013). Increased FAP expression has been observed in many cancers and is associated with invasion and metastasis in gastric cancer (Puré and Blomberg 2018; Wang et al. 2013). NF-κB activation by *H. pylori* can have additional consequences, also leading to the activation of CAFs, along with its role in inflammation (Erez et al. 2010). By producing ECM components, metalloproteinases, growth factors, and cytokines, CAFs can promote growth, angiogenesis, and inflammation, as well as immune suppression (Kwa et al. 2019; Mishra et al. 2011). In this way, *H. pylori* can have a dramatic effect on the TME through fibroblast activation. Tumor stroma is thought to be a result of a chronic, misplaced repair response from these activated fibroblasts (Kalluri 2016; Schäfer and Werner 2008). Stroma proliferates in kind with the tumor cells it populates, and CAFs secrete VEGF, and so can stimulate angiogenesis and vascular permeability (Fukumura et al. 1998). Multiple reports have also shown CAFs to produce matrix metalloproteinases (MMPs), which function to increase cell motility by facilitating the degradation of ECM proteins (Das et al. 2017). As with VEGF, expression of MMP-2 and MMP-9 is linked to tumor aggressiveness in gastric cancer (Zheng et al. 2006). Some MMPs can also be produced by gastric epithelial cells, and infection with *H. pylori* can induce the expression of MMP-1, MMP-7, and MMP-9, which have each been implicated in gastric pathogenesis (Bebb et al. 2003; Kundu et al. 2006; Mori et al. 2003; Pillinger et al. 2007; Wroblewski et al. 2003).

A large portion of tumor mass is encompassed by tumor-associated macrophages (Pollard 2004). Once recruited to tumors by chemoattractants, TAMs are often polarized to an M2-like state (see Sect. 6.2.2). Once recruited to tumors by chemoattractants, TAMs are often polarized to an M2-like state (see Sect. 6.2.2) and can promote angiogenesis, tumor cell invasion, and metastasis (Pollard 2004). Macrophages can interact with the ECM, clear apoptotic cells, and produce growth

factors (Condeelis and Pollard 2006). In normal tissue, M2 macrophages function in repair; however, their presence in the TME can interfere with T-cell activation (Coussens and Werb 2002). This has been demonstrated in a mouse model of breast cancer where genetic ablation of macrophages interfered with tumor progression, while overexpression of the macrophage growth factor CSF-1 potentiated tumor growth (Lin et al. 2001).

Regulatory T cells expressing the transcription factor Forkhead box protein P3 (FOXP3) are also found in the TME. Acting to inhibit immunological action against "self," Tregs suppress immune activity—a function that is exploited by tumors (Beyer and Schultze 2006; Jonuleit et al. 2001; Ng et al. 2001; Sakaguchi et al. 2011). Conclusions about the impact of these cells on gastric tumor progression have been mixed, with some groups correlating increased Treg expression with the poor outcome while others find the opposite (Choi et al. 2016). Regardless of the consensus on their therapeutic impact, immunohistochemical studies of gastric tumors show a larger Treg population in gastric tumors than in normal gastric tissue (Kashimura et al. 2012; Perrone et al. 2008). This is in agreement with what is found in other cancers (Heimberger et al. 2008; Hiraoka et al. 2006; Wolf et al. 2005). Indeed, as discussed above in Sect. 6.2.1, *H. pylori* infection can inhibit the memory T-cell response in vivo (Lundgren et al. 2003). Whether or not Tregs contribute to disease proliferation, the consistency of these findings suggests that the TME itself promotes Tregs in gastric cancer.

Myeloid-derived suppressor cells are immature myeloid cells that are recruited to the tumor environment by pro-inflammatory signals (Bunt et al. 2006; Ostrand-Rosenberg and Sinha 2009). MSDCs can mediate immune suppression in the tumor environment through several mechanisms, including ROS generation and Treg induction (Corzo et al. 2010; Lindau et al. 2013). It has been shown that hypoxia can induce PD-L1 expression in MDSCs through HIF-1α, providing a potential mechanism of immune evasion through hypoxic activation (Noman et al. 2014). Whether this activation can be achieved with oncogenic stabilization of HIF-1α has not been shown. As discussed previously (Sect. 6.2.3), *H. pylori* infection is associated with the induction of a differentiation factor that biases myeloid cells to a suppressive MDSC phenotype, and MDSC influx has been observed in the gastric mucosa from both *H. pylori*-infected patients and mice (Ding et al. 2016). Recent work has linked a subset of MDSCs with tumor stage and decreased survival in gastric cancer, further illustrating their importance in tumor progression (Mao et al. 2018).

Although *H. pylori* infects about 50% of the world's population, only a small percentage of these cases will go on to develop gastric cancer (Howlader et al. 2010). This indicates that additional factors contribute to the development of gastric cancer in an *H. pylori*-infected epithelium. While both genetic and macro-environmental elements have been identified as risk factors in gastric carcinogenesis, more recently the TME has also been highlighted for its importance in tumor progression and resistance to therapy (Son et al. 2017; Trédan et al. 2007; Wu and Dai 2017). Stromal cells are dynamically active in the TME and may secrete factors that contribute to tumor proliferation, angiogenesis, or metastasis.

For all cells, oxygen availability depends on the distance from the blood supply. Because the availability of essential metabolites is limited by diffusion, the tumor environment varies with distance from the vascular tissue that delivers these nutrients. However, even in tumors with nearby capillaries, significant hypoxia is detected. This decrease in O_2 tension can be detected as little as 100 um from nearby vasculature, adapting to hypoxic environments critical for tumor proliferation (Helmlinger et al. 1997). This distorted vasculature further contributes to the hypoxic microenvironment. Additionally, proliferating cells increase metabolic demand due to the persistently increasing biomass incurred with each cell division, so highly proliferative tumors can also create demands that deplete the oxygen supply of the local environment (DeBerardinis et al. 2008). With tumor development, intratumoral hypoxia becomes inevitable (Harris 2002). This hypoxia can further stimulate HIF-1α stabilization and so can promote the expression of a number of factors that promote proliferation and inhibit apoptosis, despite the hypoxic condition that might otherwise induce death. Anaerobic conditions and increased transcription of glycolytic enzymes shift the cell metabolism toward one favoring glycolysis, again enabling survival and the production of essential amino acids and lipids for cell survival. This element of the TME is critical to address, as it contributes to tumor growth and metastasis and is related to poor prognosis in a number of cancers (Lu and Kang 2010; Vaupel 2004).

6.4.2 Resistance to Immunotherapy

Immune checkpoint blockade targeting PD-1/PD-L1 has promising therapeutic efficacy in a variety of tumors, but resistance during treatment is still a major issue—Dr. Tasuku Honjo was awarded the 2018 Nobel Prize in Medicine for his work on PD-1 and checkpoint therapy. The biggest challenges for cancer immunotherapy are to understand the many complex resistance mechanisms and to develop effective combination strategies to overcome that resistance. The resistance can be primary, as in never responders, or acquired, which appears after a period of response. However, in considering resistance mechanisms to immune-based therapies, it is important to remember that the immune response is unique and constantly evolving in each patient, either as a result of the patient's own environmental and genetic makeup or as a result of treatment modalities, including surgery, chemotherapy, radiation therapy, and immunotherapy. Since tumor cells can adjust to this acquired immune response by upregulation of PD-L1 expression, synergistic effects can be expected when combining such immune stimulating therapies with anti-PD-L1 antibodies in advanced stage gastric cancer. Recent studies have shown that knocking down of PD-L1 expression in human gastric cancer cells significantly inhibited tumor growth and improved the cytotoxic sensitivity to cytokine therapies (Li et al. 2017). Kato et al. found that the median overall survival was longer with 330 nivolumab (PD-1 inhibitor)-treated patients versus 161 patients who received placebo (5.4 months vs. 3.6 months) (Kato et al. 2019).

Due to cancer immunosurveillance, immune cells usually serve as a barrier by recognizing and protecting tissues from nascent tumor cells. But there are cases where immune cells have a tumor-promoting action via cancer immunoediting. Tumor cells sometimes maintain a state of dormancy, where the immune system controls their outgrowth and alters their immunogenicity, but does not eradicate them. But when this equilibrium is broken, tumor cells successfully escape and become a poorly immunogenic tumor (reviewed in Vesely and Schreiber (2013)). Loss of tumor antigen expression or MHC-I presentation is important for successful immunoediting. Studies have shown that mouse primary sarcomas were edited in such a way that they became less immunogenic through the selective outgrowth of cells and were able to escape T lymphocyte attack (DuPage et al. 2012). Pro-tumorigenic infiltrating immune cells that cause the immune suppressive microenvironment are mainly M2 subtypes of macrophages, myeloid-derived suppressor cells (MDSCs), neutrophils, FoxP3+ T regulatory cells (Tregs), and Th17 cells, whereas the antitumor immune infiltrates are mainly antigen presenting dendritic cells (DCs), macrophages of the M1 subtype, cytotoxic T lymphocytes (CTLs), natural killer cells (NKCs), and Th1 cells. *Helicobacter pyl*ori infection has been shown to reduce Th1 polarized immunological responses (Hou et al. 2007). Several recent studies have demonstrated that *H. pylori* virulence factor vacA has the ability to reprogram DCs to develop a tolerogenic phenotype to inhibit human T-cell activation, fail to produce inflammatory cytokines, and prime Treg over Th1 or Th17 responses (Oertli et al. 2013).

After analyzing data from a large cohort of human colorectal cancers, Pages et al. documented that infiltration of cytotoxic memory T cells in the primary tumor site is the strongest predictor for recurrence and metastasis, as well as disease-free survival (Pagès et al. 2010). The Fridman group documented that inflammation is one of the major components of human tumors, and chronic inflammation generally leads to worse prognosis due to the presence of soluble IL-15 receptor in the plasma of the patients with head and neck cancers (reviewed in (Fridman et al. 2011)). In addition to MDSCs and Tregs, IDO1 is another immune checkpoint protein that promotes the inhibition of T cells and may be related to T-cell infiltration (Heeren et al. 2018). Furthermore, cytotoxic T cells can be suppressed by Tregs and MDSCs via IDO1, promoting tumor immune evasion (Ladomersky et al. 2018). A decrease in effector T cells in the tumor microenvironment also contributes to resistance to anti-PD-1 therapy. Tumors upregulate IL-6, granulocyte colony-stimulating factor (G-CSF), and CLCX1 by increasing IL-17A expression, thereby promoting tumor proliferation and decreasing CD4+ and CD8+ T cells in the tumor microenvironment. IL-17A + tumor tissues are also significantly less reactive to PD-1 antibodies in clinical samples (Akbay et al. 2017). Additionally, the absence of PTEN increases VEGF expression which promotes abnormal tumor angiogenesis, causing a hypoxic environment and inhibiting T-cell infiltration (George et al. 2017). Therefore, the absence of PTEN may reduce the infiltration of CD8+ T cells by upregulating VEGF, leading to resistance to PD-1 therapy (Zhu et al. 2017). MDSCs are negatively correlated with CD4+ and CD8+ T-cell infiltration and are an important factor in decreased T-cell infiltration (George et al. 2017). Additionally, the presence of

immunosuppressive tumor stroma, especially in some solid tumors, makes it difficult for T cells to infiltrate, limiting the efficacy of PD-1 blockade immunotherapy.

In addition to immune checkpoints and immune system activity, other techniques can improve the efficacy of PD-1/PD-L1 blockade therapy, such as activating tumor cell autophagy, and inhibit tumor angiogenesis and mesenchymal transition, in order to achieve better results in combination therapy.

6.5 Conclusions

Our knowledge of *H. pylori* pathogenesis and gastric cancer development has predominantly been based on data generated from gastric cancer cell lines or in vivo animal models of inflammation. Animal models of *H. pylori*-induced disease do not exhibit the same pathophysiological features as the human response to infection, and gastric cancer cell lines lack the cellular and architectural complexity of the gastric epithelium in vivo. There have been extensive efforts to develop patient-derived models of *H. pylori* infection and gastric cancer (Bertaux-Skeirik et al. 2015, 2019; Holokai et al. 2019). Given that organoids are capable of long-term expansion in culture and remain phenotypically and genetically stable, these cultures represent preferred preclinical models over existing immortalized cell lines and patient-derived xenografts to study cancer (Dedhia et al. 2016; Steele et al. 2019). To overcome species differences in the regional response to *H. pylori* infection, gastric organoids, whether derived from patient tissue (Bertaux-Skeirik et al. 2015, 2017; Engevik et al. 2016; Holokai et al. 2019) or induced pluripotent stem cells (McCracken et al. 2017, 2014), have allowed us to study these unique regional differences of the human stomach in response to *H. pylori* infection. Recent advances in organoid technology have also demonstrated the successful effort to establish patient-derived organoid models of *H. pylori* pathogenesis in context with the patient's immune cells (Chakrabarti et al. 2018a, b; Holokai et al. 2019). The timeline in human subjects from infection to complications arising from disease progression can span months to years. Of greater concern is the gap in our understanding of the mechanisms that shift the composition of the immune environment and subsequently the response by the epithelium. The appearance of chronic atrophic gastritis indicates that the epithelial population of the stomach is responding to the persistent inflammatory infiltrate. Subsequently, there is the development of metaplasia. Although immune cells likely initiate the epithelial changes, other stromal populations participate, e.g., fibroblasts, endothelial, and neural cells. Collectively, the inflamed microenvironment restructures the epithelium and drives a phenotype conducive of progression to neoplasia (Fig. 6.1). Studies focused around identifying the early epithelial and inflammatory changes of the gastric environment in response to infection will continue to provide information related to immune surveillance and persistence and early epithelial changes contributing to the progression of disease.

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Chapter 7
Gut Microbiome and Liver Cancer

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Abstract Liver cancer is a major cause of cancer-related death, and its incidence keeps rising. The liver is exposed to gut microbial products and metabolites via portal blood and influenced by the gut microbiome. Alteration of the gut microbiome is commonly observed in high-risk factors for liver cancer such as obesity, nonalcoholic fatty liver disease, and cirrhosis. The association between dysbiosis and liver cancer has been suggested. Importantly, animal studies provide direct evidence that the gut microbiome promotes liver cancer. The current knowledge of the gut microbiome's contribution to liver cancer and the reported mechanisms will be reviewed in this chapter.

Keywords Chronic viral hepatitis · Hepatocellular carcinoma · *Helicobacter pylori* · Gut-liver axis · Liver fluke infection · Virus · Parasites · Obesity · Nonalcoholic fatty liver disease · Microbiome

7.1 Liver Cancer Types and Risk Factors

7.1.1 Hepatocellular Carcinoma

Liver cancer is a leading cause of cancer-related death worldwide, and its incidence is rising (Tanaka et al. 2010; Mokdad et al. 2017; Forner et al. 2018; Lauby-Secretan

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et al. 2016; Villanueva 2019). Hepatocellular carcinoma (HCC), the most frequent (~80%) primary liver cancer, ranks as the sixth most common malignancy and mainly occurs in men. HCC is closely related to chronic liver diseases, arises frequently in patients with cirrhosis, and is considered a typical inflammation-linked cancer (Capece et al. 2013; Colotta et al. 2009). Although early HCC can be potentially cured by surgical resection or liver transplantation, most HCC patients are diagnosed with unresectable disease. Despite the great improvement of modern cancer treatments and increased survival in many cancers, the mortality rate of HCC is still rising (Tanaka et al. 2010; Mokdad et al. 2017; Forner et al. 2018; Lauby-Secretan et al. 2016; Villanueva 2019).

Currently, hepatitis C infection and excessive alcohol consumption are the main risk factors for HCC. In recent years, excessive body weight or obesity has been linked to higher risk of cancer at several organ sites including the liver (Lauby-Secretan et al. 2016; Tyson and El-Serag 2011; Calle et al. 2003). Significantly higher incidence and mortality from liver cancer is observed in people with high baseline body mass index (Calle et al. 2003; Campbell et al. 2016). Besides a chronic low-grade inflammation with insulin resistance and metabolic abnormalities, roughly one third of obese patients present an accumulation of large amounts of lipids inside the liver, a disease condition called nonalcoholic fatty liver disease (NAFLD). NAFLD is a spectrum of liver diseases characterized by excessive accumulation of triglycerides in hepatocytes without heavy alcohol consumption, which range from simple steatosis to hepatic triglyceride accumulation with inflammation and liver damage (nonalcoholic steatohepatitis [NASH]) and finally hepatic fibrosis and cirrhosis (Brunt 2010; Michelotti et al. 2013; Wree et al. 2013). NAFLD has been established as an important risk factor for HCC (Brunt 2010; Michelotti et al. 2013; White et al. 2017; Kanwal et al. 2018; Anstee et al. 2019). In NAFLD patients, retrospective assessments support the association between metabolic syndrome, diabetes, and HCC (Siegel and Zhu 2009). In contrast to the success in preventing and controlling of viral hepatitis with hepatitis B vaccination and curable treatments for hepatitis C, the worldwide prevalence of obesity is continuing to rise. Accompanying the increasing prevalence of obesity, NAFLD has become the most common cause of liver dysfunction globally (Li et al. 2018). The attribution of metabolic syndrome and NAFLD to HCC is expected to increase in the future (Streba et al. 2015).

7.1.2 Intrahepatic Cholangiocarcinoma

Intrahepatic cholangiocarcinoma (ICC) is the second most common (~10%–20%) primary hepatic malignancy and arises from the bile ducts within the liver parenchyma (Tanaka et al. 2010; Tyson and El-Serag 2011; Razumilava and Gores 2014; Banales et al. 2016). Established risk factors for ICC are primary sclerosing cholangitis, choledochal cysts, fibropolycystic liver disease, hepatolithiasis, parasitic infection, and toxic exposure such as the radiologic contrast agent thorotrast (Tanaka et al. 2010; Tyson and El-Serag 2011). However, the majority of ICC patients do not

present any of these risk factors. Many other less-established risk factors have been suggested such as inflammatory bowel disease, hepatitis C, hepatitis B, cirrhosis, obesity, diabetes, alcohol consumption, and tobacco use (Tanaka et al. 2010; Tyson and El-Serag 2011; Welzel et al. 2007). Surgery remains the only curative treatment option, but most ICC patients present with unresectable disease at the time of diagnosis with a median survival of less than 3 years.

7.1.3 Metastatic Liver Malignancies

Metastases are responsible for majority of cancer-related death (Lambert et al. 2017). The liver is a common site to form metastatic spread especially from cancers of the gastrointestinal tract, breast, and lung. It is often overlooked that secondary hepatic malignancies (liver metastases) account for the majority (95%) of all hepatic cancers (Disibio and French 2008). Treatment for liver metastasis is often difficult, and patients have a poor prognosis.

7.2 Carcinogenesis of Liver Cancer

7.2.1 Oncogenic Pathways in HCC

HCC tumors are highly complex and heterogenous with multiple signaling pathways contributing to hepatocarcinogenesis. Epidermal growth factor (EGF) signaling is one of the most thoroughly evaluated proliferation cascades in human HCC. EGF upregulation is an important signature for predicting late HCC recurrence after surgical resection (Hoshida et al. 2008). Insulin-like growth factor (IGF) signaling is an essential regulator of liver growth and development. Overexpression of IGF2 is frequently observed in HCC and even found in preneoplastic lesions (Breuhahn et al. 2006). Hepatocyte growth factor (HGF), the ligand for the MET receptor, is a potent mitogen for hepatocytes. Upon EGFR, IGFR, or MET activation, extracellular signals can be transduced through AKT or MAPK pathways (Johnson and Lapadat 2002). Molecules belonging to both signaling cascades (such as KRAS or AKT) have been identified as oncogenes in human cancer. Although RAS mutations are infrequent, overexpression of RAS is often found in human HCC (Calvisi et al. 2006). AKT phosphorylation has been described as a predictor of tumor recurrence after surgical resection (Nakanishi et al. 2005). Inhibition of MTOR, one of the most important molecules downstream of AKT, demonstrates antitumor function in experimental HCC models (Treiber 2009), which further increases the relevance of this pathway. The WNT/β-catenin pathway is not only involved in liver development and differentiation but also implicated in cell proliferation and metabolism (Thompson and Monga 2007). β-catenin is encoded by the *CTNNB1* gene. Mutations in *CTNNB1* and *AXIN1* (important for β-catenin ubiquitination and subsequent degradation) are frequently found in HCC (Villanueva et al. 2007). Nuclear accumulation

of β-catenin induces upregulation of genes for cell differentiation and proliferation (Boyault et al. 2007). The hedgehog pathway is involved in the embryonic liver development, and its reactivation plays a substantial role in sustaining cancer cell growth and progression in HCC (Tada et al. 2008). Chronic inflammation is closely associated with HCC. Inflammatory interleukin 6 (IL-6) signaling has been suggested to be responsible for the gender disparities of HCC incidence (Naugler et al. 2007). IL-6/STAT3 has been identified as a major pathway in maintaining stem-cell-like features in HCC (Lin et al. 2009). Substantial evidence supports the important role of NF-κB signaling in inflammation-related HCC (Elsharkawy and Mann 2007). Vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGFs) have been revealed as the major drivers for angiogenesis in HCC (Imura et al. 2004). Mutation of tumor suppressor P53 has also been associated with HCC progression (Lowe et al. 2004). The oncogene *MYC* encodes a protein which is involved in nucleic acid metabolism. The activation of the *MYC* oncogene is considered to be an important mechanism of tumor evolution in HCC. Amplification of *MYC* can be detected in all the HCC stages and is considered an important driver for HCC disease progression (Kaposi-Novak et al. 2009). Nuclear receptors are ligand-modulated transcription factors that play diverse roles in cell differentiation, development, proliferation, and metabolism. Nuclear receptors such as farnesoid X receptor (FXR) are associated with liver cancer (Huang et al. 2015). FXR is considered to be a multifunctional tumor suppressor and tightly controls bile acids synthesis (Claudel et al. 2005). Significant reduction of FXR expression was found in human HCC. $FXR^{-/-}$ mice develop spontaneous HCC with disrupted bile acid metabolism as the major defect (Yang et al. 2007). Overload of bile acids due to the depletion of the *FXR* gene is the causative factor for induction of chronic liver inflammation, enhancement of hepatocyte proliferation, and development of liver tumors. In $FXR^{-/-}$ $SHP^{-/-}$ double knockout mice, the sharply elevated bile acid levels lead to the activation of YAP protein (Anakk et al. 2013), which is a core component of the Hippo pathway and considered as a crucial promoter of hepatocarcinogenesis (Lu et al. 2010). In addition, FXR shows anti-inflammation function. Activation of FXR inhibits NF-κB transcriptional activity through decreased DNA binding of NF-κB (Wang et al. 2008).

7.2.2 *Oncogenic Pathways in Cholangiocarcinoma*

Chronic inflammation and cholestasis contribute to cholangiocarcinoma through a complex process involving multiple genomic alterations and signaling pathway deregulations. KRAS and P53 mutations are commonly found in cholangiocarcinoma with relative low mutations in BRAF and EGFR (Andersen et al. 2012; Borad et al. 2014; Simbolo et al. 2014). Overlapping molecular profile between subclasses of cholangiocarcinoma and HCC has been found, indicating that these two cancer types may share a common ancestor such as hepatic progenitor cells (HPCs) (Hoshida et al. 2009; Roskams 2006; Woo et al. 2010). Alterations in the Hippo pathway components in the liver (such as YAP, SAV1, MST1/2) expand

progenitor-like cells and lead to the development of both HCC and cholangiocarcinoma in animal models (Lu et al. 2010). Gain-of-function IDH mutations are often reported in cholangiocarcinoma (Saha et al. 2014; Wang et al. 2013). The expression of these IDH mutations inhibited hepatocyte differentiation and expanded HPCs in mice. Furthermore, the concurrence of IDH and KRAS mutations in mice shows pronounced oncogenic cooperation and led to the development of premalignant biliary lesions and subsequent progression to cholangiocarcinoma (Saha et al. 2014). Recent studies have suggested the emerging roles for NOTCH and WNT signaling in cholangiocarcinoma pathogenesis. The NOTCH signaling pathway plays an important role during embryonic development and is essential for liver regeneration and repair (Zender et al. 2013). NOTCH pathway deregulation has been implicated in the induction of inflammation and the development and progression of cholangiocarcinoma. In human cholangiocarcinoma, the upregulation of NOTCH1 and NOTCH4 has been commonly observed (Wu et al. 2014). Liver-specific expression of NOTCH1 intracellular domain in mice resulted in the formation of cholangiocarcinoma (Zender et al. 2013). The WNT pathway is highly activated in the tumor epithelium of human cholangiocarcinoma (Boulter et al. 2015). Tumor surrounding macrophages have been demonstrated to be responsible for this highly activated WNT signaling status (Loilome et al. 2014). Mimicking human cholangiocarcinoma, the progressive activation of WNT pathway during the course of cholangiocarcinoma has been demonstrated in animal models (Boulter et al. 2015). Furthermore, WNT singling inhibition successfully controlled tumor growth in the tumor-bearing animals (Boulter et al. 2015).

7.3 Infectious Disease and Liver Cancer

7.3.1 Chronic Viral Hepatitis and HCC

Currently chronic HBV and HCV infections still represent the leading cause for HCC. The majority of viral hepatitis-related HCC arise from cirrhosis and are closely associate with liver inflammation and tissue damage. However, a significant proportion of HBV-related HCC arise in the absence of liver inflammation, indicating that the virus directly contributes to hepatocarcinogenesis (Kew 1998). The HBV genome can randomly integrate into the host genome. Although random integration rarely leads to direct oncogene activation or inactivation of tumor suppressor genes, HBV integration contributes to the genetic instability (Robinson 1994). In addition, several HBV viral proteins have been identified to promote hepatocyte transformation including the HBV envelope and HBx protein (Twu and Schloemer 1987; Paterlini et al. 1995; Sunami et al. 2016). HBV envelope proteins induce endoplasmic reticulum stress and cause liver cancer when expressed in mice (Xu et al. 1997). HBV-DNA sequences coding for a C-terminally truncated envelope protein are frequently found integrated in HCC. This truncated envelope protein induces the

activation of c-Raf-1/Erk2, Ap-1, and NF-κB pathways and increases hepatocyte proliferation (Hildt et al. 2002). The HBx protein, which is essential for initiating and maintaining HBV virus replication, has been linked to chromatin modulation. With these intrinsic carcinogenic characters, up to 20% of HBV-related HCC cases occur in the absence of cirrhosis (Chayanupatkul et al. 2017).

HCV is classified into seven genotypes. Genotypes 1b and 3 are associated with an increased risk of developing HCC (Kanwal et al. 2014; Raimondi et al. 2009). Even after virus elimination by antiviral treatment, the history of infection with HCV genotype 3 confers an increased HCC risk in patients with advanced fibrosis or cirrhosis (El-Serag et al. 2016). The reported DNA methylation of enhancers in HCV-associated HCC tumors highlights a role for HCV to influence host transcription (Okamoto et al. 2014). HCV stabilizes hypoxia-inducible factor-1α to induce de-differentiation through regulating the epithelial-to-mesenchymal transition (Wilson et al. 2012). HCV core transgenic mice show an imbalance in the oxidant/antioxidant state and develop HCC. HCV-encoded polymerase NS5B has been reported to bind the tumor suppressor protein Rb and induce its degradation through host ubiquitin (Munakata et al. 2005). Despite the many potentially oncogenic features of HCV infection, unlike HBV, the HCV-related HCC almost exclusively occurs in cirrhosis. An altered gut microbiome has been discovered in chronic HBV infection (Zhu et al. 2019; Wang et al. 2019). However, whether the gut microbiome affects disease progression of viral hepatitis and its impact on HCC development is currently unknown.

7.3.2 Liver Fluke and Cholangiocarcinoma

Liver fluke infection is a well-known risk factor for cholangiocarcinoma. Liver flukes are parasitic worms that live in the bile ducts and the liver of the infected host. Currently, liver fluke infection remains a major public health problem in East Asia and Eastern Europe. The prevalence of *Opisthorchis viverrini* (Southeast Asian liver fluke) infection has a strong positive correlation with the incidence of cholangiocarcinoma in Thailand and Laos (Sripa et al. 2011; Sriamporn et al. 2004), whereas such relationship cannot be found in HCC. Liver fluke-related cholangiocarcinoma is generally considered to be caused by chronic inflammation (Holzinger et al. 1999). Fluke feeding activity and migration contribute to biliary damage (Bhamarapravati et al. 1978). Fluke eggs entrapped in the periductal tissue induce granulomatous inflammation. The liver fluke can secrete or excrete metabolic products, some of which are highly immunogenic (Wongratanacheewin et al. 1988). Oxygen radicals such as nitric oxide (NO) released from activated immune cells contribute to biliary cell damage (Pinlaor et al. 2004). Furthermore, an in vitro co-culture study found that *Opisthorchis viverrini* induces murine fibroblasts to produce growth-promoting proteins such as transforming growth factor, which contributes to cell proliferation and tumor development (Thuwajit et al. 2006). It has been reported that liver fluke infection changes the gut and biliary microbiome

(Xu et al. 2018). However, its contribution to cholangiocarcinoma development is unknown.

7.4 Overview of Gut Microbiome and Cancer

The human gastrointestinal (GI) tract is colonized with a large and immensely complex community of commensal microbials termed the gut microbiome (Donaldson et al. 2016; Gilbert et al. 2018). While the gut microbiome mostly consists of bacteria, it also contains fungi, protozoa, archaea, and viruses. It is estimated that in total there is about 10^{14} bacteria present in an adult's intestine, but that number varies greatly among individuals (Sender et al. 2016). The number and type of microbes also vary dramatically from site to site within the GI tract (Donaldson et al. 2016; Human Microbiome Project 2012; Hillman et al. 2017). Largely due to the bactericidal activity of gastric acid, microbial density increases from the stomach to the distal aspect of the GI tract (Hunt et al. 2015). In healthy individuals, stomach and proximal small intestine contain only a very limited amount of microbes with a density around 10^1 to 10^3 cells per gram of lumen content, most of which are aerobes and facultative anaerobes. In sharp contrast, the microbial density in the colon can reach up to 10^{12} cells per gram content and predominantly present as strict anaerobes. Bacterial diversity also increase along the GI tract from proximal to distal (Hillman et al. 2017).

It is estimated that a typical person harbors 500–1000 bacterial species within the GI system (Lloyd-Price et al. 2016). Surprisingly, all the bacteria belong to only a few phyla, with the majority classified under *Firmicutes* and *Bacteroidetes*. Minor representation includes *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Fusobacteria*, and *Cyanobacteria* (Donaldson et al. 2016; Human Microbiome Project 2012; Huse et al. 2008; Rajilic-Stojanovic and de Vos 2014). Due to the large variations in both taxonomic composition and abundance of shared taxa among healthy individuals, using a universally "core" set of microbial taxa to define a "healthy" gut microbiome is believed to be unpractical (Lloyd-Price et al. 2016). In contrast, the abundance of metabolic pathways or the "functional core" of gut microbiome seems considerably consistent across people and remains stable over time after establishment in early life (Human Microbiome Project 2012; Lloyd-Price et al. 2016; Turnbaugh and Gordon 2009; Abubucker et al. 2012). The combined gut microbiome genome contains more than five million genes and has a large capacity to provide diverse metabolic activities, some of which are essential for host biology such as production of essential vitamins and fermentation of polysaccharides indigestible by the host (Rowland et al. 2018). A healthy gut microbiome covers a core set of functions and is likely to be ecologically diverse. Conversely, decreased diversity within the gut microbiome is associated with diseases such as obesity, inflammatory bowel disease, and diabetes.

The gut microbiome has a profound influence on maintaining structure, function, and tissue homeostasis of the GI tract, development of the intestinal immune system,

and defense against opportunistic pathogens (Gilbert et al. 2018; Thaiss et al. 2016; Gopalakrishnan et al. 2018a). The association between the gut microbiome and cancer development was discovered a while ago (Gopalakrishnan et al. 2018a). In recent years, various cancer-associated bacteria have been identified, and both pro- and antitumor functions of these bacteria have been alluded to. So far, the most clear and direct evidence for the contribution of intestinal bacteria to carcinogenesis was the discovery of *Helicobacter pylori* as the strongest known risk factor for gastric cancer (Boland et al. 2005; Wroblewski et al. 2010; Sepulveda 2013). *Helicobacter pylori* produces a protein called cytotoxin-associated gene A, a class I carcinogen, which can induce proteasome-mediated p53 degradation in gastric epithelial cells and promote gastric cancer formation (Hatakeyama 2014). GI cancers also have a strong link with chronic inflammatory diseases that demonstrate changes in the gut microbiome. Inflammatory bowel disease, in particular Crohn's disease, is associated with the development of colorectal cancer (CRC) (Axelrad et al. 2016; Cipe et al. 2015; Jahani-Sherafat et al. 2018). Inflammatory bowel diseases present with an imbalance of gut microbial community or dysbiosis. Independent of inflammatory bowel disease, many bacteria such as *Bacteroides fragilis*, *Clostridium septicum*, *Enterococcus faecalis*, *Fusobacterium* spp., and *Streptococcus bovis* have been suggested to contribute to CRC (Gagniere et al. 2016; Purcell et al. 2017; Mirza et al. 2009; Shang and Liu 2018; Abdulamir et al. 2011). Potential carcinogenic functions of many bacterial products have been discovered such as *Fusobacterium nucleatum* effector adhesin A and *Bacteroides fragilis* metalloproteinase toxin. Both of these toxins are capable of interacting with host's epithelial E-cadherin, disrupt the intercellular junctions, and activate β-catenin signaling which triggers cell proliferation and potentially malignant transformation (Rubinstein et al. 2013; Boleij et al. 2015).

Besides their pro-tumor functions, a number of microbial-derived products show antitumor activity (Zitvogel et al. 2016). The microbial-derived short-chain fatty acids (SCFAs) such as butyrate and propionate have been found to inhibit tumor histone deacetylases and suppress CRC and lymphoma (Scheppach et al. 1995; Hinnebusch et al. 2002; Gorres et al. 2014). Monophosphoryl lipid A (MPL) from *Salmonella enterica* has been used as adjuvant in the vaccine formulation for cervical carcinoma (Monie et al. 2008). Bacillus Calmette-Guerin (BCG) vaccine, a weakened form of *Mycobacterium bovis*, has been used as immunotherapy in patients with bladder cancer (Kawai et al. 2013).

The GI tract and the liver have a close anatomical and functional relationship which is termed the "gut-liver axis." It is important to note that the communication in the gut-liver axis is bidirectional (Fig. 7.1) (Tripathi et al. 2018; Wiest et al. 2017). The liver produces and secretes bile into the intestine which contains bile acids, immunoglobulin A, and antimicrobial molecules. The bile not only helps fat digestion and absorption but also maintains intestinal hemostasis and regulates microbial number and composition (Wiest et al. 2017; Urdaneta and Casadesus 2017). On the other hand, the intestine also influences liver function by providing nutrient-rich blood via the portal vein. The single thin layer of intestinal epithelium not only facilitates nutrient absorption but also makes it easy for small microbial components

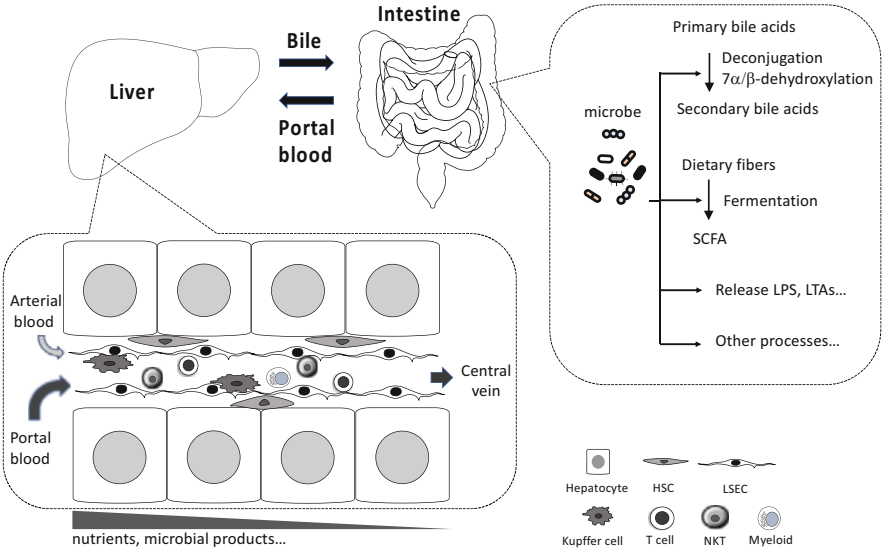

Fig. 7.1 The interaction between liver and the gut microbiome. The liver secretes bile into the intestine which not only helps fat digestion but also modulates the microbiome composition. In the intestine, the gut microbiome mediates many metabolic processes such as primary to secondary bile acid conversion and SCFA production. Bile acids, SCFA, and many microbial products such as LPS are absorbed and travel to the liver through portal blood circulation. Portal blood mixes with arterial blood and passes through liver sinusoids; during the process, nutrients are taken up, and microbial products are detoxicated by hepatocytes. The liver is heavily populated by immune cells, including Kupffer cells, NKT, T cells, and myeloid cells. Immune cells and other nonhepatic cells such as LSEC and HSC make up ~30% of the total cell population in normal liver

to cross and enter the blood stream. The blood supply to the liver carries both nutrients from digestion and also a large number of microbial components, metabolites, and even intact bacteria. Many of the intestinal metabolites function as signaling messengers regulating metabolic processes in the liver (Levy et al. 2016). In addition, the liver prevents harmful microbial products from entering the systemic circulation and thus serves as a critical "filter" to clear and detox microbial toxins such as lipopolysaccharide (LPS) (Tripathi et al. 2018; Wiest et al. 2017). Due to this close relationship, the liver is under great influence from the gut microbiome. Many important risk factors for liver cancer such as NAFLD, ALD, and cirrhosis commonly present with dysbiosis (Leung et al. 2016; Mokhtari et al. 2017; Da Silva et al. 2018; Sharpton et al. 2019; Rao 2009). It has been suggested that there is an association between alterations within gut microbiome and liver cancer (Zitvogel et al. 2016; Llorente and Schnabl 2015; Yu and Schwabe 2017). Importantly, preclinical animal models demonstrate that commensal intestinal bacteria play a critical role in the regulation of liver cancer development (Shalapour et al. 2017; Dapito et al. 2012; Yoshimoto et al. 2013; Singh et al. 2018; Ma et al. 2018). The relevant basic knowledge of the liver and its interaction with the gut microbiome,

current findings, and proposed mechanisms of intestinal bacteria in liver tumor development will be discussed below.

7.5 Relevant Liver and GI Features for the Gut-Liver Axis

7.5.1 Intrahepatic Circulation

The liver has a characteristic blood flow system (Fig. 7.1). About 75% of the blood supply to the liver is from the intestine venous system via the portal vein, which contains a significant amount of intestinal microbial products and metabolites (Abdel-Misih and Bloomston 2010). Arterial and portal vein blood mixes and passes through the thin-walled sinusoids which are lined by a single layer of liver sinusoidal endothelial cells (LSECs). Due to the small diameter, sinusoidal blood flow rate is low and often static which helps nutrient extraction and detoxification of harmful substances (Vollmar and Menger 2009).

7.5.2 Liver as an Immunological Organ

The liver is heavily populated by immune cells, and non-hepatocytes make up ~30% of the total cell population in normal livers (Fig. 7.1) (Racanelli and Rehermann 2006; Bogdanos et al. 2013; Heymann and Tacke 2016; Robinson et al. 2016). Macrophages are phagocytic innate immune cells and play an essential role in host defense. The liver harbors the largest population of tissue-resident macrophages, known as Kupffer cells, in the body. Kupffer cells comprise ~20% of the non-hepatocytes population and have multiple functions within the liver (Dixon et al. 2013; Toth and Thomas 1992; Bilzer et al. 2006). For example, they play an important role in tissue homeostasis, liver inflammation, and liver tumor progression. LSECs make up the lining of sinusoids and are in directly contact with mixed portal and arterial blood. LSECs act as efficient antigen presenting cells (APCs) and express MHC class I and II, CD1, MR1, and the co-stimulatory molecules CD40, CD80, and CD86 (Bogdanos et al. 2013; Crispe 2011; Knolle and Wohlleber 2016). The slow blood flow rate inside sinusoids lengthens the contact between immune cells and APCs, including LSECs, which promotes leukocyte extravasation. In mice, hepatic natural killer T (NKT) cells make up ~30% of the total lymphocyte population in the liver (Gao et al. 2009; Bandyopadhyay et al. 2016; Crosby and Kronenberg 2018). NKT cells are innate-like lymphocytes which recognize lipid antigens presented on CD1 molecules, which is expressed on various APCs including Kupffer cells and LSECs. Although the endogenous lipid ligand for NKTs are still elusive, lipid components of intestinal bacteria have been suggested to be able to activate NKT cells (Brennan et al. 2014; Wolf et al. 2015; Zajonc and Girardi 2015; An et al. 2014). Upon stimulation, NKT cells rapidly release cytokines to initiate

diverse immune responses and act as a bridge between innate and adaptive immunity (Terabe and Berzofsky 2008; Nishimura et al. 2000). The role of NKT cells in acute liver inflammation, alcoholic steatohepatitis, NASH, fibrosis, liver regeneration, and tumor growth has been reported (Gao et al. 2009; Bandyopadhyay et al. 2016). MAITs are MR1 molecule-restricted lymphocytes that share several characters with NKT cells (Le Bourhis et al. 2011; Toubal et al. 2019). Interestingly, MAITs recognize and are activated by metabolites derived from bacterial vitamin B2 (riboflavin) biosynthesis and are thus affected by intestinal bacteria. MAITs are specifically enriched in the intestinal system and can make up ~50% of the hepatic lymphocyte population in humans. The functional study of MAITs has just began, and the knowledge is very limited. In the lung, the potential role of MAITs in controlling *Mycobacterium tuberculosis* infection has been suggested (Gold et al. 2015).

Facing the continuous exposure of microbial products and the potential challenge of microbial infection from the GI tract, the liver local immune system is skewed toward a unique tolerance stage in order to avoid reacting with non-harmful antigens but is still able to recognize pathogens (Crispe 2003; Tiegs and Lohse 2010; Horst et al. 2016). Accumulating evidence shows that alternation of the gut microbiome has profound influences on hepatic immune cells. The role of immune cells in the gut microbiome-regulated liver tumor developed will be discussed below.

7.5.3 Pattern Recognition Receptors

One method by which the host sense the presence of microbes is through pattern recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs). PAMPs are various microbial-specific molecules including bacterial carbohydrates (such as LPS), bacterial or viral nucleic acids, bacterial peptides (such as flagellin), peptidoglycans, lipoteichoic acid, and fungal glucans (Zitvogel et al. 2016; Chu and Mazmanian 2013; Takeuchi and Akira 2010; Mogensen 2009). Based on localization, PRRs can be grouped into membrane PRRs including Toll-like receptors (TLRs) and C-type lectin receptors and cytoplasmic PRRs including NOD-like receptors and RIG-I-like receptors. PRRs recognize specific PAMPs and trigger anti-pathogenic responses through different signaling pathways. TLRs, the most well-studied PRRs, contain ten members. TLR1, 2, 4, 5, 6, and 10 are expressed on the cell membrane, while TLR3, 7, 8, and 9 are found in the endosomal compartment (Janssens and Beyaert 2003). TLR4 is a major component of the receptor complex recognizing LPS (Park and Lee 2013). TLR5 recognizes flagellin (Andersen-Nissen et al. 2007). TLR2 forms homodimers or heterodimers with TLR1, 6, and 10 to recognize protozoa, bacteria, fungi, and viruses (Oliveira-Nascimento et al. 2012). The intracellular TLRs (TLR3, 7, 8, and 9) sense nucleic acids. TLR3 recognizes double-stranded RNA and the synthetic analog polyriboinosinic polyribocytidylic acid (poly(I:C)) (Matsumoto and Seya 2008). TLR9 recognizes unmethylated CpG motifs of DNA (Ramirez-Ortiz et al. 2008).

After stimulation most TLRs induces MyD88-dependent downstream signaling often involving the NF-κb pathway to trigger various cytokine production (such as interferons) and co-stimulatory molecule expression (Kawasaki and Kawai 2014; Bagchi et al. 2007; Kawai and Akira 2007). TLRs are widely expressed in liver cells (Mencin et al. 2009; Seki and Brenner 2008). Hepatocytes and biliary epithelium express mRNA for all TLRs. LSECs constitutively express TLR4. Kupffer and hepatic stellate cells express functional TLR2 and TLR4 and produce proinflammatory cytokines upon stimulation with TLR2/4 ligands. Intrahepatic T cells and NK cells are rich in TLR1, 2, 4, 5, and 9. Although the expression and the functional role of TLRs in the liver has not been fully delineated, TLRs have been found to play a critical role in liver tissue homeostasis and various pathologic conditions including acute liver failure, ischemia-reperfusion injury, viral hepatitis, ALD, liver regeneration, fibrosis, and liver cancer (Chen and Sun 2011; Schwabe et al. 2006; Zhang and Lu 2015; Petrasek et al. 2010; Yang and Seki 2012).

7.5.4 Intestinal Barrier

The intestinal lumen microbes, especially the large amount of colon bacteria, pose a continuous threat to the host. In order to deal with this threat, a multilayer barrier has been developed to retain intestinal microbes inside the lumen (Bischoff et al. 2014; Groschwitz and Hogan 2009). The mucus layer is the first defense line separating the gut microbiome from the host, which primarily composes of a thick gel-like polysaccharide called mucin secreted from goblet cells. The colon has two layers of mucus of which the inner layer is impermeable to the luminal bacteria and has protective function. Below the mucous layer lies the intestinal epithelial layer, which is organized in crypt and villus structure to increase surface area. The intestinal epithelial cells form an extremely close lining through intercellular connections with tight and adherens junctions (Peterson and Artis 2014). Besides the physical barrier, an immune barrier exists in the lamina propria which contains gut-associated lymphoid tissue, IgA-producing plasma cells, resident macrophages, neutrophils, dendritic cells, effector cells, and regulatory T cells (Tregs) (Turner 2009). Macrophages in the intestinal lamina propria are highly phagocytic and responsible for clearing the "leaked" bacteria (Smith et al. 2011). Intestinal dendritic cells (DCs) sample lumen microbes by extending projections beyond the epithelial layer or via specialized microfold cells (Lelouard et al. 2012; Chieppa et al. 2006). Instead of immediate killing, DCs can hold living bacteria and transport them to mesenteric lymph nodes (MLN) and subsequently present microbial antigens to the immune system (Macpherson and Smith 2006). The primed local immune system can elicit quick responses against a microbial invasion if ever there is barrier dysfunction. The lamina propria also contains regulatory T cells whose development is under great influence from the intestinal bacteria (Zeng and Chi 2015; Smith et al. 2013). The presence of regulatory T cells is critical to limit unwanted inflammatory responses and avoid tissue damage.

7.5.5 *Bacterial Translocation*

The impairment of barrier function increases intestinal permeabilization which promotes paracellular transportation of microbial products such as LPS and even intact bacteria under severe conditions. The movement of bacteria across the intestinal barrier is termed bacterial translocation. Bacterial translocation is influenced by several factors including intestinal bacterial overgrowth, physical barrier impairment, and immune system functional status (Brenchley and Douek 2012). Rodent studies show that intestinal epithelia cells can uptake and transport latex particles similar in size to *E. coli*, suggesting that there is a continuous low-level trafficking of bacteria across the intestinal epithelial layer (Howe et al. 2014; Hodges et al. 1995; LeFevre et al. 1978). Most of the crossed microbes will be immediately destroyed by the intestinal phagocytes such as macrophages. Other bacteria will be taken up by DCs and transported to MLNs for training of the adaptive immune system. Clinical meaningful bacterial translocation often requires intestinal bacterial overgrowth and rarely occurs in its absence. Under bacteria overgrowth conditions and physical barrier damage, more bacteria enter the intestinal tissue and are subsequently carried to MLNs by immune cells. MLNs are often the first site to detect live translocated bacteria (Berg 1995). If there is a sufficient functional immune system, the translocated bacteria will be localized and controlled. In an immunocompromised state, uncontrolled bacteria will spread via the blood or lymphoid circulation (Berg 1995). Inside the intestinal blood stream, the translocated bacteria can move to the liver via the portal vein and even progress further to the systemic circulation. The uncontrolled bacteria inside MLNs can pass through the lymphoid vessels and enter the systemic circulation via the thoracic duct. Opposite to the low oxygen tension in the intestinal lumen, especially inside the colon, tissue and the blood stream contain relative high oxygen levels which decrease the survival of anaerobic bacteria.

Increased intestinal permeability and bacteria translocation are commonly observed in chronic liver diseases and contribute to hepatic inflammation. Consumption of high fat-containing diets are associated with increased intestinal permeabilization and elevated LPS levels in portal blood (Yoshimoto et al. 2013; Moreira et al. 2012). The critical role of elevated LPS in low-grade systemic inflammation, insulin resistance, and metabolic syndrome has been proposed. Importantly, the elevated LPS has been connected to liver carcinogenesis in animal studies (Dapito et al. 2012). The significance of bacterial translocation in liver tumors is still not clear. However, recent reports show that bacterial 16s rRNA can be detected from pancreatic cancer tumor tissue and metastatic liver tissue (Pushalkar et al. 2018; Sethi et al. 2018). Importantly, bacterial taxa composition of metastatic liver tissue can also be influenced by oral antibiotic treatment. This finding suggests that translocated bacteria can directly interact with the liver tumor environment. Its role in liver cancer development needs to be investigated further.

7.6 Gut Microbiome and Liver Cancer-Associated Conditions

Intestinal bacterial overgrowth and dysbiosis are commonly seen in risk factors for liver cancer such as obesity, NAFLD, ALD, and cirrhosis. The current knowledge of the gut microbiome in these conditions will be discussed below.

7.6.1 Obesity

Intestinal microbiomes play a critical role in regulation of energy extraction from food and in part affect obesity (Krajmalnik-Brown et al. 2012). The link between the gut microbiome and obesity was initially suggested from studies using germ-free mice, which are raised in sterile conditions and free of microorganisms. Compared to regular specific pathogen-free (SPF) mice, germ-free mice have less body fat content even though they consume more food (Backhed et al. 2004). Transferring fecal bacteria from SPF mice to germ-free mice causes a quick increase in body fat content without any change in food consumption (Ridaura et al. 2013). Importantly, germ-free condition provides a protective function against diet-induced obesity (Ley et al. 2005; Turnbaugh et al. 2006, 2008). The gut microbiome can promote energy intake through several mechanisms such as breakdown of plant polysaccharides and complex carbohydrates which normally cannot be digested by the host (Flint et al. 2012). As expected, lower caloric release from dietary plant polysaccharides was observed in germ-free mice (Turnbaugh et al. 2008). Interestingly, host metabolic processes such as energy deposition in adipocytes, hepatic fatty acid oxidation, de novo fatty acid biosynthesis, and glycogen utilization are affected by germ-free conditions and favor catabolism (Backhed et al. 2007). These studies demonstrate that the gut microbiome not only affects energy uptake from food but also influences energy expenditure and storage.

The composition of the gut microbiome has been suggested to be important in obesity development. Increase of *Firmicutes* especially some *Clostridium* clusters is involved in harvesting energy from diet (Clarke et al. 2012). Genetically obese (*ob/ob*) mice have been reported to contain higher proportion of intestinal *Firmicutes* and parallel enrichment of microbial genes for polysaccharide degradation compared to their lean siblings (Ley et al. 2005). Fecal transplantation studies demonstrate that germ-free mice who receive microbiota from obese humans develop higher adiposity compared to controls (Ridaura et al. 2013). Antibiotics, especially those with broad spectrum, affect intestinal microbiome composition. Early-life usage of penicillin causes a long-lasting effect on mouse body composition including increased fat mass and hepatic expression of adipogenesis genes (Cho et al. 2012). This supports the hypothesis that antibiotic use maybe contributing to the obesity epidemic. In humans, low fecal bacterial diversity has been found to associate with high adiposity and dyslipidemia (Le Chatelier et al. 2013). High *Firmicutes* and low *Bacteroidetes*

have been reported in obese people (Koliada et al. 2017). Interestingly, in obese volunteers who lost body weight by consuming a low fat and carbohydrate diet for a year showed a reversal of the *Firmicutes* and *Bacteroidetes* populations in the colon (Wu et al. 2011). Of note the reduction of *Firmicutes/Bacteroidetes* ratio is not always present in obese people likely due to large interpersonal variations and the large influence diets have on the intestinal bacterial community (Singh et al. 2017). Probiotics are live microorganisms and can confer a health benefit to the host. Animal research suggests that administration of various lactobacillus may reduce weight gain in response to a high-fat diet (Kobyliak et al. 2016). However, in humans the data is less consistent.

7.6.2 Nonalcoholic Fatty Liver Disease

NAFLD is an import high-risk factor for HCC. Excessive ROS production, inflammatory cytokines, endoplasmic reticulum (ER) stress, circadian dysregulation, and immune cells have been suggested to contribute to the NAFLD-promoted hepatocarcinogenesis. In a lipid-rich environment, excessive ROS causes lipid peroxidation and generation of highly reactive aldehydic derivatives including 4-HNE and malondialdehyde (MDA), which subsequently causes DNA damage and promotes hepatocyte malignant transformation. Increased ROS production in NAFLD liver and its contribution to disease progression from NASH to HCC have been described in animal models (Kathirvel et al. 2010; Sutti et al. 2014; Gandhi et al. 2015). NAFLD presents an increase of inflammatory cytokines including TNF-α and IL-6 (Dowman et al. 2010). Both TNF-α and IL-6 have been demonstrated to play a critical role in obesity-/NAFLD-enhanced HCC through enhancing cell proliferation and preventing apoptosis of hepatocytes in mice (Park et al. 2010). Hepatic ER stress is common in NAFLD and can be observed in NAFLD animal models and NASH patients (Puri et al. 2008). Its critical role in promoting NAFLD to HCC has been demonstrated in HFD-fed MUP-uPA mice through increasing macrophage TNF production and the subsequent activation of TNFR1-IKKβ-NF-κB pathway in the HCC progenitor cells (Nakagawa et al. 2014). Importantly, the HFD-fed MUP-uPA mice develop spontaneous HCC even without carcinogen treatment, which mimics the clinical disease progression from NASH to HCC. Circadian dysregulation has been demonstrated to cause dysfunction of hepatic metabolic pathways such as in mice with jet lag (Adamovich et al. 2014). Its contribution to NASH and HCC has been suggested (Kettner et al. 2016). The liver of jet-lag mice shows a genome-wide deregulation of gene expression, and a global metabolic disruption with cholesterol, bile acid, and xenobiotic metabolism are the most affected pathways. Disrupting the hepatic metabolic pathways has been shown to promote HCC in the context of NAFLD; however, the underlying mechanisms are complex. Ablation of farnesoid X receptor (FXR), one of the key regulators of bile acid metabolic pathway, increased liver bile acids and enhanced the tumor-promoting effect of jet lag in NAFLD-HCC, while the opposite effect was

found after deletion of constitutive androstane receptor (CAR), a critical modulator of xenobiotic and endobiotic metabolism (Kettner et al. 2016). The liver is rich in various immune cells. The contribution of different immune cells in NAFLD-HCC progression has been reported. An increase of hepatic NKT cells has been reported to promote steatosis through secreting LIGHT (TFNSF14), a ligand for lymphotoxin β receptor (LTβR), which acts LTβR on hepatocyte and causes enhanced lipid uptake (Wolf et al. 2014). In addition, LIGHT also activates NF-κB signaling in hepatocytes and promotes malignant transformation. The increase of IL-17-producing Th17 cells has been found in both NAFLD animal models and NASH patients (Paquissi 2016). Increased IL-17 acts on IL17RA-expressing myeloid cells and leads to release of FFAs from white adipose tissues, which promotes NASH progression and HCC formation (Gomes et al. 2016). Blocking Th17 cells decreases NASH and delays HCC (Gomes et al. 2016).

Emerging evidence suggests that the gut microbiome is an important environmental factor that contributes to NAFLD development (Leung et al. 2016; Mokhtari et al. 2017; Da Silva et al. 2018; Sharpton et al. 2019). Germ-free mice fed with high-fat diet are resistant to hepatic steatosis and dyslipidemia (Rabot et al. 2010; Cani et al. 2008). NAFLD is transmissible to germ-free mice by fecal microbial transplantation, and two bacterial strains *Barnesiella intestinihominis* and *Lachnospiraceae* have been positively associated with the development of metabolic features (Le Roy et al. 2013). NASH patients display frequent intestinal bacterial overgrowth (Augustyn et al. 2019). In humans, the association between dysbiotic environment and NAFLD has been discovered (Schnabl and Brenner 2014), and many NAFLD disease-associated bacteria have been reported. Children with NAFLD have been found to display higher presentations of *Gammaproteobacteria* and *Epsilonproteobacteria* than heathy lean and obese children (Michail et al. 2015). Increased *Proteobacteria* has also been observed in NASH patients compared to obese individuals (Zhu et al. 2013). However, so far, no single bacterial species has been identified to mechanistically associate with the development of fatty liver. In addition, some of the correlation studies yield controversial results. Lower percentage of *Bacteroidetes* was found in NASH patients compared to healthy controls (Mouzaki et al. 2013). In contrast, higher *Bacteroidetes* has also been linked with NASH patients (Boursier et al. 2016). In a different report, no change in *Bacteroidetes* was found when comparing NASH with healthy controls (Wong et al. 2013). The discrepancy is likely due to cofounding factors such as diet which plays a more important role in shaping the microbiome than genetic factors.

Several mechanisms have been suggested for the effects the gut microbiome has on NAFLD progression including regulation of intestinal protein expression, intestinal barrier breakdown, inflammatory responses, and changes in metabolites. Dysbiosis is linked with reduced synthesis and secretion of fasting-induced adipose factor (FIAF) in enterocytes, which leads to increased uptake of fatty acids in the liver and adipose tissue and ultimately favors hepatic steatosis and expansion of adipose tissue (Backhed et al. 2004; Mandard et al. 2006). NAFLD has been suggested to be associated with increased intestinal permeability. A meta-analysis comprehensively assessing the association between intestinal permeability and risk

of developing NAFLD has been performed (Luther et al. 2015). Indeed, NAFLD patients had enhanced intestinal permeability (Luther et al. 2015; Miele et al. 2009). The underlying mechanism is still not clear, but bacteria involvement has been suggested. Bacterial toxic metabolites such as acetaldehyde and ethanol are associated with gut permeability. With the increased intestinal permeability and the presence of dysbiosis, the liver is exposed to more bacterial products via portal blood, which will be recognized by liver PRRs such as TLRs and leads to the production of inflammatory cytokines. A positive correlation between plasma inflammatory cytokines and blood LPS has been found (Ceccarelli et al. 2015). In addition, plasma inflammatory cytokines have been reported to be negatively correlated with intestinal *Bifidobacteria* count (Okada et al. 2009; Cani et al. 2007). High-fructose diet is considered to be a significant risk factor for NAFLD (Lim et al. 2010). Chronic intake of fructose is associated with bacterial overgrowth and an increase in blood LPS (Vos and McClain 2009). Fat consumption promotes production of chylomicrons, which facilitate the translocation of LPS toward other organs. All these findings suggest a link between dysbiosis, microbial products, and the inflammatory component of NAFLD.

Choline deficiency is related to NAFLD pathogenesis (Corbin and Zeisel 2012). As a methyl group donor, choline contributes to the synthesis of phosphatidylcholine which is required for the synthesis and secretion of very-low-density lipoprotein (VLDL) which transports lipids from the liver to the peripheral organs (Yao and Vance 1988). Although it can be synthesized in the body, choline is considered an essential nutrient, and dietary intake is required. Reduction of choline bioavailability is associated with increased ROS, hepatic lipid accumulation, and reduced hepatic VLDL (Zhu et al. 2014). In humans, rigorously controlled choline deficiency diet leads to hepatic lipid accumulation (Spencer et al. 2011). Recently, it was discovered that intestinal bacteria can convert dietary choline to a variety of metabolites such as trimethylamine, thus reducing choline bioavailability (Romano et al. 2015). Several choline-metabolizing bacteria have been identified, and a low level of colonization of trimethylamine-producing bacteria species can induce significant reduction of host choline levels (Romano et al. 2015). In mice, high-fat diet increases choline-metabolizing microbes and leads to the development of hepatic steatosis (Boutagy et al. 2015). Interestingly, in the liver, trimethylamine can be converted to trimethylamine-N-oxide which is associated with atherosclerosis and cardiovascular disease (Janeiro et al. 2018). The association between trimethylamine-N-oxide and hepatic lipid metabolism in addition with the microbe-host interactions has been suggested, and its role in NALD needs further investigation (Chen et al. 2016a).

7.6.3 Alcoholic Liver Disease

Alcoholic liver disease is the most prevalent chronic liver disease worldwide and accounts for ~30% of HCC cases and HCC-specific death (Ganne-Carrie and Nahon 2019). Alcohol consumption is an independent risk factor for HCC and has been

associated with a high risk of serval other malignancies starting at a dose as low as 10 g/1 unit/day (Testino et al. 2014; European Association for the Study of the Liver 2018). Alcohol is classified as a group 1 carcinogen. Although not fully understood, several mechanisms have been found to contribute to the alcohol-induced hepatocarcinogenesis. Alcohol is mainly metabolized in hepatocyte cytoplasm to acetaldehyde, which is subsequently oxidized to acetate in the mitochondria (Lieber 2005). After consumption of a large amount of ethanol, cytochrome P450 2E1 (CYP2E1) also contributes to the metabolization of alcohol to acetaldehyde (Lieber and DeCarli 1968). Acetaldehyde has been shown to be a carcinogen in animal studies (Seitz and Homann 2007). Acetaldehyde interacts with DNA and proteins to form adducts which plays an important role in carcinogenesis. The formation of adducts with O6-methylguanine methyltransferase causes DNA repair system dysfunction (Collier et al. 1996). The CYP2E1-dpendent alcohol metabolization process generates various ROS, such as hydroxyethyl, superoxide anion, and hydroxyl radicals (Haorah et al. 2008). The increased ROS leads to the generation of lipid peroxidation products, such as malondialdehyde and 4-hydroxy-2-nonenal, and causes DNA damage. 4-hydroxy-2-nonenal has been found to cause a mutation at codon 249 of the p53 gene which is commonly found in HCC (Hu et al. 2002). In addition to the mutagenic effects on DNA, ROS can act as an important mediator of tumor angiogenesis and metastasis. It has been shown that alcohol-induced ROS can activate NF-kB signaling and upregulate VEGF and MCP-1 (Liu et al. 2016). Aberrant DNA methylation and protein methylation are involved in HCC development. It has been reported that alcohol inhibits the synthesis of S-adenosyl-L-methionine (SAMe), which is a universal methyl group donor. The generation of SAMe is mediated by the enzyme methionine adenosyltransferase (MAT). *MAT1A* knockout mice develop SAMe deficiency, fatty liver, and HCC (Santamaria et al. 2006). Furthermore, decreased hepatic MAT activity and *MAT1A* gene expression have been found in ALD patients (Tsukamoto and Lu 2001). ALD presents hepatic activation of innate immunity and increased proinflammatory cytokines (Kasztelan-Szczerbinska et al. 2015; Kawaratani et al. 2017). The involvement of the altered immune system in ALD-induced HCC needs further investigation.

ALD is mainly due to the accumulation of acetaldehyde, a toxic metabolite of ethanol, in hepatocytes which causes liver inflammation and fibrosis (Setshedi et al. 2010). Recently, the gut microbiome has been found to contribute to ALD development, and a growing body of evidences suggest that LPS is closely associated with ALD (Rao 2009). Chronic ethanol consumption leads to bacterial overgrowth and dramatic changes within intestinal bacterial composition (Malaguarnera et al. 2014). Specifically, there is an increase in gram-negative bacteria such as *Proteobacteria* which are the main source of LPS (Purohit et al. 2008). ALD patients display increased intestinal permeability (Zhou and Zhong 2017). Although ethanol can directly disrupt the intestinal barrier, its concentration is relatively low inside the intestine. Intestinal bacteria can metabolize ethanol to produce acetaldehyde, and a significant amount of evidences indicates that microbial-derived acetaldehyde plays a crucial role in the disruption of the intestinal barrier function in ALD (Ferrier et al. 2006). Acetaldehyde-induced disruption of tight and adherens junctions was

validated in human colonic mucosa (Basuroy et al. 2005). The increased intestinal permeability and more abundant gram-negative bacteria contribute to increased absorption of LPS. Numerous studies demonstrate that ALD patients have elevated plasma LPS compared to healthy individuals (Fujimoto et al. 2000; Bala et al. 2014; Fukui et al. 1991). LPS itself fails to mimic ethanol-induced steatosis or hepatitis. However, LPS shows a synergistic effect with ethanol to exacerbate liver damage (Pennington et al. 1997). The mechanism involves multiple factors inducing downregulation of IL-10-mediated protection, ROS production, and adrenergic stimulation (Hill et al. 2000). TLR4 knockout studies suggest that TLR4 plays a critical role in LPS-promoted live damage (Soares et al. 2010). LPS-/TLR4-mediated stimulation of different liver cells including Kupffer cells, LSECs, stellate cells, neutrophils, and hepatocytes induces secretion of proinflammatory cytokines, chemokines, and ROS which subsequently leads to liver damage and inflammation (Duryee et al. 2004; Quiroz et al. 2001; Adachi et al. 1994). In a more recent report, cytolysin, an exotoxin secreted by *Enterococcus faecalis*, has been shown to contribute to hepatocyte death and liver injury in ALD (Duan et al. 2019). ALD patients have increased level of *Enterococcus faecalis.* Importantly, the presence of cytolysin-positive *Enterococcus faecalis* correlates with the severity of liver disease. Using humanized mice colonized with fecal bacteria from ALD patients, it has been demonstrated that targeting cytolysin-positive *Enterococcus faecalis* correlates with bacteriophages and attenuates ethanol-induced liver disease. This study offers a novel therapeutic approach for ALD through precisely editing the gut microbiome.

7.6.4 Cirrhosis

Cirrhosis represents the final stage of liver fibrosis and is characterized by distortion of liver parenchyma associated with fibrous septa and nodule formation as well as alterations in blood flow. Bacterial translocation is often observed in cirrhosis, and cirrhotic patients have increased susceptibility to bacterial infections, most commonly spontaneous bacterial peritonitis (Alexopoulou et al. 2017; Bonnel et al. 2011; Jalan et al. 2014). About 10% of cirrhotic patients without selective intestinal decontamination show MLN bacterial translocation (Cirera et al. 2001). In addition, a positive correlation between cirrhosis disease severity and bacterial translocation has been reported (Alexopoulou et al. 2017; Cirera et al. 2001). Consistent with the correlation results, experimental cirrhotic animals show an increase in intestinal permeability, and ~40% of cirrhotic rats with ascites have MLN bacteria translocation (Giannelli et al. 2014). Bacteria strains isolated from MLN have been shown to be genetically identical to strains causing spontaneous bacterial peritonitis in the same animal supporting the process of bacterial translocation to infection (Bert et al. 2010).

Several pathologic changes in cirrhosis promote the bacterial translocation including bacterial overgrowth, intestinal barrier dysfunction, and impaired immune function (Pijls et al. 2013). Bacterial overgrowth and dysbiosis are often present in

cirrhotic patients (Fukui 2017). Bacterial overgrowth can even be found in the proximal small intestine likely due to a shift toward to alkaline gastric secretions (Chen et al. 2016b). In cirrhosis, a marked decrease in intestinal luminal concentration of bile acids and increased deconjugation by bacteria have been observed (Ridlon et al. 2013). In addition to its role in digestion, bile acids limit microflora proliferation and contribute to maintaining the integrity of the small intestine. Obstruction of bile flow in humans or rodents causes bacterial overgrowth and mucosal injury followed by bacterial translocation (Inagaki et al. 2006). Oral administration of conjugated bile acids in cirrhotic rats results in a reduction of intestinal bacterial overgrowth, bacteria translocation, and endotoxemia (Lorenzo-Zuniga et al. 2003). The impaired intestinal barrier function in cirrhosis is often associated with portal hypertension-related structural and functional alterations including vascular congestion, edema, widened intracellular spaces in the intestinal wall, and functional abnormalities such as reduced small bowel motility (Kalaitzakis 2014). Advanced liver diseases often display impaired chemotaxis, phagocytosis, and intracellular killing by polymorphonuclear leukocytes and monocytes (Andrews and Sullivan 2003). Cirrhosis is accompanied by impaired reticuloendothelial system (RES) which is the main defensive system against bacteremia. Most of the RES activity is located in the liver where Kupffer cells are the major component. Portosystemic shunting that bypasses the liver (escaping the action of RES) and impaired Kupffer cell phagocytic activity leads to not only the failure to clear bacteria but also failure to clear bacterial products such as endotoxins and cytokines (Moller et al. 2014; Pinzone et al. 2012).

7.6.5 Autoimmune Hepatitis

Autoimmune hepatitis (AIH) is an immunologic mediated, chronic, and progressive inflammatory liver disease of uncertain cause. Without appropriate treatment, AIH can lead to cirrhosis and HCC (Teufel et al. 2009). Genetics have been implicated as susceptibility factors for AIH. Recent evidence suggests that there is an association between the gut microbiome and AIH (Lin et al. 2015). AIH patients are reported to have increased intestinal permeability, dysbiosis, and bacterial translocation, which correlates with disease severity (Lin et al. 2015; Cai et al. 2017; Czaja 2016).

The intravenous injection of the plant lectin concanavalin A (Con A) is a well-established hepatitis model for investigating T cells and macrophage-dependent liver injury in mice (Wang et al. 2012). The model mimics pathological features of AIH patients and is considered the best experimental model for AIH research so far. Recently, several studies have shown that the gut microbiome has a profound impact on Con A liver injury (Celaj et al. 2014; Chen et al. 2014). Severity of Con A liver injury varies greatly among genetically identical mice raised in different environments harboring distinct microbiota. BALB/c mice from TAC, NCI, and JAX show clear vendor-specific Con A liver damage. Germ-free and co-housing studies show that manipulating the intestinal flora alters susceptibility to Con A liver injury (Celaj

et al. 2014). In addition, administration of pathogenic bacteria such as *Salmonella* and *Streptococcus* exacerbates Con A liver injury (Chen et al. 2014). In contrast, depletion of gut gram-negative bacteria alleviated Con A liver injury. Several mechanisms have been proposed, such as the ability of the gut microbiome to regulate the sensitivity to Fas-induced liver injury from TLR/MyD88 signaling (Celaj et al. 2014). Other proposed mechanism suggests that the presence of pathogenic bacteria stimulates dendritic cells, enhances NKT cell cytotoxicity, and exacerbates liver damage (Chen et al. 2014).

7.7 Gut Microbiome Regulates Liver Cancer

Emerging evidences suggest that there is an association between altered intestinal bacteria and the presence of HCC following advances in bacterial sequencing and profiling techniques (Yu and Schwabe 2017; Mima and Baba 2019; Gupta et al. 2019). Enriched *Actinobacteria* was observed in early HCC compared to cirrhosis (Ren et al. 2019). Increased *Bacteroides* and decreased *Bifidobacterium* were also found in HCC patients (Ponziani et al. 2019). Compared to non-HCC cirrhotic patients, HCC patients presented with high levels of fecal *E. coli* (Grat et al. 2016). Building on this evidence and the close association between dysbiosis and HCC risk factors, the gut microbiome has been suggested to play a critical role in liver cancer development.

Recently, direct evidence showing that intestinal bacteria contributes to liver cancer has emerged using preclinical animal models mainly focusing on HCC (Shalapour et al. 2017; Dapito et al. 2012; Yoshimoto et al. 2013; Singh et al. 2018; Ma et al. 2018; Sethi et al. 2018; Loo et al. 2017). A profound influence of the gut microbiome on liver cancer has been demonstrated in gut bacteria-depleted mice using antibiotic cocktails or germ-free mice. Although through different mechanisms, a consistent liver tumor-promoting effect of the gut microbiome has been observed from several research groups using various liver tumor models (Fig. 7.2 and Table 7.1). The current findings are discussed below.

7.7.1 Lipopolysaccharides

Lipopolysaccharides, also known as endotoxins or lipoglycans, are the main components of the outer membrane of gram-negative bacteria (Alexander and Rietschel 2001). LPS are a group of large molecules consisting of three parts: O polysaccharide, core oligosaccharide, and lipid A. Intestinal epithelial cells internalize LPS and transport them to the Golgi complex where LPS bind and form complex with newly generated chylomicrons (Ghoshal et al. 2009). LPS have a high affinity for chylomicrons, and chylomicron formation promotes LPS transportation. Under healthy condition, most intestinal-absorbed LPS are present on chylomicron remnants within

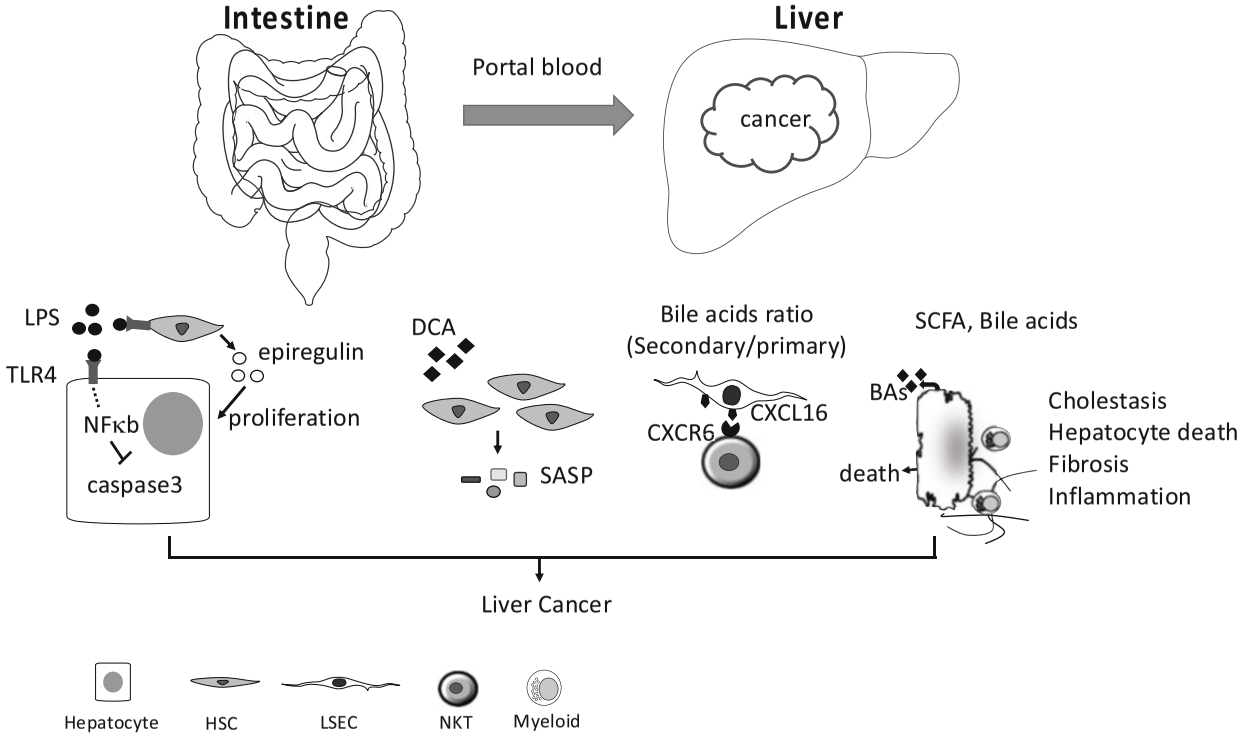

Fig. 7.2 Mechanisms by which the gut microbiome regulates liver cancer. (1) Lipopolysaccharides (LPS) produced by gram-negative bacteria activate TLR4 signaling in hepatocytes and hepatic stellate cells (HSC), which promotes cell proliferation, survival, and tumor formation. (2) Gut bacterial-metabolized secondary bile acid deoxycholic acid (DCA) induces senescence in HSC, and the senescence-associated secretory phenotype (SASP) contributes to HCC. (3) Secondary to primary bile acids ratio, which is controlled by gut bacteria, regulates chemokine CXCL16 expression on liver sinusoidal endothelial cells (LSEC) and thus modulates the level of hepatic NKT cells which express CXCR6. NKT cells control liver tumor development. (4) Short-chain fatty acids (SCFA), which are produced by bacteria through fermentation of dietary fibers, together with bile acids induce cholestasis, hepatocyte death, fibrosis, and liver inflammation which leads to HCC formation

Table 7.1 Mechanisms of how bacterial products promote liver cancer

Bacterial product	Source	Mechanisms to promote carcinogenesis
Lipopolysaccharides (LPS)	Outer membrane component of gram (−) bacteria	Induces mitogen epiregulin production and secretion from hepatic stellate cells to promote cell proliferation (Dapito et al. 2012) Decreases caspase 3 activation and inhibits hepatocyte cell death (Dapito et al. 2012)
Lipoteichoic acid (LTA)	Cell wall component of gram (+) bacteria	Increase PGE2 production through TLR2-COX2 pathway, to suppress immune responses (Loo et al. 2017)
Deoxycholic acid (DCA)	Bacteria-mediated primary to secondary bile acid metabolism, mainly in colon	Induces senescence-associated secretory phenotype (SASP) in hepatic stellate cells to promote HCC (Kang et al. 2011) Decreases CXCL16 expression of hepatic sinusoidal epithelial cells to reduce liver NKT cells (Ma et al. 2018)
Short-chain fatty acids (SCFA)	Bacteria-mediated fermentation of dietary fibers, mainly in colon	Induces live inflammation, hepatocyte proliferation, and fibrosis. The cellular and molecular mechanisms are unclear (Singh et al. 2018)

the blood. Intestinal barrier damage enhances unbound LPS absorption through paracellular movement. LPS is recognized by pattern recognition receptor TLR4 in association with protein partners such as MD2 and CD14, which signal through two major pathways: MyD88/ NF-κB and TRIF/IRF3 (Park and Lee 2013). LPS can induce a massive production of proinflammatory cytokines and even septic shock in extreme conditions (Yamamoto et al. 2011). TLR4 is expressed by hepatocytes and various cell types in the liver (Mencin et al. 2009; Chen and Sun 2011). Indeed, the liver is a critical organ responsible for clearing LPS from blood circulation. Due to its continuous exposure, the liver immune system is tolerant to low levels of LPS. The exact mechanism of LPS tolerance is still not fully understood but the immunomodulatory cytokine IL-10 and other molecules such as SHIP-1, A20, and IRAK-M are believed to be critical (Bagchi et al. 2007; Xiong and Medvedev 2011).

Elevated blood LPS is present in many conditions considered to be high risk for liver cancer. Increased serum LPS has been found in NAFLD patients (Miele et al. 2009; Harte et al. 2010). Excessive alcohol consumption is correlated with gram-negative bacteria overgrowth and high LPS in the circulation (Fujimoto et al. 2000; Bala et al. 2014; Fukui et al. 1991). Consumption of high fat-containing diets is associated with increased intestinal permeabilization with elevated LPS level in the portal blood (Pendyala et al. 2012). Importantly, the TLR4/MyD88 pathway, a major pathway downstream of LPS signaling, has been linked to carcinogenesis in colorectal cancer (Wang et al. 2010, 2018). In addition, LPS promotes liver metastasis of

colorectal cancer by directly acting on TLR4 expressed in tumor cells (Hsu et al. 2011). All these findings suggest LPS as a potential link between the gut microbiome and liver cancer.

The research group led by Robert Schwabe was the first to demonstrate the crucial role of LPS in liver carcinogenesis using animal models (Fig. 7.2) (Dapito et al. 2012). In their studies, spontaneous mouse HCC was induced by early-life exposure to the chemical carcinogen diethylnitrosamine (DEN) followed by chronic treatment with the hepatotoxin, carbon tetrachloride (CCL4) (Dapito et al. 2012). The model demonstrates a pattern of chronic liver inflammation, fibrogenesis, and increased blood LPS level which mimics features of the microenvironment from which the majority of human HCC tumors arise. To test the role of the gut microbiome in DEN-CCL4-induced HCC, a TLR4 mouse strain carrying a nonfunctional mutant TLR4 was chosen. Interestingly, a TLR4 functional deficiency causes a robust reduction in both HCC tumor number and size. The finding was recaptured in gut-sterilized mice treated with an oral antibiotic cocktail and in germ-free mice, which proves that the commensal gut microbiome is responsible. Furthermore, prolonged low nontoxic LPS exposure increases HCC size and number which directly confirms that LPS promotes HCC.

The liver is rich in immune cells and is considered a lymphoid organ (Racanelli and Rehermann 2006). TLR4 is expressed on different cell types within the liver (Mencin et al. 2009; Chen and Sun 2011; Soares et al. 2010). Besides hepatocytes, liver non-parenchymal cells such as hepatic stellate cells and various immune cells, particularly Kupffer cells, express TLR4 and can respond to LPS. The contribution of liver immune cells or parenchyma cells to gut microbiome-enhanced HCC was investigated using bone marrow chimeric study. Notably, the chimeric study was combined with Kupffer cell depletion to exclude the potential contribution of liver resident macrophages. Using this chimeric protocol, the study clearly demonstrated that the non-Kupffer resident liver cells mediate the gut microbiome-enhanced HCC.

Another interesting finding of the Dapito et al. paper was that the gut microbiome does not affect the initiation of carcinogenesis in the DEN-CCL4 HCC model (Dapito et al. 2012). Microarray analysis showed no difference in expression of cancer stem cell markers, suggesting the regulation of hepatocarcinogenesis is not likely from progenitor cells. Unexpectedly, the time frame seems to be critical for the gut microbiome's influence on hepatocarcinogenesis. Early sterilization followed by antibiotic treatment withdrawal does not affect hepatocarcinogenesis. In contrast, gut sterilization at the time when tumors start to appear greatly reduces tumor number and size. Cell proliferation was investigated, and it was demonstrated that TLR4 functional deficiency strongly reduced cell proliferation. Consistent with the chimeric results, NF-κB activation, a surrogate for LPS response, was mainly observed in the hepatic stellate cells and a large percentage of hepatocytes in DEN-CCL4 mice. Furthermore, epiregulin, a mitogen which can stimulate cell proliferation, was found to be significantly affected by TLR4 status and gut sterilization. In vivo LPS challenge significantly upregulates epiregulin in the hepatic stellate cells. As expected, epiregulin-deficiency inhibits DEN-CCl4-induced hepatocarcinogenesis. Besides the altered cell proliferation, a significant increase in cleaved-caspase3

positive cells within nontumor hepatocytes was observed in both gut-sterilized and TLR4 mutant mice. Importantly, hepatocyte apoptosis is inversely correlated with tumor number and size. Together, the data supports the mechanism that LPS act on hepatic stellate cells to produce epiregulin which enhances proliferation of malignant hepatocytes and LPS directly act on hepatocytes and promote their survival during malignant transformation.

The work described above is the first to provide direct evidence that the gut microbiome controls liver cancer. Using LPS, a common structural component of gram-negative bacteria, the gut microbiome modulates proliferation and survival of hepatocytes through targeting liver-resident cells, thus promoting HCC. It is well accepted that chronic inflammation contributes to cancer, but the underlying mechanism is still not clear (Colotta et al. 2009; Boland et al. 2005). Robert Schwabe's work demonstrates that gut bacteria-derived LPS is an important factor in malignancy and helps shed light on the contributions of chronic liver inflammation's role in hepatocarcinogenesis.

7.7.2 Bile Acids

The liver metabolizes cholesterol to produce primary bile acids which are conjugated with glycine or taurine in hepatocytes (Chiang 2013). Bile acids are secreted into the small intestinal lumen to help digestion and absorption of fat and lipid-soluble vitamins. In the presence of bacteria, the primary bile acids are converted into secondary bile acids with the main transformation steps being deconjugation and dehydroxylation (Ridlon et al. 2006, 2016; Dawson and Karpen 2015).The deconjugation step is the hydrolysis of the glycine or taurine from the steroid nucleus of primary bile acids which is catalyzed by bacterial bile salt hydrolases (BSH). BSH are mainly expressed in gram-positive bacteria but have also been found in some gram-negative bacteria such as *Bacteroides* spp. (Urdaneta and Casadesus 2017). Following deconjugation, free primary bile acids are converted into secondary bile acids through 7α/β-dehydroxylation. Unlike BSH, only a small number of bacterial species belonging to *Clostridia* have the 7α/β-dehydroxylation activity, and the 7α/β-dehydroxylation reaction mainly occurs in the colon (Ridlon et al. 2006, 2016). The body will then reuse the bacteria-modified bile acids with close to 95% of colonic bile acids being reabsorbed and shuttled back to the liver through enterohepatic circulation (Hofmann 2009).

Bile acids can activate signaling cascades and transcriptional networks and significantly influence liver function by binding to bile acid receptors such as farnesoid X receptor (FXR), pregnane X receptor (PXR), vitamin D3 receptor (VDR), constitutive androstane receptor (CAR), and membrane-bound G protein-coupled bile acid receptor1 (GPBAR1, also known as TGR5) (Copple and Li 2016; Schaap et al. 2014). Many target genes in the transcriptional networks are involved in metabolism of bile acids, cholesterol, lipid, and carbohydrates as well as inflammation, fibrosis, and carcinogenesis (Li and Chiang 2014). Each bile acid receptor

shows different affinity for individual bile acids, thus changes in bile acid composition fine tune bile acid receptor signaling. Through controlling primary-to-secondary bile acid conversion, intestinal bacteria can influence liver function. On the other hand, bile acids also regulate the size and composition of the bacterial community. Bile acids have antibacterial function through multiple mechanisms including disruption of bacterial membranes, denaturing proteins, chelation of iron and calcium, and causing oxidative damage to DNA (Urdaneta and Casadesus 2017). Overgrowth of enteric bacteria is presented in cirrhotic patients with low levels of fecal bile acids. In addition, bile acids in part control growth of pathogenic bacteria such as *Clostridium difficile* (Allegretti et al. 2016; Sorg and Sonenshein 2008).

The contribution of bile acids to carcinogenesis has long been suggested (Phelan et al. 2017). As early as the 1930s, injection of deoxycholic acid (DCA), a secondary bile acid, has been found to cause malignant tumors at injection sites in mice. High level of bile acid exposure induces the generation of reactive oxygen species in cells which leads to disruption of the cell membrane, mitochondrial dysfunction, and DNA damage (Perez and Briz 2009). In addition, by binding to bile acid receptors, bile acids can regulate the expression of a lot of genes, many of which are involved in inflammation and carcinogenesis (Schaap et al. 2014; Li and Chiang 2014). Substantial evidences suggest that bile acids, especially secondary bile acids, promote colon cancer (Ajouz et al. 2014).

Naoko Ohtani's group was the first to demonstrate that the secondary bile acid DCA acts as a critical messenger linking obesity-associated dysbiosis with liver cancer (Fig. 7.2) (Yoshimoto et al. 2013). High-fat diet (HFD)-fed mice with chemical carcinogen exposure recapitulates the liver tumor-promoting effect of obesity in humans (Park et al. 2010). In a study from Naoko Ohtani's group, mice received a single injection of the chemical carcinogen DMBA (7,12-dimethylbenz[a] anthracene), which causes oncogenic Ras mutation, and were fed HFD (Yoshimoto et al. 2013). In line with other reports, HFD-fed obese mice develop marked increase of HCC. Interestingly, depleting intestinal bacteria with an oral antibiotic cocktail greatly reduced obesity-enhanced HCC, suggesting that the gut microbiome is critical in this process. In contrast to the findings of Robert Schwabe's group (Dapito et al. 2012), TLR4 deficiency does not influence HCC in HFD-fed DMBA mice, suggesting that LPS is not involved in this setting. Obesity was associated with dysbiosis and a dramatic increase in gram-positive bacteria. Oral vancomycin treatment, which preferentially targets gram-positive bacteria, is sufficient to block HCC development. Serum metabolites were analyzed, and the secondary bile acid DCA was substantially increased in HFD mice. Lowering DCA reduces whereas DCA-feeding increases obesity-enhanced HCC. Intriguingly, DCA feeding alone is sufficient to enhance HCC in lean mice. As expected, HFD feeding causes expansion of cluster XI *Clostridium* which is a gram-positive bacteria and contains the majority of 7α/β-dehydroxylation required for primary-to-secondary bile acid conversion (Ridlon et al. 2006, 2016).

Cellular senescence is a process occurring in normal cells in response to telomere erosion or oncogene activation (Kuilman et al. 2010). This process is a barrier to tumorigenesis by acting through checkpoint activation and cell-cycle arrest. The

important role of senescence in hepatocarcinogenesis has been reported (Kang et al. 2011; Lujambio et al. 2013). The livers of HFD-fed DMBA mice show a strong increase of senescence mainly in hepatic stellate cells (HSCs), which can be blocked by either an oral antibiotic cocktail or vancomycin treatment. In recent years, senescent cells have been found to present a secretory profile composed mainly of inflammatory cytokines chemokines and proteases (Acosta et al. 2013; Kuilman et al. 2008). Some of the senescence-associated secretory phenotype (SASP) have cell-autonomous activities which reinforce cell cycle arrest and promote clearance of senescent cells, whereas other SASP factors are associated with inflammation and tumorigenesis promotion. Depending on the stage of tumor development, SASP can be tumor-inhibiting or tumor-promoting (Eggert et al. 2016). The HFD-DMBA mouse study shows that DCA provokes SASP phenotype in hepatic stellate cells with increased IL-6, Gro-a, and CXCL9. Next the role of SASP in HCC was tested. Inflammasome activation and subsequent IL1β can act as an upstream regulator of SASP. IL1β knockout greatly reduces SASP expression of HSCs and subsequent liver tumor development. In addition, depleting senescent HSC significantly reduces HCC. Importantly, the study demonstrates that it is SASP but not cell-cycle arrest that regulates obesity-associated HCC. Together, the study suggests that the DCA-SASP axis in stellate cells are a key regulator in obesity-associated HCC.

The HFD-DMBA mouse study suggests that DCA is a potential target to treat liver cancer. Lowering DCA by decreasing 7α-dehydroxylation activity with difructose anhydride III or stimulating bile acid secretion with ursodeoxycholic acid (UDCA) inhibits HCC in HFD-fed DMBA mice. Cholestyramine, a bile acid sequestering resin that promotes bile acid excretion, has been reported to inhibit HCC in a different mouse model (Singh et al. 2018). The findings have clinical implication since UDCA is commonly used to treat patients with primary sclerosing cholangitis and cholestatic diseases (Lindor et al. 2009). UDCA usage has been reported to be associated with reduced mortality in colorectal cancer patients (Pardi et al. 2003). A possible association between UDCA usage and lower HCC incidence has been reported in patients with hepatitis C-associated cirrhosis (Tarao et al. 2005). The potential benefit of bile acid-targeting therapy approaches for liver cancer treatment should be investigated.

7.7.3 Short-Chain Fatty Acids

Short-chain fatty acids (SCFAs) are a group of saturated fatty acids containing less than six carbon molecules and include acetate, propionate, butyrate, pentanoic acid, and hexanoic acid. Intestinal SCFAs are produced by bacteria through fermentation of dietary fibers such as non-starch polysaccharides and oligosaccharides that cannot be digested by host enzymes (den Besten et al. 2013). Lacking a gut microbiome causes the significant reduction of SCFAs in germ-free mice (Hoverstad and Midtvedt 1986). The proximal colon has the highest concentration of SCFAs (70–140 mM) with acetate, propionate, and butyrate being the major components

(Topping and Clifton 2001; Cook and Sellin 1998). A wide range of bacteria can generate acetate, whereas the production of propionate and butyrate seems more specific. *Akkermansia muciniphila* is believed to be the major propionate-producing bacteria (Louis and Flint 2017). In the colon, the molar ratio of acetate, propionate, and butyrate is roughly 60:25:15, respectively. This ratio, however, can be affected by many factors such as diet and bacterial composition. SCFAs are readily absorbed by the host. Butyrate serves as an energy source for colon epithelial cells (Hamer et al. 2008), and propionate is metabolized by hepatocytes. Acetate can pass through the liver in portal blood and reach the systemic circulation (Bloemen et al. 2009).

SCFAs influence host systems both at the cellular and molecular levels through two major mechanisms. First, SCFAs can directly inhibit histone deacetylases (HDACs) to regulate gene expression (Waldecker et al. 2008). Second, SCFAs regulate signaling through G-protein-coupled receptors (GPCRs) (Husted et al. 2017). The major GPCRs activated by SCFAs includes GPR41, GFPR43, and GPR109A. Extensive evidence shows that SCFAs are important for host health and affect the pathogenesis of a wide range of diseases including allergies, metabolic disorders, neurological diseases, and cancer (Koh et al. 2016). SCFAs promote the differentiation of anti-inflammatory regulatory T cells (Smith et al. 2013). GPR43 activation by SCFAs is necessary for the normal resolution of certain inflammatory responses (Maslowski et al. 2009). Butyrate and propionate at low amounts exert beneficial effects, including prevention of oxidative stress, inflammation, and lipid oxidation in hepatocytes (McNabney and Henagan 2017).

In contrast to the numerous reported beneficial effects of SCFAs, recently Matam Vijay-Kumar's team demonstrates that SCFAs can exert a detrimental function and promote HCC (Fig. 7.2) (Singh et al. 2018). Initially, the team investigated the influence of dietary fiber fermentation and SCFA production on metabolic changes in TLR5 knockout mice which are prone to develop metabolic syndrome. Inulin, a soluble fiber, was supplemented to the mice in the diet. Consistent with other reports that inulin ameliorates low-grade inflammation, insulin resistance, and obesity (Zou et al. 2018), inulin feeding lowered obesity incidence and improved the indices of metabolic syndrome in the TLR5 knockout mice. Unexpectedly, some inulin-fed mice had hyperbilirubinemia, hepatic injury and inflammation, and impaired liver detoxification functions. Perhaps most interesting, prolonged inulin feeding caused primary HCC in the TLR5 knockout mice. Dietary fiber can be broadly categorized as insoluble or soluble. By comparing different types of dietary fibers, the study concluded that soluble fibers, but not fermentable insoluble fibers, induce HCC. Importantly, soluble fiber-induced HCC only occurs in mice with dysbiosis, not in TLR5 mice with balanced gut microbiome. Cohousing and cross-fostering studies demonstrate that the soluble fiber-induced HCC is transmissible between mice exchanging intestinal microbes. Germ-free TLR5 KO mice fed with irradiated soluble fiber diet failed to develop hyperbilirubinemia and HCC, confirming that it is gut microbiome dependent. A high-fat diet causes dysbiosis in mice (Murphy et al. 2015). Consistently, combining soluble fiber with HFD induced HCC in a small portion of wild-type mice, suggesting the phenomena is generalizable. Unlike insoluble fibers, soluble fibers can be fermented by the gut bacteria to produce

SCFAs. HCC-prone TLR5 KO mice display gut dysbiosis characterized by an increase in fiber-fermenting bacteria and *Proteobacteria*. As expected, blocking microbial fermentation by depleting SCFA-consuming bacteria or plant-derived β-acids protects mice from soluble fiber-induced HCC. Although it causes hyperbilirubinemia, liver inflammation, enhanced hepatocyte proliferation, and fibrosis, single SCFA butyrate feeding fails to induce HCC, suggesting other factors are required.

In this model HCC is closely associated with hyperbilirubinemia caused by chronic liver inflammation. Mice with hyperbilirubinemia show higher liver expression of pattern recognition receptor NLRC4, the important inducer of inflammasome (Duncan and Canna 2018), and TLR4 signaling. However, knockout of TLR4 or NLRC4 has no influence on HCC incidence, suggesting that LPS and the inflammasome pathway are not important in this setting. Higher serum levels of bile acids were found in mice that developed HCC following soluble fiber feeding. Furthermore, cholestasis is closely associated with dietary fiber-induced HCC, suggesting the contribution of bile acids in HCC development. *Clostridia* members, particularly *Clostridium* cluster XIVa, are the main producers of butyrate and secondary bile acids (Van den Abbeele et al. 2013; Riviere et al. 2016). Supporting these findings, bacterial taxa analysis shows that *Clostridia* predominantly distinguishes hyperbilirubinemia mice, which develop HCC, from other groups. Indeed, lowering bile acid levels using cholestyramine suppresses HCC formation from dietary fiber feeding. The study is in line with the finding from Naoko Ohtani's group that the gut bacteria-controlled secondary bile acid DCA promotes HCC (Yoshimoto et al. 2013).

The finding is surprising since dietary fibers and SCFAs are known to have antitumor function. It has been well recognized that dietary fiber consumption is associated with reduced risk of colon cancer (Hinnebusch et al. 2002). Importantly, two clinical studies reported that high intake of fiber has a protective effect against HCC (Fedirko et al. 2013; Yang et al. 2019). In the EPIC cohort of Western Europeans, dietary fiber from cereals and cereal derivatives has been found to have a significantly inverse association with HCC risk (Fedirko et al. 2013). In a separate study using large NHS and HPFS cohorts of US adults, increased intake of whole grains and possibly cereal fiber has been suggested to be associated with a reduced risk of HCC (Yang et al. 2019). However, this study showed that in the presence of a dysregulated intestinal microbiome, soluble fibers and SCFAs have opposite effects and promote HCC development, suggesting that the benefit of soluble fibers and SCFA is context dependent. The finding has important implications. Half of US adults consume dietary supplements to improve health. However, adverse effects caused by dietary supplement consumption have been reported (Ronis et al. 2018). More studies are needed to improve the safety and appropriate usage of dietary supplements.

7.7.4 Immune Cells

The liver contains a large number of immune cells with particular concentrations of Kupffer cells, NKT, and MAIT cells (Racanelli and Rehermann 2006). HCC is a typical inflammation-related cancer, and HCC patients often have underlying chronic liver inflammation (Michelotti et al. 2013; Anstee et al. 2019; Ganne-Carrie and Nahon 2019; Ponziani et al. 2019). The important role of immune surveillance in liver cancer has been recognized. In mice, removing the adaptive immune system promotes carcinogen in DEN-induced HCC (Schneider et al. 2012; Mossanen et al. 2019). Together with macrophages, CD4 T cells remove senescent hepatocytes to prevent malignant transformation (Kang et al. 2011). In addition, the liver immune cells play a critical role in obesity-/NAFLD-promoted HCC. Several mechanisms have been identified including hepatic activation of NKT, CD8 T cells, IgA-producing B cells, Th17 cells, and loss of liver CD4 T cells (Shalapour et al. 2017; Wolf et al. 2014; Gomes et al. 2016; Ma et al. 2016). In humans, adoptive T-cell therapy using tumor-specific CD4 T cells induced a strong antitumor response and complete eradication of tumor lesions in a late-stage cholangiocarcinoma patient (Tran et al. 2014). The patient is still alive 10 years after diagnosis. Based on the promising results, immune checkpoint inhibitors nivolumab and pembrolizumab have recently been approved by FDA to treat HCC patients (Okusaka and Ikeda 2018).

The gut microbiome has been established as a modulator for antitumor immunity (Gopalakrishnan et al. 2018a). Emerging evidences suggest that the gut microbiome plays a critical role in regulating liver local antitumor immune responses. In a follow-up study by Naoko Ohtani's group, lipoteichoic acid (LTA), a gram-positive bacterial component, has been shown to contribute to obesity-enhanced HCC in accordance with the bile acid DCA (Loo et al. 2017). Unlike DCA, LTA is a major ligand for TLR2. Indeed, TLR2-deficient mice have reduced HCC in the HFD-MDTA model. Interestingly, the binding of LTA to TLR2 leads to increased COX2 activity, which subsequently enhances the production of prostaglandin E2 (PGE2). Tumor tissues upregulate PTGER4, the receptor for PGE2 (Sugimoto and Narumiya 2007), which is predominately expressed on immune cells particularly on T cells, but not on hepatocytes or HSCs. In vitro culture experiments show that PGE2 attenuates the proinflammatory cytokines IFNγ and TNFα but increases anti-inflammatory cytokines IL10 and IL6. High production of COX2 and PGE2 can be detected in noncirrhotic NASH-associated HCC patients. To our knowledge, it is the first publication which links the gut microbiome and liver tumor development through inhibition of the immune system.

A recent mouse study from Vikas Dudeja's group investigated the effects of the gut microbiome on tumor growth (Sethi et al. 2018). An oral antibiotic cocktail was applied to remove intestinal bacteria, and the subsequent influence on growth of both subcutaneous tumors and liver metastases was tested. Enhanced tumor development in both compartments were found after removing the gut microbiome. Interestingly, the tumor-promoting function of the gut microbiome depends on adaptive immunity

and disappears in Rag1 knockout mice which lack mature T, B, and NKT cells. Removing intestinal bacteria induces higher T-cell activation and production of proinflammatory cytokines such as IFNγ in the tumor microenvironment, which correlates with improved survival. This report provides direct evidence that the adaptive immune system in involved in the gut microbiome-modulated HCC.

Our group also studies the influence of the gut microbiome on liver cancer (Fig. 7.2) (Ma et al. 2018). Consistent with previous observations, removing gut bacteria using oral antibiotic cocktail reduces liver tumor formation (Shalapour et al. 2017; Dapito et al. 2012; Sethi et al. 2018). The effect is not only observed in primary HCC but also seen in liver metastatic models with nonhepatic tumors. Interestingly, removing the gut bacteria causes a seemingly location-dependent opposite effect on the growth of metastatic lesion from the same tumor line in the liver compared to the lung. This observation suggests that the gut microbiome-influenced liver environment is crucial for controlling tumor growth. We focused on the changes within the liver immune system to understand the mechanism. A prominent increase in liver $CXCR6^+$ NKT cells was found in the oral antibiotics-treated mice or germ-free mice. Importantly, either NKT cell deficiency or disrupting liver accumulation of NKT cells by knocking out CXCR6 eliminated the influence of the gut bacteria on liver tumor growth. In addition, hepatic NKT cells from antibiotic-treated mice are more active and produce higher levels of IFNγ, an important cytokine to induce antitumor immunity, after in vivo stimulation. Together these results suggest that there is a critical role of NKT cells in gut microbiome-controlled liver tumor growth.

NKT cells are a group of innate-like lymphocytes and serve as a bridge between the innate and adaptive immune system (Gao et al. 2009; Bandyopadhyay et al. 2016; Terabe and Berzofsky 2008; Nishimura et al. 2000). With limited TCR repertoire, NKT cells recognize lipids presented on CD1 molecules, which leads to a quick release of a larger amount and various cytokines that help initiate a variety of immune responses. NKT cells are enriched in the liver and make up ~30% of intrahepatic lymphocytes in mice (Bandyopadhyay et al. 2016; Terabe and Berzofsky 2008). The endogenous lipid ligands for NKT are still elusive, but studies have shown that the lipid components of gut bacteria can activate NKT cells (Brennan et al. 2014; Wolf et al. 2015; Zajonc and Girardi 2015), which suggest a potential role for gut bacteria regulation of liver NKT cells. It is well-known that NKT cells have antitumor functions (Terabe and Berzofsky 2008). NKT cells are reported to be important in controlling liver metastasis (Cullen et al. 2009). A recent publication demonstrates that NKT cells play an important role in preventing DEN-induced HCC (Mossanen et al. 2019). NKT cells can exert antitumor function by either directly killing tumor cells or an indirect function through secreting cytokines. The exact mechanism of how liver NKT cells mediate gut microbiome-controlled liver tumor formation is not clear and still under investigation.

Next, we focused on how the gut microbiome regulates the intrahepatic NKT cell population. Removing gram-positive bacteria is sufficient to increase NKT cells. Liver accumulation of NKT cells is mediated by CXCR6 receptor which recognizes its ligand CXCL16 (highly expressed on LSECs) (Geissmann et al. 2005) in the

lining of liver sinusoids. Removing gut bacteria or selectively targeting gram-positive bacteria increases CXCL16 expression in LSECs. Bacteria responsible for primary-to-secondary conversion of bile acids are gram-positive. For this reason, we tested the possible link between bile acids and NKT cells. Interestingly, secondary bile acids decreased whereas primary bile acid increased CXCL16 expression. Feeding secondary bile acids to mice inhibits liver CXCL16 expression and reduces NKT cells in the liver. Colonization of mice with *Clostridium scindens*, a bacteria species well-known for primary-to-secondary bile acid conversion (Studer et al. 2016), accelerates the dropping of liver NKT cells following antibiotic withdrawal. Importantly, *Clostridium scindens* colonization increases liver tumor burden. Next, we checked whether the finding in mice can also be replicated in humans. Indeed, in nontumor liver tissues of HCC patients, a positive association between primary bile acid CDCA and CXCL16 mRNA level was found. In contrast, secondary bile acid DCA is negatively associated with CXCL16 mRNA levels, suggesting the bile acids/CXCL16 axis also exists in humans. Our study clearly demonstrated that the gut bacteria use bile acids as a messenger to regulate liver NKT cells thus influencing liver antitumor immunity.

Our understating of the gut microbiome's effects on liver cancer is still in its infancy. Most of the studies still focus on HCC, the major type of primary liver cancer. The influence of the gut microbiome on cholangiocarcinoma has not been reported, although there are reports of the association between intrahepatic cholangiocarcinoma and chronic inflammatory bowel diseases which display dysbiosis (Tyson and El-Serag 2011). In addition, the majority of patients with primary sclerosing cholangitis, a well-established risk factor for cholangiocarcinoma, have a concomitant inflammatory bowel disease (Gulamhusein et al. 2016). Our study with liver metastatic models suggests that the gut microbiome regulates liver antitumor immunity and the tumor regulatory function is not limited to HCC but also apply to other liver cancer types including liver metastasis.

7.8 Gut Microbiome and Immunotherapy

Immunotherapies, particularly immune checkpoint inhibitors, have shown promising effects in treating patients with solid tumors (Khalil et al. 2016). The gut microbiome plays a fundamental role in the development and functional regulation of the host immune system. Importantly, alternation of the gut microbiome has been found to influence the efficacy of many types of immunotherapies for cancer including immune checkpoint inhibitors (Routy et al. 2018a). In patients with metastatic melanoma, non-small cell lung cancer, or renal cancer, the presence of intestinal bacteria such as *Faecalibacterium prausnitzii*, *Bifidobacterium longum*, or *Akkermansia muciniphila* has been linked to better anti-PD-1 responses (Routy et al. 2018b; Matson et al. 2018; Gopalakrishnan et al. 2018b; Frankel et al. 2017; Pinato et al. 2019). As expected, usage of antibiotics, especially the ones with broad spectrum, has been connected with impaired efficacy of checkpoint blockade in

these studies. The timing of antibiotic application seems to be important. Prior, but not concurrent, administration of broad-spectrum antibiotic with anti-PD1 therapy is associated with worse overall survival and a higher risk of tumor refractory (Pinato et al. 2019). Of note, no close taxonomic relationship has been associated with improved efficacy of anti-PD1/PD-L1 therapy, suggesting that multiple bacteria-controlled pathways are involved or these bacteria are functionally related. Besides immunotherapy, proper immune responses are required for many chemotherapies to reach full-treatment potency such as cyclophosphamide- and platinum-based therapies. In mouse studies, the optimal antitumor efficacy of cyclophosphamide and oxaliplatin has been found to be dependent on intact gut commensal bacteria and is significantly dampened in germ-free mice or mice received antibiotic cocktails (Iida et al. 2013). Although the crucial role of intestinal bacteria in tumor immunotherapy has been established, the underlying mechanisms are still largely unknown.

Following the advancements of immunotherapy for solid tumors in the past few years, there is a global interest in immunotherapies for HCC (Hou et al. 2019; Johnston and Khakoo 2019; Floudas et al. 2019; Xie et al. 2018). Most HCC patients diagnosed with advanced disease are not eligible for curative approaches such as surgical resection, liver transplantation, or local percutaneous tumor ablation. Systemic treatments for HCC constitute mostly of multitargeted tyrosine kinase inhibitors (TKIs) with sorafenib being the only FDA-approved drug until 2017 (Johnston and Khakoo 2019; Floudas et al. 2019). Although additional TKIs have been approved for HCC treatment recently, its overall survival benefit is limited. Recently, nivolumab and pembrolizumab, two immune checkpoint inhibitors, have been approved by the FDA to treat HCC patients. Besides immune checkpoint inhibitors, other immune-based approaches for HCC are under investigation including DC vaccines, oncolytic viruses, adoptive cell therapy, antigen-targeting antibodies, and blocking inhibitory cytokines (Floudas et al. 2019; Xie et al. 2018). Currently, the influence of the gut microbiome on immunotherapy for liver cancer is unknown. A recent mouse study reported the impact of the gut microbiome on anti-PD1 therapy for pancreatic adenocarcinoma (PDA) which is a deadly GI malignancy (Pushalkar et al. 2018). Similar to liver cancer, the gut microbiome promotes PDA development. Interestingly, removing the gut bacteria sensitizes PDA tumors to anti-PD1 therapy accompanying an enhanced adaptive T-cell response. The observation is contradictory to many reports that the gut microbiome is needed for the success of anti-PD1 therapy (Routy et al. 2018b; Matson et al. 2018; Gopalakrishnan et al. 2018b; Pinato et al. 2019). Further investigations are needed to clarify the potential influences the gut microbiome has on immunotherapy against GI cancers.

7.9 Summary

Liver cancer is a deadly disease, and its incidence is rising. Following the success of controlling viral hepatitis and global pandemic of obesity, the major risk factors for HCC are shifting toward obesity and chronic metabolic dysregulation conditions

such as NAFLD. Obesity and NAFLD are closely associated with the alteration of the gut microbiome. Together with the finding of liver cancer-associated bacteria, it has been suggested that the gut microbiome contributes to liver cancer formation. Importantly, animal studies prove direct evidence that the gut bacteria promote HCC development. The potential strategies targeting the gut microbiome to treat liver cancer, and the influence of the gut microbiome on available liver cancer treatment, especially immunotherapies, should be further investigated.

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Chapter 8
The Microbiome and Urologic Cancers

In "Inflammation, Infection, and Microbiome in Cancers: Evidence, Mechanisms, and Implications"

Mithunah Krishnamoorthy, Saman Maleki Vareki, and Jeremy P. Burton

Abstract Cancers of the urinary system are likely to be influenced by both the urinary and gut microbiotas. While commensal organisms do not densely colonize the urinary system, those present still play a significant role in health and disease. Like other organs, there is always a potential for malignancy to occur when microorganisms cause chronic inflammation. Additionally, the urinary system is commonly exposed to the waste products in the body, including those microbial metabolites from the gut which have entered the circulation. It is, therefore, possible that the microbiota of different sites contributes to the process of carcinogenesis through metabolic toxification and detoxification as well as immune interactions. The emerging clinical evidence also suggests a prominent role for microbiota in affecting the efficacy of cancer therapeutics, including chemotherapy and immunotherapy agents.

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8.1 Introduction

The microbiome is quickly emerging as an essential factor in determining the prognosis of various cancers (Routy et al. 2018; Riquelme et al. 2019; Gopalakrishnan et al. 2018). The microbiome refers to not only the organisms inhabiting an environment but also includes the factors that they produce (Whiteside et al. 2015). As there are thought to be approximately ten times more bacterial cells than host cells in the human body, the human microbiome has significant impacts on overall health (Turnbaugh et al. 2007; Round and Mazmanian 2014). Notably, without the microbiome, the development of the immune system is impaired. Germ-free mice were found to have deficits in antibody production and are more susceptible to bacterial infections (Round and Mazmanian 2014). The immune system has evolved to protect the body against foreign entities and neoplastic cells; therefore, the microbiome is needed to support the antitumor capabilities of the immune system. The microbiome is initially populated by the flora of the mother's vaginal canal in neonates, where it is then shaped by diet and the environment. Bacteria encounter the host at several sites, including the mucous membranes and the skin. To date, most research has focused on the gut microbiome, and much less is known about the microbiome of the urinary tract. The urine shares 23.6% of the same bacteria as the gut microbiome (Morand et al. 2019). However, bacteria present in the urine can only give an idea of the bacterial composition of the entire urinary system. A recent study suggests that the bladder tissue and the urine have different compositions of bacteria, and these compositions change when the tissue becomes neoplastic (Pederzoli et al. 2020). Urological cancers refer to the family of cancers that include bladder, kidney, prostate, and testicular cancer. Here, we briefly discuss how the microbiome impacts the process of tumorigenesis and cancer progression and affects the treatment of urological cancers.

8.2 The Urinary System

The intestinal tract is designed for nutrient absorption for the human body and then waste disposal of the exhausted components. The urinary tract's role, however, is only to remove waste products from circulation, and it subsequently has a one-way flow out of the body. While both the urinary and intestinal tracts have exposure to bacteria at their epithelial barriers, these occur at different bacterial densities, types of microbes, and in the presence of various host cell types. The urinary system has specially evolved to filter waste from the blood then followed by systems to collect and then expel the liquid waste without leakage. Despite being the size of a human fist, both kidneys receive 20% of the cardiac output and filter approximately 125 mL

of blood per minute (Scott and Quaggin 2015). With the highest blood flow per 100 g of tissue, the kidneys have a disproportionately higher exposure to toxins than other organs (Vervaet et al. 2017). The nephrons within the kidneys retain any molecules above ~50 KDa, filtering out water, salt, amino acids, and certain metabolites (Kurts et al. 2013). These metabolites are in contact with the urothelium for extended periods of time; if carcinogenic, they may also lead to tumorigenesis. The gut microbiota is a significant source of metabolites, and many of these enter hepatic circulation and ultimately are filtered out through the urinary system. It illustrates why bacteria at other sites can influence the urinary system, even if they are distant to this site, as the small metabolites easily disseminate beyond where they originated.

Several carcinogenic metabolites are produced by gut microbes, including polyamines, ammonia, and *N*-nitroso compounds (Louis et al. 2014). These are produced during the breakdown of proteins by microbes in the gut, where some are transferred to the bloodstream via the intestinal mucosa (Tofalo et al. 2019). These compounds can induce inflammation which may lead to malignancies (Louis et al. 2014). Metabolites such as polyamines are also produced endogenously and are necessary for eukaryotic cell growth. However, the overabundance of polyamines is toxic. The carcinogenic effects of polyamines are due to its catabolism that releases large amounts of reactive oxygen species (ROS) which can lead to destructive DNA damage (Casero et al. 2019). Notably, the polyamine spermine concentration in urine was found to be a biomarker for prostate cancer (Tsoi et al. 2016). This study, however, did not determine if the source of spermine was bacterial or host derived. Bacteria also produce *N*-nitroso compounds (NOCs) in the gut, which correlated with a high incidence of colorectal cancer in European populations (Loh et al. 2011; Kobayashi 2018). As NOCs can enter the urinary system via the blood, they can potentially induce urinary cancers as well. Indeed, and increase in nitrate (NOC precursor) consumption correlated with incidence of renal cell carcinoma (Dellavalle et al. 2013). On the other hand, some metabolites such as short-chain fatty acids (SCFAs) are anti-inflammmatory and limit tumorigenesis by suppressing inflammation. SCFAs are the by-products of insoluble carbohydrate fermentation in the gut and promote the differentiation of $CD4^+$ T cells into immunosuppressive regulatory T cells (Tregs) (Park et al. 2015). Tregs play a key role in suppressing inflammation in tissues by limiting the proliferation of pro-inflammatory immune cells (Corthay 2009).

The urinary system, especially in women, is proximal to the terminal gastrointestinal tract and carries a greater risk of infection given the high number of microbes at this site. Many gut microbes end up in the urogenital tract, especially in women via transfer across the perineum, vagina, and then to the urethra, which in women is closer to the bladder than men. This closer proximity contributes to the higher risk of infection in women. Some of these bacteria include uropathogenic *Escherichia coli* (UPEC), a highly heterogenous group especially adapted to survive in the urinary tract (Foxman 2010). Notably, a study found a higher prevalence of mucosal binding UPEC in tissue samples from colorectal cancer patients than patients with diverticulosis (noncancerous pouches that form on the colon wall) (Buc et al. 2013). These strains of *E. coli* produced cyclomodulin and genotoxin, which regulate cell cycle

progression and damages DNA, respectively. Therefore, the presence of these bacterial strains in the urinary tract may induce tumorigenesis as well. Toxic metabolites may also be degraded by specific microbiota that inhabit the urinary system. Potentially, by altering the microbiota, one can remove toxic compounds, thus limiting exposure. Alternatively, specific bacterial metabolites may pose a threat and promote tumorigenesis once in the hepatic system. Future studies must explore how microbial composition affects the breakdown of compounds and how these breakdown products impact overall health.

Microbiomes have been implicated in the transformation of healthy cells into malignant cells, particularly in the gastrointestinal tract. The most well-known being the chronic infection of *Helicobacter pylori* and the development of gastric cancer. Three percent of patients with *H. pylori* infections develop gastric adenocarcinoma (Wroblewski et al. 2010). *Helicobacter pylori* secretes virulence factors that induce chronic inflammation, which subsequently leads to the production of ROS and then carcinogenesis (Wroblewski et al. 2010). As a similar phenomenon occurs in the colon as well, one might assume that infections in the urinary system may also lead to formation of cancers. Indeed, meta-analysis of eight studies showed that exposure to urinary tract infections (UTI) favored in risk of non-schistosomiasis-related urinary bladder cancer (UBC). Surprisingly, analysis of female only data did not show a correlation between UTIs and UBC (Bayne et al. 2018). Nevertheless, the occurrence of UBC or other urinary cancers has not yet been associated with specific bacteria. One study did find that the genus *Streptococcus* was more abundant in the urine samples of females with urothelial carcinoma compared to healthy individuals (Xu et al. 2014). Whether the abundance of *Streptococcus* induced an inflammatory response prior to tumor formation was not investigated in this study. These studies suggest that chronic inflammation of the urinary tract may predispose individuals to tumor development.

The epithelial cells along the urinary tract are the first line of host defense against pathogens, expressing pattern recognition receptors (PRRs) that trigger anti-inflammatory signaling cascades upon binding bacterial components like lipopolysaccharide. The epithelial cells secrete cytokines such as IL-6 and IL-8 in order to recruit macrophages and dendritic cells to the site of infection. These cells are crucial for phagocytosing bacteria and clearing infection. At homeostasis, the kidney is residence to dendritic cells and macrophages, which are restricted to the space external to the nephrons (Kurts et al. 2013). Upon infection, resident dendritic cells secrete chemokines to attract neutrophils which directly kill pathogens via phagocytosis or release of antimicrobial agents. The dendritic cells themselves can also uptake apoptotic cell remnants and present the peptides which activates T cells. Activated T cells can secrete cytokines that support macrophage activity. Unlike the intestines, the urinary system does not have a dedicated set of lymphoid organs that can continuously monitor bacterial populations. Instead, it depends on the ability of the epithelial cells to recruit immune cells. An essential subset of immune cells are cytotoxic T cells, which can detect non-self cells and directly kill them. Studies have shown that mice with depleted cytotoxic T cells are unable to slow tumor growth,

highlighting the importance of cytotoxic T cells in tumor control (Fan and Edgington 1989; Sivick et al. 2018).

8.3 Bladder Cancer and Microbes

The disproportionate members of men with bladder cancer is thought to be due to industrial or lifestyle exposure, rather than other causes such as infection. However, there is mounting evidence that microbes may play a role in the precancerous conditioning of tissue (Burger et al. 2013). Bladder cancer is the fourth most common cancer in men and the eleventh most common malignancy in women (Kamat et al. 2016). From the 1800s until 2012, a healthy bladder was believed to be devoid of any microorganisms, as most bacteria from the urine could not be cultured using techniques that were available at the time (Wolfe et al. 2012). These techniques were unsuitable to identify the slow-growing anaerobic bacteria that are now known to exist in the bladder. However, with the development of 16S rRNA sequencing technology and fluorescent microscopic techniques, bacteria in the bladder could be readily identified, disproving the paradigm of sterility (Wolfe et al. 2012; De Nisco et al. 2019).

While there is some variation from study to study, notably, males and females are thought to harbor different compositions of microbiota. While female bladders were colonized largely by *Lactobacillus* and *Gardnerella*, males' bladder were described to be colonized by *Corynebacterium*, *Staphylococcus*, and *Streptococcus* (Pearce et al. 2015; Shrestha et al. 2018). The difference in bacteria between the sexes may contribute to the increasing instances of bladder cancer observed in males compared to females. In females, a large proportion of bacteria may originate from the vagina, where the most prevalent genus of bacteria is *Lactobacillus*, which is usually a marker of good health (Zhong et al. 2013). Notably, *Lactobacillus casei*, an intestinally associated lactobacilli, had been shown to reduce tumor size in mice inoculated with the TC-1 (lung cancer model). This study showed that *L. casei* had antitumor properties as it induced dendritic cells to secrete IL-2 (Jacouton et al. 2019). IL-2 is a pro-inflammatory cytokine that can recruit cytotoxic T cells and natural killer cells which can then directly kill tumor cells (Mortara et al. 2018). Also, IL-2 is an FDA-approved therapy for melanoma when administrated intravenously (Tsao et al. 2004). In a study where *L. casei* was administered orally to males with superficial bladder cancer in a double-blinded study, the 50% time-to-recurrence rate was 1.8 times longer than untreated patients, suggesting that *L. casei* may be used as a probiotic to reduce bladder cancer recurrence. Of note, certain species of lactobacilli like *L. casei* in the bladder of women can lower the concentration of ATP in their environment (Abbasian et al. 2019). Since the tumor interstitial space has a high concentration of ATP that is used by cancer cells to drive cell growth and proliferation, *L. casei* may potentially slow tumor growth by decreasing ATP concentrations (Qian et al. 2016).

In preliminary studies with a relatively limited number of subjects, male bladder cancer patients were shown to have a different composition of bacteria than those who are healthy (Bučević Popović et al. 2018). Although the species diversity was not significantly different, urine collected from bladder cancer patients had increased proportions of *Fusobacterium*, *Actinobaculum*, *Facklamia*, and *Campylobacter* (Bučević Popović et al. 2018). Interestingly, *Fusobacterium nucleatum* has been prevalent in colorectal cancer tissues as well (Castellarin et al. 2012). When $Apc^{min/+}$ mice that lack the tumor suppressor gene *APC* are fed *F. nucleatum*, the development of tumors is accelerated in the colon. This was accompanied by an increase of myeloid-derived suppressor cells in the tumor, which have been shown to suppress T-cell activity (Kostic et al. 2014). Thus, *F. nucleatum* may induce an environment that fosters the growth of tumors and may only require small pockets of persistence in the bladder tissue to induce tumorigenesis.

It is well-known that infection with the parasite *Schistosoma haematobium*—schistosomiasis—is associated with an increased risk of bladder cancer. *S. haematobium* implants its eggs into the bladder wall, leading to inflammation and urothelial hyperplasia, a potentially precancerous lesion (Ishida and Hsieh 2018). However, not all who have schistosomiasis developed carcinomas. Whether or not schistosomiases will lead to carcinogenesis may depend also upon microbiota present in the bladder. Bacterial taxa such as *Fusobacterium*, *Sphingobacterium*, and *Enterococcus*, which are also known immunostimulants, have been found in patients with urogenital schistosomiasis-induced bladder pathologies (Adebayo et al. 2017). Additionally, NOCs were commonly found in high concentrations in patients with schistosomiasis (Mostafa et al. 1999). NOCs are highly carcinogenic and are produced by certain strains of bacteria which may induce tumor formation (Markowski et al. 2019).

Inflammatory events involving bacteria can induce crystallizations that can form the initial nidus of bladder and kidney stones. The occurrence of bladder stones may also predispose patients to bladder cancer (Fernando et al. 2017; Cho and Holley 2013; Chung et al. 2013). The mechanical irritation caused by stones can increase the proliferation of cells in the bladder wall, leading to an increase in the mutation frequency (Takahashi et al. 2000). This ultimately may lead to the formation of bladder cancers (Takahashi et al. 2000). Biofilms of bacteria may also grow on the stones themselves. Whether these are disease causing or not is yet to be understood (Schwaderer and Wolfe 2017). However, the bacteria do seem to promote the aggregation of crystals. One study showed that the growth of calcium oxalate crystals increased in the presence of *Escherichia coli* and *Klebsiella pneumoniae* (Chutipongtanate et al. 2013). Perhaps the presence of these bacteria promotes the growth of crystals, which subsequently promotes tumorigenesis.

Surprisingly, the administration of bacteria can have therapeutic effects. For the past 50 years, Bacillus Calmette–Guérin (BCG) has been used to treat non-muscle-invasive bladder cancer (NMIBC) and remains the most effective intravesical treatment; even with a 50% recurrence rate. BCG is a live, attenuated form of the slow-growing, aerobic bovine tuberculosis bacterium, *Mycobacterium bovis* (Kamat et al. 2015). For the BCG immunotherapy to be effective, the bacterium is postulated

to bind to the bladder wall in a fibronectin-facilitated manner (Ratliff et al. 1987). The attachment of BCG to the bladder wall immediately recruits different types of lymphocytes, including $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells (Kates et al. 2018). This influx of lymphocytes is transient and returns to pretreatment levels 3 weeks after BCG treatment (Kates et al. 2018). Whether or not the patient responds to the treatment is dependent on which populations of lymphocytes are recruited to the tumor microenvironment. Compared to responders, nonresponders of BCG treatment had threefold more $FOX3P^+$ regulatory T cells and tumor-associated macrophages recruited to the tumor. Both $FOX3P3^+$ regulatory T cells and tumor-associated macrophages contribute to the suppression of T-cell activation (Pichler et al. 2016; Pathria et al. 2019). BCG is often used in combination with other therapies, such as chemotherapy and transurethral resection, to maximize progression-free survival (Deng et al. 2017; Hinotsu et al. 2011).

Given the high occurrence rate with BCG therapy, we ponder the relationship that the microbiome has on the efficacy of this treatment. Members of commonly found urinary system inhabitants interact with proteins such as fibronectin (McMillan et al. 2013) This may competitively prevent the adhesion of the *Mycobacterium bovis* to the bladder, thus reducing the efficacy of the vaccine. More importantly, commensal and probiotic bacterium are known to reduce pro-inflammatory responses at various exposed epithelial surfaces including urogenital tract, oral cavity, and gut which may also reduce vaccine efficacy (McMillan et al. 2013; Cosseau et al. 2008; Plaza-d et al. 2017). There may be other interactions such as co-aggregation with commensal species which removes its ability to interact with the host surface directly.

8.4 Renal Cell Carcinoma

Renal cell carcinoma (RCC) is a malignancy of the proximal convoluted tubule in the kidney. RCC is less often diagnosed in its early stages due to the lack of clinical symptoms, leading to 20% of patients presenting with metastatic disease (Choueiri and Motzer 2017). Several different variations of RCC exist, clear cell renal cell carcinoma (CCRCC) being the most prevalent; 70% of all RCC patients have metastatic CCRCC. Notably, urinary tract infections positively correlate with instances of RCC. Male current smokers with recurrent urinary tract infections (UTIs) constitute the highest proportion of RCC patients (Parker et al. 2004). This might imply that constant inflammation may lead to tumor formation.

Targeted therapies, such as vascular endothelial growth factor receptor-targeted, tyrosine kinase inhibitors (VEGF-TKIs), have been the standard line of treatment in the past decade. While VEGF-TKIs decrease tumor size quickly, patients often become resistant to treatment within months and on average have a median survival of 26.9 months (Heng et al. 2014; Beacham and Deatrick 2008). A large proportion of patients undergoing VEGF-TKI treatment have diarrhea as a side effect. Notably, 16S RNA gene sequencing of patient stool samples showed a difference between the bacterial composition of those who have diarrhea and those who do not. *Bacteroides*

spp. was enriched in stool samples from patients with diarrhea, while *Prevotella* was enriched in the non-diarrhea group (Pal et al. 2015). Additionally, another study has shown that administering a *Bacteroides*-specific antibiotic prevents diarrhea while also prolonging progression-free survival by 10 months in RRC patients treated with VEGF-TKIs (Hahn et al. 2018). This suggests that the dysbiosis of gut microbiota can cause diarrhea which may be managed by the administration of a bacteria-specific antibiotics or probiotics. Colitis-like side effects may also be managed by fecal microbiota transplant (Wang et al. 2018).

More recently, combination treatment with checkpoint immunotherapies against programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) has become the first line of treatment for intermediate- and poor-risk RCC. These immunotherapies work by blocking the receptors that trigger T-cell inactivation (Pardoll 2012). When compared to sunitinib, a VEGF-TKI, the combination of nivolumab (PD-1 inhibitor) and ipilimumab (CTLA-4 inhibitor) was much more effective as the median overall survival was not reached with nivolumab and ipilimumab. In contrast, the median overall survival for sunitinib was 26.0 months (Motzer et al. 2018). The effectiveness of immunotherapy was recently associated with the gut microbiome (Routy et al. 2018; Riquelme et al. 2019). Indeed, a study following a large cohort of RCC patients showed that the median progression-free survival was 2.9 months in antibiotic users and 8.1 months in non-antibiotic users that were treated with anti-PD-1 therapy (Lalani et al. 2019). This suggests that disturbing the gut microbiota with antibiotics is detrimental to the effectiveness of immunotherapy. Surprisingly, antibiotics have a less profound impact on VEGF-TKI therapy, interferon therapy, and mammalian target of rapamycin (mTOR) inhibitors than immunotherapy (Lalani et al. 2019). Antibiotics can drastically change the gut microbiome, lowering the number of observed species by 60–75% and may selectively eliminate helpful bacteria while allowing for the expansion of less desirable microbiota (Suez et al. 2018). It is unclear what composition is needed to elicit the best response to immunotherapy, but, generally, patients with more diverse microbiota are better responders. Stool samples from RCC patients who responded better to anti-PD-1 therapy were found to have enriched populations of *Akkermansia muciniphila* (Routy et al. 2018). When fecal matter from responding patients was transplanted into germ-free mice, tumor burden decreased more when treated with anti-PD-1 compared to mice transplanted with nonresponder feces. In these mice, there was an upregulation of the Th1 response, evidenced by the increase of tumor-infiltrating $CXCR3^{+}CD4^{+}$ T cells (Routy et al. 2018). $CXCR3^{+}CD4^{+}$ T cells are essential as they secrete cytokines that activate dendritic cells, which in turn promotes the proliferation of cytotoxic T cells (Yoon et al. 2009). The underlying mechanism of the protective effect of *A. muciniphila* is still unclear.

8.5 Prostate Cancer

One probable risk factor for developing prostate cancer is inflammation. As the prostate is in close proximity to the urinary tract, inflammatory pathogens may enter due to urine reflux and lead to infections. In a study that compared the urinary microbiome between prostate cancer patients and healthy volunteers, patients with prostate cancer had pro-inflammatory uropathogenic bacteria. This population included *Streptococcus anginosus*, *Anaerococcus lactolyticus*, *Anaerococcus obesiensis*, *Actinobaculum schaalii*, *Varibaculum cambriense*, and *Propionimicrobium lymphophilum* (Shrestha et al. 2018). These bacteria are associated with prostatitis, UTIs, bacterial vaginosis, and sexually transmitted infections (STIs). Pathogenic bacteria may initially recruit macrophages and neutrophils that release reactive oxygen species and pro-inflammatory cytokines that destroy DNA. Repeated bacterial infections may lead to proliferative inflammatory atrophy, which is recognized as a benign lesion that has the potential to become cancerous (Woenckhaus and Fenic 2008).

Whether the prostate itself has a microbiome or not has been debated. Some studies argue that in a healthy prostate, prostatic fluid is antibacterial, which prevents the growth of bacteria (Porter et al. 2018; Com et al. 2003). Other studies have found bacterial DNA in prostatic fluid. Ma et al. have shown that the prostatic fluid showed differences in bacterial composition between healthy volunteers and prostate cancer patients. While the species present were similar between the two groups in this study, prostate cancer patients had less microbial diversity and evenness (Ma et al. 2019).

The growth of some prostate cancers is dependent on androgens, such as testosterone. Therefore, androgen ablation therapy has been the conventional treatment in preventing the growth of tumors (Feldman and Feldman 2001). Commensal bacteria in the gut have been found to catabolize testosterone. In one study, the comparison of free testosterone levels in the gut between germ-free and conventional mice showed that germ-free mice have higher testosterone levels (Colldén et al. 2017). Conversely, certain bacteria can also promote the production of testosterone. *Lactobacillus reuteri* increased serum concentrations of testosterone when supplemented in the diets of mice (Poutahidis et al. 2014). Besides, members of the intestinal microbiota, such as *Clostridium scindens*, can produce androgens from compounds often administered to cancer patients such as glucocorticoids such as cortisol (Ridlon et al. 2013). We have shown that bacteria can directly utilize chemotherapeutic drugs such as abiraterone acetate, an inhibitor of the androgen receptor CYP17A, to suppress the production of testosterone (Abdur-Rashid et al. 2019). Potentially, the gut microbiota composition can determine the growth rate of prostate cancer and impact the efficacy of hormone therapies (Porter et al. 2018).

8.6 Gut Microbiome and Urinary Cancers

The urinary microbiome can be influenced by the gut microbiome. Indeed, a 1% abundance of *Enterococcus* in the gut microbiota of kidney transplant patients increased the risk of *Enterococcus* bacteriuria and urinary tract infection (Magruder et al. 2019). How pathogenic bacteria from the gut initially seeds the urinary tract was not described in this study. Whether an increase in gut bacteria that support tumor growth (ex. *F. nucleatum*) can subsequently change the urinary microbiome composition and induce malignancies in the urinary system should be explored in future.

The gut microbiome can also have indirect impacts on the urinary system. As mentioned previously, effectiveness of cancer immunotherapy for renal carcinoma was correlated to enrichment of *A. muciniphila* in the gut. The presence of *A. muciniphila* can induce immune activation that subsequently reduces tumor burden (Routy et al. 2018). This suggests that modifying the microbiome of cancer patients may improve responses to immunotherapy. Currently, several clinical trials are using fecal microbiota transplant (FMT) to test this (NCT03341143, NCT03353402, NCT03772899, and NCT04116775). *A. muciniphila* was also found to be increased in the feces of prostate cancer patients undergoing androgen receptor therapy compared to those who were not. This suggests that future lines of treatment, like immunotherapy, may have better antitumor responses in patients undergoing androgen receptor therapy (Sfanos et al. 2018).

8.7 Conclusion

While not the only factor that affects prevention, causation, and treatment of urological cancers, the microbiota from the urinary system plays a critical role in health and disease. These microbes contribute to the toxification and detoxification of compounds, including pharmaceutical agents which may enter hepatic circulation. The microbiota is also becoming more recognized as an important regulator of the immune system and antitumor immunity. While there is still more to elucidate about the role of the microbiota in urinary cancers, new developments are being made, especially in the context of immuno-oncology and how we can manipulate the microbiome to improve existing therapies.

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Chapter 9
Role of Infections and Tissue Inflammation in the Pathology of the Fallopian Tube and High-Grade Serous Ovarian Cancer

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Abstract High-grade serous ovarian cancer (HGSOC) is the deadliest gynecological malignancy, with a 5-year survival rate of 30–40%, caused by late detection, high recurrence rate, and pervasive resistance to platinum chemotherapy. Moreover, the heterogeneous clinical presentation of this occult disease and failure to determine actionable molecular and genetic subtypes hampers the development of targeted novel therapies. A majority of HGSOC cases originate from the neighboring fallopian tube epithelium (FT), but the exact mechanisms of cellular transformation and early metastasis to the ovary remain elusive, and our knowledge about the interplay of risk factors remains rudimentary. Recent data from the novel in vitro models of chronic *Chlamydia trachomatis* infection in human fallopian tube organoids and patient-derived cultures from cancer tissue deposits suggest there is a molecular link between the regulation of stemness and differentiation in the epithelium and tumor drivers of HGSOC. The chapter focuses on this complex relationship between pathogens, tissue renewal, and inflammation in the upper genital tract and provides an overview of current knowledge about the cellular mechanism of cancer development. The inflammatory microenvironment influences both early carcinogenesis and the progression of advanced disease, thus these discoveries have important implications for the development of diagnostic tools and innovative lines of therapy.

Keywords Cellular origins of ovarian cancer · Adult stem cells · Breast cancer · Patient-derived organoids · *Chlamydia trachomatis* · Chronic infections · Inflammation · Reactive oxygen species · Stem-cell niche · Tumor microenvironment

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9.1 Introduction

Despite great improvements in cancer treatments in recent years, ovarian cancer remains a great challenge for medical professionals in the twenty-first century, especially its most aggressive and occult type, high-grade serous ovarian cancer (HGSOC). While a range of factors has been associated with increased risk for this malignancy, there is no clear understanding of when and why it develops. During their lifetime 1 in 75 women will develop ovarian cancer, and a vast majority of cases are sporadic without clear hereditary risk. Only 2.5% of women diagnosed with cancer are ovarian cancer patients, but the disease is responsible for over 5% of cancer deaths due to extraordinarily high mortality (Torre et al. 2018). Several comprehensive reviews in recent years covered different aspects of advances in HGSOC research and therapy, including the development of new models, signaling interactions between tumor and the surrounding healthy tissue, mechanisms of platinum resistance development, and testing of new lines of immunotherapy (Maru and Hippo 2019; Pogge von Strandmann et al. 2017; Damia and Broggini 2019; Lee et al. 2013). This chapter outlines current research concepts and the most important questions relating to the early stages of HGSOC carcinogenesis and focuses on the role of the local microenvironment in this process. This includes the interplay of physiological, hormonal factors, ageing, and the potential involvement of pathogens and other inflammatory microbial agents. Infections by pathogenic bacteria and viruses and the presence of the microbiota as part of the normal flora recently became a focal point of the broader field of cancer research. Several studies in recent years revealed previously overlooked signatures that are indicative of a microbial contribution to cellular transformation in human malignancies and could explain the context that favors the expansion of specific malignant phenotypes in human colon (Pleguezuelos-Manzano et al. 2020; Wilson et al. 2019; Cougnoux et al. 2014). This chapter pays special attention to the potential contribution of sexually transmitted disease (STD) pathogens to the carcinogenesis of HGSOC. In this context, the chapter reviews advances in using novel patient-derived organoid models to identify and assess in vitro the critical molecular events that cause the development of malignancy. Organoid models, a pioneer methodology, made it possible to preserve and maintain in vitro the inherent capacity of mucosal surfaces to regenerate over time, thus creating an opportunity to study key features of human tissue homeostasis. Thereby, early cellular changes in the fallopian tube epithelium that precede the development of tubal pathology sequels can be analyzed in a controlled experimental system, previously impossible feat due to the inaccessibility of the fallopian tube. Also, the realization that epithelial progenitor stem cells of the fallopian tube and the ovarian mucosa regenerate the epithelial surface in response to tissue damage has provided a new angle of research. An increasing body of experimental data is emerging, which supports a model that adult tissue stem cells and tissue mechanisms that control their function are likely central players in the process of cellular carcinogenesis in general. Cancer tissue maintains a hierarchical organization, and the population of cancer stem cells powers its growth, a common

characteristic of all solid malignancies (Kreso and Dick 2014). Though further studies are necessary, it is becoming increasingly clear that epithelial cells "remember" infections and that these changes extend to the level of genome regulation and regulation of stemness. Therefore, frequent exposure to the genital pathogens of the fallopian tube mucosa should be considered as a risk factor within postulated models of HGSOC development. In addition to infection-related changes in the epithelial homeostasis, inflammatory processes associated with infections and long-term consequences likely contribute to the immunogenic profile of the tumor. A better understanding of the biology of the inflammation and immune system activation in the genital tract is essential to improve the response to checkpoint inhibitor-based immunotherapies in HGSOC patients. In this context, the chapter will discuss the role of tissue inflammation as a potential niche that confers selection pressure and favors the survival of the malignant cells in the fallopian tube at the expense of the healthy epithelium. Deciphering early steps in HGSOC carcinogenesis is also of pivotal importance to identify sensitive and specific biomarkers of tissue pathology and thereby improve strategies for timely cancer detection and diagnosis. Taken together, the chapter aims to provide a comprehensive overview of different perspectives on HGSOC development based on the data from epidemiology, clinical practice, and fundamental molecular studies. With a focus on the complex relationship between inflammation, infection, microbiome, and cancer, the goal is to describe the current knowledge and discuss new research directions that could help to improve the management of this deadly disease.

9.2 Classification of Epithelial Ovarian Cancer

Ovarian cancer is a heterogeneous malignant disease of highly versatile clinical presentation, unspecific symptoms that lead to delayed diagnosis, and limited therapeutic options. Each year around 240,000 women worldwide are diagnosed with a disease. The 5-year survival rate remains between 30–40% despite improvements in surgical management and best efforts to optimize therapeutic regimens. The vast majority of ovarian cancers originate from the epithelium (90%), and the remaining 10% derives from the stromal compartment (5–6%) or germ cells (3–4%). Epithelial ovarian cancer (EOC) includes five histological subtypes which are characterized by substantial differences in tissue morphology and molecular profile: high-grade serous (70%), endometrioid, (10%), clear-cell (10%), mucinous (3%), and low-grade serous borderline (5%). Variability is likely caused by distinct etiologies and mechanisms of cellular transformation of each subtype (Fig. 9.1). Based on the mutational profile, the existence of known precursors and clinical progression EOC can be classified into (1) low-grade serous and (2) high-grade serous. Low-grade ovarian cancer originates from the surface of the ovary, develops slowly in a gradual transition from benign neoplastic tissue toward borderline tumors and slow invasive malignancy. Endometrioid and clear-cell carcinoma are typical representatives of type I cancers that likely develop as the sequel of benign cysts of the ovary or

a

stage	localization	5-yrs	incid.
I	limited to ovaries: one or both; intact /ruptured capsule	75-90%	9%
II	pelvic extension; spread to FT, uterus, bladder, rectum	45-60%	7%
III	spread to abdomen; deposits in peritoneum +- lymph node	30-40%	51%
IV	distant metastaes pleural effusions, liver paren.	<20%	29%

FIGO classification of ovarian cancer

b

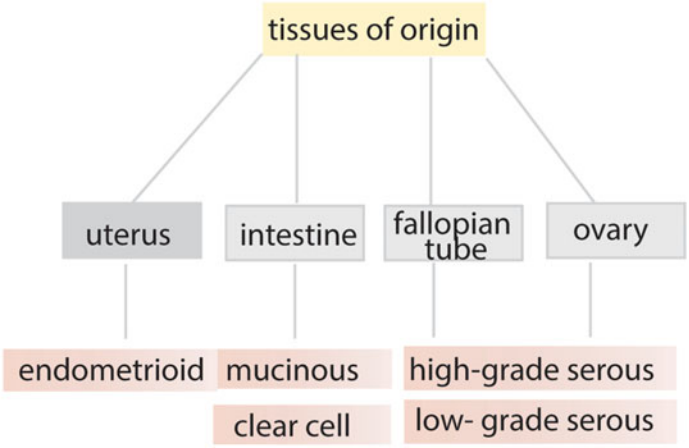

Fig. 9.1 (**a**) Classification of epithelial ovarian cancer based on stages. International Federation of Gynecology and Obstetrics (FIGO) criteria. (**b**) Classification of epithelial ovarian cancers based on the tissue of origin

underlying endometriosis, a prevalent, benign condition of hyperproliferative and ectopic endometrium (Dawson et al. 2018; Banet and Kurman 2015). Mucinous carcinoma and low-grade serous cancer also belong to the type I group low-grade malignancies (Kelemen and Kobel 2011) and are histologically similar to colonic and tubal epithelium, respectively. On the genomic level, type I EOCs are characterized by the frequent presence of a set of somatic mutations: PTEN, K-RAS, BRAF, ERBB2, ARIDA, and specific for endometrioid CTNNB1 (Koshiyama

et al. 2014). Low-grade ovarian cancers normally express wild-type TP53, and this property can be used in the differential diagnosis as a distinction from high-grade EOC. HGSOC, type II epithelial ovarian cancer, is almost uniformly mutated for TP53 and harbors a set of other genomic alterations that are highly diverse between patients. Genomic or epigenomic inactivation of BRCA1/2 genes is found in ~50% of cases. HGSOC is the most frequent (~67%) and aggressive form of EOC as >70% of patients are diagnosed >stage III (Fig. 9.1). At this stage, the 5-year survival rate is <40%, making HGSOC the most lethal gynecological malignancy.

9.3 HGSOC: Molecular Characteristics, Origins, and Main Risk Factors

Late diagnosis of the primary tumors and chemoresistant phenotypes in recurrent disease strongly limit therapeutic options and cause high mortality in HGSOC patients. The inability to identify clearly defined premalignant precursors on the surface of the ovary raised fundamental questions about the origins of this cancer type. Several competing models were proposed during the past decades to explain carcinogenesis of HGSOC: the transformation of cortical inclusion cysts, direct transformation of ovarian epithelial cells, and transformation of fallopian tube epithelial cells. Today, experts in the field, scientists and clinicians, have reached a broad consensus that accepts the central importance of the fallopian tube for the development of HGSOC. It is now presumed that HGSOC is in essence a peritoneal malignancy of complex etiology that originates from the fallopian tube epithelium, with ovarian localization representing the preferable route of the metastasis. Due to the exceptional heterogeneity of the disease, it cannot be excluded that some HGSOC cases do arise from the ovarian surface epithelium (OSE) or metastasize from other regions of the genital tract, but fallopian tube involvement appears essential in the majority of cases (Vaughan et al. 2011; Bowtell et al. 2015).

Advances in next-generation sequencing (NGS) and other omics technologies (proteomics, transcriptomics, phenomics, etc.) enabled detailed molecular profiling of human cancers and big data analysis of cellular networks that drive cancer growth. The Cancer Genome Atlas (TCGA) Program, a large platform funded by the National Institute of Health (NIH) and the National Cancer Institute (NCI), performed detailed molecular profiling of over 20,000 cancer samples from 33 different cancer types to date, among them 489 HGSOC cases (Cancer Genome Atlas Research, N 2011). Sequencing confirmed the great variability in the landscape of somatic mutations in HGSOC tissues, as only TP53 (96%) and BRCA1/2 (20%) are mutated in more than 10% of the patients. However, broader network analysis that integrated transcriptomics data, as well as genomic copy number variations, revealed that PI3 kinase and RB1 pathways are deregulated in 67% and 45% of cases, respectively. The study classified HGSOC samples into four subtypes based on their global gene expression profile: differentiated, immunoreactive, mesenchymal,

and proliferative. However, despite the progress made in the understanding of the biological variations in HGSOC phenotypes, the direct clinical relevance of this study remained modest. Except for BRCA 1/2 mutation status, all attempts to stratify HGOSC patients based on the tumor characteristics, including the effort by the TCGA, failed to show a clear benefit in the clinical setting. As a result, the main components of the treatment in HGSOC patients have not changed for decades, and all patients receive platinum-based therapy in combination with debulking or interval surgery.

Numerous studies of candidate risk factors associated with HGSOC development failed to establish a clear causative relationship for any of them or explain the cascade of molecular events that lead to malignancy. The occurrence of HGSOC is more likely in women who had many undisrupted ovulatory cycles, suggesting that hormonal milieu is an important factor during carcinogenesis. Nevertheless, HGSOC is a disease of menopausal age (average age of >55 years at the time of the first diagnosis), usually detected at a time where the active ovarian function has already subsided. The inaccessible location of the ovaries and fallopian tubes, tucked within the abdomen, represents a major hurdle to develop noninvasive surveillance strategies. Also, available biomarkers such as CA125 have limited specificity. CA125 belongs to the mucin protein family (MUC16), expressed in the healthy fallopian tube epithelium, but patients with advanced stages of HGSOC have strongly increased levels of the secreted form in the circulation. However, CA125 is increased in only ~60% of stage I HGSOC cases and is also found elevated in benign conditions such as endometriosis (van Haaften-Day et al. 2001; Kitawaki et al. 2005). Consequently, screening in the general population causes a high rate of false positives leading to unnecessary invasive treatments without improvement in early HGSOC detection rates (Henderson et al. 2018). Thus there is no effective medical procedure to screen for HGSOC in asymptomatic women, and in many countries mandatory ultrasound examinations combined with CA125 levels have been abandoned. There is also no systematic effort to follow the impact of genital infections on the pathology in the upper genital tract including the development of HGSOC. The high prevalence of sexually transmitted infections (STI) means that the majority of women have been exposed to genital pathogens at some point during their reproductive active years. Harald zur Hausen was awarded Nobel Prize in medicine in 2008 for the discovery that HPV viruses are the central culprit behind the development of cervical cancer (Boshart et al. 1984; zur Hausen 2002). This breakthrough in cancer research had an immensely positive impact on women's health. Regular screenings, the Pap smears, and vaccination against oncogenic HPV strains represent a successful example of efficient public health policy which resulted in declining rates of cervical cancer. By contrast, for a myriad of reasons, improvement in the diagnosis and management of serous ovarian cancer has been minimal. The role of infections in HGSOC etiology has for a long time been a matter of controversy. The unique methodological challenges of STI serology in connection with HGSOC will be discussed in more detail below, but inconsistencies led to wide skepticism about a role for pathogens in HGSOC development.

Still, regardless of these problems, the role of pathogens causing tubal pathology, in general, is supported by a large body of experimental and epidemiological evidence and as such has to be revisited in light of the "tubal origins" of HGSOC hypothesis.

Independent of the type of risk studied, hormonal, hereditary, or infection-related, it would be exceptionally important to identify molecular events that connect risk factors with the process of cellular transformation in a causative fashion. To achieve that, understanding of the basic regulatory mechanisms of the healthy fallopian tube mucosa is necessary.

9.4 The Fallopian Tube as a Tissue of Origin of Ovarian Cancer

Histological characteristics of the HGSOC tissue such as serous papillary structure and PAX8 expression are reliable diagnostic tools to distinguish ovarian cancer from other mesothelial malignancies (Laury et al. 2010). Also, its phenotype is much more similar to the healthy fallopian tube epithelium than to the ovarian surface epithelium. The fallopian tubes develop from an embryonic structure called Müllerian tract, which is Pax8 positive. They connect the ovary to the uterus and provide a niche for the first phase of human embryo development. Conception occurs in the fimbrium, the distal segment of the tube. Afterward, the embryo takes 3–4 days to travel toward the uterus where it implants, 7–10 days after fertilization of the oocyte. Fallopian tube mucosa is made of polarized epithelial cells which are either secretory (PAX8+) or ciliated (PAX8−). The columnar monolayer is organized into many longitudinal folds (plicae) particularly in the distal region facing the ovary. The proper function of the fallopian tube mucosa, the beating of the ciliated cells, and tubal peristalsis are important for the transport of the gametes and reception of the released oocyte (Lyons et al. 2006). The surface of the ovary, which develops from the gonadal ridge (intermediary mesoderm), is covered with flat cuboidal PAX8— epithelium. Despite the failure to identify precursor lesions or clear preneoplastic stages on the surface of the ovary, many models of HGSOC carcinogenesis postulated that the malignancy originates locally due to a clear presentation of tumor masses at the time of the diagnosis. Diverging cellular phenotypes between the healthy ovary and the cancer were explained by the presumptive conversion/transdifferentiation from simple cuboidal ovarian surface epithelium (OSE) into the more structured columnar epithelium of HGSOC. Proof of concept that such transdifferentiation is possible was shown in transgenic mice by ectopic expression of HOXa9, HOXa7, and HOXa11 genes in OSE cells (Cheng et al. 2005) that drove differentiation into distinct histological types. The cortex of human ovaries is known to include cortical cysts (CIC) that contain either PAX8+ epithelial cells or OSE cells. CICs likely develop by a process of epithelial invasion at the time of post-ovulatory injury (Banet and Kurman 2015). CICs are considered as prime candidates

among putative locations where transdifferentiation could occur. One study reported mixed types of cysts identified in BRCA1/2 carriers based on incidental co-expression of Pax8 and the mesothelial marker calretinin (Park et al. 2018). Yet, no cascade of cellular events and transition phenotypes in patient samples has been identified so far that could fully explain the carcinogenesis of HGSOC at the surface of the ovary, although OSE epithelium remains a candidate tissue for at least a subset of cases.

At the turn of the century, carriers of the BRCA1/2 mutation who had cancer risk-reducing surgeries were shown to have distinct neoplastic regions in the epithelium of the distal fallopian tubes. The existence of these atypical cellular clusters termed *s*mall *t*ubal *i*ntraepithelial *c*arcinoma (STIC), was confirmed in numerous multicentric studies at a frequency of 2–8%, providing the first comprehensive and consistent clinical evidence of HGSOC precursor lesions (Powell et al. 2011). STICs are characterized by a TP53 mutant phenotype, polymorphonuclear atypia, high Ki-67 index, and several additional markers that distinguish them from the surrounding healthy fallopian tube epithelium (Lee et al. 2007). It remains unclear if all STICs progress to cancer and if an isolated STIC finding warrants clinical intervention (Meserve et al. 2017), but the majority of patients with advanced malignant disease have STICs in their fallopian tubes (Carlson et al. 2008). A recent detailed analysis by NGS sequencing confirmed continuity in the lineage between STICs and genomic profiles of the advanced cancer tissue in the HGSC patients (Labidi-Galy et al. 2017). Interestingly, based on the analysis of somatic single-copy DNA alterations, lineage continuity could be established between fallopian tube epithelium and malignant HGSOC deposits even in the patients where STICs were not found. Interpretation of these findings implies that STIC formation is not a necessary intermediary step in the HGSOC development and that independent transformation routes of fallopian tube epithelium exist (Ducie et al. 2017).

Competing models of HGSOC development have been tested in the mouse model. Induction of HGSOC malignant transformation by concomitant mutant TP53 and SV40 expression, in parallel setup, revealed that cancer can originate from both ovarian surface and fallopian tube epithelium. Experiments with targeted tissue-specific in vivo genetic inactivation of the RB pathway (by expression of SV40) in a TP53 mutant background of the ovary or fallopian tube epithelium showed that in both locations genetic manipulation leads to the development of malignancy (Zhang et al. 2019). The existence of different tissues of origin could also explain the great heterogeneity of the disease that is observed in clinical practice. Interestingly, although the underlying genomic perturbation was identical, tumors arising from the FT epithelium and OSE epithelium exhibited differences in phenotype, gene expression pattern, and response to drug treatment. Fallopian tube tumors grew faster and disseminated more profusely compared to ovarian tumors but appeared to be more sensitive to carboplatin which is the first line of treatment for HGSOC patients. Again, it can be concluded that tumor development has to be analyzed in the broader context of cell-cell communication and the local tissue environment. Nevertheless, critical pieces of the puzzle are missing to understand under which conditions transformation occurs and what are the key molecular events

that lead to the development of malignancy from either of these two locations. Also, the complexity of the ovarian surface epithelium reprogramming remains difficult to prove. Notably, although PAX8 is one of the most reliable diagnostic markers of HGSOC, OSE-induced tumors in this study showed only weak PAX8 positivity. Thus it is not clear if this animal system adequately recapitulates the mechanism of the human disease.

9.5 Epidemiology Studies of HGSOC Prevalence and Main Risk Factors

9.5.1 Model of "Incessant" Ovulation as the Main Driver of HGSOC

During the process of spontaneous ovulation, the oocyte release is accompanied by follicular fluid release. This fluid contains a complex mixture of gonadotropins (FSH, LH), estradiol, progesterone, prolactin, inhibin, corticoids, growth factors of the TGF-β family, interleukins, and reactive oxygen species (ROS) (Revelli et al. 2009). The fluid is released into the abdomen, in a distinct recto-uterine region, termed pouch of Douglas, and can be used as reliable sonographic evidence of ovulation. Due to the anatomical proximity, a fraction of the fluid is flushed into the distal fallopian tube along with the oocyte. Thus, the epithelial surface of the ovary and the lining of the fallopian tube are exposed to direct stimulation with hormones and ROS regularly in each cycle. This was recognized early as a potential risk factor for cancer development in the upper genital tract and led M.F. Fathalla to postulate the theory of "incessant ovulation" as the main driver of the HGSOC carcinogenesis as early as 1971 (Fathalla 1971). More than four decades later, epidemiological studies have provided ample evidence that the hypothesis is valid at least as an important contributing factor in the multistage process of HGSOC development. Virtually no other individual physiological event is as consistently connected with an increased risk for ovarian cancer than ovulation. Early menarche and late menopause, ovulation-inducing treatments, and nulliparity all cumulatively result in a higher number of ovulatory cycles and have consistently been shown to be independent factors that elevate risk (Titus-Ernstoff et al. 2001). In agreement with this, long periods of suppression of ovulation such as pregnancies and hormonal birth control that inhibit the function of the ovary have protective effects and reduce the risk (Tworoger et al. 2007). There are many potential explanations and interpretations of these epidemiological facts. The exact mechanism of how ovulations promote transformation is still poorly understood and participation of ROS is likely to be only one of the contributing factors.

The regeneration potential of the ovarian epithelium surface is activated monthly in cycling women, to repair the epithelial injury triggered by follicular rupture. This somewhat abrupt process, is associated with a cascade of physiological events that

resemble a strong inflammatory reaction. It has been shown that ROS play an essential role in ovulation, as exposure of the follicles to antioxidant agents at the time of the LH surge strongly reduces the number of released oocytes (Shkolnik et al. 2011). The study also convincingly showed in the mouse model that inhibition of ROS signaling prevents the LH-induced rise in progesterone synthesis, a critical luteinization signal in the preovulatory follicle. Indeed, the presence of ROS in the follicular fluid likely has a direct effect on the oocyte itself influencing fertilization and early embryo development. Studies in the field of assisted reproduction that investigated the putative association between levels of ROS in the follicular fluid of retrieved oocytes and the outcome of the subsequent in vitro procedure could show that there is indeed a window of ROS concentration that supports optimal fertilization and early embryo development (Attaran et al. 2000; Jozwik et al. 1999). While a certain basal level of ROS in the follicular fluid is necessary for the development of a healthy oocyte, the elevation of ROS above a defined threshold has a strong negative effect (Wiener-Megnazi et al. 2004). The study also found a correlation between rising ROS levels and the age of the women, which could help to explain the mechanism behind a sharp reduction in the quality of produced oocytes in patients over 40 years of age (Huang et al. 2015).

The detrimental effect of ROS exposure on the fallopian tube epithelium could be demonstrated in a study that analyzed the in vitro physiological properties of 11 samples of follicular fluid from IVF patients. Treatment with samples containing high ROS concentration caused an increase in double-stranded DNA breaks and intracellular ROS production in the culture of primary human fallopian tube explants. The study also confirmed that follicular fluid has a general tumor-promoting potential as injection of ROS high fluid triggers faster onset of tumor formation in TP53−/− mice. The second study from the same group established a positive effect of hemoglobin in the follicular fluid on the survival of fallopian tube cells that have been damaged by ROS or have depleted p53 levels (Huang et al. 2016). This report proposes a potentially interesting and significant concept of pro-tumorigenic action via the rescue of damaged cells.

9.5.2 The Inheritable Risk Associated with BRCA1/2 Status

In the general population, HGSOC is a rare disease that affects approx. 1 in 70 women during their lifetime. In stark contrast, women who carry germline mutations in the BRCA1 or BRCA2 genes that regulate DNA homologous recombination have a >50% lifetime risk of developing HGSOC and breast cancer, depending on the exact type and position of the nucleotide changes (Miki et al. 1994). Also, BRCA1/2 mutation carriers have a 2–5 times higher risk to develop pancreatic cancer in comparison to the general population (Greer and Whitcomb 2007). BRCA1 and BRCA2 proteins are involved in one of the core mechanisms that preserve genome integrity, with each having their own unique and non-redundant function. BRCA1 is recruited to DNA double-*s*tranded *b*reaks (DSB) and involved

in the regulation of the G2/M and S—checkpoints and correction of mistakes in nucleotide base incorporation during DNA replication. BRCA2 is a component of the core machinery of homologous recombination which is the main mechanism for correcting DSBs (Roy et al. 2011). HGSOC cancers in germline BRCA1/2 carriers exhibit almost uniform loss of heterozygosity (LOH) (Maxwell et al. 2017). This is a process whereby a wild-type copy of the gene initially present at one of the chromosomes in the germline is eliminated from the genome in the cancer tissue. This convincingly demonstrates the critical importance of inactivation of DSB repair in the process of carcinogenesis of HGSOC (Hilton et al. 2002). The sensitivity of the "BRCAness" cancer phenotype to the increase in DSBs was confirmed by the discovery of PARP1 inhibitors. The PARP1 protein regulates single-strand DNA repair constituting the first line of the defense that protects genome integrity. Its inhibition unavoidably leads to an increase in unrepaired single-strand DNA breaks, and thus the higher activity of DSB repair mechanisms is required to maintain DNA integrity. Cells competent for DSB repair function with wild-type BRCA1/2 can functionally compensate for the defect, while mutant cells die. This genetic phenomenon called "synthetic lethality" has been successfully adopted for the implementation of PARP inhibitors in clinical practice for the treatment of BRCA1/2-deficient HGSOC patients (Lord et al. 2015; Fong et al. 2009).

The enormous and specific inheritable burden that germline carriers of BRCA1/2 mutation have for the development of breast and ovarian cancer is suggestive of a role of endocrinological factors in the selection process of transformed clones. Although DNA homologous repair is part of the core housekeeping functions in the cell, heterozygote defects in the BRCA gene can be functionally compensated in most tissues. The mechanism behind this extraordinary context-specific risk is yet to be understood, although it has recently been reconfirmed by a large-scale NGS study across a set of malignancies. Genomic profiling of more than 17,000 patients of 55 different cancers and corresponding germline controls revealed that BRCA1 and BRCA2 mutations occur sporadically in other cancer types (2.7% and 1.8%, respectively) but follow a clear pattern of passenger mutations. Loss of heterozygosity, biallelic inactivation, and sensitivity to PARP inhibitors are detected exclusively in the cancers for which BRCA1/2 is known to be a heritable risk. This is also confirmed by enrichment in the genomic homology-directed repair (HDR) signature. Exon sequencing conclusively showed that defects in DNA homologous repair undergo positive selection in HGSOC, breast, and to some extent in pancreatic cancer (Jonsson et al. 2019) while other malignancies remain HDR competent, even in the presence of BRCA1/2 heterozygous mutations.

The folliculogenesis phase of the menstrual cycle is associated with rising levels of unopposed estradiol. Nevertheless, experience from hormone replacement therapy treatments also shows an increased risk for HGSC development regardless of the type of hormones used (Zhou et al. 2008) (single-agent estradiol or double agent in combination with progestins). This suggests that there is a more complex connection of the hormonal milieu with this malignancy and no definitive classification of any of the hormones as "carcinogenic" is justified. It is necessary to point out that hormones not only regulate homeostasis of the mucosal surfaces of the uterine tract but also

have an impact on immune system activity. Therefore, a whole range of indirect effects related to changes in the endocrinological environment may play a role and would require focused studies to determine their relevance for HGSOC development.

In breast cancer, the role of estradiol has been extensively studied. A subtype of this malignancy, tumors that express estradiol receptor ER are strongly dependent on the estradiol signaling to maintain growth. Overall, roughly two thirds of all breast cancers are ER-positive, and while the majority of BRCA1-mutated cancers are triple-negative (ER-/PR-/HER-), 10–35% do express ER (Atchley et al. 2008). This was recognized early and has been exploited successfully for over five decades by the addition of tamoxifen in breast cancer treatment (Jaiyesimi et al. 1995). Tamoxifen is a nonsteroidal triphenylethylene derivative that blocks the ER receptor function by competitive binding and thereby inhibits proliferative estradiol signals in the breast tissue (Coezy et al. 1982). The molecular action of tamoxifen is complex and involves targeting both epithelial cells and the stromal compartment (Colletta et al. 1990). One of the most potent growth restricting actions of tamoxifen is the induction of TGFβ1 in the stromal compartment (Butta et al. 1992). Interestingly this phenomenon is not restricted to ER + patients and could perhaps explain the certain protective effect of tamoxifen also in the hormone receptor-negative breast cancers (Yang et al. 2012).

Antitumorigenic properties of tamoxifen in the breast have also been confirmed in the early stage of cancer development, as continuation of therapy greatly reduces the risk of occurrence of the contralateral malignancy in BRCA mutation carriers (Narod et al. 1998). Wider implementation of tamoxifen as a prophylactic therapy has been contraindicated due to significant side effects such as endometrial cancer or pulmonary embolism (van Leeuwen et al. 1994).

Despite significant parallels between breast and ovarian cancer in the profile of risk factors for malignant transformation, including the hereditary risk mediated by BRCA1/2, tamoxifen is not effective at suppressing the growth of the serous ovarian cancer (Shirey et al. 1985). And although endocrine therapy has been continuously evaluated and could provide limited benefits for some advanced patients, this is not comparable to the high effectiveness of tamoxifen in breast cancer (Paleari et al. 2017). This is the case despite the fact the ERα receptor is expressed in >50% of HGSOC cases (Pujol et al. 1998). And despite of a clear association between the follicular phase of the menstrual cycle, which is high in estradiol signaling, and the risk for HGSOC development. This stark difference in the response of these two malignancies to endocrinological therapy is a powerful illustration of the complex molecular etiology of cancer development and progression in each tissue.

9.5.3 *Recurrent Episodes of Infection and the Risk of HGSOC Development*

Sexually transmitted diseases, caused by pathogenic bacteria and viruses that frequently colonize the lower genital tract, are extremely frequent with more than *377 million new infections* annually, according to estimates of WHO (Newman et al. 2015). The most prevalent causative agents are *Chlamydia trachomatis* (>100 million), *Neisseria gonorrhoeae*, *Treponema pallidum* (syphilis), trichomoniasis, and herpes simplex virus 2 (HSV-2). STIs represent a major burden for public health, as problems in timely diagnosis and treatment can lead to the development of more complex pathological sequels, with infertility being the most widely recognized long-term morbidity.

Chlamydia trachomatis (Ctr), a gram-negative obligatory intracellular pathogen, is the most frequent STD-causing bacterium and the major causative factor of preventable infertility in women today. *Ctr* infection is efficiently cleared with the administration of doxycycline or azithromycin therapies (Lau and Qureshi 2002), but the absence of symptoms in the majority of cases prevents appropriate diagnosis and therapeutic intervention. It is estimated that roughly 10% of *Ctr* infections pass the cervix and colonize the uterus and fallopian tubes, causing inflammation, scarring, and sustained tissue damage. Asymptomatic chronic salpingitis, inflammation of the fallopian tubes, is mainly caused by *Ctr* and is the main risk factor for the development of tubal occlusion that prevents conception. Accordingly, past *Ctr* infection strongly increases the odds of ectopic pregnancy (Chow et al. 1990). The complication of salpingitis, termed hydrosalpinx, where the closure of the distal tube causes a characteristic accumulation of fluid represents a strong negative prognostic factor for the success of in vitro fertilization cycles, thus surgical removal is indicated before the procedure (Strandell et al. 2001). Recurrent episodes of pelvic inflammatory disease (PID) and *Ctr*-driven salpingitis are well documented; however, there are indications that a certain degree of protective immunity develops during *Ctr* infection, at least in some individuals. This conclusion is based on the fact that despite improvements in screening and rising numbers of early intervention and treatments, the number of infections continues to rise (Batteiger et al. 2010), sometimes at levels that surpass initial infection rates. Interpretation of these data from large population cohorts suggests that early detection and antibiotic treatment interferes with effective adaptive immunity reaction, making reinfections upon new exposures more likely than in patients where the infection is encountered only with natural immunity.

9.5.4 The Serologic Evidence of Chlamydia Infection in Cancer Patients

The adaptive immune response to a *Ctr* challenge shows significant individual variability among patients, while a decline in the IgG titers over time complicates epidemiological studies and interpretation of data (Henry-Suchet et al. 1994). There is a strong correlation between the level of *Ctr* antibodies and the probability of tubal pathology (Mardh 2004). However, the statistical analysis of the putative connection between *Ctr* infection and diseases that occur later in life such as ovarian cancer remains challenging. Overall, past studies have provided mixed results. The key obstacle for epidemiological analysis is the selection of suitable serological biomarkers to discriminate the patients who had only vaginal *Ctr* infection from the group that developed ascending infections and associated pathology sequels, pelvic inflammatory disease (PID), and salpingitis.

A large retrospective population study from Thailand determined an increased risk for the development of ovarian cancer in patients with recurrent PID episodes (hazard ratio O.R 2.46 for 5 episodes) (Lin et al. 2011). Repeated analysis with a longer follow-up period and slightly altered inclusion criteria failed to establish the connection between the two (Shen et al. 2016). One of the critical differences discussed by the authors is the inclusion of patients who had cervical, vaginal, and vulvar inflammatory disease in the first study and shorter follow-up periods that could explain the discrepancy in the outcome.

Epidemiological studies that directly analyzed serology parameters related to *Ctr* infection also provided conflicting accounts. Ness and colleagues performed two studies and compared the frequency of positivity to *Ctr* antibodies against elementary bodies and heat shock protein (HSP60) among high-grade serous patients and healthy controls. The first study involving 117 subjects found a significant correlation (Ness and Cottreau 1999), but the effect was exactly the opposite in the second larger cohort of 521 patients (Ness et al. 2003). The problem of specificity and sensitivity of the selected serological markers is highlighted as a potential confounding factor. Also, these studies involved ovarian cancer patients without classification of the cancer histological type. Considering the large differences in clinical presentation and disease characteristics between low-grade and high-grade cancers, it is likely that this could also contribute to the fluctuations in the results. A genome-wide screen of the *Ctr* proteome based on recognition by sera of *Ctr* infected patients revealed 27 candidates as immunodominant antibodies that were recognized in >50% of cases (Wang et al. 2010). Among them, an antibody against Pgp3, plasmid-encoded secreted protein emerged as one of the most stable biomarkers of the sustained host response to *Ctr* despite failure to provide protective immunity in humans (Chen et al. 2010). A new study of two independent patient populations, one retrospective and one prospective nested-case control study, established an increased risk for ovarian cancer development by using Pgp3 seropositivity (Trabert et al. 2019). Interestingly, the study also compared Ab-Pgp3 against other *Ctr* antibodies (anti-MOMP, anti-Tarp, and anti-HSP60) and found it

superior in its predictive power. Analysis of risk odds for ovarian cancer in infections with other pathogens (*Mycoplasma genitalium*, HPV, herpes simplex virus 2, hep-B, hep-, EBV, and CMV) failed to show any significant positive association in these two study groups.

9.5.5 Coinfections and HGSOC

STIs are frequently being transmitted not as a single-agent disease but include simultaneous co-infection with different pathogens. In some cases, this has significant ramifications for the development of disease and the risk of further transmission. For example, the risk of HIV transmission is much greater in individuals who are positive for STD pathogens than in noninfected individuals (Galvin and Cohen 2004). There is also no clear understanding of what are the critical factors that mediate ascending vaginal STI infections and the passing of the cervical barrier.

The potential contribution of co-infections with different pathogen species further complicates association studies in connection with HGSOC carcinogenesis. Several studies reported an increased incidence of HHV-6 virus sequences integration in ovarian cancer tissue in comparison to matched healthy controls (Banerjee et al. 2017). It has also been shown that *Ctr* infection has the potential to reactivate the expression of HHV-6 sequences from the genome of host cells (Prusty et al. 2013). At the same time, co-infection with HHV-6 in 2D Hela cells model promoted *Ctr* persistency, a form of aberrant infection process that reversibly arrests bacterial development (Prusty et al. 2012). More studies in primary cell culture infection models and better characterization of available clinical samples are needed to assess the significance of these phenomena for the development of tubal pathologies in vivo. But the data raises the important concept of interference and synergy between different pathophysiological mechanisms. *Mycoplasma genitalium* and *Neisseria gonorrhea* (*N. gonorrhea*) are also known as potent colonizing pathogens of the fallopian tube that cause salpingitis, scarring, and infertility (Svenstrup et al. 2008).

Currently, *Ctr* is significantly more prevalent than *N. gonorrhea*, as the gonococcal disease declines worldwide, but there is compelling evidence that the two bacteria thrive in the co-infection setting. A prospective 2-year study in five STD clinics in the USA found that among patients with a confirmed positive diagnosis for *Neisseria*, the incidence of *Ctr* co-infection was 19% in men and 42% in women, much higher than in the control group negative for *Neisseria* (7% in men and 9% in women, respectively) (Lyss et al. 2003). This finding is in line with similar studies in other centers (Dicker et al. 2003; David et al. 1997). The observed difference among sexes is especially interesting. It is suggestive of increased susceptibility to *Ctr* in the presence of *N. gonorrhea* and the existence of specific co-infection conditions in the female genital tract.

Moreover, the incidence of *M. genitalium* was found to be twofold higher in *Ctr*-positive patients than in the control *Ctr* negative group (Chernesky et al. 2017; Harrison et al. 2019). The common belief is that this epidemiological correlation

could be an important factor in the recent dramatic increase in the resistance of *M. genitalium* to macrolide therapy (average 40% globally). Because *Ctr* is usually treated with single-dose azithromycin, when co-infection exists, selection pressure builds for the propagation of *M. genitalium* mutations which confer resistance to macrolides (Mondeja et al. 2018), due to the higher minimal inhibitory concentration that is required to eliminate this pathogen. As is the case with *Ctr*, *M. genitalium* infections are usually asymptomatic, and there are no medical procedures that investigate effects on the host tissue in the patient groups beyond the eradication of the bacteria. Consequently, there are no available studies on potential differences in the tubal pathology depending on the type and combination of pathogens that caused salpingitis.

Also, co-infections have so far not been tested in in vitro models of the fallopian tube. Bacterial pathogens co-exist together in all compartments of the genital tract, and it is entirely possible and indeed likely that one would need to identify niche-specific aspects to establish a definitive connection with the development of ovarian cancer.

9.5.6 Contribution of the Microbiota to the Inflamed Environment

The vaginal microbiome is essential for the establishment and maintenance of the healthy physiological niche in the lower female genital tract. Species of gram-positive *Lactobacillus* (*L. crispatus*, *L. gasseri*, *L. inners*, and *L. jensenii*) are responsible for the acidification of the local environment (pH 4.5) which has an important protective function. Changes in the composition of the vaginal microbiome have been investigated as part of the infertility evaluation. A prospective cohort study identified a prediction model to stratify patients based on types and frequency of *Lactobacillus* species to predict the patients with a low chance of embryo implantation (accuracy 94%, sensitivity 26%, specificity 97%) in IVF- and IVF/ICS cycles (Koedooder et al. 2019). The second study identified changes in bacterial vaginosis scores (BV) to be predictive of the cycle outcome (Haahr et al. 2016, 2019). The prospective case control trial compared microbiomes of ovarian cancer patients, healthy controls, patients with benign gynecological conditions, and healthy BRCA1/2 mutation carriers (Nene et al. 2019). Two types of microbiota, community type L (>50% Lactobacillus species) and community type O (<50% of Lactobacillus species), are found to be unequally distributed within the groups. O type is found to be significantly more frequent in cancer patients and BRCA1/2 women than in age-matched controls. These examples reveal the great potential of microbiome characterization for improving or complementing diagnostic procedures in gynecology. Much more work remains to be done to elucidate the putative involvement of the microbiome in the development of infertility or cancer. As microbiota are always present and changes are often only subtle quantitative changes

in proportions and dynamic of expansion of certain strains, it is challenging to distinguish with certainty coincidence from correlation or causative relationship. Early neoplastic changes in cellular phenotypes could enhance the proliferation of one microbiota species at the expense of the other, while at the same time change in the microbiome could provide selective pressure and create an environment that favors the growth of cancer.

9.5.7 Infertility and Risk of HGSOC Development

Among the potential candidate factors that were studied in association with HGSOC development, infertility was found to be consistently positively correlated. In the age of the great expansion of assisted reproduction technologies (ART), much of the focus has been placed on the potential link between hormonal stimulation protocols that are the central part of IVF protocols and the risk for ovarian cancer. However, similar to other questions related to ART outcomes, it is exceptionally difficult to distinguish to which extent procedure or underlying infertility contributes to the risk. Indeed, when IVF patients are compared with a control group of infertile patients instead of a healthy fertile group, the increase in HGSOC risks often disappears (Siristatidis et al. 2013). Analysis of medical records of the large group of infertile patients (12,183) concerning the causes of infertility found a strong association between endometriosis and tubal disease as risk factors for ovarian cancer development (RR = 2.3 and RR = 2.2, respectively). Also, it needs to be noted that a relatively high risk has been identified for the group of women with "unexplained" infertility (Runnebaum and Stickeler 2001) (odds ratio 2.76) illustrating great gaps in our knowledge of the links between physiological functions of reproductive organs and molecular mechanisms of disease development.

9.6 Infection of the Fallopian Tube Pathogen-Host Interaction and Long-Term Changes in Homeostasis

The ability of *Ctr* to colonize fallopian tube epithelium and cause protracted asymptomatic inflammation of the tube has been well-known for many decades, but its clinical relevance was only considered important in the field of reproductive biology. As advances in assisted reproduction technology development and the broad availability of IVF treatments rendered tubal patency nonessential to achieve pregnancy, the biology of the tubal disease became less relevant. The discovery that high-grade serous cancer originates from the tubal epithelium fundamentally altered this attitude. Retrospective analysis of the long term follow-up of patients after hysterectomies with and without the removal of both adnexa (tubes) clearly showed that a great risk reduction for ovarian cancer development later in life occurs in the

subset of patients with removed tubes. This led to the gradual change in clinical guidance to mandate it whenever is possible in postmenopausal patients (Tamhane et al. 2019; Falconer et al. 2015). A prophylactic salpingo-oophorectomy, complete removal of ovaries and fallopian tubes, is now offered as the standard of care to BRCA1/2 mutation carriers even earlier in life, due to their extraordinarily high risk for cancer development (Kauff et al. 2002). The procedure efficiently reduces the cancer risk but is considered a radical solution due to serious side effects. Oophorectomy triggers immediate menopause and thereby causes considerable morbidity. The inaccessibility of the upper genital tract and absence of any specific biomarkers make better stratification of the patients in whom surgery could be safely postponed virtually impossible. In general, it can be said that the cytological and histological status of the fallopian tube mucosa of any patient is unknown prior to the surgery and pathological analysis of sections. Therefore, it is impossible to know when the first premalignant changes occur in BRCA1/2 mutation carriers.

The relatively low prevalence of HGSOC in the general population does not justify any invasive procedures before menopause because of the disproportional risk-benefit analysis. As a consequence, there is almost no data about the types and distribution of tissue phenotypes in the fallopian tube from salpingitis patients, although ascending infections are relatively frequent.

As the majority of *Ctr* salpingitis cases are either asymptomatic or receive antibiotic therapy, pathogen-host interaction in the human model could only be studied in ex vivo infection systems. One of the first such studies in organ culture of FT fragments infected with *Ctr* reported structural damage of the epithelium, loss of microvilli, and disruption of intact junctions as observed by electron microscopy (Cooper et al. 1990). The inflammatory cytokine IL-1 has been identified as a major regulator of structural damage in the fallopian tube (Hvid et al. 2007) and upstream of IL-8 production which serves as a potent chemoattractant of neutrophils. Infection in ex vivo tissue models provided a novel opportunity to investigate bacterial infection in the context of the fully differentiated and intact epithelial monolayer. Intact cell–cell communication plays a central role in the regulation of tissue homeostasis; thus, a response to infection involves the reaction of both directly infected cells and neighboring cells in the epithelium. Indeed, strong activation of paracrine pathways is found to be one of the hallmarks of *Ctr* infection of the fallopian tube tissue (Kessler et al. 2012). Disruption of epithelial integrity evident by the loss of apicobasal polarity is compensated by the increase in proliferation of noninfected epithelial cells. The induction of the Wnt signaling pathway in response to *Ctr* is shown to be an important regulator of the changes in global gene expression and a homeostatic defense mechanism—as the addition of porcupine inhibitor IWP2 enhances *Ctr* replication and suppresses induction of EpCAM and OLFM4. The study highlighted the necessity to analyze the consequences of *Ctr* infection in the broader tissue context. The infected epithelium also represents a changing microenvironment in which cell communication and long-distance signals coordinate the response. Although important and informative, ex vivo tissue infection models have serious limitations, mostly due to the short time window of viability in culture (max 96 h) and the inability to apply molecular biology and gene editing techniques. Thus,

the discovery of the stem cells in the adult mucosa and the development of the organoid model was of pivotal importance for studying the properties of human primary tissues in vitro.

9.7 Patient-Derived Organoids: In Vitro Diseases Modeling and Translational Applications

The limited life span and expansion potential of primary cell culture isolates represented for many decades a great methodological limitation for utilization of human tissue samples in the lab. Fundamental requirements for high reproducibility, robust design, and permissivity for experimental manipulation of the studied models could only be fulfilled by immortalized or cancer-derived cell lines which found universal usage in almost all areas of biomedical research. Analysis of human tissue probes was restricted to largely descriptive approaches that capture properties of the cellular state at the time of the sample collection (imaging, proteomics, NGS) without the possibility to analyze the functional properties of the cells. This paradigm was changed by John B. Gurdon who showed that cell differentiation state can be reversed and the discovery of induced pluripotency by Shinya Yamanaka who showed that any somatic cell in the body can be reprogrammed to revert to the pluripotent state by transitional expression of a set of cellular factors (Takahashi et al. 2007). This research created a basis of iPS cell technology, a revolutionary approach of de novo in vitro development of any human tissue of choice whose importance was acknowledged in 2012 as Yamanaka and Gurdon were awarded Nobel prize in medicine. In parallel, the lab of Hans Clevers was successful in the identification and molecular characterization of Lgr5+ positive long-lived stem cells in the intestine of mice that give rise to all different epithelial cell types as shown by lineage tracing experiments. It was the work of Toshiro Sato in the same lab that demonstrated that these unique features of adult stem cell longevity and differentiation potential can be preserved in vitro by seeding in 3D matrix and supplementing with an optimal paracrine signaling environment to generate mini epithelial organs in the dish, termed organoids. Patient-derived organoid models have since been successfully established for tissues of the gastrointestinal tract (intestine (Sato et al. 2011), stomach (Bartfeld et al. 2015), liver (Huch et al. 2015), pancreas (Wang et al. 2020)), reproductive tract (prostate (Drost et al. 2016), breast (Rosenbluth et al. 2020), fallopian tube (Kessler et al. 2015), endometrium (Turco et al. 2017)), and others. Importantly, it was found that malignant tissue that originates from the same organ also maintains the underlying concept of tissue renewal, thus leading to the establishment of organoid cultures for many solid tumors: colon cancer, breast cancer, prostate cancer, stomach cancer, etc. Organoids can be expanded in long-term culture while preserving genomic and phenotypic stability, cryopreserved, and, importantly, modified by gene editing methodology (Matano et al. 2015), all properties which are essential for downstream experimental applications. While long-term stable expanding organoids from healthy mucosa enable in vitro studies of

disease development such as the design of new infection models, cancer organoids provide an opportunity to generate "living biobanks" of patient samples (van de Wetering et al. 2015; Yan et al. 2018; Sachs et al. 2018) as a valuable tool to study personalized therapy approaches and test system of novel targeted therapies.

9.8 Regulation of the Epithelial Renewal in the Upper Genital Tract

9.8.1 Stem Cells of the Ovary

The Wnt signaling pathway plays a central role in the regulation of epithelial homeostasis in the fallopian tube and the ovary, just as in the tissues of the intestinal tract and skin (Barker et al. 2007; Snippert et al. 2010). Since these tissues originate from different embryonic layers, this illustrates the degree of conservation of the process of epithelial regeneration programs across germ layers of ectoderm, endoderm, and mesoderm. Stem cells of the ovary (Lgr5+ ALDH1+) are localized in the hilum region, an anatomic structure where nerves and blood vessels enter the organ and give rise to the cells of the surface epithelium as shown by lineage tracing experiments in the mouse (Flesken-Nikitin et al. 2013). The existence of Lgr5+ adult stem cells in the junctional region between the ovary and the fallopian tube was also confirmed in the study of Ng and colleagues who investigated in more detail the pattern of Lgr5 expression at different stages of embryonic development, neonatal period, and in adult animals (Ng et al. 2014). The study, however, could not confirm the role of ALDH1 as a specific stem cell marker, rather showing its broader expression in the epithelial cells on the surface of the ovary. Moreover, detailed analysis of Lgr5+ cell populations by Lgr5_EGFP_ires_CreERT2 (Lgr5-KI) reporter mice revealed broad expression of Lgr5+ in the ovary and fallopian tube epithelia during development but the more localized expression in the adult tissue to the regeneration active domains. Interestingly, in addition to the hilum region, Lgr5+ cells are localized at the surface of the adult ovary around pre-ovulatory growing follicles and corpora lutea suggesting a functional importance for the repair of tissue injury after the oocyte release. Lgr5+ cells are also described to be exclusively ID2 negative which indicates the low activity of BMP signaling in these cells, as ID2 is one of the classical target genes of the BMP pathway. While the importance of the Wnt pathway for the development of the fallopian tube was well-known for a long time since Wnt4 was identified as one of the determiners of the female sex in the early embryo (Vainio et al. 1999), it was not clear which role it plays in the maintenance of homeostasis in the adult epithelium. As models of ovarian cancer development postulate expansion of the secretory cells as the hallmark of the initial transformation events, it was of interest to see which function they have in the tissue hierarchy of the healthy mucosa.

9.8.2 *Stem Cells of the Fallopian Tube- Pax8+ Progenitors*

Lineage tracing experiments in the mouse model (Ghosh et al. 2017) demonstrated that Pax8+ secretory cells represent the progenitor population capable of self-renewal that gives rise to the differentiated ciliated cells. Stem-cell progenitor potential of the secretory cells is dependent on Wnt pathway activity as ablation of the β-catenin gene leads to a reduction of the secretory cells, and accordingly overexpression induces proliferation.

The existence of bipotent cells in the mucosa of the human fallopian tube adult tissue has been confirmed, as single EpCAM+ cells sorted from mucosal tissue give rise in vitro to organoids (Fig. 9.2a) comprised of two different cell types: ciliated and secretory cells (Kessler et al. 2015). Indeed, early organoids contain only Pax8 positive cells, while ciliated cells differentiate during the second week in culture. The generation and growth of organoids in vitro are supported by exogenous supplementation of Wnt agonists Wnt3a and RSPO1. Inhibition of the BMP signaling cascade by noggin, which chaperones BMP molecules away from receptors, is also required for the preservation of longevity, similar to organoid models from the gastrointestinal tract (Sato et al. 2011). This high-Wnt, low-BMP environment in addition to an active EGF and FGF10 axis and blockade of TGFβ signal via Alk4/5 receptor ensures that the progenitor potential can be preserved in vitro almost indefinitely (>1 year) and continuously produces differentiated progeny, mimicking the in vivo renewal mechanisms of the epithelial homeostasis. The organoid epithelium closely resembles the cellular composition (Fig. 9.2b) of the native fallopian tube mucosa, and the number of ciliated cells depends on the activity of NOTCH signaling. Organoid cultures were also successfully derived from endometrial mucosa, with the same underlying growth requirements (Turco et al. 2017). It remains to be determined which LGR receptor regulates progenitor populations in the adult fallopian tube as functional redundancy is well-known among family members (de Lau et al. 2011).

9.9 Chronic *Chlamydia* Infection in Human Fallopian Tube Organoids

The development of the organoid model from human fallopian tube provided a unique opportunity to establish in vitro infections to study long-term interaction between *Ctr* and the tubal mucosa. This type of longitudinal experimental setting under controlled conditions was able to provide insight into the infection process extended beyond the initial 72 h of the *Ctr* life cycle for the first time. The ability of organoids to preserve critical features of epithelial homeostasis also enables studies of the effect of pathogen-host interactions on tissue renewal mechanisms. *Ctr* readily infects organoids and established a productive replicative niche comparable to standard 2D models. However, in addition to a robust inflammatory response at

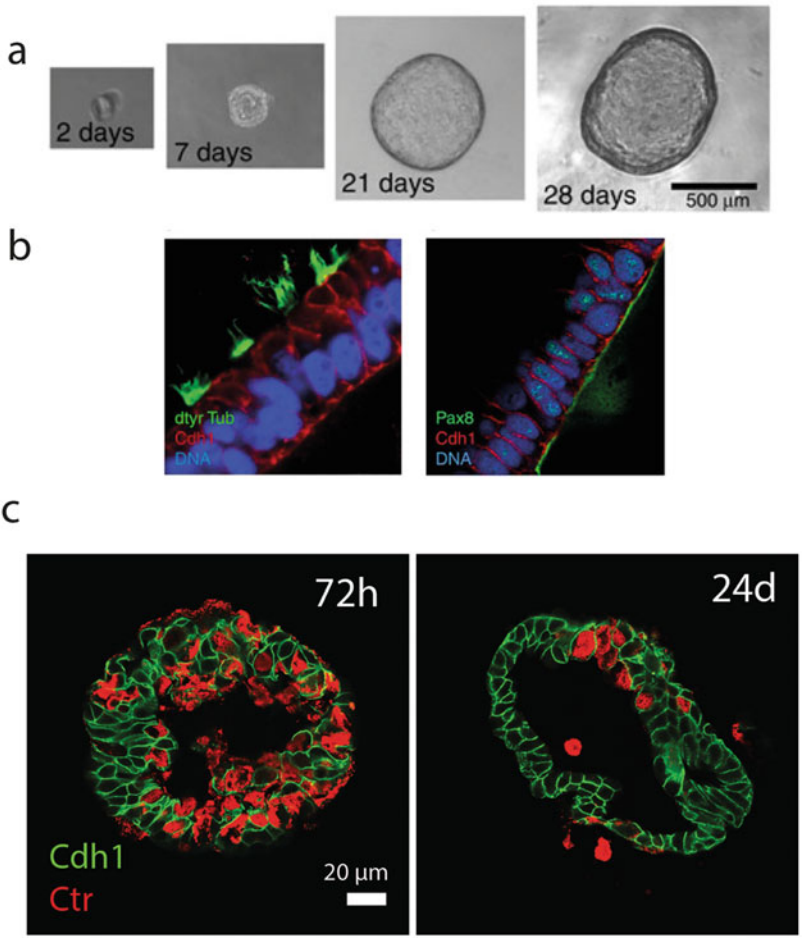

Fig. 9.2 (**a**) Fallopian tube organoids originate from single EpCAM+ bipotent cells isolated from tissue. (**b**) Organoid epithelium is polarized and contains ciliated (dTub) and secretory cells (PAX8). (**c**) *Ctr* readily infects organoids and establishes replicative niche within inclusions. Inclusions containing bacteria can be found for months after initial infections took place. Images were taken from original publications (Kessler et al. 2015, 2019) and adapted based on Creative Commons Attribution 4.0 International License

72 h post-infection, organoids react by activation of paracrine signaling networks that control cell proliferation, cell fate, and integrity of the epithelial surface. In this model, the extrusion of inclusions from infected cells, previously described in 2D as an occasional phenomenon, represents the major mechanism of bacterial clearance and tissue defense. Within 72–96 h post-infection, the vast majority of the bacteria-containing inclusions are being expelled into the lumen of the organoid prior to rupture of the membrane and release of infectious elementary bodies (EBs). In this model, *Ctr* undergoes multiple infection rounds, mimicking chronic infection that

occurs in vivo (Fig. 9.2c, showing presence of inclusions at 24 days post-infection). The analysis of infected *Ctr* organoids that were continuously expanded for 9 months in culture revealed several important and previously unknown consequences of chronic infection of the FT epithelium on cellular phenotypes (Kessler et al. 2019). Phenotypic analysis showed that chronic infection causes a significant shift in the cell-type composition and reduction in the number of ciliated cells. These findings strongly suggest the existence of *Ctr*-driven changes in epithelial homeostasis toward a less differentiated state. Indeed, chronically infected organoids acquired increased organoid-forming capacity consistent with an increase in stemness potential. Analysis of the changes in the distribution pattern of methylated CpGs over time by the BeadChip method showed that the genome of the organoids ages in culture. Differentially methylated CpGs are not randomly distributed but rather belong to repressed genomic regions that are known to be regulated by the polycomb complex. It can be concluded that organoid genome ages during the 9-month-long cultivation and that strikingly this process appears to be accelerated in the presence of *Ctr* infection. These highly significant molecular data strongly suggest that the consequences of chronic *Ctr* infection are much more far-reaching than structural damage to the tube. Moreover, a permanent fingerprint in the genome of the host epithelial cells, as *Ctr*-infected organoids age faster, raises the prospect that salpingitis episodes could leave patients with a substantially altered mucosa that is more vulnerable to carcinogenic stimuli. This certainly warrants further molecular studies on the contribution of chronic *Ctr* infection as a risk factor for the development of ovarian cancer. Of course, although epithelium is the primary targeted tissue for *Ctr* infection, and HGSOC is an epithelial tumor, the broader context of inflammatory tissue phenotype also needs to be considered. In the organoid model, global analysis of the gene expression changes that persisted after chronic *Ctr* infection has been cured revealed sustained deregulation candidates that are likely to affect the interaction of the epithelium with the immune system (SPP1, TNFSF14 up, and CCR7, IGF1, IL17 RB, SULF1 downregulated). This data, which needs to be followed up within a more complex co-culture experimental model including the presence of immune cells, could be helpful to understand the complex field of *Ctr* infection-associated immunity and explore the potential involvement of past infections in the development of ovarian cancer via altered immunosurveillance (Swann and Smyth 2007).

9.10 Patient-Derived HGSOC Organoids: Evidence of Early Changes in Regulation of the Stem-Cell Niche

Ctr infection of the in vitro organoid model confirmed the potential of this pathogenic bacterium to achieve a sustained impact on the fallopian tube epithelium. Despite this, the cultivation conditions required to facilitate long-term expansion of the infected organoids remained unchanged. This suggests that core cellular

mechanisms of stemness maintenance and controlled epithelial differentiation remained intact despite the shifts in phenotype and increase in stemness potential.

Interestingly, simultaneous depletion of the three tumor suppressor genes p53/PTEN/RB1 by lentivirus-mediated shRNA knockdown does cause growth arrest of fallopian tube organoids. Phenotypic characterization revealed that the process is accompanied by the progressive loss of stemness markers and irreversible cell differentiation. This important observation implies the existence of a functional connection between the tumor driver genes p53/PTEN and RB1 and cellular pathways that control the maintenance of stemness potential in the fallopian tube epithelium. Indeed, systematic testing of conditions for the stable in vitro expansion of patient-derived organoids from HGSOC solid deposits revealed a substantial change in the composition of paracrine signals that support cancer organoid growth in vitro in comparison to the healthy epithelium. Most significantly, exogenous activation of canonical Wnt signaling by the addition of agonists (Wnt3a and RSPO1) which is an essential component of the adult stem cell-based organoid cultures from healthy mucosa has a negative effect on the preservation of stemness in HGSOC cancer organoids. The negative impact of conditional medium containing Wnt3a, initially observed during the establishment of the first biobank of ovarian cancer organoids (Kopper et al. 2019), was more closely investigated in a parallel study focused on HGSOC deposits from patients who have not been exposed to chemotherapy (Hoffmann et al. 2020). A fundamental shift in the requirement for growth factors between healthy organoids and cancer organoids were discovered and characterized in detail, revealing an important novel biological concept. In contrast to healthy epithelium organoid cultures, the low-Wnt environment is proved essential for stable expansion of HGSOC organoids, all of which were cultured in the absence of exogenous Wnt ligand and only two required RSPO1. It was shown that the addition of Wnt agonists leads to the reduction in surface expression of the stemness marker CD133, strongly induces differentiation, and causes growth arrest (Hoffmann et al. 2020). Moreover, BMP signaling, which is actively suppressed by the presence of noggin in the medium for healthy organoids, promotes the formation and long-term expandability of HSGOC cultures. Indeed, addition of exogenous BMP2 ligand further amplifying BMP signalling was found to improve organoid forming efficiency even further in some primary isolates. These important changes in the paracrine signaling environment, required for the formation and expansion of HGSOC organoid cultures in vitro, indicate that analogous changes in in vivo tissue could potentially provide selection pressure and create conditions that favor initial tumor outgrowth. Further analysis of the in vivo tissue microenvironment at different stages of the cancer development is necessary to discover the mechanism and cell types that regulate this shift in signaling niche composition. This is a rather difficult task, keeping in mind the complete absence of exploratory invasive gynecological procedures in asymptomatic women. Interestingly, WNT4 and RSPO1 were identified among six new susceptibility loci for ovarian cancer development as a result of the large GWAS study, in agreement with findings from the organoid model. Kuchenbaecker and colleagues investigated the association of 11 million different genomic variants with EOC, based on computed data from the 1000 genomes project

on samples from over 15,000 patients and 30,000 controls. The WNT4 locus showed increased association with EOC in general and the RSPO1 variant conferred risk for the development of HGSOC (Kuchenbaecker et al. 2015). The identified shift in a niche requirement is a highly significant finding as it alters the concept of cancer as a tissue of unrestricted growth potential, obtained at the time of the transformation that is achieved in a cell-autonomous intrinsic fashion. Loss of dependence on niche factors has been previously described as a late-phase event in the metastatic spread of colon and pancreatic cancer (Fujii et al. 2016; Seino et al. 2018). Data from engineered fallopian tube organoids, by contrast, strongly argues that in HGSOC this change occurs early in carcinogenesis and could be part of a critical mechanism that allows for the initial expansion of transformed cells at the expense of surrounding healthy epithelium.

9.11 Wnt Signaling in Health and Disease

Implications of the findings of reduced Wnt pathway activity in HGSOC cancer tissue are far-reaching. The Wnt paracrine pathway is well-known as an essential component of embryo development in eukaryotes and is indispensable for the development of the female genital tract. WNT4 has been identified as the master regulator of the formation of the reproductive system in females. WNT4 mutant mice don't have organs deriving from the Mullerian duct (Vainio et al. 1999), and WNT4 is required for the normal folliculogenesis in the ovary (Boyer et al. 2010). Its expression in early gonads is regulated by RSPO1 and represents an early marker of female sex determination in developing embryos which are initially indistinguishable regardless of the genetic sex. Indeed RSPO1 mutations can lead to sex XX reversal phenotype (Parma et al. 2006) syndrome which has been described in humans in 46, xx males in affected families or, depending on the mutation type, be a monogenic cause of true hermaphroditism (Tomizuka et al. 2008; Tomaselli et al. 2008).

Robust advances in basic cancer research in the last half of the century were propelled by key discoveries in the field of molecular and cell biology. It became clear that in many cases tissue malignancy occurs because of changes in the pathways that control normal development. Among them, perturbations of Wnt signaling are proven to be the culprit behind almost 90% of colorectal cancer cases. Constitutive hyperactivation of the Wnt signaling pathway due to impaired function of the beta-catenin degradation complex has been identified in families with adenomatous polyposis coli (APC) mutations and causes familial adenomatous polyposis (FAP) syndrome which greatly increases the risk for colon cancer development (Jasperson et al. 2010). In addition to a large spectrum of different APC mutations, other Wnt-activating mutations have been found to be associated with sporadic colon cancer cases (RNF43, TCF4, AXIN1, AXIN2), as well as epigenetic inactivation by methylation of Wnt inhibitors, DKK2, and WIF1 (Segditsas and Tomlinson 2006). Increased activity of Wnt signaling is also found to be significant

for the progression of breast cancer, leukemia, and other malignancies mostly in the context of the recurrent disease and resistance to therapy (Khramtsov et al. 2010; Lu et al. 2004). In contrast to endometrioid adenocarcinoma where Wnt signaling activating mutations are found in 40% of the cases (Schwartz et al. 2003), somatic activating mutations in Wnt pathway components in HGSOC cases are exceptionally rare. There is some evidence that in aggressive forms of advanced disease, and patients resistant to platinum therapy, Wnt pathway activity is increased (Nagaraj et al. 2015). Correlation is found between the stemness potential of CSC defined by expression of CD117+ with an increase of resistance to the chemotherapy and activation of the Wnt pathway (Chiu et al. 2015). In vitro experiments in low attachment spheroid cultures of cancer cell lines and ascites cells demonstrated that β-catenin directly transcriptionally regulates ALDH1A1, an enzyme associated with CSC phenotype (Condello et al. 2015). However, analysis of the properties of patient-derived organoids from primary HGSOC tissue from chemo-naive patients convincingly demonstrated the negative influence of high-WNT environment on the formation and growth of cancer organoids in culture. Though these findings might appear contradictory, they likely illustrate the complexity of the cellular organization of the HGSOC tissue and growth mechanisms at different stages of disease progression. This underlines the necessity to analyze cancer biology not as an isolated entity of individual cells but as an integral part of the microenvironment which changes over time driven by novel selection pressures. Indeed, defects in HDR DNA repair mechanisms are a major driver of HGSOC carcinogenesis, while recurrent disease stages are frequently accompanied by the diverse mechanisms of reacquired HDR proficiency which greatly limits therapeutic options (Sakai et al. 2008; Weigelt et al. 2017).

9.12 Tissue Inflammation as a Precursor of the Tumor Microenvironment

Carcinogenesis is frequently described as a complex multistage process driven mainly by the mutations in a small number of key tumor-driving genes that accumulate in the cell until the transformation threshold is reached (Vogelstein et al. 2013). Other numerous mutations and genomic alterations abundant in all cancers are passenger hits that contribute to cancer diversity but do not provide a growth advantage. Interestingly, several recent NGS studies that analyzed clonal populations of epithelial cells from healthy individuals revealed the prominent occurrence of tumor-driving mutations in healthy tissues (skin, esophagus, colon) at a much higher rate than the incidence of malignancy (Martincorena et al. 2018; Lee-Six et al. 2019). It has been shown that around 1% of colonic crypts in each individual between 50 and 60 years of age carry hot spot colorectal cancer mutations even though colorectal cancer develops in only 5% of the people. These studies convincingly demonstrated that many critical conceptual questions regarding the

early stages of cancer development remain unanswered, in particular questions regarding the influence of the microenvironment, clonal selection, and the mechanisms underlying metastasis and immunosurveillance. Though the model of driver mutations introduced by Vogelstein remains an important pillar of our understanding of carcinogenesis, it is also clear that the process is not linear and that there are multiple complex layers of regulation of tissue homeostasis and protective mechanisms which act to prevent malignant transformation. In the case of HGSOC, investigation of the disease origins is particularly difficult, due to the occult nature of the malignancy and the inability to detect or sample the early stages of the neoplastic changes. While STICs are considered the first stage of the neoplastic disease, the incidental finding does not warrant a change in the clinical management of the patients, as no data is available about frequency and risk of the progression to HGSOC. The occurrence of TP53 signatures, sets of at least 12 secretory cells harboring distinct p53 nuclear staining and increased DNA damage, was identified as a potential first stage of an "abnormal" histological phenotype (Lee et al. 2007) in the distal fallopian tube. However, it is found with equal frequency in tissue sections of BRCA germline mutation carriers and healthy controls, raising the prospect that the p53 "signature" falls within the scope of normal physiological responses and that additional events are needed to drive the development of the tumor (Folkins et al. 2008). It also shows that the selection of p53 mutations in the FT epithelium is independent of BRCA status, which needs to be taken into account when considering putative transformation sequels and the exact cellular mechanism behind early carcinogenesis. Profiling of uterine lavages (Utl) by ultrasensitive deep sequencing demonstrated high efficiency for the detection of cancer-related P53 mutations (80%) in the group of ovarian cancer patients. However, pathogenic mutations were also detected in the healthy control group albeit at very low frequency. This is an important finding, in line with the previously cited studies from other tissues, and demonstrates that the occurrence of pathogenic TP53 driver mutations alone is not sufficient to trigger malignant transformation in the genital tract.

Taken together, histological evidence and molecular genomic data support the conclusion that a complex yet undetermined set of physiological stimuli/environmental factors provide critical selection pressure that leads to the expansion of mutant cells in the fallopian tube epithelium and development of HGSOC. Especially interesting is the very low incidence of the primary tubal cancer, estimated to be ~100× fold less frequent than HGSOC (40 vs. 4500 cases, respectively, per year in the UK) (Pectasides et al. 2006). Primary fallopian tube cancer is rarely asymptomatic, in contrast to HGSOC, which could reflect the fact that a fast-growing mass within a narrow tube is more disruptive than malignant tissue on the surface of the ovary.

How and why HGSOC cells metastasize to the ovary remains one of the most significant open questions. The proximity of the distal fimbria and ovarian surface exposes tubal epithelium not only to the follicular fluid but could also be within a range of the local gradients of signaling molecules. The frequent bilateral clinical presentation of the malignancy, already involving both ovaries at the stage IB, is also an interesting phenomenon that needs to be understood to explain the mechanisms of

monoclonal origin and early growth. The main route of HGSCOC dissemination is thought to be via direct shedding of the transformed fallopian tube cells to the ovary and/or peritoneum. Clinical data supported by experiments in animal models also showed a significant contribution of hematogenous spread and lymphovascular invasion (LVI) to the progression of the advanced disease (stage III and IV). At an early stage of the disease, a minority of patients have LVI (17% and 32% at stage I and stage II, respectively), and this group has significantly worse progression-free survival (PFS) and overall survival (OS) than LVI negative cases (Chen et al. 2015). The ability of the cancer cells to disseminate to the omentum by hematogenous metastasis has been shown to correlate with the expression levels of the ErbB3 receptor on the tumor cells and its activating ligand neuregulin-1 on the surface of the omentum in the parabiosis mouse model by injecting SKOV3 ovarian cancer line. Endoglin (CD105) a member of the TGF beta receptor family has also been identified as a potential mediator of the hematogenous spread to ovary (Bai et al. 2019). It has been found that STIC lesions show an increased level of CD105 compared to the surrounding epithelium, which raises the interesting possibility that this pathway is also activated during early tumorigenesis. The propensity of CD105+ cells to metastasize to the ovary through the bloodstream is demonstrated in the mouse model, again by using the SKOV3 cancer line. These findings create new potentially promising therapeutic opportunities to specifically target dissemination mechanisms, although more comprehensive studies are needed to confirm the functional relevance of these signaling pathways for disease progression. The induction of a pro-inflammatory environment is considered an important mechanism that enhances the metastatic potential of the tumor (Wu and Zhou 2009). Experiments in an orthotopic xenograft mouse model that tested the peritoneal spread of cancer cells to the omentum showed the accumulation of neutrophils in the target tissue before metastasis (Lee et al. 2019) occurs. Moreover, neutrophils were activated and underwent netosis, i.e., releasing NETs—fibers of extracellular chromatin and proteins. It was shown that NETs facilitate homing and attachment of the incoming cancer cells to the omentum, and depletion of the neutrophil pool strongly reduces the rate of cancer metastasis (~70%). Significantly, the study could validate the principle findings of neutrophil accumulation in paraffin sections from omentum in patients diagnosed with stage I–II HGSOC.

9.13 Tumor Heterogeneity and Local Microenvironment

Broad and early dissemination across the peritoneal cavity is one of the hallmarks in the clinical presentation of the HGSOC. Due to the lack of symptoms, or their unspecific nature, cancer is rarely detected before FIGO stage III and >75% of diagnosed patients are > stage III when large tumor deposits are already present outside of the primary localization to the ovary. Interestingly despite the extensive dissemination potential within the peritoneal cavity and large tumor masses that usually require large, high-risk debulking surgeries to achieve “tumor-free”

resection, serous ovarian cancer metastasizes to distant organs only in a minority of cases (35%) even in the absence of any therapy. The main locations of extraperitoneal spread are the lungs and liver (Dauplat et al. 1987). It remains unclear which cellular mechanisms are responsible for this remarkable restriction of malignant potential. The TP53 mutation status has been shown to correlate with a propensity for distant metastasis. As the sequence of this important tumor suppressor gene is altered in almost all HGSOC cases (TCGA study), more than two third involve point mutations that cause overexpression, while one third results in the complete absence of the protein termed "null mutants". It is this latter group of missing TP53 which is at significantly higher risk for distant metastasis as well as overall worse prognosis (Sood et al. 1999; Shahin et al. 2000). The differential effect was confirmed in the mouse model of tumorigenesis, as p53 null mutants showed significantly stronger metastatic potential than point mutations alleles when crossed in the $APC^{-/-}/Pten^{-/-}$ genomic background as histopathology showed spread to the parenchyma of the liver and the kidney. On the other hand, several studies found a significant association between the presence of the overexpressing p53 mutant and the development of resistance to platinum chemotherapy as well as shortened disease-free survival period after first-line therapy. Aggregation of p53 molecules, either by modification of the cellular turnover machinery or by expression of stabilized mutants that obtain prion-like properties (Silva et al. 2018) are proposed to be conditions that promote resistance to chemotherapy (Yang-Hartwich et al. 2015) in the group of affected patients. Nevertheless, the exact clinical classification and prediction of disease progression solely based on the type of detected TP53 mutation remain elusive (Reles et al. 2001).

Numerous studies confirmed great tissue heterogeneity of ovarian cancer at different sites in the body, and functional importance of the local tumor microenvironment has been asserted by many experts in the field. Tumor deposits vastly differ in the composition of the infiltrating immune cells and stromal compartment, although clonal evolution of the mutational profile of the epithelial cancer cells remains relatively straightforward. A study of 135 samples from 14 patients, using genome-wide SNP arrays, showed that all main populations detected in the relapse are already present in the primary tumor (Schwarz et al. 2015). Detailed phylogenetic analysis by whole genome sequencing (WGS) of 68 samples from 7 patients showed that the majority of patients exhibited monoclonal and unidirectional seeding, while 2 patients had polyclonal composition and mode of spread indicating the existence of at least 2 different mechanisms how HGSOC spreads in the body (McPherson et al. 2016). Despite fast progress in documenting variations in tumor composition by implementing new tools of molecular analysis and expansion of bioinformatics and system biology approaches to integrate the data, there is still only limited understanding of how this heterogeneity is generated, which are the organizing principles, and most importantly which unifying common properties should be targeted to improve clinical responses. Novel immunogenomic approaches made it possible to perform detailed analysis on the single-cell level of the diverse metastatic niches in an HGSOC patient over the extended course of 2 years (Jimenez-Sanchez et al. 2017). This heavily treated patient had several metastases

that regressed in response to therapy, while others were resistant to treatment. It was shown that advanced metastases exhibited low immunogenic profile and oligoclonal expansion of T cells was prominent in regressing tumors. However, exome sequencing has not confirmed the relationship between the increased mutational burden of the tumor and local immune response, questioning one of the popular hypotheses that novel neoepitopes that arise in heterogeneous malignant disease define the immunogenic profile of the particular deposit.

9.14 Inflammation and Response to Immunotherapy

Chronic infection processes in the fallopian tube mucosa raise the prospect of subtle yet sustained changes in the adaptive immunity repertoire which could also have significant implications in the long-term immunosurveillance capacity of tissue and influence metastatic capacity of the tumor. A recent study in a mouse model showed that a range of inflammatory stimuli (wound healing, ovulation, and aging) increases the efficiency of cancer cell metastasis (Jia et al. 2018). Interestingly, however, PD-1 and PD-L1 molecules are expressed in HGSOC tissue, and expression level correlates with a stage of the disease (Drakes et al. 2018) making it a potentially good candidate for checkpoint immunotherapies. The clinical response rate so far has been rather modest, and the administration of single-agent PD-L1 therapy achieved a complete response in only 15% of patients (Hamanishi et al. 2015). Thus, it can be assumed that the biology of HGSOC cancer includes additional potent mechanisms for immunoevasion which render the blockade of the PD1/PDl-1 signaling ineffective. A putative connection between inflammatory and infection processes in the upper genital tract and increased risk for cellular transformation warrants investigating interferon signaling and its contribution in the context of the pathology development (Zitvogel et al. 2015). Adaptations of interferon signaling in cancers have been implicated as one of the important cellular mechanisms that can influence response to immunotherapies and could explain resistance to treatment and successful immune evasion strategies that enable disease progression.

Type I interferon signaling is a cell defense mechanism mediated by interferon α and β upon activation of pathogen recognition receptors (PRR) after recognition of intracellular pathogens, usually viruses. The stimulator of interferon genes (STING) is a transmembrane protein localized to the intracellular organelle membranes (e.g., endoplasmic reticulum), a potent activator of the interferon type I (IFNI) response upon recognition of foreign DNA and/or invading pathogens. Notably, STING plays a crucial role in the innate immune response to tumor development and the establishment of efficient adaptive T-cell response (Woo et al. 2014) as it recognizes tumor DNA released into the environment. In this context, the function of the STING complex is unique, as other upstream mediators of the INF I response MY88, TLR4, TLR9, P2X7R, TRIF, or MAVS did not influence T CD8+ antitumor population. Activation of the INF I response and strong chemokine profile, together with T-cell infiltration, are hallmarks of an “inflamed” cancer phenotype and have

positive clinical prognostic value in melanoma, breast cancer, gastrointestinal stromal tumor, renal cancer, and ovarian cancer (Gajewski et al. 2013). Numerous clinical trials are ongoing that try to exploit this important correlation to improve response to checkpoint inhibitor therapies, by improving the immunity of the tumor and convert "cold" to "hot-inflamed" tumors. Experimental models of ovarian cancer suggest that the STING pathway can be induced by various means including standard cisplatin chemotherapy (Grabosch et al. 2019). Treatment with PARP inhibitors increases the amount of unrepaired single-strand DNA, a signal that also triggers STING activation (Shen et al. 2019). Therefore, a combination of existing lines of therapy and new immunotherapeutics could be a promising possibility to improve the response of ovarian cancer to checkpoints inhibitors, but this hypothesis is yet to show a clear benefit in large clinical trials. Functional redundancy between innate immunity pathways activated during infections and the immune response to malignancy is being investigated for the development of novel therapeutic strategies in a form of new vaccines as adjuvants to checkpoint inhibition. A new class of cyclic lipids has been identified as a vehicle for delivery of mRNA vaccines that specifically induce STING pathway response and thereby improves the effectiveness of PD-L1 treatment in melanoma and E6/E7 mouse tumors models (Miao et al. 2019).

Big data analysis also revealed a potential link between expression of human endogenous retroviral elements (hERV) in renal cancer tissue and cancer immunogenicity (Smith et al. 2018). In silico characterization based on two mechanisms, activity of RIG-I receptor signaling for pathogen recognition and retroviral activation of adaptive immunity, correctly predicted response to immunotherapy. Interestingly, high expression of hERV elements is identified in ovarian cancer, but in the group of low-grade histotype while HGSOC tissues showed a lower median level than healthy tissue control (Wang-Johanning et al. 2007). Studies are ongoing to explore if it is possible to improve the responses to immunotherapy by reactivating hERV sequences in advance disease (Kong et al. 2019).

9.15 Contribution of the Microbiota to Disease Progression and Response to Immunotherapy

Analysis of the involvement of microorganisms in the development of malignancies or their influence on patient outcomes by influencing patients' response to therapy requires the distinction of two separate classes based on their location within the organism. Throughout this chapter, numerous examples have been provided about the effects of local microorganisms that colonize the genital tract both as the pathogens and commensal species. Besides, it is increasingly clear based on accumulating experimental evidence in different models that the composition of the gut microbiome has a powerful influence on the response of the body to cancer progression and response to therapy. For example, the gut microbiome strongly influences

the effectiveness of the cancer drug cyclophosphamide which induces antitumor activity of the immune system as demonstrated in a mouse model. Cyclophosphamide in combination with cisplatin was used as the first-line therapy for ovarian cancer before the discovery of paclitaxel and is today used in late-stage maintenance therapy. Also, healthy commensal bacteria species such as *Bifidobacterium* or *Akkermansia muciniphila* appear to be a positive factor in determining response to new checkpoint inhibitor-based immunotherapies in the kidney, lung tumor, and melanoma patients (Routy et al. 2018). However, studies also give a picture of rather complex and sometimes contradictory findings where more detailed and better controlled clinical studies are needed before the microbiome can be used as a reliable biomarker or point of intervention in new therapeutic strategies (Gong et al. 2019).

In the research field of malignancies in female tissues that are responsive to estradiol stimulation, there is a great interest in the potential modulating role of the gut microbiota by species that produce the β-glucuronidase enzyme. This enzyme deconjugates estradiol from the bile excretion products leading to its reabsorption and return to the circulation, thereby influencing the level of this hormone in the circulation. Thus, intestinal dysbiosis can enhance the pathologic effects of low estrogen levels or, conversely, increase hyperestrogenic states such as endometriosis or PCOS (polycystic ovarian syndrome) in cases of increased proliferation of these species Baker et al. 2017. *Bacteroidetes* and *Firmicutes* phyla among others have been shown to express β-glucuronidase at dynamic levels that can vary based on the diet or density of bacteria in the gut (Lampe et al. 2002). The effect of the gut microbiota on estradiol levels, termed in the literature as estrabolome, thus represents an important field of research that is likely to provide valuable insight and a better understanding of complex physiological factors that participate in hormone-driven disease and potentially affect tumorigenesis, disease progression, and immune response.

9.16 Future Directions in the Research of Tubal Pathology and HGSOC Development

Robust induction of an interferon type I response is a hallmark of acute *Ctr* infection, as observed in infected FT organoids. As an obligatory intracellular pathogen that establishes a replicative niche with a cytoplasmic vacuole, it can be argued that *Ctr* elicits host responses that are very similar to the response to virus infections in other models. Interestingly, the requirement of Wnt signaling for optimal induction of the INF I pathway is a part of the antiviral response for vesicular stomatitis virus (VSV), herpes simplex virus HSV-1, and Sendai virus (Yang et al. 2010; Khan et al. 2015). The consequences of beta-catenin activation during the infections appear to be context-dependent. Bearing in mind the characteristics of the patient-derived HGSC organoids model, and the requirement of low-Wnt environment for the maintenance of long-term growth upon depletion of key tumor drivers in fallopian

tube organoids, the molecular link between Wnt signaling and the interferon response could have exceptionally important implications for cancer development. STING pathway activation and the subsequent INF I response, as discussed above, are important components of the cancer "inflamed" phenotype as a mediator of T-cell recruitment. Therefore, a putative shift toward a low-Wnt microenvironment in the HGSOC tissue could lead to reduced INF I pathway activity and thereby contribute to the modest response to checkpoint inhibitor immunotherapy, but at this stage, this hypothesis remains a theoretical possibility and requires in-depth experimental research. Currently, there is no data about the cellular mechanisms that mediate in vivo the change in the microenvironment in the fallopian tube tissue at the time of malignant HGSC transformation. The organoid models discussed here provide an important in vitro readout about changes in the regulation of the epithelial renewal mechanisms in response to infection or depletion of the tumor suppressors in vitro. Patient-derived HGSOC organoids reveal and preserve the intrinsic cellular composition of malignant tissue of each patient and pave the way for personalized therapy approaches. Nevertheless, it remains unclear which cell types provide Wnt ligands in vivo in healthy tissue and under which circumstances the paracrine environment changes to favor outgrowth of cancer clones. It is tempting to speculate that chronic infection of the fallopian tube, by *Ctr* and other pathogens and microbiota, leading to the shift in epithelial homeostasis, also alters the way the mucosa interacts with the surrounding environment, both parenchyma and immune cells, and thereby additionally affects broader tissue response to other physiological or environmental challenges. Comprehensive studies are needed to investigate several different scenarios that could plausibly occur in vivo regarding the interplay of infection, microbiota, and interferon signaling. Also, it is of critical importance to identify the factors that provide a growth advantage to the cells that harbor preexisting mutations. As discussed above, rare p53 mutant cells do occur spontaneously in the mucosa of healthy women without direct negative consequences. It would be interesting to know how and if the occurrence of this "mosaic" phenotype influences the outcome of chronic infections and local inflammation, both in terms of priming of the immune response and potential consequences on the dynamics of epithelial renewal and distribution of different clones.

Finally, new patient-derived organoid models create an opportunity to sample the primary tissues and analyze the functional properties of the epithelium in vitro. This greatly expands the possibilities for improvements in the diagnostic procedures and could lead to the discovery of reliable early phenotypic markers of the neoplastic changes. This line of research is still in its infancy but could pave the way for the long-awaited improvement in early detection of HGSOC.

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Chapter 10
Commensal Microbes and Their Metabolites: Influence on Host Pathways in Health and Cancer

Roberto Mendez, Sulagna Banerjee, and Santanu Banerjee

Abstract Commensal microbial homeostasis has been associated with the health of an individual for centuries. Microbial dysbiosis (alteration of microbial composition, with expectation of adverse outcome) in disease, infection, and chronic metabolic disorders are associated with distinct comorbidities, hitherto known, and yet poorly understood. With extensive use of sequence-based molecular phylogeny, we now understand the commensal microbial association with the diseases, their manifestation, and pathogenesis. Microbiome and its metabolic products continuously interact with the host, both immunologically and biochemically in health and disease. This interaction can be beneficial or adversarial, depending on its nature of participation in the host metabolic process. In this chapter, we have discussed the major microbial metabolites that dominate the metabolic landscape of gut microbiota (the cancer microbiome) and influence cancer pathogenesis and chemotherapeutic efficacy.

Keywords Microbiome · Microbial metabolism · Cancer microbiome · Inflammation · Pathways · Cancer

10.1 Introduction

The human gut microbiome consists of all microbial organisms within the gastrointestinal tract. The role of the gut microbiome in health has been elucidated by recent developments in sequencing technology. The effects of microbial composition in

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this regard extend beyond those of direct infections by pathogenic species. Commensal bacteria have an important role in host health as modulators of immune response and producers of vital metabolites. In mammals, initial shaping of the gut microbial composition is dependent on the dietary components of mother's milk (Al Nabhani et al. 2019). The transition from mother's milk to solid food marks a significant remodeling of gut microbiome yet again, with associated changes in microbial metabolism (Al Nabhani et al. 2019; Beaumont et al. 2020), leading to adjustments and eventual maturation of the intestinal barrier (Beaumont et al. 2020; Oliphant and Allen-Vercoe 2019). Throughout the age of the mammal, depending on geography, diet, and hygiene, gut microbes and their metabolites continually influence and modulate host systemic and immune functions in health and disease (Oliphant and Allen-Vercoe 2019). As ecological studies have shown, microbial metabolic processes can be powerful enough to reshape components of specific ecosystems (Lu et al. 2020). Within the body, microbes have a similar level of control on the health of their host through the production of metabolites. The microbiota of the human gut is especially important in production of biomolecules, such as polyamines, trimethylamine (TMA), short-chain fatty acids (SCFAs), and kynurenine, to mention a few (Oliphant and Allen-Vercoe 2019; Jacobs and Braun 2014).

Understandably, the microbiota of the gut is not static in composition. This community is in a constant flux as it interacts with incoming chemical components of diet, new bacteria introduced by the environment outside the host, and immune responses generated by the host. As the composition and environment of the microbiota change, so do the metabolites produced by the microbiome. However, there may be instances where change in overall composition of microbiome triggers changes in metabolic "behavior" in microbes that may or may not be altering in their relative abundance. In any case, the metabolic influence of the microbiota on the host is manipulatable and, possibly, exploitable for amelioration of primary disease conditions and associated comorbidities (Jacobs and Braun 2014).

Alteration of gut microbiota ("microbial dysbiosis") is strongly implicated in systemic inflammation, leading to carcinogenesis and cellular dysplasia (Helmink et al. 2019). Microbial dysbiosis and resultant inflammation leads to immune escape and production of pro-tumorigenic metabolites (Silbergleit et al. 2020), often associated with mucosal barrier compromise and bacterial translocation and associated innate immune activation. In fact, gut microbial dysbiosis is always associated with low- to high-level systemic inflammation, depending on the primary cause of the said dysbiosis (Jacobs and Braun 2014). It is hence perceivable that microbial dysbiosis and associated inflammation create an environment within the host that promotes the initiation and onset of carcinogenesis, maintenance of cancer through immune escape, and resistance to therapy. All of these factors are achieved by changes in microbial composition (gram-positive or proteobacterial skew), exposure of surveilling immune cells to microbial cell-wall products (lipopolysaccharides, LPS; lipoteichoic acid, LTA; etc.), and specific flux of metabolites that can affect immune cells in terms of polarity and activity. For both gastrointestinal (GI)-associated and non-GI-associated malignancies, there is strong evidence of antibiotic

use (and microbial dysbiosis by association) and disease onset and progression (Boursi et al. 2015). In colorectal cancer (CRC), where the major premise of "cancer microbiome" was established, studies have shown a distinct dysbiotic microbial composition compared to controls (Sears and Garrett 2014; Kwong et al. 2018; Hale et al. 2018; Allen and Sears 2019; Yoon and Kim 2018). Additionally, it has been shown that engraftment of CRC-associated dysbiotic microbiome in naïve animals can induce colonic polyp formation that goes on to establish symptomatic CRC in the recipients (Wong et al. 2017). Similarly, many bacterial genera are now established as carcinogenic, notably *Salmonella* and *Helicobacter*, that are implicated in biliary cancer, gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma (Di Domenico et al. 2017; Wang et al. 2014). Apart from being the initiator of tumorigenesis, there is ample evidence that dysbiotic microbiome can exacerbate tumorigenesis. Work from our own laboratory has shown that bacterial polyamines can promote tumorigenesis in pancreatic ductal adenocarcinoma (PDAC) and that bacterial metabolites do play a role in diabetes-induced chemoresistance in PDAC (Mendez et al. 2019; Kesh et al. 2020).

In this chapter, we have discussed major commensal microbial metabolites that have known influence in various cancers. The influence of commensal microbial metabolites on cancer biology (both progression and therapy) is a rapidly evolving field. Hence, while the current knowledge base is limited, the possibilities and its ramifications on cancer biology are enormous.

10.2 Microbe-Derived Metabolites

Metabolic interactions within gut microbes and that between microbes and multicellular host are one of the predominant modes of interspecies interaction in nature. In fact, interspecies participation of metabolites in homeostatic physiological processes is the foremost denominator for the establishment of mutualistic and commensal relation between two different species. In the current context, metabolite sensing among different microbial species determines the composition of biofilms on the mucosal surfaces, and their sensing and uptake by the host establish the threshold of commensalism between the two in healthy conditions (McCarville et al. 2020). The microbial presence within the mammalian gut is an example of an overtly complex ecosystem. The constituents are at continuous flux with the chemical environment presented by proximal host tissue, as well as the metabolic secretome of the surrounding microbes (Sanchez and Gore 2013; Sung et al. 2017). At homeostasis, the microbes thrive by utilizing host, diet, and other microbe-derived metabolic products (Sung et al. 2017; Russell et al. 2013). In disease-associated microbial dysbiosis, there are profound changes in the metabolic landscape, with clear consequences for disease progression, manifestation, and therapy (Jacobs and Braun 2014). This section is dedicated to the discussion of few of the metabolites that are essential for homeostasis and influence disease processes in host upon microbial dysbiosis and thematically summarized in Table 10.1.

Table 10.1 Microbial metabolites and interactions with host pathways

Microbes	Metabolites	Interacting pathways in host signaling	References
Salmonella typhi	Produces variety of glucuronidases from primary bile acids	Bile acid metabolism pathways	Di Domenico et al. (2017), Tsuchiya et al. (2018)
Salmonella typhi	Produces nitroso compounds from primary bile acids	Bile acid metabolism pathways	Di Domenico et al. (2017), Tsuchiya et al. (2018)
Salmonella typhi	Cytolethal distending toxin (CTD)	Bile acid metabolism pathways; promotes carcinogenesis in proximal host tissue	Di Domenico et al. (2017), Tsuchiya et al. (2018)
Gut bacteria like *Firmicutes*	Deoxycholic acid	Secondary bile acid metabolism that promotes hepatocyte inflammation and liver carcinogenesis	Jia et al. (2018), Jones et al. (2014), Ridlon and Bajaj (2015), Hylemon et al. (2006), Ridlon et al. (2014, 2016)
Gut bacteria like *E. coli*	Produce queuosine	Altered T-RNA base, promotes proliferation of cancer cells by inducing unfolded protein response	Ishiwata et al. (2001), Pathak et al. (2008), Fergus et al. (2015), Zhang et al. (2020)
Firmicutes and *Proteobacteria*	Trimethylamine (TMAO)	Choline metabolism pathway; involved in atherosclerosis and kidney diseases as well as increased risk of colorectal cancer	Koeth et al. (2013), Romano et al. (2015)
Gut bacteria	Kynurenine	Tryptophan metabolism pathway	Puccetti et al. (2015)
Bacteroides and *Fusobacteria*	Polyamine (putrescine and cadaverine)	Nucleic acid synthesis, chromatin modification	Kesh et al. (2020)
Akkermansia, *Bacteroides*, *Bifidobacterium*, *Prevotella*, and other gut bacteria	Acetate	Acetylation of proteins and chromatin modification	Morrison and Preston (2016), Rey et al. (2010)
Bacteroides, *Salmonella*, and other gut bacterial	Propionate	Inflammatory pathways	Louis et al. (2014)
Coprococcus and other gut bacteria	Butyrate	Energy homeostasis, HDAC inhibition, and regulation of gene transcription	Zeng et al. (2017)

10.2.1 Bile Acids

In certain well-studied instances (e.g., hepato-enteric recirculation of bile acids (Dawson and Karpen 2014, 2015; Banerjee et al. 2016)), microbial fermentation of host-derived metabolites (in this instance, primary bile acids) is an essential step to complete the metabolic cycle (Dawson and Karpen 2014, 2015). On the other hand, any perturbation in the composition of bile-converting microbial genera would essentially disrupt bile recirculation cycle (Banerjee et al. 2016). In gallbladder (GB) cancer, for instance, colonization and biofilm production by commensal *Salmonella typhi* have been particularly implicated for disease onset and progression (Di Domenico et al. 2017; Tsuchiya et al. 2018). Once colonized, *S. typhi* utilizes primary bile acids to produce a variety of glucuronidases, nitroso compounds, and a group of cytolethal distending toxins (CTDs), that promote carcinogenesis in the proximal host tissues (Fowler et al. 2017; Nath et al. 2010). Treatment of mice with antibiotics has shown marked reduction in GB, colon, and liver cancer tumorigenesis (Jia et al. 2018). One of the major secondary bile acids, deoxycholic acid (DCA), is a microbial fermentation product of cholic acid (CA) within the gut. DCA is known to promote hepatocyte inflammation and, by association, liver carcinogenesis (Jia et al. 2018). Recent report suggests that the primary to secondary bile acid balance and the resultant CXCL16 could be a determinant of liver tumor growth. Indeed, depletion of gram-positive phylum *Firmicutes* decreased CXCL16 levels, deactivating CXCR6 on liver sinusoidal endothelial cells, thereby enhancing recruitment of NKT cells and reduction in tumorigenesis (Ma et al. 2018). Additionally, the role of bile acids in hepatocellular carcinoma (HCC) and colorectal cancer (CRC) is well described and currently the target of research in the context of microbial dysbiosis (Jia et al. 2018; Jones et al. 2014; Ridlon and Bajaj 2015; Hylemon et al. 2006; Ridlon et al. 2014, 2016).

10.2.2 Mediators of Oxidative Stress

Our recently published study shows that in models of type 2 diabetes, there was a distinct enrichment of bacterial population that metabolize antioxidants and thus contribute to therapy resistance in pancreatic cancer (Kesh et al. 2020). Other studies implicated glutathione, an antioxidant that is responsible for reactive oxygen species scavenging, in reducing oxidative stress in the intestine (Wanders et al. 2020). Studies have shown that germ-free mice have lower levels of intestinal glutathione synthesis compared to conventionally raised mice (Mardinoglu et al. 2015). Deficiency of queuosine, another microbial metabolite, has been shown to promote Warburg metabolism and reversal of mitochondrial ATP synthase in HeLa cells (Hayes et al. 2020). Role of queuosine in cancer is not well understood, but studies have shown its role in affecting growth and progression of multiple cancers (Ishiwata et al. 2001; Pathak et al. 2008; Fergus et al. 2015; Zhang et al. 2020).

Trimethylamine (TMA) is a bacteria-derived metabolite that is converted to trimethylamine-*N*-oxide (TMAO) in the host liver by flavin-containing monooxygenase 3 (FMO3). TMA is produced in the gut by bacterial conversion of dietary choline and L-carnitine (Koeth et al. 2013). TMAO has been associated with atherosclerosis and kidney disease. Inhibition of gut microbial TMA showed significant improvement in atherosclerosis model (Wang et al. 2011). FMO3, the enzyme responsible for conversion of TMA to TMAO, has been closely linked with gut microbiome. There are five functional FMO (FMO1-5) in humans. Among these, FMO3 is instrumental in catalysis of TMA to TMAO (Lang et al. 1998). FMO3 is thus a host enzyme that participates in the metabolic interactions between the host-gut microbiome (Shih et al. 2015). In a study by Romano et al. (2015*)*, it was observed that upon in vitro incubation in mice gut medium supplemented with choline, a significant part of gut bacteria produced TMA. These bacteria were predominantly of the *Firmicutes* and *Proteobacteria* phyla since they were the ones that had the enzymes required for conversion of L-carnitine to TMA. Interestingly, even though *Bacteroidetes* is among the most common bacterial phyla, they were absent in this study since they lacked this enzyme, and production of TMA was is not only reliant on the choline and L-carnitine intake, but also the relative ratio of *Firmicutes* and *Bacteroides* in the gut.

There have been a few studies linking TMA and cancer. Plasma TMAO levels have been positively correlated with CRC risk in women (Bae et al. 2014) as well as in prostate cancer (Mondul et al. 2015) and oral squamous cell carcinoma (Bag et al. 2015). The exact mechanism that would link cancer to TMA/TMAO is unknown. One hypothesis is that the link is merely a correlation and that the true increased risk factor is disruption of the gut barrier, which allows for the transportation of TMA from the gut to the liver, where it is oxidized. Studies also show that cell cycle progression is tied to TMAO production (Janeiro et al. 2018), which can suggest a possible link to carcinogenesis. Oxidative stress is strongly associated with cancer. It has also been implicated as one of the factors linking TMAO and cancer. Increased level of circulating TMAO was shown to promote oxidative stress by inducing production of superoxides. Accumulation of reactive oxygen species or ROS and induce superoxide production, a reactive oxygen species (ROS) is linked to oxidative stress (Li et al. 2017). Studies have shown TMAO to be associated with generation of *N*-nitroso compounds that can lead to DNA damage and contribute to carcinogenesis (Chan et al. 2019).

Another metabolic pathway that is instrumental in modulation of oxidative stress and maintenance of the host-microbe symbiosis is the tryptophan metabolism pathway. Tryptophan catabolism by indoleamine-2,3-dioxygenase-1 (IDO1) feeds into the kynurenine pathway and is vital for the production of NAD. Kynurenine is associated with oxidative stress, DNA replicative stress, and modulator of mitochondrial function (Castro-Portuguez and Sutphin 2020). Activity of this pathway in increased in HIV-positive individuals and correlates to increased relative abundance of specific bacteria genera in these patients (Favre et al. 2010; Vujkovic-Cvijin et al. 2013). While this correlation is in part due to the upregulation of the kynurenine pathway in host cells, some of these bacteria possessed homologs of human

tryptophan catabolism genes and are likely directly converting tryptophan to kynurenine (Vujkovic-Cvijin et al. 2013). Kynurenine, itself, has been identified as an oncometabolite (Venkateswaran and Conacci-Sorrell 2020). The kynurenine has been shown to accumulate in breast and colon cancer. Additionally, this increase was not associated with an increase in host IDO1 expression, which may indicate bacterial production (Puccetti et al. 2015).

Lignans are polyphenols derived from dietary fibers. Conversion of dietary lignans to enterolignans by gut bacteria has been investigated for its impact on health, with several studies indicating a role in cancer development. Gut microbes are responsible for metabolism of lignans to enterolactone and enterodiol (Adlercreutz 2007; Wang et al. 2010). Intestinal and urine enterodiol and enterolactone were undetectable in germ-free rats, but after the introduction enterolignan-producing bacteria, these metabolites were present (Mabrok et al. 2012). This indicates a strong role for the gut microbiome in production of enterolignans.

10.2.3 Polyamines

Polyamines are low-molecular-weight aliphatic polycations, highly charged and ubiquitously present in all living cells. Interest has been increasing during the last 30 years in the naturally abundant polyamines *putrescine* (diamine), *spermidine* (triamine), and *spermine* (tetraamine), which were demonstrated to be involved in a large number of cellular processes. In eukaryotic cells, the polyamines support cell growth and survival. Additionally, they are associated with nucleic acid biosynthesis, maintenance of chromatin conformation, regulation of specific gene expression, ion-channel regulation, maintenance of membrane stability, provision of a precursor in the synthesis of eukaryotic translation initiation factor 5A (EIF5A), and free-radical scavenging. In addition to their de novo polyamine synthesis, cells can take up polyamines from extracellular sources, such as cancer tissues, food, and intestinal microbiota. Decreased levels of polyamines are associated with disturbance of the cell cycle and induction of apoptosis. High levels of polyamines are associated with tumor growth, and inhibitors of polyamine biosynthesis have been used to treat cancer (Polyamines 1999). Main bacterial contributors of polyamines in the human gut include members of the *Bacteroides* and *Fusobacterium* genera (Noack et al. 2000; Tofalo et al. 2019). In a spontaneous mouse model for pancreatic cancer using the $KRAS^{G12D}TP53^{R172H}Pdx^{Cre}$ (KPC) mice, we observed development of a dysbiotic microbiome over the course of their disease progression. Predictive metabolomic analysis of this altered microbiota indicates an increase in microbe-derived polyamines (Mendez et al. 2019). This finding correlates with increased plasma polyamine levels in KPC mice. As previously stated, increased polyamine levels are associated with cancer development and tumor progression, since they are actively involved in nucleic acid metabolism. These findings highlight the gut microbiome as drivers of pancreatic cancer progression. In breast cancer, studies

have indicated that breast tissue has its own microbiome in healthy and diseased states (Hieken et al. 2016; Urbaniak et al. 2014). Additionally, new evidence is emerging that shows the gut microbiome is an important player in disease progression. Cadaverine, a polyamine produced by gut bacteria, was found to be effective in treating cancer in a mouse model, as well as reducing stemness in cultured breast cancer cell lines (Kovács et al. 2019). Additionally, breast cancer patients had lower fecal DNA content for cadaverine-producing enzymes compared to healthy controls. Thus, cadaverine is considered to be a regulator of early breast cancer.

10.2.4 Short-Chain Fatty Acids

Short-chain fatty acids (SCFAs) like acetate, propionate, and butyrate are produced from dietary fiber including non-starch polysaccharide and oligosaccharides through bacterial fermentation in the colon. They are a fundamental energy source for gut epithelial cells. Acetate constitutes about ~60% to 75% of the total SCFAs and is generated by many bacterial groups via reductive acetogenesis (Morrison and Preston 2016). A large spectrum of bacteria produce acetate. These include *Akkermansia*, *Bacteroides* spp., *Bifidobacterium* spp., *Prevotella* spp., *Ruminococcus* spp., *Blautia hydrogenotrophica*, *Clostridium* spp., and *Streptococcus* spp. (Rey et al. 2010) that are commonly present in the gut. The other SCFAs like butyrate and propionate are produced by more specialized bacteria. The main propionate-producing bacteria, apart from the commonly occurring *Bacteroides* spp., are *Phascolarctobacterium succinatutens*, *Megasphaera elsdenii*, *Veillonella* spp., *Dialister* spp., *Coprococcuscatus*, *Roseburia inulinivorans*, *Salmonella* spp., and *Ruminococcus obeum* (Louis et al. 2014). The main butyrate-producing bacteria are *Coprococcus comes*, *Coprococcus eutactus*, *Eubacterium hallii*, *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Anaerostipes* spp., *Coprococcuscatus*, and *Roseburia* spp. While the primary role of the SCFA are to produce energy, studies have shown that SCFAs can influence colonocyte growth and differentiation.

Butyrate is selectively taken up by the monocarboxylate transporters (MCT1) in the colonic epithelium and is used by colonocytes for their energy homeostasis at a low concentration. At higher concentration, butyrate acts as a HDAC inhibitor and regulates gene transcription by influencing the epigenetic machinery of the cell (Zeng et al. 2017). This further influence multiple signaling pathways and affects the cell cycle of the colonocytes. In osteosarcoma cells, butyrate acts in an antiproliferative role and induces expression of anti-inflammatory mediators (Perego et al. 2018). Role of butyrate as an antineoplastic agent is being evaluated. Like butyrate, propionate also affects proliferation of cells. Studies have shown that gut microbiome-produced propionate can induce cell cycle arrest and apoptosis in lung cancer cells in vitro (Kim et al. 2019). Independent studies show that gut microbiota derived propionate can also abrogate experimental colitis in mice via regulation of Reg3 mucosal lectins. Dietary fructose is converted to acetate by the gut bacteria and

is routed to the liver. In the liver, it is used for hepatic lipogenesis. Studies using in vivo isotope tracing show that liver-specific deletion of ATP citrate lyase or ACLY in mice is unable to suppress fructose-induced lipogenesis, confirming the involvement of microbial metabolites in this process (Zhao et al. 2020).

While in vitro studies indicated that SCFAs may have antineoplastic properties, recent studies indicate that SCFAs may be associated with immune evasion and resistance to checkpoint inhibitor therapy. In mouse models, butyrate was found to inhibit anti-CTLA4 induced upregulation of CD80/CD86 on dendritic cells as well as inhibit ICOS on T cells. Further, butyrate also prevented accumulation of tumor-specific T cells and memory cells. Similarly, in patients, high blood butyrate levels ameliorated ipilimumab-induced accumulation of memory and ICOS + CD4 + T cells and IL-2 infiltration. These observations indicated that SCFAs like butyrate may confer immune suppression to cancer cells and negate the effect of checkpoint inhibitor therapy (Coutzac et al. 2020).

10.3 Future Directions

In the last two decades, the role of gut microbiome in regulating health and disease has become clear. However, the specific implications of the changes in diversity of the microbial population in the disease stage has still remained an enigma. It now appears that the microbiome influences the host inflammatory and immune pathways in multiple ways. The most obvious modulators are the microbial metabolites. These compounds arising from breakdown products of the dietary components synergize with the host metabolism to fuel critical signaling pathways. In cancer, the need for heightened proliferation requires efficient nucleic acid and protein biosynthesis; efficient means to combat the oncogenesis-induced stress and upregulation of resistance mechanisms. These needs are often met by the microbial metabolites produced in the gut. Increased polyamine production assists purine and pyrimidine production that feeds into the nucleic acid biosynthesis pathway. The bacterial polyamines supplement the host polyamines in cancer to meet the increased need. Similarly, oncogenesis-associated enhanced proliferation leads to activation of stress pathways in the transformed cells. Bacterial metabolites like queuosine and kynurenine help in protection from these altered redox state by decreasing accumulated reactive oxygen species. Thus, it is possible that during tumor progression, the host and its microbiome enter into a positive synergy that is conducive for tumor growth and progression. The effectiveness of chemotherapeutic drugs has been linked to microbial presence, and some drugs, such as gemcitabine, have been found to have increased effectiveness when used in combination with antibiotics (Imai et al. 2019). A 2017 study found that human colorectal cancer cells grown in culture with bacteria were resistant to gemcitabine treatment. Further inquiry revealed that the bacteria responsible for this possessed a form of the cytidine deaminase gene that is capable of directly degrading gemcitabine (Geller et al. 2017). The combination of this finding with the previously known combinatory

effect of the drug with antibiotics tells the story of microbial influence in chemotherapeutic response variation. The previously mentioned presence of bacteria in the tumor microenvironment also indicates the potential for microbial degradation of chemotherapeutic agents in the gut or at the tumor site (Pushalkar et al. 2018).

Interestingly, depleting microbiome has not always resulted in tumor regression. In a study published in Science in 2017, Routy et al. found that antibiotic administration to patients in conjunction with immunotherapy was associated with shorter progression-free survival (PFS) and shorter overall survival (OS) (Routy et al. 2018). Similarly, the use of probiotics as treatment for microbial deficiencies has been met with mixed results. Fecal microbiome transplantation has emerged as another mechanism for modulating gut microbiome and microbial metabolome. This has proven effective as method of microbial community engraftment. This method has been used clinically to significantly improve the clinical cure rates of recurrent *Clostridium* difficile infection (Kelly et al. 2016; Khoruts and Sadowsky 2011). Given the previously discussed alterations of the gut microbial communities associated with the diseased state and potential of these altered communities to impact pathology and treatment efficacy, engraftment of a microbial community may be an effective measure in the treatment.

These observations indicate that more in-depth research is required to specifically understand the role of microbial metabolites on host signaling pathways. Despite a large number of studies being done in this area, the relationship between the different bacterial communities in the gut and their collective relationship with the host remains an enigma. Without understanding this, attempts to modulate the microbiome in hope of tackling diseases like cancer may be premature. Another difficulty in the field is that the whole paradigm of microbiome and microbial metabolism is dependent on high-throughput platforms and expensive and requires extremely specific analytical skillsets. Both microbial sequencing and mass spectrometry-based metabolomic analysis have numerous analytical variables, that may lead to slightly different results from a single dataset. In recent times, the Human Microbiome Project (https://www.hmpdacc.org/) has specified the parameters that are recommended for end-to-end analysis of microbiome for both 16s pyrosequencing and shotgun metagenomics. However, the metabolomics landscape in the context is still very chaotic and requires a concerted effort for formulating unifying guidelines for measuring and annotating microbial metabolites. While the San Diego GNPS effort (https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp) is a positive step in this regard, additional streamlining of the processes is required to improve quality and consistency of analysis pipelines to generate meaningful data.

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Chapter 11
Diet, Microbiome, Inflammation, and Cancer

Xiaotao Zhang and Carrie R. Daniel

Abstract Interactions between host nutrition, microbiota, and inflammation is an area of intense and growing interest to prevent or halt cancer. In complement to recent reviews focusing on the experimental evidence and the potential mechanisms underlying these relationships, this book chapter focuses on human observational and interventional studies of the microbiome as it relates to dietary patterns and key dietary factors with established links to inflammation and inflammation-driven cancers. Toward establishing causality in humans and the development of broadly beneficial targets, human prospective and interventional data bolster experimental models demonstrating that dietary components or patterns, at least in part, shape the composition of the gut microbiome and that diet and the gut microbiome, independently and collectively, modulate localized and systemic inflammation, as well as other critical pathways to cancer initiation and progression. Thus, dietary interventions, if capable of achieving timely and sufficient long-term behavior change, could theoretically be used to "improve" microbiome community structure and function and/or displace or reduce bacteria that promote cancer. Research focused on various parameters of microbiome-based personalized nutrition in cancer prevention and treatment is likely to continue to inform important targets and interactions that will shape clinical and public health practice.

Keywords Dietary patterns · Inflammatory biomarkers · Human gastrointestinal microbiome · Cancer prevention · Human prospective studies

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11.1 Introduction

The human gut harbors diverse and abundant microbes, creating a complex ecological system that interfaces with both the host and the environment and facilitates biologically relevant interactions between the two. The gut microbiome (collective genome of microorganisms in an environment) or microbiota (a community of microorganisms) comprise the "hidden" organ supporting host immunity, energy homeostasis, and nutrient metabolism (Klement and Pazienza 2019). The breadth of the gut microbiome's capacity is reflected in the over three million genes it encodes, as well as the thousands of metabolites it produces (if not more, yet unknown). These microbial metabolites facilitate various physiological functions including modulating oxidative stress, the integrity of the intestinal barrier, and inflammation in the host (Singh et al. 2019).

The role of the microbiome in cancer risk and outcomes, and how to modulate this process, is an area of intense and growing interest (Scott et al. 2019; Schwabe and Jobin 2013; Daniel and McQuade 2019), with estimates suggesting that microbial agents may cause ~20% of the global cancer burden (Pevsner-Fischer et al. 2016). The relationship between the microbiome and cancer is multifactorial and, likely, bidirectional. Cancer-associated changes in the microbiome may occur as a result of the emergence or presence of a tumor and may also contribute to cancer progression—both early and over the course of treatment (Vivarelli et al. 2019).

The link between host nutrition or dietary habits, microbiota, inflammation, and cancer risk is also growing stronger (O'Keefe 2016). In a groundbreaking trial, O'Keefe and colleagues found that reciprocal changes in traditional diets (modulating both fiber and fat) in African Americans and native Africans over just 2 weeks resulted in dramatic changes in the composition and structure of the gut microbiome, as well early markers of cancer risk or cell proliferation within the colorectal epithelium (O'Keefe et al. 2015). In prospective studies using tissue samples and data from the Nurses' Health Study and Health Professionals Follow-Up Study, Ogino and colleagues found that a prudent diet rich in plant food sources of dietary fiber, as compared to a typical Western diet, is associated with lower risk of *Fusobacterium nucleatum*-positive colorectal tumors (Mehta et al. 2017), but no association was observed with cancer arising from *F. nucleatum*-negative tumors. Ogino and colleagues also recently reported that the inflammatory potential of the diet modulates risk of F. *nucleatum*-positive colorectal tumors, particularly in the proximal colon (Liu et al. 2018). The presence of *F. nucleatum* has also been linked to a microenvironment that promotes the progression of colorectal neoplasia (Wu et al. 2019) by inhibiting T cell-mediated immune responses against colorectal tumors (Hamada et al. 2018).

Although several studies suggest that the microbiome plays an important role in diet, inflammation, and cancer development, the mechanisms involved in these processes are not fully understood or established as causal in human data. In complement to recent reviews focusing on the experimental evidence and potential mechanisms underlying these relationships (Zitvogel et al. 2017a; Whisner and

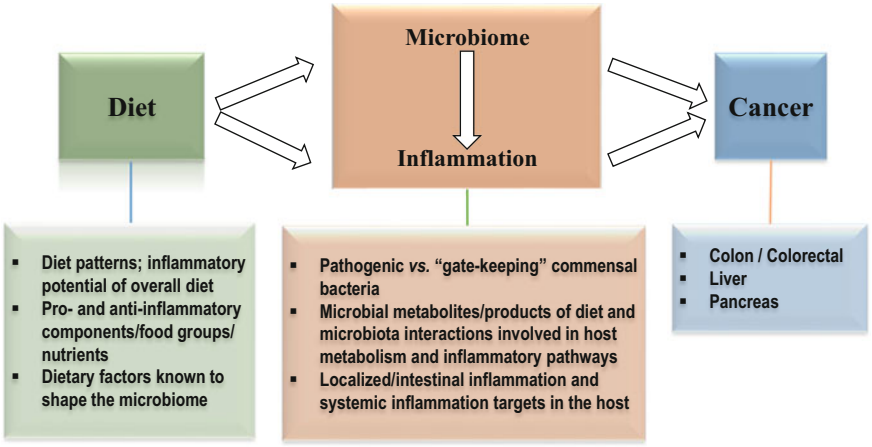

Fig. 11.1 Diet, microbiome, inflammation, and cancer: working model for chapter

Aktipis 2019; Bultman 2017; Tilg et al. 2020), this book chapter focuses on human observational studies and clinical trials to examine the association between diet, microbiome, inflammation, and cancer (Fig. 11.1). We searched the literature for the following terms: "diet," "nutrition," "dietary pattern," "dietary index," "microbiome," "microbiota," "inflammatory," "inflammation," "cancer," "carcinogenesis," "carcinoma," and "adenocarcinoma" with respect to studies conducted in 2009–2019. We focused on dietary factors or eating patterns (not supplements) that have previously been linked to cancer and inflammatory mechanisms (World Cancer Research Fund and American Institute for Cancer Research 2018).

11.2 Microbiome, Inflammation, and Cancer

The gut microbiome plays an essential role in gut and systemic inflammation. Chronic inflammation is a maladaptive version of acute inflammation in which the immune response persists for extended periods of time and is characterized by elevated levels of tumor necrosis factor alpha (TNF-a), interleukin-6 (IL-6), and C-reactive protein (CRP) (Buford 2017). Markers of gut dysbiosis have been linked to several circulating inflammatory cytokines in human and experimental data and suggest that a sustained dysbiotic state leads to dysregulation of various key functions, which, in turn, contribute to the development of autoimmune conditions such as inflammatory bowel disease, systemic inflammatory arthritis, multiple sclerosis, and systemic lupus erythematosus (Clemente et al. 2018).

Inflammatory diets may contribute to the development of dysbiosis by decreasing beneficial microorganisms or their metabolites and/or promoting the growth of harmful bacteria (Fig. 11.1) (Brown et al. 2012; Lachnit et al. 2019). Interactions between inflammatory, or high-fat and low-fiber diets, and microbes modulate the

balance of functional metabolites, including short-chain fatty acids (SCFA), bile acids, and products of mucin degradation (den Besten et al. 2013; Koropatkin et al. 2012). Several commensal bacteria, including members of the *Enterobacteriaceae* family, the *Bacteroides* and *Porphyromonas* genus, *Akkermansia muciniphila*, and *Clostridium ramosum* species have been associated with inflammation (Belkaid and Hand 2014; Wang et al. 2018).

Several gram-positive and opportunistic pathogenic bacteria directly induce inflammation. A recent randomized double-blinded, placebo-controlled crossover trial conducted in healthy adults, found that consumption of *E. coli*-targeting bacteriophages, is associated with reduced levels of circulating IL-4. The researchers postulated that phage treatment resulted in lower levels of bacterial lipopolysaccharide (LPS), which decreased IL-4 expression and, consequently, reduced systemic inflammation (Febvre et al. 2019). Facultative aerobe, *Escherichia coli*, and strictly anaerobic *Bacteroides fragilis* (described below), are linked with the development of inflammatory bowel disease (IBD), as well as colorectal cancer (Wang et al. 2007). *Enterococcus*, a frequent contaminant in foods, is also associated with inflammation (Gonzalez-Navajas et al. 2008).

Conversely, certain fractions of gut microbiota are less prone to inducing inflammation and may even inhibit systemic inflammation. In particular, bacteria with the capacity to produce butyrate, short-chain fatty acids (SCFA) and preferred energy source for other potentially beneficial and "gate keeping" bacteria, exhibit anti-inflammatory properties. *Faecalibacterium prausnitzii* exerts anti-inflammatory effects by secreting metabolites, including butyrate, that block the NF-κB pathway and IL-8 secretion (Quévrain et al. 2016). *Eubacterium spp.*, another butyrate producer, is involved in stimulating enterocyte turnover and in maintaining tight barrier junctions (Gobert et al. 2016). Butyrate-producing *Clostridium* cluster XIVa is also inversely correlated with systemic inflammation (Duytschaever et al. 2013; Van den Abbeele et al. 2013). Significant reductions in these bacterial populations are associated with (and may precede) the development of inflammatory conditions within the gastrointestinal tract (and beyond). When a healthy and stable gut microbial community is compromised, gram-negative bacteria meet little resistance and are able to "take over." These changes in the microbiome increase intestinal permeability and/or the release of proinflammatory endotoxins, leading to the development of leaky gut syndrome and chronic inflammation (Hakansson and Molin 2011).

Microbiota exert proinflammatory and immunosuppressive effects to subvert anticancer immunosurveillance (Vivarelli et al. 2019; Zitvogel et al. 2017b). A dysregulated microbiome and its products favor the generation of trophic growth factors with microbiota-mediated alterations in circulating metabolites initiating a cascade that promotes tumor growth (Fessler et al. 2019). Pathobiotic and pathogenic bacteria promote CRC by inducing colonic chronic autoimmune inflammation. In both cases, chronic inflammation elicits repeated cycles of tissue damage and regeneration and then generates oxidative stress. This results in accumulated DNA damage in epithelial cells, eventually leading to tumor development in the gut (Chen et al. 2017). For example, infection with *H. pylori* increases the risk of non-cardia

gastric cancer by injecting a toxin (produced by cytotoxin-associated gene A) and altering the structure of epithelial cells lining the stomach, allowing the bacteria to infiltrate or attach (Polk and Peek 2010; Ryoo et al. 2019). Long-term exposure to the toxin causes chronic inflammation. Similarly, *B. fragilis* and *E. coli* produce colibactin, a bacterial genotoxin that promotes colon tumors (Wilson et al. 2019; Dejea et al. 2018). Several other bacterial pathogens have been associated with colorectal cancer (Shmuely et al. 2014; Wei et al. 2016), including *Fusobacterium nucleatum* (Nosho et al. 2016; Ye et al. 2017; Yu et al. 2016; Kostic et al. 2013), *Peptostreptococcus anaerobius* (Tsoi et al. 2017; Hibberd et al. 2017), and sulfidogenic bacteria (Yazici et al. 2017; Hale et al. 2018). Chronic/persistent *Salmonella* infection has also been linked to the development of colitis-associated colorectal cancer (Lu et al. 2014).

The gut microbiota also encounters antigens, carcinogens, or their substrates via diet (Zhernakova et al. 2016; Falony et al. 2016). Diet has long been linked to the development of different human gastrointestinal tract (GIT) tumor types and appears to do so largely via effects on microbial metabolites or via pro-carcinogenic activities of specific pathogens (Louis et al. 2014). For example, secondary bile acids induce inflammation and DNA damage (Nguyen et al. 2018; Zeng et al. 2019), while butyrate pathways suppress inflammation and inhibit neoplastic changes (Morrison and Preston 2016).

11.3 Diet and Microbiome Interactions

Recent observational studies and dietary intervention trials targeting the gut microbiome have shown that of all exogenous factors affecting the gut microbiome in healthy individuals, long-term diet appears to exert one of the strongest effects (Xu and Knight 2015). While investigating changes in the microbiota during and after dietary intervention may ultimately inform the design of effective nutrition therapies (Xu and Knight 2015), the microbiome is a resilient environment (Xu and Knight 2015). Extreme changes in diet can induce rapid alterations in the relative abundance of different bacteria with the gut; however, predominant phyla and overall structure of the microbial community are largely determined by inter-individual variation and long-term diet (David et al. 2014). Therefore, dietary interventions designed to establish long-term behavior change or maintainable dietary habits could theoretically be used to modulate microbiome community structure and function to improve health and, through healthy competition, to displace or decrease bacteria previously demonstrated to be causally related to disease.

In this book chapter, we focus on the key diets or dietary factors known to shape the microbiome with links to both inflammation and cancer (Fig. 11.1). These include measures of overall dietary patterns or diet quality, as well as key tenants of a high-quality and microbiome-enriching diet, including dietary fiber and other plant-derived bioactive components. Various types of fat and protein and the food

groups they arise from are also reviewed. As detailed in major reports and evidence-based recommendations from the World Cancer Research Fund/American Institute for Cancer Research and American Cancer Society, as well as our recent commentary, a diet that supports a reduced risk of inflammation and cancer is largely composed of plant foods (vegetables, fruits, whole grains) and low in processed foods (refined grains, added sugars, trans fats) with protein primarily derived from fish and plant sources (pulses and legumes) (Daniel and McQuade 2019; World Cancer Research Fund and American Institute for Cancer Research 2018; Kushi et al. 2012).

11.3.1 Diet Pattern

Shifts in multiple dietary components or patterns, e.g., Western, Mediterranean, prudent, animal-based, and plant-based diet patterns, are linked to shifts in the diversity and composition of the gut microbial community (Bhat and Kapila 2017; Telle-Hansen et al. 2018). The Western dietary pattern, characterized by high intake of sweets, refined grains, and red and processed meat, is associated with increased levels of inflammatory proteins, gut dysbiosis, and a dysregulated immune signature (Song and Chan 2017; Hills et al. 2019). Several observational studies have examined how different diet patterns or diet quality indices contribute to the inter-individual variability in microbiome composition and diversity.

A large cross-sectional study of 2070 participants from the TwinsUK cohort examined associations of fecal microbiota diversity with three different defined dietary indices representing food diversity and adherence to US dietary recommendations (Healthy Eating Index [HEI]) or the Mediterranean diet (MD). They observed that each of these diet indices explained a reasonable proportion of the observed variation in α diversity (microbial richness and/or evenness), but the HEI performed the best (Bowyer et al. 2018). Maskarinec and colleagues also examined diet quality as assessed by HEI-2010, Alternative HEI-2010, MD, and Dietary Approaches to Stop Hypertension (DASH) in relation to fecal microbial diversity and community structure within a subset of the Multiethnic Cohort study. Across all four indices, higher diet quality was associated with higher microbial diversity and lower levels of *Collinsella*. Fiber-fermenting bacteria, such as *Faecalibacterium*, *Lachnospira*, and *Ruminococcus* were also consistently associated with higher diet quality (Maskarinec et al. 2019). Another recent cross-sectional study assessed overall diet quality (HEI) in relation to the colonic mucosa-associated gut microbiome of 34 healthy participants who provided 97 samples. They found that *Alistipes*, *Barnesiella*, *Bifidobacterium*, *Fusicatenibacter*, and *Odoribacter* were associated with high diet quality. Conversely, low diet quality was associated with lower α-diversity; lower abundance of *Roseburia*, *Subdoligranulum*, and *Parabacteroides*; and higher abundance of *Fusobacterium* and *Escherichia* (Liu et al. 2019).

Adherence to the MD in relation to the gut microbiome has been examined in several observational studies and limited trials. A European cross-sectional study of healthy adults found that adherence to MD diet was not associated with microbial diversity as measured by the Shannon index (representing biodiversity or both richness and evenness) but was positively associated with gut microbial richness, alone (as measured by the Chao index). Garcia-Mantrana and colleagues conducted a cross-sectional study to determine the effect of nutrient compounds, as well as adherence the MD, on the gut microbiome of healthy adults living in the Mediterranean region. They found that a higher MD score was related to a lower *Firmicutes*/*Bacteroidetes* ratio, as well as higher levels of *Catenibacterium* genus. Lower adherence to the MD and higher consumption of animal proteins, saturated fats, and simple sugars was associated with decreased microbial richness and diversity, while higher consumption of plant-based nutrients, such as plant protein, polysaccharides, and dietary fiber, was associated with higher levels of *Bifidobacterium* and total fecal SCFA (Garcia-Mantrana et al. 2018). A cross-sectional study among healthy Italians compared a group of individuals following a modern Paleolithic Diet (PD) for at least a year to those following a Mediterranean Diet (MD) (Barone et al. 2019). *Faecalibacterium*, *Bacteroides*, and *Prevotella* were higher in the PD, as compared to the MD group. Microbial diversity was much higher in the PD group than MD group and comparable to that observed among Hadza hunter-gatherers from Tanzania. These results indicate that returning to a modern PD composed of natural (and region-specific) foods but excluding dairy, grains, refined sugar, and other processed foods could counteract the loss of microbiome diversity observed in Western societies.

Zimmer and colleagues compared fecal samples from omnivores, vegetarians, and vegans compared to omnivores and found that *Bacteroides* spp., *Bifidobacterium* spp., *Escherichia coli*, and *Enterobacteriaceae* spp. were significantly lower in vegan samples than in controls (Zimmer et al. 2012). An omnivorous diet versus vegetarian diet has also been linked to increased urinary levels of trimethylamine *N*-oxide (TMAO), a gut microbiota-derived choline metabolite associated with increased risk for heart disease and colorectal cancer (CRC) (Wu et al. 2016).

Within the last decade, groundbreaking controlled feeding studies showed that short-term, dramatic swaps or shifts in diet patterns resulted in significant shifts in the gut microbiome. As described previously, the O'Keefe trial found that administration of a traditional African, high-fiber and low-fat diet among African Americans was associated with a significant decrease in the levels of *Bilophila wadsworthia*. Conversely, a Western, low-fiber, high-fat diet administered to native Africans was associated with increased levels of *Fusobacterium nucleatum* (O'Keefe et al. 2015). David and colleagues conducted a controlled 30-day crossover interventional study and found that a change in diet to either an exclusively animal-based or plant-based diet resulted in significant changes in gut microbiota diversity within 5 days (David et al. 2014). A 2-week dietary intervention trial conducted among a Russian urban population targeted increased consumption of specific healthy food products underrepresented in the volunteer's long-term diet and decreased consumption of

overrepresented "junk foods." They found that a higher intake of vegetables and fruits was associated with increased levels of butyrate-producing Clostridiales and community richness. In addition, the dietary intervention produced profound changes in community structure, and *Methanobrevibacter*, *Bifidobacterium*, *Clostridium*, and *Lachnospiraceae* increased (Klimenko et al. 2018).

11.3.2 Key Components of Inflammation-Related Diet

Numerous studies have examined how individual dietary components, such as fiber, fat, and protein type, affect the gut microbiome.

11.3.3 Dietary Fiber

Dietary fiber influences gut transit (Muller et al. 2018) and shapes the composition and metabolic or functional capacity of the gut (fecal) microbiome (Vandeputte et al. 2016). High dietary fiber intake is associated with increased levels of butyrate-producing genera such as *Clostridium*, *Eubacterium*, *Roseburia*, and *Anaerostipes* (Bach Knudsen et al. 2018). The gut microbiome exhibits a strong preference for fiber-based substrates. In the absence of dietary fiber, a compensatory shift involving increased levels of mucin-degrading bacteria and enzymes occurs (Desai et al. 2016). Garcia-Mantrana et al. observed that higher intake of dietary fiber in the context of the MD was associated with increased levels of *Methanobrevibacter* genus, whereas low consumption of dietary fiber was related to a predominance of *Blautia* and *Bulleidia* (Garcia-Mantrana et al. 2018). Another observational study evaluated associations of dietary fiber intake with the gut microbiome among colorectal adenoma patients vs. healthy controls and reported that a high-fiber dietary pattern in conjunction with bacteria that produce butyrate is associated with lower risk of advanced adenomas (Chen et al. 2013).

In the O'Keefe trial, a high-fiber/low-fat diet was associated with increased levels of potential butyrate producers (such as *Eubacterium rectale* and *Clostridium symbiosum*) and bacteria associated with the utilization of complex carbohydrates, such as *Oscillospira guilliermondii* (O'Keefe et al. 2015). A recent randomized crossover clinical trial conducted in 60 Danish adults at risk for metabolic syndrome testing an increase in whole grains reported that while a whole grain-rich diet did not appear to significantly affect the diversity and composition of the gut microbiome, it reduced body weight and systemic low-grade inflammation (Roager et al. 2019).

11.3.4 Fat

Accumulating evidence in animal models and limited human data suggests that overall, a high-fat diet, particularly from animal sources, promotes an inflammatory milieu in the gut, characterized by an overgrowth of inflammatory and bile-tolerant bacteria, decreased numbers of beneficial or butyrate-producing bacteria, and concurrent promotion of tumorigenesis via activation of the TGFB1/SMAD3 and NF-κB signaling pathway (Zhang et al. 2018; Agus et al. 2016). Types of fat or fatty acid composition also affect microbiome diversity and composition. Omega-3 polyunsaturated fatty acids (PUFA) from seafood, nuts, and seeds are associated with higher diversity of intestinal microbiota. An omega-3 PUFA-rich diet may also be useful to ameliorate gut dysbiosis induced by antibiotics or omega-6 PUFA (Menni et al. 2017). Other studies suggest that the ratio of saturated fatty acids and MUFAs, rather than overall fat amount, may also affect the microbiome (Lang et al. 2018).

A total of 88 UK adults at increased risk of metabolic syndrome were fed a high saturated fat diet (HS) for 4 weeks (baseline) and then randomized to one of five experimental diets for 24 weeks: HS, high monounsaturated fat (MUFA)/high glycemic index (GI) (HM/HGI); high MUFA/low GI (HM/LGI); high carbohydrate (CHO)/high GI (HC/HGI); and high CHO/low GI (HC/LGI). High-MUFA diets did not affect individual bacterial population numbers but reduced total bacteria and plasma total and LDL cholesterol. The low-fat, HC diets increased fecal *Bifidobacterium* and reduced fasting glucose and cholesterol compared to baseline (Fava et al. 2013).

11.3.5 Protein

The effects of protein intake vary with respect to the type and the source, e.g., plant-based protein versus animal-based protein and intake of red/processed meats versus poultry or fish. Meat is a rich source of sulfur-containing amino acids such as cysteine and methionine. Red meat provides a bioavailable source of heme iron and processed meat typically contains inorganic salt curing agents or preservatives including nitrites and sulfites (Brosnan and Brosnan 2006).

When comparing meat-eaters to non-meat eaters (vegetarians/vegans), individuals that derive majority of their protein from animal sources exhibited reduced levels of butyrate-producing bacteria such as *Roseburia*, *Ruminococcus bromii*, and *Eubacterium rectale* (David et al. 2014; Riviere et al. 2016), while a diet high in red meat was associated with increased fecal levels of *Bacteroides*, *Fusobacterium*, *Streptococcus bovis*, *Clostridium*, and *Helicobacter pylori* (Zimmer et al. 2012).

The David et al. short-term feeding study reported that consumption of plant protein was associated with increased *Lactobacillus* and *Bifidobacterium*, decreased levels of *Clostridium* and *Bacteroides*, and increased production of fecal SCFAs

(David et al. 2014). In the Animal and Plant PROtein and Cardiovascular Health (APPROACH) trial, results suggested that inter-individual differences outweighed the influence specific 4-week dietary changes on the microbiome and that moderate changes in saturated fat level and protein source correspond to modest changes in the microbiome (Lang et al. 2018).

11.3.6 Micronutrients and Bioactive Components of Plant Foods

In addition to providing dietary fiber, plant-based protein, and healthy fatty acid profiles of mono- and polyunsaturated fatty acids, plant foods, such as colorful fruits and vegetables, whole grains, legumes, nuts, and seeds, contain other bioactive components with varied effects on the gut microbiome and metabolites. These include numerous vitamins (A, B, C, D, E, K), flavonoids, indoles, inositol, polyphenols, terpenes, and carotenoids, to name a few (Pandey and Rizvi 2009). As reviewed previously, several observational studies examined the associations of micronutrients (Biesalski 2016); however, gut microbiota associated with fat-soluble vitamins, such as vitamin A, K, and D, remain unclear.

Walnuts are rich in omega-3 fatty acids, phytochemicals, fiber, phenolic compounds, folate, and vitamin E, and their effect on the gut microbiota was assessed in a randomized crossover controlled feeding study in 18 healthy participants. Walnut consumption was associated with increased *Firmicutes*, including butyrate-producing *Clostridium* clusters XIVa and IV, *Faecalibacterium*, and *Roseburia*, and with reduced levels of microbially derived proinflammatory fecal secondary bile acids and LDL cholesterol. However, walnut consumption was also associated with decreased *Ruminococcus*, *Dorea*, *Oscillospira*, and *Bifidobacterium* (Holscher et al. 2018).

Other common polyphenol-rich foods include tea, cocoa products, and wine. Polyphenols have been linked to enrichment of *Bifidobacterium* and *Lactobacillus*, yielding increased production of fecal SCFA (Sun et al. 2018) with anti-pathogenic and anti-inflammatory effects (Singh et al. 2017). A recent cross-sectional study of three large—TwinsUK, the Flemish Gut Flora Project (FGFP), and American Gut Project (AGP)—has shown that red wine consumption is associated with higher α diversity of the gut microbiome. This association may be attributable to high content of polyphenols, such as anthocyanin, resveratrol, and gallic acid (Le Roy et al. 2020).

11.3.7 Diet and Oral Microbiome

Less in known about the oral microbiome that inhabits the gateway to the body and GI tract, representing the first encounter with food, antigens, and early carbohydrate digestion. In one cross-sectional study, intake of saturated fatty acids (SFA) was positively associated with the relative abundance of oral phyla *Proteobacteria* and Fusobacteria; and higher glycemic load was positively associated with *Lactobacillaceae* (Kato et al. 2017). In a larger cross-sectional study of the American Cancer Society Cancer Prevention Study II (ACS CPS-II) and the National Cancer Institute Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial (NCI PLCO) cohorts, high tea intake was associated with increased richness and diversity of oral microbiota, as well as differences in overall community composition. However, no such associations were observed with coffee intake. Additionally, tea intake was associated with increased abundance of *Fusobacteriales* and *Shuttleworthia satelles* and decreased abundance of *Bergeyella and Kingella oralis.* Coffee intake was associated with greater abundance of *Granulicatella* (Peters et al. 2018).

11.4 Cancer Related to Diet, the Microbiome, and Inflammation

Cancers known to be related to diet, inflammation, and the microbiome include colorectal, liver, and pancreatic cancer (Fig. 11.1). Several studies suggest that inflammatory diets trigger local intestinal inflammation, which eventually leads to the breakdown of epithelial barriers that separate microbiota from immune cells in the lamina propria. This causes translocation of intestinal microbiota and exposes immunogenic microbial components to both antigen-presenting and epithelial cells. These immunogenic vesicles and enterotoxins lead to mutations in tumor-suppressing and DNA repair genes involved in colorectal carcinogenesis (Zitvogel et al. 2017a; Brennan and Garrett 2016; Tabung et al. 2017). Anti-inflammatory diets suppress tumorigenesis by activating chloride channels and increasing the abundance of beneficial bacteria (Liu et al. 2018).

11.4.1 Colorectal Cancer

Gut microbiota plays a key role in mediating the influence of diet on CRC risk. Dramatic differences are observed in gut microbial structures of populations consuming different diets (Conlon and Bird 2014). High abundance of *Bacteroides fragilis* and *Fusobacterium nucleatum* is also associated with poor survival in patients with CRC (Wei et al. 2016), while the presence of *Faecalibacterium prausnitzii* is associated with a reduced CRC risk (Huang and Liu 2019; Ganesan

et al. 2018). Although inflammation is an essential trigger for CRC, inflammation alone is not sufficient to promote tumorigenesis—complex interactions among gut microbiota, host genetics, inflammation, and environmental exposure are involved (Armstrong et al. 2018). In the colon, excess chronic exposure to hydrogen sulfide is associated with key drivers of carcinogenesis including DNA damage, inflammation, epithelial hyperproliferation, changes in the populations and function of immune cells, and impaired colonocyte nutrition (Singh and Lin 2015). Considering the critical role of diet in the production of bacterial metabolites and configuration of gut microbiotal communities, diet likely influences the risk for CRC by modulating the composition and metabolic activities of the gut microbial community. This, in turn, shapes immune response, leading to chronic inflammation and tumor development (Song and Chan 2017, 2019; Tilg et al. 2018).

11.4.2 Liver Cancer

The liver is uniquely exposed to intestinal bacterial components and to their metabolites and byproducts via the portal venous system. These factors are associated with inflammatory changes and hepatotoxicity, which can eventually lead to carcinogenesis (Tripathi et al. 2018). Microbiotal modification of primary bile acids, produced by the liver, and conversion to secondary bile acids, such as deoxycholic acid (DCA), can cause hepatotoxicity, DNA damage, and carcinogenesis (Chiang and Ferrell 2018; Wahlstrom et al. 2016). Moreover, gut microbiota are also associated with response to infectious hepatitis, development of obesity and nonalcoholic steatohepatitis (NASH), and other pathologies, all of which can lead to cirrhosis and contribute to the development of HCC, the most common type of liver cancer (Minemura and Shimizu 2015; Meng et al. 2018). Increased levels of *Proteobacteria* and decreased levels of *Firmicutes* are observed during progression of nonalcoholic fatty liver disease (NAFLD) (Loomba et al. 2017), while patients with NASH show considerably lower levels of *Bacteroidetes* compared with patients with simple steatosis and healthy individuals (Mouzaki et al. 2013). This suggests that gut dysbiosis is associated with NAFLD/NASH, both of which are major risk factors for HCC. Heavy alcohol intake is also associated with unique features of the microbiome and is a major risk for HCC. Patients with alcoholic hepatitis (AH) exhibit high levels of *Streptococcus*, *Bifidobacterium*, *Enterobacter*, and *Atopobium* species (Llopis et al. 2016).

11.4.3 Pancreatic Cancer

Pancreatic cancer, one of the most aggressive and deadly cancer types, is the quintessential example of an inflammation-driven cancer (Ilic and Ilic 2016). Two meta-analysis studies have shown that *H. pylori*, a common pathogen residing in the

upper gastrointestinal tract, is associated with pancreatic carcinogenesis (Trikudanathan et al. 2011; Signoretti et al. 2017). However, the causal relationship between infection with *H. pylori* and pancreatic inflammation in the upper gastrointestinal tract remains unclear. A recent population-based nested case-control study within the ACS CPS-II and NCI PLCO cohorts found that oral pathogens *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are associated with higher risk of pancreatic cancer, while Fusobacteria and its genus *Leptotrichia* are associated with lower risk (Fan et al. 2018). Toll-like receptors (TLR) play a vital role in pancreatic cancer, and gut microbe, *Parabacteroides distasonis*, exerts anti-inflammatory and anticancer effects by reducing TLR signaling/Akt activation in mouse models (Zambirinis et al. 2014). Alcohol consumption is linked to dysfunction of the intestinal barrier function and overgrowth of gram-negative bacteria in the intestine. This leads to elevated systemic levels of lipopolysaccharide (LPS), a gut microflora metabolite, which increase the risk for pancreatic cancer (Yan and Schnabl 2012). Overall alterations in oral microbial composition linked to pancreatic cancer are predominantly attributable to *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and the *Cytophaga-Flavobacterium-Bacteroides* group (Farrell et al. 2012). *Fusobacterium* species in the oral cavity are also prevalent in patients with pancreatic cancer. Although these species are not associated with any disease-related gene mutation or epigenetic alterations, their presence is independently associated with poor prognosis (Mitsuhashi et al. 2015).

11.4.4 Other Malignancies

Several studies have shown a link between gut dysbiosis and other malignancies. A recent nested case-control study, which included 122,004 participants from ACS CPS-II and NCI PLCO, found that oral abundance of commensal *Corynebacterium* and *Kingella* is associated with decreased risk of head and neck squamous cell carcinoma (HNSCC) (Hayes et al. 2018). The presence of periodontal pathogen *Tannerella forsythia* is associated with higher risk of esophageal adenocarcinoma (EAC), while depletion of *Neisseria* and *Streptococcus pneumoniae* is associated with lower risk of EAC. Periodontal pathogen *Porphyromonas gingivalis* is associated with esophageal squamous cell carcinoma (ESCC) (Peters et al. 2017). A secondary analysis of stool samples collected from prostate cancer patients enrolled in a randomized pre-surgical weight-loss trial reported that *Clostridium* and *Blautia* were associated with Gleason sum (a clinical predictor of progression). *Blautia* was also associated with increased red meat intake from baseline (Fruge et al. 2018).

In summary, advancements in microbiome screening techniques may uncover more links between diet, microbiota, and various types of cancer. Further preclinical, epidemiological, and clinical studies together will ultimately uncover the relationship between diet, dysbiosis, and cancer.

11.5 Conclusions and Clinical Implications

In this book chapter, we discussed the microbiome and its relationship with diet, inflammation, and cancer and described recent human observational and interventional studies evaluating these relationships. Overall, this research suggests that diverse microbial and metabolic responses to different dietary patterns or dietary interventions, as well as high inter-individual variability, may continue to challenge our ability to find clear and consistent results across studies. Similar to the progress made in understanding the impact of human genetic and metabolic variation in nutrition and cancer, basal microbiome composition and inter-individual variation may also influence the physiological effects of specific foods and responses to dietary intervention (Zeevi et al. 2015; Korem et al. 2017). Research focused on various parameters of microbiome-based personalized nutrition in cancer prevention, and treatment is likely to be important for future applications and overall impact (Hills et al. 2019). Numerous ongoing studies are focused on diet, microbiome, inflammation and cancer. In the next decade, we will likely have more information on these important targets and interactions to inform clinical and public health practice.

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Chapter 12
Autophagy and Cancer: Current Biology and Drug Development

Arianne L. Theiss

Abstract Autophagy is a catabolic pathway that degrades and recycles cytoplasmic components through lysosomal degradation to sustain survival in response to cellular stressors. Autophagy defects are associated with the etiology of numerous diseases including cancer. The role of autophagy in cancer is complex and context-dependent, with opposing roles of preventing tumor initiation and promoting tumor progression. Recent evidence suggests involvement of selective autophagy of mitochondria, called mitophagy, in tumorigenesis. Understanding the roles of autophagy and mitophagy in cancer stem cells, cancer immunosurveillance, cancer metabolism, and iron homeostasis will influence development of emerging anti-cancer therapeutics targeting autophagy.

Keywords Macroautophagy · Mitophagy · Ferroptosis · Cancer stem cells · Metabolic reprogramming

Abbreviations

AMPK	5′-AMP-activated protein kinase
ATG	Autophagy-related
ATP	Adenosine triphosphate
CAF	Cancer-associated fibroblast
CQ	Chloroquine
CSCs	Cancer stem cells
EMT	Epithelial-to-mesenchymal transition
ER	Endoplasmic reticulum
HCQ	Hydroxychloroquine
LC3	Microtubule-associated light chain 3
mTOR	Mammalian target of rapamycin

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OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
PI3-P	Phosphatidylinositol 3-phosphate
ROS	Reactive oxygen species
VDR	Vitamin D receptor

12.1 Introduction

Since autophagy was first described in 1963 (Klionsky 2007), we have gained important insights into how this evolutionarily conserved catabolic pathway modulates health and disease states. Basal autophagy plays an important role in cell homeostasis through quality control removal of damaged organelles or protein aggregates that can cause genotoxic stress. This role of autophagy plays an important role in tumor suppression, preventing cell transformation and the initiation of tumorigenesis. Autophagy also serves as a cellular mechanism for adaption to stresses, such as starvation or oxidative stress, rendering the cell able to tolerate hostile conditions. Autophagy adaption response to cellular stress is a well-established feature of cancer cells key to their survival, proliferation, and metastasis in the hypoxic and nutrient deficient tumor microenvironment. In this regard, the tumor suppressive properties of autophagy are context-dependent, occurring at the initial stage of tumor development, which in turn facilitate tumor aggression after malignant transformation has occurred. Autophagy of mitochondria, called mitophagy, enhances tumorigenesis by influencing cancer cell metabolism and protecting against mitochondrial dysfunction and immune cell recognition.

Therapeutic strategies aimed to manipulate autophagy in many types of cancer are the focus of multiple current clinical trials, with most testing the pharmacological inhibitors of autophagy, chloroquine (CQ), and its derivative hydroxychloroquine (HCQ), as adjuvant to chemotherapeutic drugs. A current limitation of these clinical trials in understanding the anti-cancer potential of autophagy inhibition is that CQ and HCQ target pathways beyond autophagy. At this time, autophagy- or mitophagy-specific drugs are lacking. However, preclinical models utilizing genetic deletion of essential autophagy genes demonstrate the potential of targeting autophagy as cancer therapy. Here, we will discuss the current understanding of the role of autophagy in tumorigenesis and how this relates to drug development.

12.2 Autophagy Pathway

12.2.1 Autophagy Overview

Autophagy is a "self-eating" catabolic pathway that removes cytoplasmic components through lysosomal degradation (Mizushima et al. 2008). Three types of autophagy have been described in mammalian cells: macroautophagy, microautophagy, and chaperone-mediated autophagy. Although each of these has distinct molecular pathways, all three lead to delivery of cargo to the lysosome for degradation and recycling of the breakdown products back into the cytosol. Microautophagy and chaperone-mediated autophagy sequester cargo directly into the lysosome. During microautophagy, the lysosomal membrane extends or envaginates to engulf a portion of cytosol to be degraded (Oku and Sakai 2018). Chaperone-mediated autophagy only degrades protein as cargo identified by the pentapeptide KFERQ motif. Specific chaperones transport these proteins directly across the lysosomal membrane one by one thereby playing a central role in protein quality control (Kaushik and Cuervo 2018). Macroautophagy (herein referred to as autophagy), which is the best characterized type of autophagy, involves de novo synthesis of double-membrane lipid bilayer vesicles that sequester cargo, called autophagosomes, and fuse with the lysosome where the cargo is degraded (Fig. 12.1).

Autophagy is controlled by complex signaling events and occurs in a multistep process. Basal levels of autophagy occur in all cells. Autophagy is induced to promote cell survival and restore homeostasis in response to cellular stressors such as starvation, damaged organelles, or the invasion of microorganisms. Autophagy can also eliminate apoptotic cells (Qu et al. 2007). The molecular mechanisms and signal induction pathways for basal autophagy and stress-induced autophagy likely have distinctions but are not well-characterized. Investigation predominantly in yeasts identified autophagy-related (*Atg*) genes involved in the formation and recycling of the autophagosome. The fundamental mechanism of autophagy is conserved across organisms such as yeast, plants, and mammals and involves related *Atg* genes (Klionsky 2007). The autophagic process can be divided into stages such as initiation of phagophore formation, expansion, and elongation of the autophagosome membrane, cargo selection, and fusion with the lysosome.

12.2.2 Initiation of Phagophore Formation

A cellular stress response, such as loss of nutrients, will initiate the autophagy pathway. Mammalian target of rapamycin (mTOR) kinase is a sensor of cellular stressors and is the major negative regulator of autophagy. Cellular stress inactivates mTOR kinase. 5′-AMP-activated protein kinase (AMPK), another nutrient sensing kinase, can activate autophagy directly or by inactivating mTOR (Lee et al. 2010).

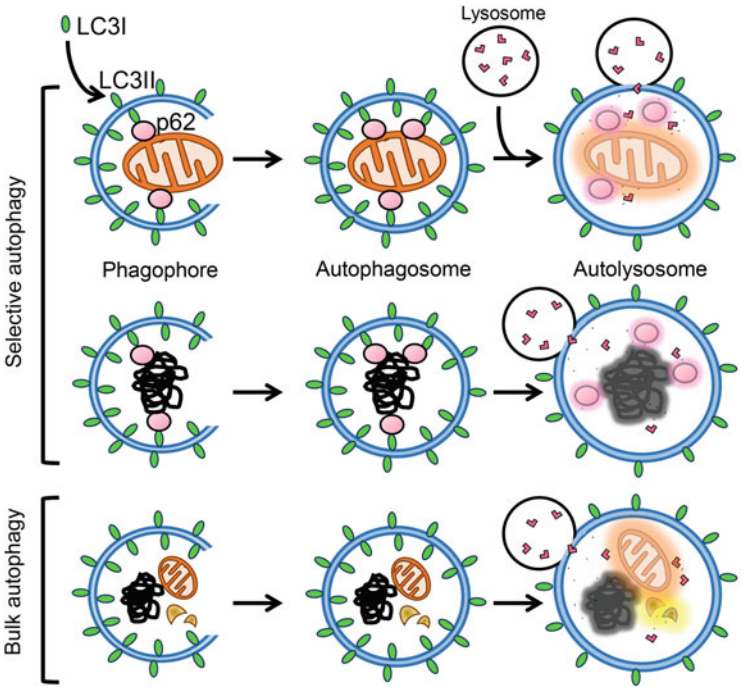

Fig. 12.1 Stages of the autophagic process. During autophagy, cytosolic cargo is sequestered into a double-membrane lipid bilayer vesicle formed via de novo synthesis through conjugation of proteins and lipids in a complex, multistep process. Upon the induction of autophagy, the phagophore membrane begins to form and LC3I is conjugated to phosphatidylethanolamine, producing the lipidated form LC3II that is incorporated into the forming membranes. Selective autophagy targets specific cargo to be incorporated into the autophagosome for degradation. Selective autophagy is mediated by adapter molecules such as p62/Sequestosome 1 (SQSTM1), which recognizes and binds to polyubiquitinated cargo such as damaged mitochondria or protein aggregates and also binds to forming vesicle membranes via its direct binding to LC3II. Bulk autophagy is a nonselective process, engulfing cytoplasmic material at random. The phagophore membrane elongates, and once the vesicle is closed, it is called an autophagosome. The autophagosome fuses with the lysosome, called an autolysosome, where the cargo and adapter molecules such as p62 are degraded and recycled or used as metabolic fuel to promote cell survival

Both mTOR and AMPK regulate autophagy via inhibition or activation, respectively, of the ULK1 complex composed of ULK1 [also known as autophagy-related 1 (ATG1)], ULK2, ATG13, RB1-inducible coiled coil 1 (RB1CC1), and ATG101. ATG1 binds ATG17, which, along with ATG13, regulates the kinase activity of ATG1 (Suzuki et al. 2014). ATG1 regulates the transmembrane protein ATG9 which acts as a phagophore initiator by recruiting lipids from cellular sources such as the endoplasmic reticulum (ER), mitochondria, and endosomes (Simonsen and Tooze 2009). ATG1, ATG13, and RB1CC1 may further facilitate autophagy machinery recruitment via LC3 interaction regions (LIR) (Corona Velazquez and Jackson 2018).

12.2.3 Expansion and Elongation of the Autophagosome Membrane

Elongation of the phagophore occurs when the Beclin 1 complex is activated to produce phosphatidylinositol 3-phosphate (PI3-P) lipids. The Beclin 1 complex is composed of Beclin 1, ATG14, phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3), and phosphatidylinositol 3-kinase regulatory subunit 4 (PIK3R4). The ULK1 complex phosphorylates Beclin 1 and ATG14, promoting their association and the formation of PIK3C3 complexes to convert phosphoinositide (PI) to PI3-P lipids. Microtubule-associated light chain 3 (LC3) is processed by ATG4 to produce LC3I. ATG3 interacts with LC3I producing the lipidated form conjugated to phosphatidylethanolamine, called LC3II (Levy et al. 2017). This membrane-bound LC3BII is incorporated into the autophagosome membrane and is often used to mark or measure autophagosome formation. The autophagosome is completed when two distinct inner and outer bilayers are formed and the double-membrane vesicle is closed.

12.2.4 Cargo Selection

Autophagy was long thought to be an indiscriminant process, engulfing cytoplasmic material at random. In more recent years, molecular mechanisms have been identified whereby specific cargo is selected during autophagy (Fig. 12.1). Autophagy machinery and the growing phagophore membrane are recruited to sites of bacterial invasion, damaged organelles, or protein aggregates, promoting their uptake as cargo and degradation by autophagy. Emerging studies have identified adaptor molecules that serve to flag and facilitate the uptake of specific cargo. Through interactions with autophagy machinery, adaptor molecules bring specific cargo into the forming phagophore to be eventually surrounded by the expanding membrane in a completed autophagosome. The best characterized adaptor molecule involved in selecting cargo is p62/Sequestosome 1 (SQSTM1) which is an ubiquitin-binding scaffold protein and promotes degradation of polyubiquitinated cargo such as protein aggregates or damaged mitochondria via its direct binding to LC3II (Sanchez-Martin and Komatsu 2018). In this way, p62 links ubiquitinated proteins to the autophagic machinery. Since p62 is degraded along with the cargo during autophagy, its accumulation indicates inhibition of autophagy, whereas decreased levels indicate induction of autophagy, making it a common marker to measure autophagic flux (Sanchez-Martin and Komatsu 2018).

NOD1 and NOD2 provide another example of adapter proteins involved in the autophagic response with a role specific to invading bacteria. NOD1 and NOD2 are intracellular receptors that recognize muramyl dipeptide, a component of the bacterial cell wall, and interact with and recruit ATG16L1 to the site of bacterial entry into the cell (Travassos et al. 2010). This interaction facilitates the incorporation of

invading bacteria into the forming autophagosome. Mutant NOD2 is unable to recruit ATG16L1 and bacterial engulfment into autophagosomes is impaired (Travassos et al. 2010).

12.2.5 Fusion with the Lysosome

The fully formed autophagosome migrates along microtubules to a lysosome where small GTPases such as Rab7, SNAREs, and endosomal sorting complex required for transport (ESCRT) facilitate the fusion of the outer autophagosome membrane with the lysosome, known as an autolysosome. In certain circumstances such as starvation, autophagosomes may fuse with late endosomes (called amphisomes) prior to fusion with lysosomes. The acidic lysosomal components degrade the cargo as well as adapter molecules such as p62 and LC3II present on the inner autophagosome membrane. LC3II located on the autolysosome membrane can be converted back to LC3I. The degraded contents are recycled back into the cytosol or used to fuel metabolic pathways, thereby promoting cell survival.

12.3 Dual Roles of Autophagy in Cancer Initiation Versus Progression

12.3.1 Autophagy and Cancer Suppression

Like many molecular pathways in cancer, the role of autophagy in cancer is complex and demonstrates tumor suppressor and tumor promoter functions (Fig. 12.2). Such opposing outcomes are attributed to context dependency including stage of tumor, type of tumor, presence of tumor genetic mutations, and therapies. The tumor suppressive function of autophagy is largely summed up as preventing malignant transformation. As the outcome of autophagy is quality control via the elimination of damaged organelles, invading bacteria, or protein aggregates or via “self-eating” to produce metabolic fuel during starvation, autophagy function is crucial in maintaining cell homeostasis. Loss of quality control provided by autophagy leaves cells susceptible to genotoxic stress, a known inducer of tumor initiation. This has been demonstrated in various mouse models deficient in autophagy exhibiting enhanced tumor initiation (Cianfanelli et al. 2015; Marino et al. 2007; Mathew et al. 2009; Qu et al. 2003; Rao et al. 2014a, b; Takamura et al. 2011). Furthermore, autophagy plays an essential role in the maintenance of stem cells, including embryonic, hematopoietic, neural, and mesenchymal stem cells (Rodolfo et al. 2016). Autophagy is required for the maintenance of stemness in normal stem cells and cancer stem cells (CSCs). The role of autophagy in CSCs will be discussed in Sect. 12.3.3.

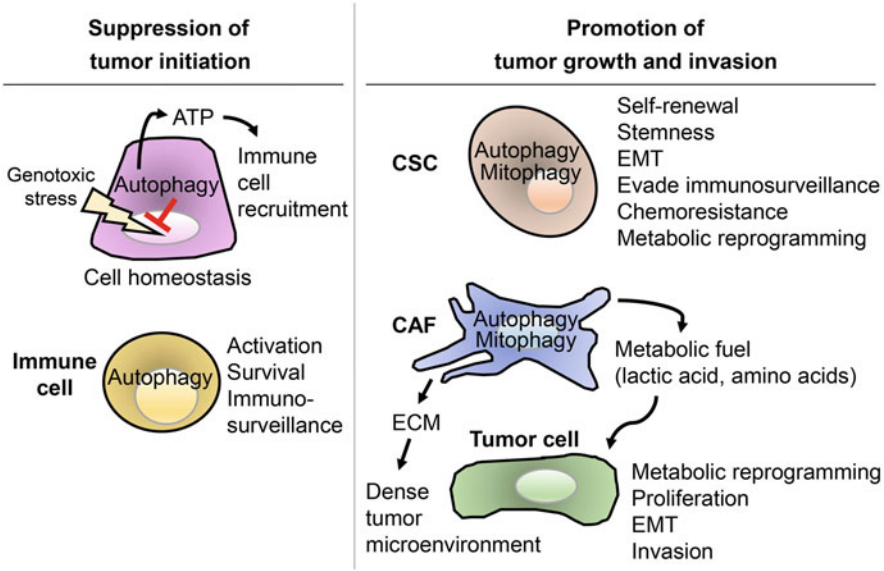

Fig. 12.2 Dual roles of autophagy in cancer. Autophagy suppresses tumor initiation by providing cellular quality control and defense against genotoxic stress. Extracellular ATP release generated via autophagy stimulates and recruits immune cells, initiating an anti-cancer immune response. Autophagy in immune cells modulates the release of cytokines and danger signals and is important for activation and survival of immune cells crucial for tumor immunosurveillance. Once malignant transformation has occurred, autophagy promotes tumor growth and invasion. Enhanced autophagy and mitophagy in CSCs drives characteristic features of CSCs such as self-renewal potential, stemness, epithelial-to-mesenchymal transition (EMT), and chemoresistance. Autophagy and mitophagy in CAFs enhance secretion of metabolic fuel for neighboring tumor cells, which in turn drives their aggressive behavior via metabolic reprogramming, proliferation, EMT, and invasion. Additionally, enhanced extracellular matrix (ECM) secretion including collagen contributes to a dense tumor microenvironment that can hinder anti-cancer drug access and efficacy

The molecular mechanism whereby autophagy protects against early oncogenesis involves regulation of the capacity of the immune system to eliminate or retard the growth of cancers. The immune system constantly surveils for cancer cells for their destruction, called immunosurveillance. Autophagy has been shown to influence the abundance of T-cell subtypes, specifically FoxP3$^+$ regulator T cells (Tregs) and CD8$^+$ cytotoxic T cells, in the tumor microenvironment. Deletion of *Atg5* in the *Kras*^{G12D} mouse model of non-small cell lung cancer increased infiltration of early adenomas with FoxP3$^+$ Tregs (Rao et al. 2014b). Tregs function as anti-inflammatory T cells, resulting in immunosuppression and accelerated oncogenesis. Enhanced autophagic flux in human breast cancer cells was associated with favorable prognosis, increased abundance of CD8$^+$ cytotoxic T cells, and decreased abundance of FoxP3$^+$ Tregs (Ladoire et al. 2016). A key stimulant of the immune response is extracellular adenosine triphosphate (ATP), which is generated via autophagy through lysosomal ATP release (Martins et al. 2014). Extracellular

ATP recruits dendritic cells, natural killer cells, and $CD8^+$ cytotoxic T cells to attack the cancer. During autophagy deficiency, this ATP release and immune stimulation are deficient, and cancer cells evade immune recognition. The anti-cancer immune response can be reestablished by restoring extracellular ATP release via alternate pathways (Michaud et al. 2011; Rao et al. 2014a). Immunosurveillance is also essential for the induction of anti-cancer immune responses by chemotherapy treatments anthracyclines or oxaliplatin. Cancer cells that develop means to inhibit autophagy, thereby preventing release of ATP and immunosurveillance, are refractory to these chemotherapeutic drugs (Pietrocola et al. 2017).

12.3.2 Autophagy and Cancer Progression

Once malignant transformation has occurred, autophagy takes on a fundamental role in cancer cell survival, proliferation, and metastasis allowing adaptation to cellular stress present in the tumor microenvironment, including hypoxic, metabolic, and inflammatory stress. In addition, autophagy affects cell motility and EMT, important contributors to cancer invasion and metastasis (Marcucci et al. 2017). This provides rationale for targeting autophagy as a chemosensitizing agent alongside current cancer treatments to improve therapeutic response. Autophagy is upregulated in human cancers, but this is generally not driven by genetic mutations in essential autophagy genes which are infrequently mutated in tumors (Lebovitz et al. 2015). Instead, commonly occurring cancer mutations in tumor suppressor genes, such as RAS and p53, are thought to drive autophagy upregulation in tumor cells (Zhang et al. 2017).

Various mouse models of spontaneous tumorigenesis have demonstrated the crucial role of autophagy in promoting cancer progression. Crossing these mouse models of spontaneous tumorigenesis with mice deficient in crucial autophagy genes provide a method to determine the role of autophagy under physiological conditions including a functional immune system and tumor microenvironment. Deletion of *Atg5* in the $Kras^{G12D}$ mouse models of pancreatic ductal adenocarcinoma or lung cancer decreases tumorigenesis (Rao et al. 2014a; Rosenfeldt et al. 2013; Yang et al. 2014a). Similar results were demonstrated during *Atg7* deletion in the $Braf^{V600E}$/$Pten^{-/-}$ model of melanoma or $Pten^{-/-}$ model of prostate cancer (Santanam et al. 2016; Xie et al. 2015). *Atg7* deletion in the $Braf^{V600E}$ non-small cell lung cancer model compromised tumor growth and progression due to defects in mitochondrial metabolism (Strohecker and White 2014). Deletion of *Atg7* in intestinal epithelial cells prevented tumorigenesis in the $Apc^{min/+}$ mouse model of colorectal cancer due to metabolic stress in tumor cells but not normal cells (Levy et al. 2015). In *Lkb1*-deficient *Kras*-driven lung cancer, deletion of *Atg7* decreased tumor initiation and growth due to altered mitochondrial metabolism and energy crisis normally sustained by autophagy (Bhatt et al. 2019).

A pitfall of these studies is the use of models with complete loss of autophagy, which rarely occurs in human patients. A recent study used heterozygous $Atg5^{+/-}$

mice alongside *Atg5*$^{-/-}$ mice to determine the dose effect of autophagy in the *Kras*^{G12D} mouse model of pancreatic ductal adenocarcinoma (Gorgulu et al. 2019). Surprisingly, loss of 1 allele of *Atg5* enhanced tumorigenesis and metastasis, whereas complete loss of *Atg5* inhibited tumorigenesis. Monoallelic loss of *Atg5* induced cell intrinsic changes involving mitochondrial homeostasis and enhanced autophagy that altered the tumor microenvironment to favor aggressive pancreatic cancer (Gorgulu et al. 2019). A similar result was demonstrated during heterozygous intestinal epithelial cell-specific deletion of *Sirt1*, which activates autophagy by deacetylating *Atg5*, *Atg7*, and *Atg8* (Lee et al. 2008), in the azoxymethane-dextran sodium sulfate mouse model of colorectal cancer. *Sirt1*$^{+/-}$ mice exhibited increased colorectal cancer development, whereas homozygous *Sirt1* deletion suppressed tumorigenesis (Ren et al. 2017). These findings suggest a dose-dependent regulation of tumorigenesis by autophagy.

Autophagy in multiple cell types present in the tumor microenvironment influences tumor progression. Induction of autophagy in cancer-associated fibroblasts (CAFs) increases their secretion of amino acids such as alanine used as metabolic fuel in neighboring cancer cells (Sousa et al. 2016) and secretion of extracellular matrix such as collagen, contributing to density of the tumor microenvironment that can hinder anti-cancer drug access and efficacy (Chen et al. 2019b). Autophagy in immune cells modulates the release of cytokines and danger signals and is important for activation and survival of myeloid and lymphoid cells crucial for tumor immunosurveillance (Germic et al. 2019). In addition, ample evidence has emerged demonstrating the important role of autophagy in promoting survival of self-renewing and chemoresistant CSCs.

12.3.3 Autophagy and Cancer Stem Cells

CSCs are a population of tumor cells that are resistant to apoptosis and conventional chemotherapeutics, are highly invasive and tumor-propagating, and give rise to cancer heterogeneity through their immense self-renewal and limited differentiation capabilities. The origin of CSCs is not completely elucidated, but it has been demonstrated that EMT can give rise to CSCs (Mani et al. 2008). Furthermore, it is well-established that CSCs are often characterized by upregulation of autophagy that in turn drives pluripotency, resistance to hypoxia and nutrient deficiency in the tumor microenvironment, resistance to anti-cancer therapies, enhanced migration and invasion, and immunosurveillance evasion. A seminal role of autophagy in CSC maintenance and expansion was demonstrated by loss of tumorigenicity in nude mice by breast CSCs deficient in Beclin 1 (Gong et al. 2013). Autophagy upregulation has been demonstrated in many types of CSCs including from breast, colon, ovarian, pancreatic, gastric, esophageal, hepatic, lung, renal, and glioblastoma cancers (Lee et al. 2015; Nazio et al. 2019; O'Donovan et al. 2011; Singla and Bhattacharyya 2017). In fact, upregulated autophagy has been proposed as a distinguishing trait for CSCs versus EMT tumor cells (Marcucci et al. 2017).

Many signaling pathways have emerged as drivers of autophagy upregulation in CSCs. FOXO transcription factors, known to be central regulators of cellular quality control pathways, modulate transcription of many genes involved in autophagy such as *ATG5*, *ATG8*, *Beclin 1*, *LC3*, and *ULK1*. FOXO3 deficiency increased self-renewal of prostrate, ovarian, breast, liver, colon, and glioblastoma CSCs (Nazio et al. 2019). Interestingly, FOXO3a/VEGF/CCL2 signaling has been shown to stimulate the transformation of normal fibroblasts into CAFs, enhancing tumor growth and metastasis in lung cancer (Shen et al. 2016). Signal transducer and activator of transcription 3 (STAT3) regulates autophagy via transcriptional control of autophagy genes as well as interaction with autophagy-related signaling molecules including FOXO1 and FOXO3. STAT3 also localizes to the mitochondria depending on its phosphorylation status and modulates mitophagy of damaged mitochondria (You et al. 2015). STAT3 is overexpressed in CSCs and is a proposed molecular marker of autophagy dependency in triple-negative breast cancer (Hajimoradi et al. 2016; Liu et al. 2018; Maycotte et al. 2014; Shiraiwa et al. 2019). Other studies suggest that cross talk between molecular pathways controlling EMT and autophagy also plays an important role in CSC maintenance (Chen et al. 2019a). For instance, TGFβ, a cytokine often elevated in the plasma and the tumor microenvironment, induces CD44, a CSC marker, and vimentin expression giving rise to a mesenchymal phenotype (Cufi et al. 2011). EMT signaling is a key feature of enhanced migration of CSCs and is modulated, at least in part, by autophagy as demonstrated by loss of CSC migration and invasion capacity during autophagy inhibition (Apel et al. 2008).

12.4 Mitophagy: Adaptation to Drive Tumor Progression

12.4.1 Mitophagy Overview

Selective autophagy of mitochondria, termed mitophagy, is crucial in mitochondrial quality control via elimination of damaged or dysfunctional mitochondria and restoration of the mitochondrial pool. Mitochondrial dysfunction [depolarization, excessive misfolded proteins, mitochondrial-derived reactive oxygen species (ROS)] stimulates outer mitochondrial membrane (OMM)-localized autophagy receptors to incorporate mitochondria into the autophagosome (Fig. 12.3; Jin and Youle 2013; Wang et al. 2012). For instance, the kinase PINK1 is stabilized on the OMM of damaged mitochondria and recruits the E3 ubiquitin ligase Parkin. Some autophagy receptors are dependent on Parkin, which forms polyubiquitinated chains on OMM proteins (such as MFN1, MFN2, and VDAC1) that are then recognized by an autophagy receptor, thereby incorporating the mitochondrion into the autophagy pathway for degradation. Other autophagy receptors act independently of Parkin and localize to the OMM and bind to autophagy machinery such as LC3 via their LC3-interacting region (Ding and Yin 2012).

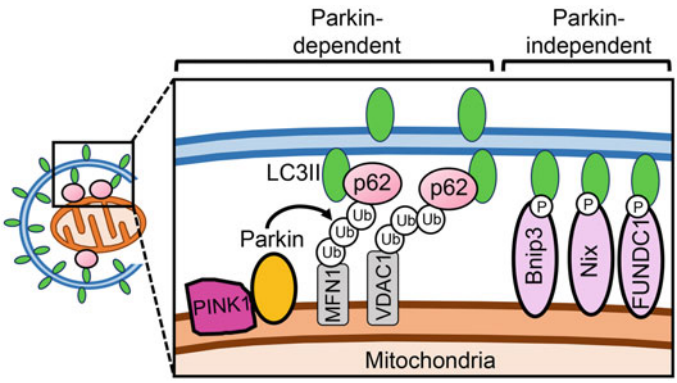

Fig. 12.3 Targeting mitochondria for mitophagy. In Parkin-dependent mitophagy, Pink1 is stabilized on the OMM of damaged mitochondria, which recruits the E3 ubiquitin ligase Parkin to add polyubiquitin chains to OMM proteins such as MFN1 or VDAC1. The autophagy adapter proteins p62, NBR1, or AMBRA1 function as a bridge between binding the polyubiquitin chains and LC3II, thereby incorporating damaged mitochondria into the forming autophagosome. Parkin-independent mitophagy involves upregulation of expression of Bnip3, Nix, or FUNDC1 upon mitochondrial damage, which constitutively localizes to the OMM and directly binds LC3II, with enhanced affinity for LC3II binding by phosphorylation in their LIR domain

Currently, nine autophagy receptors have been identified to target mitochondria and are categorized into two groups: (1) PINK1/Parkin-dependent, (1) p62, (2) NBR1, (3) AMBRA1, (4) optineurin, and (5) NDP52 bind polyubiquitinated targets (which include mitochondria and many other targets) via their ubiquitin-binding domain, and (2) Parkin-independent, (6) Bnip3, (7) Nix/Bnip3L, (8) FUNDC1, and (9) BCl2L13 contain transmembrane domains and upon expression constitutively localize to the OMM and interact with autophagy factors such as LC3II, ULK1, DFCP1, and WIPI1 (Hamacher-Brady and Brady 2016; Lazarou et al. 2015). Expression of BNIP3 and Nix is controlled by the transcription factors Hypoxia-inducible factor 1 alpha (Hif1α), NF-κB, or FOXO3, suggesting hypoxia, inflammation, or autophagy quality control pathways, respectively, are involved in mitophagy induction (Bellot et al. 2009; Chaanine et al. 2016; Dhingra et al. 2013; Sowter et al. 2001). Given the hypoxic and inflammatory tumor microenvironment, alteration of these transcription factors during tumorigenesis could result in powerful effects on mitophagy. Additional posttranslational regulation of BNIP3, Nix, and FUNDC1 by phosphorylation in the LIR domain increases their affinity for LC3II binding, suggesting that phosphorylation regulates their pro-mitophagy activity (Lv et al. 2017; Rogov et al. 2017; Zhu et al. 2013).

12.4.2 Mitophagy and Cancer Metabolism

Alteration of cellular metabolism is fundamental to transformation, selected for during tumorigenesis, and does not occur passively as a response to damaged mitochondria or reduction in ATP concentration (Ward and Thompson 2012). Mitochondrial reprogramming alters cellular metabolism to enhance anabolic precursors such as reduced carbon, reduced nitrogen, and cytosolic NADPH critical in supplying the increased requirements of tumor growth. Cancer cells exhibit a hybrid metabolic state, utilizing both oxidative phosphorylation (OXPHOS) and glycolysis allowing adaptation to changing microenvironments (Yu et al. 2017). In a dynamic process, the function of tumor mitochondria is complemented to the metabolic needs necessary to achieve rapid cell growth and enhance metastatic potential.

Mitochondrial metabolism, function, and ROS generation (as a by-product of OXPHOS) play critical roles in stem cell fate and self-renewal (Berger et al. 2016; Ito et al. 2006; Khacho et al. 2016; Morshead et al. 1994). Active stem cells of frequent turnover tissues requiring more bioenergetic activity, such as the intestinal epithelium, rely on OXPHOS metabolism (Rodriguez-Colman et al. 2017). Similarly, CSCs rely on OXPHOS for enhanced energy production that is linked to stemness, self-renewal, and anti-cancer resistance (Peixoto and Lima 2018). Blockade of OXPHOS alters CSC properties in pancreatic ductal adenocarcinoma (Fabian et al. 2019), esophageal cancer (Liu et al. 2019), breast cancer (Gao et al. 2018), and ovarian cancer (Nayak et al. 2018). Increased ROS production derived from enhanced OXPHOS can cause genotoxic damage; however, CSCs elicit upregulated antioxidant defenses to prevent ROS-induced cell death. For instance, inhibition of Nrf2, the master transcriptional antioxidant activator, sensitizes mammospheres (breast cancer cells with stem/progenitor cell properties) to chemotherapeutics and inhibits growth (Wu et al. 2015).

In addition to antioxidant defenses, CSCs rely on mitophagy as protection against mitochondrial dysfunction. A recent study demonstrated that endolysosomal Rab5- and Rab7-mediated mitophagy is essential for survival and chemoresistance of colorectal cancer CSCs (Takeda et al. 2019). Mitophagy is crucial for the maintenance of stemness and self-renewal capacity of hepatic CSCs by directly removing the tumor suppressor p53 that localizes to mitochondria and suppresses transcription of NANOG, a key transcription factor driving CSC properties (Liu et al. 2017). Inhibition of mitophagy in leukemia stem cells results in loss of self-renewal potential and stem cell properties (Pei et al. 2018). Chemoresistance of colorectal cancer CSCs is dependent on Bnip3-mediated mitophagy (Yan et al. 2017). Survival of glioblastoma CSCs requires Nix-induced mitophagy regulated by Hif1α in the hypoxic tumor microenvironment (Jung et al. 2019). In fact, hypoxic signaling mediated by Hif1α as a response to conditions inside the tumor is a key signaling pathway driving CSC metabolic reprogramming and mitophagy induction via transcriptional control of Bnip3 and Nix (Sowter et al. 2001).

Emerging evidence suggests that mitophagy in cells beyond CSCs is important in tumorigenesis. In colorectal cancer, mitophagy in intestinal epithelial cells controls

the immunosurveillance response by $CD8^+$ T cells (Ziegler et al. 2018). Mitophagy in intestinal epithelial cells induces iron (II)-accumulation in epithelial lysosomes causing permeabilization of the lysosomal membrane and release of proteases (cathepsins) into the cytoplasm. This, in turn, enables antigen processing directly in intestinal epithelial cells and contributes to enhanced $CD8^+$ T-cell activation (Ziegler et al. 2018). In this way, mitophagy in intestinal epithelial cells enhances antitumor immunity in colorectal cancer patients, in which increased tumor $CD8^+$ T cells is associated with prolonged survival (Fridman et al. 2012). A recent study demonstrated that Nix-dependent mitophagy upregulation in breast cancer CAFs drives metabolic reprogramming favoring lactate production (Sung et al. 2019). Conditioned medium from these CAFs promoted proliferation, EMT, and invasion of breast cancer cells (Sung et al. 2019). Thus, mitophagy in multiple cell types present in the tumor microenvironment influences tumorigenesis.

12.4.3 Mitophagy and Iron Homeostasis

Iron is essential for cellular homeostasis and growth. Cellular iron levels are tightly regulated with excess cellular iron stored in ferritin. Mitochondria play a central role in iron homeostasis as the site of heme synthesis and iron/sulfur clusters. Emerging evidence suggests that cancer exhibits dysregulated mitochondrial iron trafficking. Many types of cancer accumulate iron to sustain cell proliferation. A recent study demonstrated that mice with deletion of PINK1 and Parkin exhibited enhanced KRAS-driven pancreatic tumorigenesis mediated by metabolic reprogramming induced by mitochondrial iron accumulation via importers SLC25A37 and SLC25A28 (Li et al. 2018a). Using the STAT3 loss of function sporadic intestinal tumorigenesis model, mitophagy was shown to drive iron accumulation in lysosomes of intestinal epithelial cells, which in turn lead to lysosomal membrane permeabilization, antigen processing by dendritic cells, and $CD8^+$ T-cell antitumor immune response (Ziegler et al. 2018). These studies support the role of mitophagy in iron trafficking which influences cancer cell fate such as metabolic reprogramming and susceptibility to tumoral immune cells.

Mitochondria are also involved in ferroptosis, a form of cell death distinct from apoptosis or necrosis driven by iron-dependent oxidative degeneration of lipids (lipid peroxidation) causing the accumulation of lipid ROS. Sensitivity to ferroptosis is dependent on multiple cellular processes including redox signaling, metabolism of amino acids, iron, polyunsaturated fatty acids, and the biosynthesis of glutathione, NADPH, and phospholipids (Stockwell et al. 2017). Lipid peroxidation can be initiated by hydroxyl radical and hydroperoxyl radical, which are formed by the Fenton reaction (ferrous iron (Fe^{2+}) interacts with hydrogen peroxide). Ferroptosis occurs as a result of lipid peroxidation, although the exact mechanism whereby this leads to cell death is not fully elucidated (Dodson et al. 2019). Treatment of leukemia with dihydroartemisinin or typhaneoside induces ferroptosis dependent on mitochondrial dysfunction, mitochondrial-derived ROS accumulation, and autophagy

degradation of ferritin (Du et al. 2019; Zhu et al. 2019). p53 has been shown to promote ferroptosis via repression of transcription of *SLC7A11*, a gene that encodes system Xc^- cysteine/glutamate antiporter and is overexpressed in several forms of cancer including colorectal, liver, and kidney cancers (Jiang et al. 2015). Interestingly, the CSC marker CD44v associates and stabilizes system Xc^-, and therefore it has been proposed that tumor cells with high CD44v expression may be susceptible to ferroptosis induction via system Xc^- inhibition (Toyokuni et al. 2017). In colorectal cancer, a transcription-independent role of p53 in inhibiting ferroptosis via blockade of DPP4 and NOX1-induced lipid peroxidation was recently reported (Xie et al. 2017). Given that p53 localizes to mitochondria, it is tempting to speculate that p53 may play a role in ferroptosis from within the mitochondria by regulating iron trafficking, but this has yet to be demonstrated.

12.5 Autophagy-Targeted Drug Development for Cancer Therapy

12.5.1 Clinical Trials Targeting Autophagy for Cancer Therapy

Many compounds inhibit autophagy at different stages of the autophagic process; however, the only FDA approved autophagy inhibitor is chloroquine (CQ) and its derivative hydroxychloroquine (HCQ), which inhibit autophagosome fusion with the lysosome. Preclinical and early-stage clinical trials using CQ or HCQ have been reported for pancreatic, breast, liver, and lung cancers (Marinkovic et al. 2018). HCQ monotherapy in metastatic pancreatic cancer demonstrated negligible therapeutic efficacy (Wolpin et al. 2014). Current Phase I and II clinical trials are testing the combination of HCQ with the chemotherapeutic drugs capecitabine or Abraxane in pancreatic cancer. Results from the CQ-gemcitabine combination Phase I study reported a clinical response in patients with metastatic or unresectable pancreatic cancer (Samaras et al. 2017). Phase I and II clinical trials in metastatic breast cancer are currently testing CQ or HCQ in combination with ixabepilone or taxane. Single-agent CQ treatment was not associated with any effect on breast cancer cell proliferation in a recently reported Phase II clinical trial (Arnaout et al. 2019). HCQ in combination with transarterial chemoembolization or sorafenib for unresectable or advanced hepatocellular cancer, respectively, is being tested in current Phase I and II trials, but results have not yet been reported. HCQ in combination with erlotinib in advanced non-small cell lung cancer was shown to be well-tolerated in a Phase I trial (Goldberg et al. 2012). A Phase II trial was recently completed in non-small cell lung cancer patients testing HCQ in combination with paclitaxel, carboplatin, or bevacizumab (NCT01649947). CQ in combination with 5-FU, vorinostat, or bortezomib has demonstrated promising results in preclinical mouse models of

colorectal cancer and cell lines, but this has not yet advances to clinical trials (Marinkovic et al. 2018).

Sirolimus and everolimus, which are mTORC1 inhibitors and therefore activators of autophagy, are being tested in clinical trials for various cancers. Sirolimus and everolimus are being tested in Phase II and III clinical trials for advanced hepatocellular cancer in patients whose disease progressed while on sorafenib or to prevent recurrence. Results reported so far suggest that everolimus did not improve overall survival (Zhu et al. 2014), efficacy (Koeberle et al. 2016), or recurrence-free survival beyond 5 years (Geissler et al. 2016) compared to sorafenib alone. Combined sirolimus and gemcitabine therapy did not alter clinical response in a Phase I/II trial of patients with metastatic pancreatic cancer (Karavasilis et al. 2018). BOLERO-2, BRAWO, and 4EVER clinical trials reported that in postmenopausal women with $HR^+/HER2^-$ advanced breast cancer, everolimus in combination with exemestane increased median progression-free survival versus exemestane alone and was well-tolerated (Fasching et al. 2014; Tesch et al. 2019).

12.5.2 Targeting Autophagy in CSCs

It is proposed that relapse following anti-cancer therapy can be driven by intrinsically chemoresistant CSCs. This forms the basis of logic to combine cytotoxic drugs and autophagy inhibitors as an adjuvant treatment to sensitize CSCs to chemotherapy. Using preclinical models or cell culture experiments, multiple studies have shown autophagy blockade to enhance therapeutic efficacy via decreased survival of CSCs. Combined 5-FU and CQ treatment decreased cell viability of gastric CSCs (Li et al. 2018b). In fact, CQ ablates CSCs from multiple types of gastrointestinal cancers including esophageal, gastric, and colon cancers (Kim et al. 2017). JAK2-induced autophagy was shown to preserve stemness and drive resistance to cisplatin by bladder cancer CSCs; combined treatment with JAK2 inhibitors and CQ induced cell death of cisplatin-resistant bladder cancer CSCs (Ojha et al. 2016). Atg7 deficiency or treatment with CQ renders breast CSCs susceptible to salinomycin or carboplatin (Bousquet et al. 2017; Liang et al. 2016; Yue et al. 2013). Autophagy inhibition by CQ inhibited stemness and cisplatin resistance in lung CSCs, resulting in immense tumor growth suppression (Hao et al. 2019). Similar results were found in pancreatic CSCs during autophagy inhibition resulting in chemosensitivity to gemcitabine (Yang et al. 2015; Zhang et al. 2019).

It is important to note that although CQ and HCQ are well-established inhibitors of late stage autophagy, recent evidence demonstrates that CQ ability to suppress tumor growth is not dependent on its autophagy inhibiting activities. Instead, it has been proposed that the mechanism of CQ anti-cancer activity involves altered tumor cellular metabolism (Weyerhauser et al. 2018). Unfortunately, the only autophagy inhibitor approved for clinical trials is CQ/HCQ so comparison to other autophagy inhibitors is lacking. Despite multiple actions of CQ in cancer cells, studies utilizing models deficient in essential autophagy genes (as discussed in Sects. 12.3.2 and

12.3.3) support the essential role of autophagy in tumor progression and CSC stemness and survival. Further studies are necessary to elucidate the importance of autophagy inhibition in adjuvant anti-cancer therapies in human clinical trials.

Metallic nanoparticles, including silver, gold, zinc oxide, iron oxide, and copper oxide, are potent modulators of autophagy and are emerging as a strategy for anti-cancer therapy (Cordani and Somoza 2019). The nanoparticle itself has direct effects to modulate autophagy and can also promote the efficacy of chemotherapeutics carried as nanoparticle cargo via enhanced delivery to bulk tumor cells as well as CSCs (Sun et al. 2016a).

12.5.3 Targeting Mitophagy

Drugs such as CQ and HCQ that inhibit late stage autophagy will also inhibit mitophagy, and therefore it is difficult to distinguish the specific role of mitophagy inhibition using these drugs. However, genetic deletion or knockdown studies specific to essential mitophagy regulators provide evidence to support targeting mitophagy as anti-cancer therapy. Parkin deficiency enhanced the sensitivity of breast cancer cells to radiation during hypoxia (Zheng et al. 2015). Knockdown of Rab9a, which localizes to the mitochondrial membrane and is essential for mitophagy induction during loss of Atg7 expression, induced apoptosis of leukemia cells associated with elevated ROS levels and DNA damage (Wang et al. 2016). Colorectal CSCs resistant to doxorubicin were shown to exhibit enhanced mitophagy, which when suppressed by silencing of Nix caused CSC chemosusceptibility (Yan et al. 2017). Similar results were reported during knockdown of FUNDC1 in cervical cancer cells which decreased proliferation, increased apoptosis, and enhanced the sensitivity to cisplatin and radiation (Hou et al. 2017). Lung cancer cells commandeer PINK1-mediated mitophagy to promote tumor resistance to cisplatin treatment (Villa et al. 2017). A recent study revealed that multidrug resistant cancer cells HepG2/ADM and MCF-7/ADR were rendered susceptible to apoptosis by a betulinic acid analog, B5G1, during knockdown of PINK1 (Yao et al. 2019). Thus, mitophagy provides a mechanism whereby cancer cells can resist anti-cancer therapy. It has been proposed that mitophagy accomplishes this pro-cancer role by preserving mitochondrial fitness to match metabolic needs of tumor cells (Vara-Perez et al. 2019). It remains unknown which specific mitophagy proteins play an essential role in chemotherapy and radiation resistance, and further studies are necessary to develop mitophagy-specific therapeutic strategies against cancer.

12.5.4 Targeting Ferroptosis

Since drug resistance cancer cells are particularly vulnerable to ferroptosis, influencing iron homeostasis via mitophagy is an emerging pathway for cancer therapy. Small molecules that activate ferroptosis such as erastin, sulfasalazine, artesunate, typhaneoside, dihydroartemisinin, or sorafenib may be effective against a variety of cancers such as leukemia, pancreatic, kidney, and liver cancers (Du et al. 2019; Wang et al. 2019; Yang et al. 2014b; Yu et al. 2015; Zhu et al. 2019). The majority of these ferroptosis-inducing agents are inhibitors of system Xc^- or glutathione peroxidase 4 (GPX4), a phospholipid hydroperoxidase that prevents ferroptosis by reducing lipid peroxides to lipid alcohols (Dodson et al. 2019). A complexity of this strategy presents with cancer cell adaptation to ferroptosis by increasing antioxidant responses to quench elevated ROS and lipid peroxidation (Toyokuni et al. 2017). Recent studies suggest that the master antioxidant gene transcriptional activator, Nrf2, plays a central role in cancer cell evasion of ferroptosis as demonstrated by knockdown of Nrf2 sensitizing cancer cells to ferroptosis (Roh et al. 2017; Sun et al. 2016b). A combination of pharmacological inducers of ferroptosis and inhibitors of Nrf2 may show therapeutic promise against resistance cancer cells (Dodson et al. 2019). A recent study demonstrated that autophagic degradation of the circadian clock protein ARNTL prevented activation of the transcription factor Hif1α and favored ferroptosis tumor cell death (Yang et al. 2019). This too suggests that combination therapy of inhibiting ARNTL and activating ferroptosis may provide a more effective anti-cancer outcome.

12.5.5 Vitamin D and Autophagy

Epidemiological data suggests that sufficient vitamin D is protective against cancer (Garland et al. 2006). This is not a recent observation, and more current studies have elucidated the molecular mechanism of vitamin D protection. Activation of the vitamin D receptor (VDR) by the hydroxylated, biologically active form of vitamin D, 1,25-dyhydroxyvitamin D_3, is an important regulator of cancer cell proliferation and death by modulating autophagy (Tavera-Mendoza et al. 2017). This effect of vitamin D was shown to involve its modulation of autophagy in non-transformed cells such as macrophages, neurons, and intestinal epithelial cells and in breast and skin cancer cells (Bristol et al. 2012; Jang et al. 2014; Lu et al. 2019; Tavera-Mendoza et al. 2017). VDR was shown to be an important transcriptional regulator of autophagy genes including *ATG16L1* and *MAP1LC31B* (encodes LC3) (Sun 2016; Tavera-Mendoza et al. 2017). Interestingly, vitamin D induced an autophagy gene signature in normal mammary gland cells, thereby increasing basal autophagy that was lost during the progression to breast cancer (Tavera-Mendoza et al. 2017). Overexpression of VDR is associated with *PIK3CA* or *KRAS* mutations in colorectal cancer (Kure et al. 2009). Expression of VDR can be regulated by the

tumor suppressor gene p53, with mutated p53 altering the effect of vitamin D on breast cancer cells to be anti-apoptotic (Stambolsky et al. 2010). Similar results based on p53 mutation status were demonstrated in colorectal cancer cells treated with a combination of vitamin D and metformin resulting in decreased proliferation via a mechanism involving increased autophagy that was specific to wild-type versus mutant p53 status (Abu El Maaty et al. 2017). Although the role of vitamin D in cancer prevention is not fully elucidated, these studies suggest vitamin D levels should be considered in patients enrolled in clinical trials testing therapeutics that modulate autophagy.

12.6 Conclusions/Perspectives

The role of autophagy in cancer is complex, and our current understanding remains vastly incomplete. A dual role of autophagy in suppressing tumor initiation and in promoting tumor growth and invasion are supported by current investigations. However, why is autophagy inhibition monotherapy not effective to induce clinical response in clinical trials of various cancer types? If autophagy serves to fuel cancer cells during nutrient depletion and protect from hypoxia and inflammatory stress, why does autophagy-targeted monotherapy fail? A likely answer is our limited understanding of even basal autophagy in various tissues, which is further complicated in tumors due to the genetic heterogeneity of cancers. We currently do not know how complex genetic mutations common in cancer alter autophagy or response to autophagy-targeted therapeutics. Future studies aimed at better understanding the molecular mechanisms of autophagy and influence of genetic mutations will facilitate more effective, personalized therapies targeting autophagy. Furthermore, an understanding of the role of selective autophagy, such as mitophagy, may provide additional therapeutic targets that may be more specific than CQ or HCQ.

Currently, 68 international clinical trials are testing autophagy modulation as therapy for cancer, with the majority investigating autophagy inhibition as a chemosensitizing agent alongside current cancer treatments to improve therapeutic response. Most preclinical and Phase I and II clinical trials have reported promising results, but further study is needed. This again reflects the complexity of cancer in which the targeting of more than one molecular pathway is often necessary for effective therapy. Ideally, these studies will combine clinical outcome data with genetic mutation status and analyses of tumor cell and molecular biology to better understand the role of autophagy in patients who respond to autophagy modulation versus patients who are non-responders. Considering the influence autophagy plays on cell fate decisions in various cell types during health and disease, autophagy provides an exciting target in the pursuit of cancer therapies that we are only beginning to harness.

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Chapter 13
Mitochondrial Regulation of Inflammation in Cancer

Joseph Inigo, Jordan Woytash, Rahul Kumar, and Dhyan Chandra

Abstract Inflammation can occur in response to transient or chronic conditions. Transient inflammation is beneficial during injury or invasion of pathogens. Chronic inflammation presents an unresolved response which can have harmful consequences to the host system. Inflammation is prevalent in multiple human diseases. Current studies provide a strong association between inflammation and cancer. Mitochondrial dysfunction augments the production of mitochondrial reactive oxygen species (mtROS) to support inflammation and proliferation via release of various cytokines. Inflammation, in turn, can further induce mitochondrial dysfunction and reactive oxygen species (ROS) to create a feedback loop of inflammatory insult, which supports the growth and survival of tumor cells. Current pharmacological agents seek to exploit this process by targeting either the mitochondria or downstream targets of the mitochondria which promote inflammation. This chapter delves into the origins of mitochondrial dysfunction and the corresponding signaling pathways that regulate inflammation in cancer.

Keywords Mitochondrial dysfunction · Inflammation · Reactive oxygen species · Hypoxia · Cytokines

13.1 Introduction

Inflammation is a physiological response to infection and tissue injury, which is typically mediated by immune cells, cytokines, and chemokines (Medzhitov 2008). Once activated, immune cells act in tandem to mobilize and eliminate the infectious threat. For example, neutrophils can eliminate pathogens by releasing toxic contents such as ROS (Rosin et al. 1994; Nathan 2006). However, due to the lack of discrimination between foreign agents and local cells, damage to host tissue is

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Table 13.1 Causes of inflammation

Mitochondrial mutations	Accumulation of mutations in mtDNA leads to irregular activity of the electron transport chain, creating an abundant pool of mtROS which can directly or indirectly induce inflammation
Mitochondrial ROS	Inhibit MAPK phosphatases and promote the transcription of IL-6, IL-8, IL-10, and TNF-α
	When damaged mitochondria gather, mtROS oxidizes mtDNA. This mtDNA is released from the mitochondria into the cytosol to activate inflammasomes
Local immune cells	Directly release ROS into the tumor microenvironment (e.g., macrophages and neutrophils) to induce DNA damage in neighboring cells including cancer cells
	Release pro-inflammatory cytokines which stimulate mtROS production by the electron transport chain
Hypoxic conditions	Under low-oxygen conditions, mitochondria produce greater quantities of mtROS that stabilize HIF-1α which, in turn, increases levels of pro-inflammatory cytokines and chemokines

inevitable (Nathan 2002). The inflammatory response must then be resolved, or chronic inflammatory insult will develop serious consequences (Medzhitov 2008) such as development of various types of cancer. Colorectal and lung cancers are the most extensively studied cancers with ties to chronic inflammation (Kamp et al. 2011). There are various mechanisms/conditions that contribute to inflammation (Table 13.1), and some key causes are discussed in this chapter.

13.2 Mitochondrial ROS

ROS provides the association between chronic inflammation and cancer (Ohshima and Bartsch 1994; Rosin et al. 1994; Weitzman and Gordon 1990). Mitochondria are believed to be the major source of ROS (Murphy 2009; Balaban et al. 2005), mainly due to the activity of the electron transport chain (ETC). Complexes of the ETC couple the transfer of electrons with the transfer of protons across the mitochondrial membrane to create an electrochemical proton gradient, which helps produce ATP (Huttemann et al. 2007). However, electrons can escape from the mitochondria to interact with oxygen and form mtROS as a byproduct (Murphy 2009). These highly reactive species include superoxide, hydrogen peroxide, and hydroxyl radicals (Murphy 2009). These reactive species can each cause oxidative damage to DNA, proteins, and lipids (Evans et al. 2004). Enzymes of the tricarboxylic acid (TCA) cycle, present in the mitochondrial matrix, have been shown to generate mtROS. For example, 2-oxoglutarate dehydrogenase, branched-chain 2-oxoacid dehydrogenase, and pyruvate dehydrogenase complexes are also capable of producing superoxide and hydrogen peroxide (Quinlan et al. 2014).

Various systems act to regulate the levels of ROS, as both normal and cancer cells require a redox balance to preserve cellular health. Antioxidant enzymes such as

superoxide dismutase (SOD), catalase, and glutathione peroxidase contribute to the attenuation of various toxic ROS (Weydert and Cullen 2010). SODs catalyze the dismutation of superoxide radicals into hydrogen peroxide and oxygen, while catalase and peroxidases further process hydrogen peroxide into water (Weydert and Cullen 2010). However, when levels of ROS exceed the capacity of these antioxidant systems, oxidative damage is more likely to occur (Evans et al. 2004).

13.3 Mitochondrial Dysfunction

Throughout the lifespan of an organism, mitochondrial DNA (mtDNA) is a key target of oxidative damage due to its proximity to mtROS production (Wei et al. 2001). The vulnerability of mtDNA is compounded by a lack of protection offered by histones as well as a deficiency in repair mechanisms relative to nuclear DNA (Alexeyev et al. 2013). Consequently, the rate of mutations formed in mtDNA is greater than those found in nuclear DNA and can include point mutations, insertions, deletions, and changes in mtDNA copy number (Larsen et al. 2005). This produces dysfunctions in the mitochondria which can give way to further mutations in mtDNA as well as nuclear DNA (Escames et al. 2012). Mutations in mtDNA can alter the function of the ETC to exacerbate mtROS generation. For example, mutations which decrease the activity of Complex IV of the ETC can impact the efficiency of previous Complexes, raising the probability of superoxide production from Complexes I, II, and III (Shidara et al. 2005). In leukemic cells, a missense mutation in the *ND1* gene of Complex I is believed to disturb electron transfer and increase mtROS formation (Piccoli et al. 2008). In Lewis lung carcinoma, mutations in the *ND6* gene of Complex 1 lead to a reduction in Complex I activity and overproduction of ROS (Ishikawa et al. 2008). A heteroplasmic mutation can interfere with the synthesis of the ND5 subunit of Complex I and consequently disrupts the assembly of Complex I (Hofhaus and Attardi 1995). This specific mutation was previously identified in colorectal tumors (Polyak et al. 1998) and later found to increase mtROS levels (Park et al. 2009). These mutations, particularly those found in mtDNA encoding proteins of the ETC, ultimately result in mitochondrial dysfunction and enhanced mtROS production (Theurey and Pizzo 2018).

13.4 Mitochondrial ROS and Dysfunction Promote Inflammation

Due to the higher ROS content in cancer cells relative to normal cells (Liou and Storz 2010), the events described above in which mitochondrial dysfunction occurs is exacerbated in tumors. This sets the stage for the creation of an abundant supply of mitochondrial ROS, which can then contribute to inflammatory activity (Sabharwal

and Schumacker 2014), as will be discussed in the following sections. This is further escalated by the presence of local immune cells, as tumorigenesis involves the recruitment of inflammatory immune cells to the tumor microenvironment. These cells can directly contribute to oxidative stress by releasing ROS into the tumor microenvironment (Frenkel 1992; Shacter et al. 1988). For example, macrophages and neutrophils can release superoxide, hydrogen peroxide, and hydroxyl radicals to cause DNA damage in neighboring cells (Trush and Kensler 1991). Furthermore, these immune cells can also secrete pro-inflammatory signals via cytokines to further impair mitochondrial functions. Cytokines such as Il-1β, TNF-α, and interferon-γ (IFN-γ) can stimulate production of mtROS by the mitochondria (Cao et al. 2013; Yang et al. 2007) through various mechanisms. IL-1β induces depolarization of the mitochondria and inhibits Complex I of the ETC (Lopez-Armada et al. 2006). Increases in mtROS, in turn, can increase the expression of IL-1β (Shi et al. 2018). Chronic exposure to TNF-α alters mitochondrial energetics to decrease mitochondrial membrane potential, decrease ATP turnover, and increase proton leak (Hahn et al. 2014). Additionally, TNF-α can decrease the mRNA expression of transcription factors crucial to mitochondrial biogenesis, such as peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and endothelial nitric oxide synthase (eNOS) (Hahn et al. 2014). IFN-γ induces the loss of mitochondrial membrane potential and subsequent release of cytochrome c to drive cells toward apoptosis (El Jamal et al. 2016). Overall, the resulting mitochondrial dysfunction then gives way to highly enhanced levels of mitochondrial ROS, which can play a role in various signaling and transcriptional processes to propagate a tumor-supporting inflammatory scenario (Fig. 13.1).

13.5 Mitochondria and Cellular Signaling During Inflammation

The ensuing mitochondrial dysfunction has shown to further increase the response to cytokine-induced inflammation through ROS production and NF-κB activation (Vaamonde-Garcia et al. 2012). High-mobility group box 1 (HMGB1) is upregulated in tumor cells and supports inflammation once released into the extracellular space (Tang et al. 2010). Upon interaction with the receptor for advanced glycation end products (RAGE), ERK1/2 is phosphorylated and prompts the mitochondrial localization of RAGE where it can phosphorylate Complex I to enhance ATP production and meet the metabolic demands of tumor growth (Kang et al. 2014). A high-fat diet (HFD) accelerates inflammation-associated pancreatic intraepithelial neoplasm (PanIN) development in mice with oncogenic KRAS activation (Khasawneh et al. 2009). To keep pace with the increased energetic needs of early tumor promotion, a rise in mitochondrial fatty acid β-oxidation is observed (Khasawneh et al. 2009). In patients with inflammatory bowel disease, protein kinase R (PKR) amplifies the phosphorylation of eukaryotic translation initiation

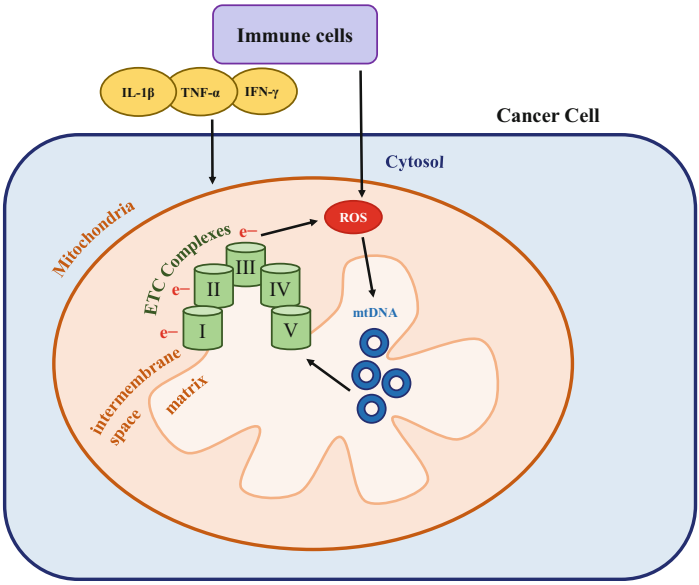

Fig. 13.1 Mitochondrial dysfunction in cancer cells. Complexes of the electron transport chain, mainly Complexes I and III, generate ROS, which damage mtDNA. This event promotes mutations in mtDNA, which encode proteins of the ETC, leading to mitochondrial dysfunction and further increased ROS. Immune cells in the tumor microenvironment contribute to ROS and release cytokines, which can further impair the mitochondria and augment ROS production

factor (eIF) 2α and c-Jun to induce transcriptional activation of stress-related genes such as the mitochondrial component heat shock protein 60 (HSP60) and preserve mitochondrial health (Rath et al. 2012).

MtROS itself can directly participate in cellular signaling events. Hydrogen peroxide can oxidize the thiol side chain of cysteine groups to produce conformational and functional changes in proteins (Garcia-Santamarina et al. 2014). For example, phosphatases such as the tumor suppressor PTEN and MAPK phosphatases can be inactivated by hydrogen peroxide in this manner (Kwon et al. 2004; Kumar et al. 2018). The inhibition of constitutively active PTEN and MAPK phosphatases can assist with the accumulation of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) and MAPK to a level which is sufficient for triggering downstream signaling (Kwon et al. 2004), thereby influencing targets such as the Akt and MAPK pathways to promote the growth and survival of cancer cells (Chalhoub and Baker 2009; Chetram and Hinton 2012). Production of mtROS, specifically by Complex III, is necessary for KRAS-mediated MAPK/ERK signaling for cell proliferation and tumorigenicity (Weinberg et al. 2010). The forkhead box, class O (FoxO) family of transcription factors, is also reported to regulate antioxidant levels through crosstalk with mtROS (Klotz et al. 2015). Oxidation of protein tyrosine phosphatases (PTPs) by ROS inhibits FoxO and promotes tumorigenesis (Harris et al. 2014).

13.6 Hypoxia and Inflammation

Hypoxia, defined as a deficiency in oxygen levels, is common in various malignancies (Muz et al. 2015). Hypoxia-inducible factor-1α (HIF-1α) is a key component of these activities (Semenza 2010). HIF-1α is destabilized by prolyl hydroxylases (PHDs) during oxygen-rich conditions (Huang et al. 1998). Consequently, hypoxic conditions lead to the inhibition of PHD activity and stabilization of HIF-1α, which in turn can promote angiogenesis, metastasis, and resistance to therapy, leading to disease progression (Muz et al. 2015). During inflammation, various mechanisms such as those involving signal transducer and activator of transcription 3 (STAT3) (Pawlus et al. 2014) and NF-κB (van Uden et al. 2008, 2011) lead to HIF-1α induction. HIF-1α itself can directly enhance the inflammatory response by regulating the levels of pro-inflammatory cytokines and chemokines (D'Ignazio et al. 2016). This cross talk between inflammation and hypoxia is partially mediated by the mitochondria. Mitochondria increase the production of mtROS during hypoxia, largely at Complex III, which helps stabilize HIF-1α and activate the expression of several cytokines including vascular endothelial growth factor (VEGF) (Chandel et al. 1998, 2000a) and platelet-derived growth factor (PDGF) (Yoshida et al. 2006). Furthermore, mtROS is shown to induce NF-κB DNA-binding and increase TNF-α gene transcription during hypoxia (Chandel et al. 2000b).

13.7 Mitochondria and Cytokine Production via Inflammasomes

Cell death can lead to release of damage-associated molecular patterns (DAMPs), thereby propagating an inflammatory response (Krysko et al. 2011). These DAMPs act upon receptors termed pathogen-associated molecular patterns (PAMPs), which include NOD-like receptors (NLRs) (Krysko et al. 2011). Mitochondria have been identified as a source of DAMPs, which may be mediated by mtROS and mtDNA.

Several studies demonstrate that mtROS can drive the production of pro-inflammatory cytokines. In a TNFR1-associated periodic syndrome (TRAPS) model, mtROS functions to induce the transcription of IL-6, IL-8, IL-10, and TNF-α by inhibiting negative regulators of mitogen-activated protein kinase (MAPK) signaling such as MAPK phosphatases (Bulua et al. 2011). Together, findings by Nakahira et al. (2011) and Zhou et al. (2011) illustrate that when damaged mitochondria accumulate in the absence of autophagy, mtROS increases and activates the NLRP3 inflammasome, one of the most well-studied NLRs. NLRP3 catalyzes the activation of pro-caspase-1, leading to cleavage of pro-IL-1β to form mature IL-1β which can be exported from the cell. A further study clarifies the method by which mtROS influences NLRP3 to induce IL-1β production. During mitochondrial dysfunction, mtDNA is oxidized by mtROS and released into the cytosol where it binds and activates NLRP3 (Shimada et al. 2012).

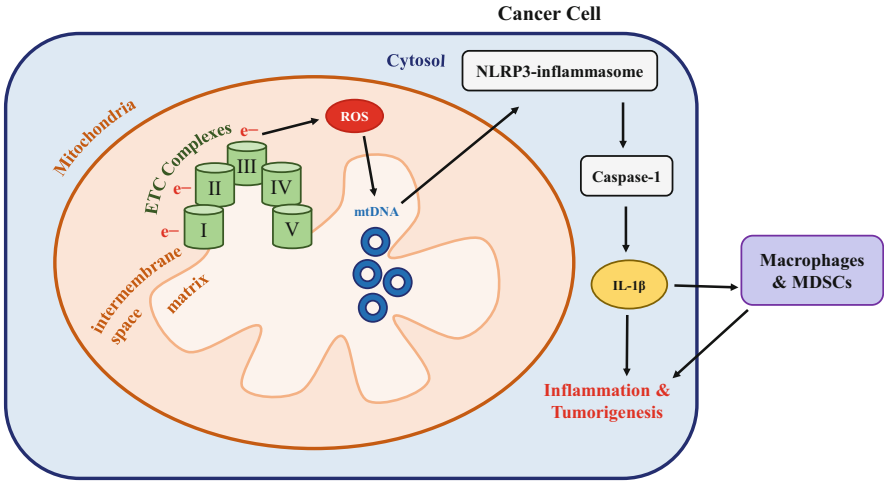

Fig. 13.2 Mitochondrial induction of inflammasomes. Mitochondrial ROS oxidize mtDNA. Once released into the cytosol, mtDNA activates NLRP3 inflammasomes, which in turn, activate caspase-1. Caspase-1 cleaves pro-IL-1β to form IL-1β. IL-1β attracts immune cells such as macrophages and myeloid-derived suppressor cells (MDSCs), which enhance angiogenesis and support tumor growth

Cytokine products such as those derived from inflammasomes display oncogenic effects. In a murine model, transgenic expression of fibroblast growth factor receptor 1 induces breast cancer in association with localized IL-1β production (Reed et al. 2009). In another model, stomach-specific expression of IL-1β drives gastric inflammation and tumorigenesis (Tu et al. 2008). IL-1β recruits and activates myeloid-derived suppressor cells (MDSCs) through the interleukin-1 receptor (IL-1R) and NF-κB pathway. IL-1R antagonism consequently suppressed the mobilization of MDSCs and development of gastric cancers. IL-1β expression in lung cancer induces infiltration of macrophages to the tumor microenvironment, where these cells can enhance angiogenesis and tumor growth (Nakao et al. 2005). Constitutive activation of the NLRP3 inflammasome in human melanoma cells leads to continual release of IL-1β (Okamoto et al. 2010). IL-1β signaling was again shown to attract macrophages and promote angiogenesis due to secretion of cytokines IL-6, IL-8, and MCP-1. Overall, the mitochondria can activate inflammasomes by various means to ultimately promote inflammation and tumorigenesis (Fig. 13.2).

13.8 Targeting Inflammation and the Mitochondria

Targeting of inflammasomes and corresponding cytokines is currently being studied for the prevention and treatment of human cancers (Thi and Hong 2017). The cytokine release inhibitory drug 3 (CRID3) inhibits NLRP3 activation and reduces IL-1β and IL-18 production (Ludwig-Portugall et al. 2016). A selective

small-molecule inhibitor of NLRP3, MCC950, is another potential therapy for NLRP3-associated diseases (Coll et al. 2015). Monoclonal antibodies and recombinant derivatives which neutralize both IL-1α and IL-1β are available (Dinarello 2011). Anakinra, an IL-1β receptor antagonist, inhibits IL-6 production in vitro and increases progress-free survival in myeloma patients (Lust et al. 2009). Anakinra reduces the growth of mammary tumors and metastatic lesions due to inhibition of proliferation and angiogenesis (Holen et al. 2016) and was recently utilized in a pilot clinical trial of breast cancer patients (Wu et al. 2018). It has also shown to block IL-1β-mediated IL-6 production in Castleman's disease, a rare lymphoproliferative disorder (El-Osta et al. 2010).

Interestingly, IL-1 inhibitors may prove to be useful in moderating the side effects of chemotherapy. Anthracyclines such as doxorubicin can activate the NLRP3 inflammasome to produce undesirable reactions. Anakinra reduces doxorubicin-induced cardiac dysfunction and apoptosis (Zhu et al. 2010; Sauter et al. 2011). Treatment with 5-fluorouracil increases IL-1β expression, leading to intestinal mucositis and apoptosis. The use of anakinra attenuates these effects (Wu et al. 2011). Anakinra diminishes bleomycin-induced inflammation and pulmonary fibrosis (Gasse et al. 2007).

Studies suggest that dietary intake of antioxidants may lower the incidence of inflammatory-associated diseases such as cancer (Griffiths et al. 2016). In line with this ideology, the grape-derived antioxidant, resveratrol, mitigates oxidative stress to improve mitochondrial function and reduce inflammation (Catalgol et al. 2012). The effects of resveratrol can be attributed to the ability to restore the proper activity of Complex III, a major source of mtROS, and upregulate the expression of antioxidants SOD2 and glutathione (Xu et al. 2012; Ungvari et al. 2009). As a result, resveratrol has been shown to have chemopreventive and chemotherapeutic effects in various cancers (Catalgol et al. 2012). Isothiocyanates (ITCs), derived from the metabolism of glucosinolates, can act as anti-inflammatory agents (Waterman et al. 2014; Davaatseren et al. 2014) and are believed to be a source of cancer-preventive effects of cruciferous vegetables such as broccoli, cauliflower, cabbage, and kale (Keck and Finley 2004; Murillo and Mehta 2001). Studies demonstrate that ITCs accelerate the metabolism of carcinogens to promote their elimination (Wattenberg 1981), inhibit NF-κB (Jeong et al. 2004; Youn et al. 2010), induce cell cycle arrest (Singh et al. 2004; Chen et al. 2010), and promote apoptosis in association with decreases in mitochondrial membrane potential (Park et al. 2007; Choi and Singh 2005; Sehrawat et al. 2017). The *Withania somnifera* plant is commonly used in Ayurveda medicine (Baliga et al. 2013). An extract from the roots and leaves of this plant, withaferin A, displays cancer-preventive properties (Hahm et al. 2013; Li et al. 2016; Samanta et al. 2017; Sehrawat et al. 2017, 2019). Withaferin A promotes apoptosis by reducing mitochondrial membrane potential to cause release of cytochrome c and activation of caspase-3 (Mandal et al. 2008) and by inducing chromosome instability in cancer cells (Das et al. 2014). Withaferin A impedes maturation and reduces secretion of pro-inflammatory cytokines such as IL-1β and IL-18 (Dubey et al. 2018). One potential use of withaferin A is highlighted by a study in which chemotherapy-induced fatigue was reduced in patients with breast

cancer (Biswal et al. 2013). Altogether, dietary use of vegetables and herbs to obtain the anticancer and anti-inflammatory benefits of derived metabolites such as ITCs and withaferin A is recommended.

Another strategy is to further impair the mitochondria as a form of treatment. Metformin, a first-line therapy for diabetes (Rojas and Gomes 2013), displays anticancer effects in breast cancer (Alimova et al. 2009), ovarian cancer (Rattan et al. 2011), and colorectal cancer (Nguyen et al. 2019). This effect has shown to be associated with a suppression of the inflammatory response (Hirsch et al. 2013; Nguyen et al. 2019). And there is evidence that this occurs due to the ability of metformin to directly act upon and inhibit Complex I of the mitochondria (Hirsch et al. 2013). Sulindac, classically known as an anti-inflammatory agent (Huskisson and Scott 1978), inhibits tumorigenesis in colorectal cancer (Boolbol et al. 1996; Rao et al. 1995) and has shown pro-apoptotic effects in breast cancer cells (Sui et al. 2018). Studies suggest that sulindac enhances the selective targeting and elimination of cancer cells by promoting mitochondrial dysfunction and ROS production (Ayyanathan et al. 2012).

13.9 Conclusion

Inflammation is a normal biological process which, when prolonged, can compromise the health of an organism. This condition is prevalent in various cancers and can aid both tumorigenesis and tumor progression. Inflammation can be mediated by the mitochondria, largely via mitochondrial ROS, to influence various signaling pathways and regulate targets such as NF-κB, Akt, MAPK/ERK, PTEN, HIF-1α, VEGF, PDGF, TNF-α, IL-6, IL-8, IL-10, IL-18, and Il-1β. As a result, inflammation is further propagated, and a tumor-supporting inflammatory environment is generated. Due to the extensive role of inflammation and the mitochondria in cancer, corresponding therapeutics are under investigation. These include agents which either stabilize or further impair the mitochondria or agents which target components downstream of the mitochondria such as inflammasomes and cytokines.

However, caution is advised as we move forward with these studies. Direct targeting of mitochondria using metformin has the potential to cause mitochondrial dysfunction and lactate overproduction of human platelets (Protti et al. 2012). Use of monoclonal antibodies presents several challenges in the clinic. Due to the molecular size of antibodies, uptake of antibodies by solid tumors has been a prevailing issue (Thurber et al. 2008). Nevertheless, developments in the engineering of antibodies such as site-directed mutagenesis of the Fc region and defucosylation are now being used to increase the affinity of antibodies for their intended targets (Desjarlais et al. 2007; Yamane-Ohnuki et al. 2004; Wang et al. 2018). Bioavailability of natural compounds such as withaferin A also remains a key concern (Devkar et al. 2015). But synthesis of receptor-targeted nanoparticles housing withaferin A holds great promise in pacifying this concern (Agarwalla et al. 2016). Finally, modulating inflammasomes could predispose patients to infections and auto inflammatory

diseases (Karki et al. 2017). For example, human studies using inflammasome inhibitors such as canakinumab and rilonacept shows adverse events such as urinary tract infections and upper respiratory tract infections (Thompson and Nidorf 2018; Kapur and Bonk 2009). MCC950, a potent inhibitor of the NLRP3 inflammasome, displays less immunosuppressive effects compared to canakinumab and rilonacept (Coll et al. 2015; Ren et al. 2018). MCC950 has a shorter half-life, and treatment could therefore be discontinued more rapidly if unwanted reactions such as infections were to occur (Coll et al. 2015). Further studies are necessary to uncover the full breadth by which inflammasomes affect cancer progression, with a focus on how the tumor microenvironment is affected in conjunction with the tumor. Therefore, defining the role of mitochondria in inflammation and cancer biology will provide unique advantages to developing novel techniques and therapies.

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Chapter 14
Modern Germ-Free Study Designs and Emerging Static Housing Technology in a Growing "Human Microbiome" Research Market

Alex Rodriguez-Palacios

Abstract Germ-free (GF) animals and the housing systems necessary to maintain animals as GF during breeding, or as "gnotobiotic" (GN) during specifically designed experiments, have been based on a traditional electrically pressurized ventilation system that influx HEPA-filtered air into the housing units that have existed since at least the 1940s. Since their proposition, these systems remain in principle unmodified, and thus they abound as gold-standard GF housing system in research institutions. Initially used as metallic "pressurized bubbles" to house a set of cages where animals were maintained and handled through rubber portable gloves, these systems were made more significant in size, during the 1960s, to allow the entrance of humans into GF spaces (e.g., "trailers") to conduct experiments and perform animal husbandry. After a period of decline, great learning, and the near disappearance of the word GF from the literature, the importance of GF research (GFR) resurged during the late 1990s with the realization that the human microbiome drives health or promotes disease, often as a secondary factor dependent on dietary habits, genetics, geography, and behavior. An increased number of studies and the resurgence of GF research were facilitated by the HEPA pressurization of individually ventilated cages, which are maintained on rack systems as traditionally occurs with specific-free pathogen (SFP) animals in research facilities. This chapter serves as a source of key referent publications in GF and emphasizes fundamental concepts of biology that are relevant to GF animals and modern study designs. Lastly, this chapter introduces a novel simplified concept of ventilation based on

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nonelectrical non-pressurized passive filtration, enabling the expansion of GF studies to a broader range of laboratory settings.

Keywords GF · Gnotobiotics · Gnotobiology · Nested Isolation · NesTiso · Isolators · Husbandry · Mice

14.1 Introduction

The term "human microbiome" refers to the aggregate of all microbes (e.g., bacteria, viruses, fungi) that reside on, or within, the human organs or fluids and which are increasingly known to determine how we feel and how healthy we are. Acquired especially during birth, the human microbiome has become a hallmark for research worldwide to find the cure to many diseases. Laboratory animals, especially mice, have been critical for scientists to understand how the human microbiome works (Shek et al. 2015). Of all laboratory animal models, the best to study microbes is when scientists raise mice in "isolation bubbles" where unwanted germs (microbes) cannot enter. These so-called germ-free (GF) animals have become the workhouse of microbiome scientists (Kubelkova et al. 2016; Yuan et al. 2017), since their first attempts in the early 1900s. Germ-free science reached its peak in the 1960s (Heneghan 1973; Luckey 1963) but declined thereafter. After a resurgence, a second peak, occurring with hundreds of papers published on a yearly basis (which has been steadily growing over the last 10 years), indicates there is a need to broaden the implementation of modern GF models to decipher how microorganisms (individually and as communities) modulate mental health (Grover et al. 2019) and diseases such as cancer and immune-mediated chronic inflammatory conditions, namely, inflammatory bowel diseases and even rheumatoid arthritis (Maeda et al. 2016a, b) and conditions mediated via the gut-immune-skeletal axis (McGinty and Mallon 2018).

Germ-free facilities are rare in general, but growing interest exists in implementing more of them worldwide. GF facilities are always at risk of contaminations due to breaches in the GF system or microbial barrier integrity, a problem that (sporadically and often unpredictably) occurs in all facilities (Rodriguez-Palacios 2016). GF housing technologies seek to prevent the contamination of GF animals with environmental germs. If contaminated, the airborne or otherwise spread of such microbes into, and within, GF colonies is catastrophic (Rodriguez-Palacios 2016; Yuan et al. 2017). Re-derivation of animals and the reinitiation of colonies are costly both in terms of material losses and time. To date, virtually all GF facilities rely on technologies that use pressurized "germ-free isolator bubbles" or animal "isolation cages" with highly efficient (HEPA) filtered pressurized air (Heneghan 1973). Unfortunately, such GF isolation systems require pressurization via costly laborious instruments, which limits access for most scientists cyclical (Rodriguez-Palacios et al. 2018a, b). High costs accompanied with the need of specialized facilities and training limit large-scale production of GF animals (Fig. 14.1).

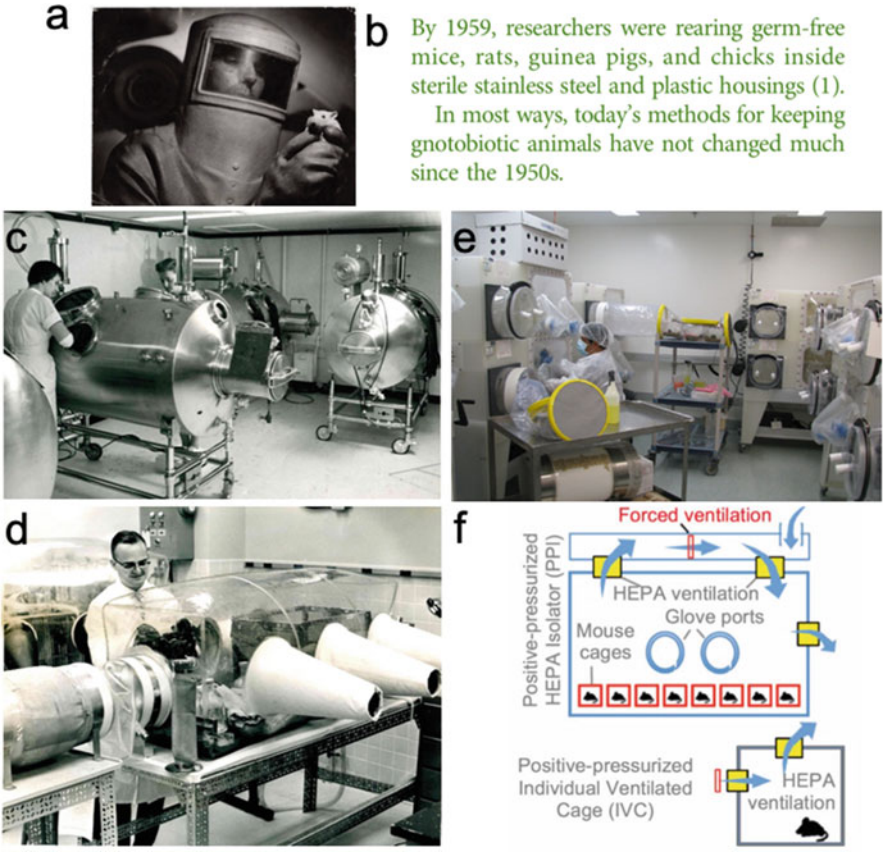

Fig. 14.1 Since the 1930s, GF housing technologies based on pressurized electrical ventilation (to provide GF air to animals) had been the only housing strategy available to scientists until 2018 when we reported that passive ventilation can be used effectively to maintain GF animals. In HEPA-pressurized multi-cage "bubble isolators," technicians handle GF animals with gloves attached to isolators. (**a**) Photograph from W. Eugene Smith, (Man in protective helmet and rubber gloves holding mouse, J.A. Reyniers' Laboratories of Bacteriology at the University of Notre Dame, Indiana), 1949. Source, International Center of Photography (Smith 1949). (**b**) Excerpt from article in the Proceedings of the Natural Academy of Sciences 2014 (Williams 2014). (**c**) First isolators were designed in steel. Original photograph, 1960 press photo of technicians working at St Petersburg FL Germfree Life Research Center Isolator. Source, A. Rodriguez-Palacios. (**d**) Appearance of first flexible film isolators. Original photograph, 1967 press photo Dr. James Heneghan at a germ-free unit at Louisiana State University. Dr. Heneghan was editor of a collection of 97 studies published as proceedings in 1973 (Heneghan 1973). (**e**) GF mice facility at the National Institute of Allergy and Infectious Diseases, initiated by Yasmine Belkaid and Randy Elkins in 2008 with 12 isolators, for a capacity of 5 cages/each, and 5 mice/cage (60 mice total), at the Comparative Medicine Branch to support investigators on campus. Public domain image, downloaded from reference (Wu 2011). (**f**) Illustration of ventilation and air filtration in housing systems commercially available for mice. Use unmodified from Rodriguez-Palacios et al., under Creative Commons Attribution 4.0 International License http://creativecommons.org/licenses/by/4.0/ (Rodriguez-Palacios et al. 2018a, b)

Driven by the market and scientific demands, scientists, especially those with no access to GF facilities, could benefit with alternatives for improving GF research efficiency while reducing the risk of catastrophic contaminations common with traditional GF systems, while reducing husbandry costs. This chapter serves as a non-exhaustive source of peer-reviewed published modern concepts on GF biology and study designs and illustrates the novel contributions that GF experiments have made to our understanding of immunity and microbiome in nutrition, intestinal health, microbiota, and various cancers. Since GF research requires housing of such GF animals using specialized isolators which are primarily based on a design originating from the 1940 to 1950s (Miyakawa and Luckey 1968), this chapter discusses the available technologies and introduces a novel simplified concept of animal housing and isolation based on nonelectrical non-pressurized passive filtration/ventilation (Rodriguez-Palacios 2016; Rodriguez-Palacios et al. 2018a, b) that is enabling the expansion of GF studies to a broader range of laboratory settings, outside of specialized GF facilities (Basson et al. 2019a, b; Menghini et al. 2019).

14.2 Market Value and Exponential Growth of the GF and the Human Microbiome Industry

A 2019 report from the Business Communications Company Research on "Laboratory Animal Models, 3D Cultures, and Organoids," predicts that by 2023, the global market for laboratory animal models will reach $7.8 billion, from $5.9 billion in 2018, indicating a compound annual growth rate of 5.6% (Kubeš 2019). Of this total, mouse models alone (which provide a valuable tool for studying diseases since humans and mice have >99% of DNA similarity) is expected to reach US$1.75 billion by 2023.

The unprecedented modern growth rate for GF research and science worldwide can also be evidenced by the number of publications on "germ-free," "mouse," and "microbiome" data. Comparatively, the number of publications using GF has had a much faster growth rate compared to publications on microbiome and mice, as illustrated by the curve slopes computed over the 10 last years in Fig. 14.2. The number of peer-reviewed publications focusing on GF animal research has historically reached two peaks in history, one in the 1970s and one in 2019, the latter of which is expected to project far beyond 2020. Continuous growth is expected owing to the support arisen from national governments via programs of federal funding for microbiome research and the emergence of global consumer markets (e.g., on probiotics and "microbiome and health") supported via startups and significant venture investments. According to a 2019 study report, the human microbiome market will have a 21.7% compound annual growth rate by 2014 in terms of revenue considering the market share of vital companies in the human microbiome business. The same study also estimated that the global market size will reach US$481.9 million in 5 years, from US$219.9 million estimated for 2019 (Market 2019). Similar

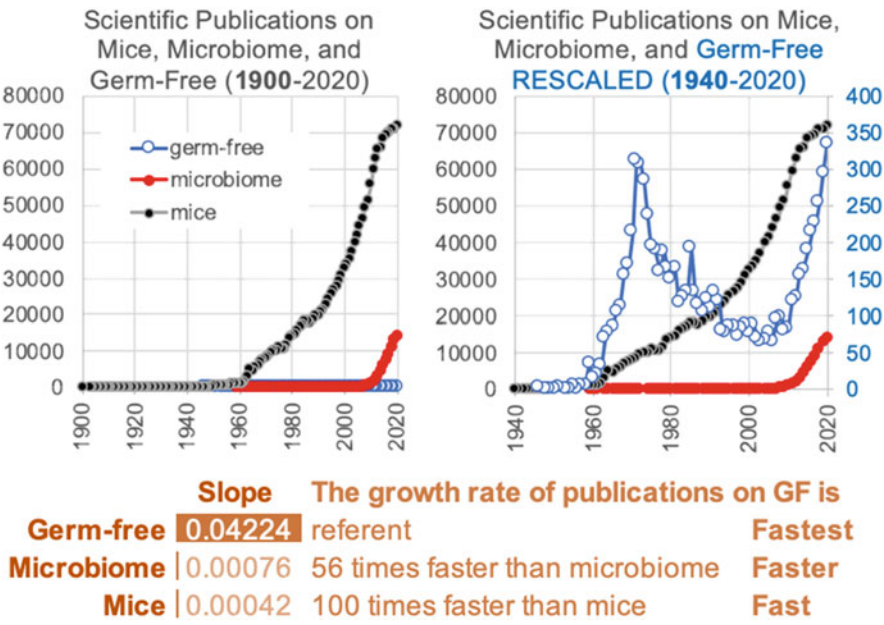

Fig. 14.2 Unprecedented modern growth rate for germ-free (GF) research worldwide. Count statistics of peer-reviewed publications in PubMed. The number of publications by scientists in GF has two peaks in history, one in the 1970s, and one in 2019. Comparatively, the number of publications using GF has had a much faster growth rate compared to publications on microbiome and mice (see slopes, computed over 10 last years)

projections indicate that a compound annual growth rate of 22.5% will lead to an estimated global value of US$1.731 billion by 2027, evenly expanding in major regions, namely North America, Europe, and Asia-Pacific, with a slower participation by other regions of the world (Market 2020).

Favoring the academic and entrepreneurial expansion of human microbiome research, it is the continued interest of scientists to engage in more GF studies (Carter and Foster 2006; Shek et al. 2015; Yuan et al. 2017). Their ultimate goal is to identify patentable innovations that could be translatable into the commercialization of products or methods that could alleviate diseases or improve human and animal health. In a recent survey of professionals conducted by our group Basson et al., in 2019, interrogating members affiliated to three professional organizations of scientists, veterinary, and laboratory animal practitioners (DDRCC, AALAS, and The Gnotobiotic Listserv), demonstrated that 89% of active users of GF want to continue using GF, while approximately 40% with no access want to start conducting GF research, for a net growth and expected positive retention of scientists attracted to GF research and innovation (Fig. 14.3) (Basson et al. 2019a, b). Considering that every mouse needs housing, GF cages/husbandry requires physical space and is a major fraction of research expenses, which is steadily growing as a GF

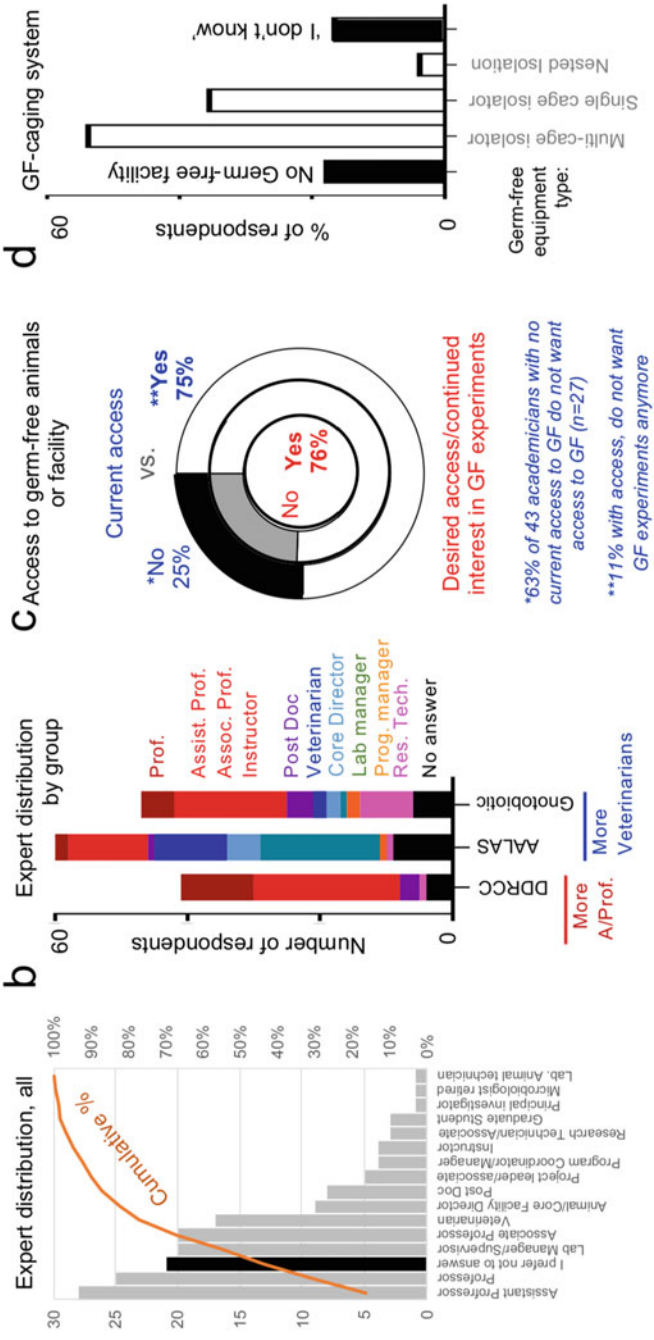

Fig. 14.3 Survey of professionals in organizations specialized in basic science and laboratory animal science and gnotobiotic/GF research. Unmodified illustration of demographic data used unmodified under Creative Commons Attribution 4.0 International License http://creativecommons.org/licenses/by/4.0/ from the author's laboratory (Basson et al. 2019a, b). (**a**) Job descriptions categorized based on information provided by all respondents surveyed. (**b**) Distribution of job descriptions by the three largest groups of participants. (**c**) Our 2019 survey of GF scientists shows that 89% of active users want to continue using GF, while ~40% with no access want to do GF research, for a net growth/retention of scientists attracted to GF. (**d**) The portable cost-effective Nested Isolation system described below is an emerging strategy for GF housing, which simplicity may attract scientists deterred by pressurized systems/high costs

field worldwide. More space-efficient and portable GF housing systems that are financially viable and cost-effective for all researchers are in growing high demand. Cost-efficient scalable GF systems are relevant given the increased need of GF research to understand and promote health. For practical insights on the development of a business plan for GF and gnotobiotic facilities, refer to George Langan and Betty Theriault's discussion from The University of Chicago (Langan and Theriault 2017).

At the institutional level, the market and research opportunity for ~5300 colleges in the USA, with 3026 dedicated to 4-year programs, and an average of one animal facility per university, combined with an estimated of 7750 active research facilities in the USA alone (Liljegren 2016), the number of potential studies based on GF models worldwide is too large to summarize in this manuscript. The sections below provide representative examples of modern study designs that may serve as referent for future studies. With very few recent exceptions using the emerging portable non-pressurized housing (Basson et al. 2019a, b; Menghini et al. 2019), most GF studies have been conducted using anchored pressurized systems.

14.3 Basic Animal Germ-Free Biology and Gnotobiology from Studies in the 1960s

In terms accessible to the public, the Encyclopedia Britanica, in its 250th anniversary described a "germ-free life" as a "biological condition characterized by the complete absence of living microorganisms. While 'Gnotobiology' comprises the study of GF plants and animals, as well as living things in which specific microorganisms, added by experimental methods, are known to be present. When one or more known species of microorganisms are added experimentally to a GF plant or animal, the host, of course, is no longer GF; both the host and the introduced species are gnotobiotic, however, since all added species are known to the investigator. Precise comparisons between germfree and conventional animals cannot be made unless both are isolated from the environment and fed the same sterile diet" (Parrott-Sheffer et al. 2018).

The first attempts to propagate GF animals date back to 1895 at the Hygiene Institute of Berlin, with guinea pigs (Parrott-Sheffer et al. 2018; Smith 1949). However, successful GF vertebrate experiments were conducted with chicks only toward 1912. Shortly thereafter, GF goats were kept alive for 2 months. Advances during the 1920–1930s led to the routine raising of GF animals. By the 1960s internationally established associations and meetings existed where world-renowned groups discussed experiments. Findings were regularly published as formal book format proceedings. Available examples include the 1967 proceedings, which gathered scientist contributing to 97 studies on various aspects of technology, implementation in the practice of human medicine and surgery, and preclinical medicine expanding our knowledge on the biology of animals living without germs. Some of the key discoveries during initial GF studies include the identification of nutritional

deficits that occur as a consequence of autoclaving of diets and the lack of microbial metabolites (Luckey 1963; Miyakawa and Luckey 1968). Initial observations revealed that GF animals have larger ceca, smaller livers, and seemingly under sized and developed lymphoid organs, which are used today to help phenotype GF animals (Luckey 1963; Rodriguez-Palacios et al. 2018a, b).

In context, the interest in GF research started a few decades after Louis Pasteur and Robert Koch collectively fathered the era of bacteriology and the infectious principles of various epidemic diseases of plants and animals. Koch's postulates describe the necessary steps to experimentally define a microorganism as a causal agent responsible for the occurrence of a pathological effect in a host. Louis Pasteur's pasteurization experiment illustrates the fact that the spoilage of liquid was caused by particles in the air rather than the air itself. These experiments were important pieces of evidence supporting the germ theory of diseases in which the central dogma states that infectious microorganisms need to be in direct contact with the host to cause disease and that air is necessary for airborne microbes. Therein, air filtration and provision of GF air to the animals in their GF environment are the hallmarks of GF facilities.

14.4 Retroviruses and *mdr1a* May Influence Pharmacodynamic/Microbiome Studies in GF Mice

The only infectious agents acceptable in GF settings are primarily retroviruses, because they self-integrate in the host genome, and there are no accessible means to eradicate them once integrated. Although retroviruses are challenging to grow in vitro, their presence can be indirectly inferred using serum antibody titers. Retroviruses referred to as "endogenous viruses" depend on the host to replicate. Therefore, retroviruses can influence the biological performance of GF models. Retrovirus insertions may affect the GF physiology if insertions affect genomic elements directly interfering with gene sequences or indirectly affecting epigenetic mechanisms.

Several examples of animals affected with endogenous viruses exist, one of which includes AKR/J mice, a genetic line that has been used to generate numerous sublines (Takeda et al. 1981). AKR/J mice are viremic from birth and express the ecotropic retrovirus AKV in all tissues (Jackson-Laboratory 2020). The AKR/J line (carriers of the Thy1.1 "theta AKR" haplotype antigen) suffers from leukemia (60–90% incidence), and, thus, it is one of the lines with the shortest life span. AKR/J were one of the first mouse lines raised under GF conditions, but the worsening of disease susceptibility and ("preleukemic syndrome") morbidity experienced by AKR mice under GF conditions, first reported at the Lobund Laboratory, Department of Microbiology, University of Notre Dame, makes raising productive GF AKR/J mouse colonies challenging (Pollard 1967, 1969). Such limitations are particularly notable when GF AKR are required as a putative control, such as for

senescence-accelerated prone/resistant (SAMP/SAMR) mouse, a mouse line derived from inbreeding and selection of the AKR/J (Pizarro et al. 2011). Senescence-prone strains (SAMP) have earlier onset of loss of passivity and reactivity, loss of skin glossiness and increased coarseness, hair loss, periophthalmic lesions, and increased lordokyphosis of the spine (Takeda et al. 1981).

As a second example, AKV retroviruses have also been reported triggering sudden deleterious effects in the genome of various SAMP lines, which can be now studied under GF conditions (Zhang et al. 2008). Zhang et al. explained that the enhanced susceptibility that some SAMP sublines to suffer drug toxicity is due to a retroviral insertion affecting the drug transported gene *mdr1* (currently designated as *abcb1a*). The *mdr1* gene is clinically relevant because retroviral-disrupted *mdr1* leads to a dysfunctional transmembrane pump (a P-glycoprotein) in the blood-brain barrier that excretes medications outside of the brain cells to prevent neurocytotoxicity. Such dysfunctionality in several SAMP lines causes increased susceptibility to ivermectin, making animals prone to suffer neurologic signs because the neuronal cells are prone to toxicity. Ivermectin and similar moxidectin and selamectin are considered "parasite- specific" macrocyclic lactones, medically derivative from soil microorganisms of the genus *Streptomyces*, used as antiparasitic to treat mites in mice as they act as potent agonist of glutamate-gated chloride ion channel activity in the CNS of the parasite, which leads to parasite paralysis and death (Nashat et al. 2018).

Increased susceptibility to topical moxidectin has also been described in SAMP8 and SAMR1 sublines (Takeda et al. 1981), with 14–18 times higher concentrations of the drug in the brain, while normal concentrations are present in the serum. Since ivermectin may affect behavioral and consumption or preference phenotypes (Davis et al. 1999; Yardley et al. 2012), it is important to consider that in any given GF experimental design, the susceptibility mechanism that explains ivermectin-dependent neurological signs (Kanwar and Varshneya 1995) may unknowingly affect other neuropharmacological aspects of GF research, for instance, the response to stress or to altered gut-microbiome-brain axis. The importance of this P-glycoprotein in study designs also should be considered in all the lines known to have this 8.5-kb spontaneous retroviral insertional mutation right before exon 23 in the ATP-binding cassette, subfamily B (MDR/TAP), member 1A (*Abcb1a*). Specifically, the AKR/J sublines SAMR4, SAMR5, SAMP1, SAMP6, SAMP7, and SAMP9, and the mice Crl:CF1-*Abcb1a* mice, which may have a possible distant common ancestor (Zhang et al. 2008). Of interest, this mutation is absent in the other contemporary sublines SAMP3, SAMP8, SAMP10, and SAMP11, and their putative parental genetic line AKR/J, and also in other common laboratory strains including 129/SvJ, SJL/J, SWR/J, A/J, BALB/c, C3H/He, C57BL/6J, DBA/2, NZB/N, ddy, CD-1/Crj, and the ICR/Jcl.

Of relevance to study designs, the induction of a homozygous disruption of this gene (replacement of exons 6–7 by a hygromycin phosphotransferase cassette) in C57BL/6 mice has resulted in threefold increased susceptibility to carcinostatic drug vinblastine, which in context is less pronounced than the 100-fold increased susceptibility conferred to ivermectin (Schinkel et al. 1994). Relevant to cancer and

microbiome GF studies, it is important to consider, as described by Schinkel et al., that P-glycoproteins confer multidrug resistance by actively extruding a wide range of structurally unrelated amphiphilic hydrophobic drugs from the cell (Schinkel et al. 1994). Many of such compounds can be toxic in nature and occur naturally in plant fungi and bacteria or be semisynthetically originated for immune suppression and chemotherapy purposes and which can be studied in GF systems, namely, vinca alkaloids, actinomycin D, taxanes, epipodophyllotoxins, cyclosporin A, and FK506, or FK866, among several others which can influence cancer research and GF biology (Ogino et al. 2019; Schinkel et al. 1994; Čižmáriková et al. 2019).

As an advancement to our understanding of *mdr1* glycoproteins and GF biology, more recently, Cao et al. in Mark Sundrud's laboratory at The Scripps Research Institute determined that the *mdr1* gene is essential for specific ($CD4^+$ effector) T-cells, outside of the nervous system (Cao et al. 2017), in a study that has been elegantly summarized as "Mdr1 saves T cells from bile" (Izcue and Pabst 2017). Relevant to IBD, dysfunctional *mdr1* expression in T cells drives in part the susceptibility to toxicity to bile acid exposure, which occurs readily in the gut wall of the ileum as T cells migrate into the tissue to complete differentiation and gain immunotolerance. As a consequence, T-helper cells with abnormal *mrd1* gene upon exposure and before dead release signaling molecules that trigger an excessive inflammatory reaction by other cells in the proximity. Our groups, at Case Western Reserve University, are currently examining the role of bile transport blockade in the prevention of inflammatory bowel disease, and its dependence on the animal microbiome. As we improve our understanding of retrovirus integration in new GF lines, it is essential to determine how these retroviral mediated mechanisms could modulate diseases that may co-occur independently of the gut microbiome. Of importance, SAMP1-IBD-ileitis, which occurs in both SPF and GF conditions (therefore it is independent of gut microbiota), is not believed to be due to retroviral insertions, since other SAMP lines, having the same *mrd1* mutations, do not develop IBD (i.e., Crohn's disease-like ileitis) (Rodriguez-Palacios et al. 2015a, b).

14.5 Mechanism of Disease in Modern GF Study Designs

Germ-free animals are an excellent disease model tool for studying the biology of different conditions in both animals and humans, including cancer and gastrointestinal disorders or their interdependence with environmental microbes. Studies have demonstrated the value of GF animals in deciphering the role of the human gut microbiota in gut physiology and development of the wall integrity in preterm infants and in cancer development (Sommer and Bäckhed 2013; Yu et al. 2016). The number of manuscripts published on GF and cancer, every year, has been steadily increasing over the past decade. The use of conventional mice for the study of microbiome principles derived from the human microbiome, although critical to increase external validity, may produce confounded data (which may be irreproducible across laboratories due to large microbiome differences across animal

facilities and seasons) and makes it difficult to precisely identify mechanisms solely linked to human-derived microbes. Below is a series of contributions made to modern biology enabled or strengthened by the use of GF mice. Several of the referent studies cited below may serve as guide to design modern experiments requiring GF systems to study several diseases including colorectal cancer (CRC) and IBD. Several examples of study designs and basic concepts of GF biology can be found in all-time referent books from the 1960 to 1970s (Heneghan 1973; Luckey 1963).

14.5.1 The Gut Microbiota of Preterm Infants Has a Unique Effect in the Gut of GF Animals

Germ-free animals, namely, mice and piglets, owing to their genetic and omnivorous functional/anatomical proximity to the human biology have been used as surrogates to understand diseases in children and preterm infants. Bovine calves have also been used as GF models, despite their larger size, for the study of enteric viruses, namely rotavirus and coronavirus. Of historical relevance, after GF research was well-perceived for experimental purposes using a wide variety of animals in the early 1960s, pressurized GF technologies were used in clinical application (Heneghan 1973). The most remarkable clinical justification was their use to protect infants with severe combined immunodeficiency (SCID). In the USA, the most historical case was when a newborn (David Vetter, the 'Bubble Boy') was transferred and maintained in isolation in a pressurized GF isolator until he died 12 years later as a consequence of receiving a bone marrow transplant from a donor (his sister) infected with Epstein-Barr virus (Fig. 14.4) (Kirk 2012a, b). Today, GF technologies are primarily used for research combined with animal models to help determine the role of the microbiome in infant infectious diseases and malnutrition and increasingly important in conditions linked to severe immune disorders.

Using body weight gain as a marker for gut health in GF C57BL/6 J mice, Yu et al., at the University of Chicago, recently documented that microbial communities from infants have differential effects on intestinal development (Yu et al. 2016). Specifically, microbiota from a preterm infant with normal weight gain induced desirable microscopic phenotypes in the gut lining with increased villus height and crypt depth, active cell proliferation, more goblet and Paneth cells, and tight junctions compared to the microbiota from a preterm infant affected with poor weight gain. Microdissection and gene expression showed that the normal infant induced in GF mice increased stem cell activity (*Lgr5*), Paneth cells (*Lyz1*) and crypt populations (*Cryptdin5*), and more goblet cell and mature enterocytes (Muc3) in the villi. In contrast, the ill-thrift infant failed to promote such microbiome-mediated changes in the gut wall, making the gut wall more similar to the GF status.

In addition to determining that GF mice have decreased numbers of Paneth and goblet cells (Yu et al. 2016), earlier studies with GF mice led by McMaster

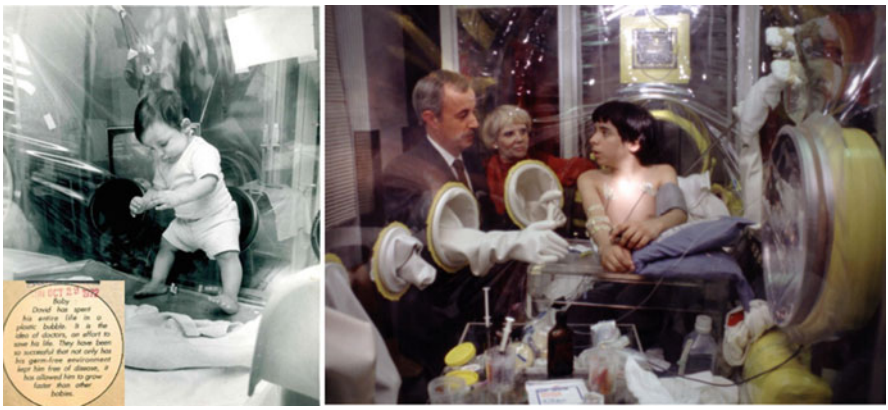

Fig. 14.4 In the 1970s GF pressurized systems were promoted and used for human clinical and surgical purposes (Heneghan 1973). Left, original photograph. Source, A. Rodriguez-Palacios [1972 Press Photo Baby David (Bubble Boy) lives entire life in plastic bubble. By Tom Colburn, for Chronicle Magazine, October 29, 1972]. Excerpt on back of photo reads: "It was the idea of doctors, an effort to save his life. They have been so successful that not only has his germ-free environment kept him free of disease; it has allowed him to grow faster that other babies". Right photo, [David Vetter speaks with Dr. William Shearer during preparations for the experimental bone marrow transplant in 1983. (Photo Credit: Baylor College of Medicine) Source, Houston Public Media] Public domain, downloaded from reference (Feibel 2016)

University and Harvard University identified that the lack of microbial stimulus (via maternal epigenetics or colonization at birth) leads to increased intestinal permeability (Smith et al. 2007) and decreased intestinal epithelial cell migration, proliferation, and renewal (Rakoff-Nahoum et al. 2015). Neonatal piglets and calves have also provided tremendous insights into the biology of human infectious diseases and increasingly common their interaction with the human microbiome (Vlasova et al. 2019; Wen et al. 2012). How the lack of certain immunological features or the microbiota in GF animals affects the resistance to parasitic infections is currently under investigation. Using as example the tapeworm *Hymenolepis diminuta*, Shute et al., at the University of Calgary (Shute et al. 2020), determined in vitro analysis that the worm extract enhances the growth of anaerobic bacteria and that infection in mice promoted Th2 immune responses, with increased colonic levels of IL10, IL-25, Muc2, trefoil factor 3, and β2-defensin mRNA. However, despite the effect of the tapeworms, the study found no evidence that resistance to parasitic infestation is due to the gut microbiota or the unprimed/primed gut immunity in GF mice (Shute et al. 2020).

14.5.2 The Human Gut Microbiota from Cancer Patients Induce Cancer in GF Animals

The pathogenesis of CRC is complex, progressive, and multifactorial. Evidence suggests that environmental factors, chronic inflammation, gut microbiota, and genetic predisposition influence CRC progression, with ~95% of lesions originating from adenomas (Housseau et al. 2016; Li et al. 2019). The use of azoxymethane (AOM) as a mutagenic agent, which can be injected aseptically to GF mice, has facilitated the study of inflammation-dependent and independent mechanisms of colon carcinoma (Neufert et al. 2007) and its modulation by the human gut microbiota. In 2017, a study of fecal microbiota from patients with colorectal cancer (CRC) transplanted to mice by oral gavage twice a week for 5 weeks, at the Chinese University of Hong Kong, demonstrated that cancer lesions are exacerbated in conventional (male C57BL/6) mice by the human gut microbiota in animals pre-treated with azoxymethane (10 mg/kg) and oral antibiotics to induce colon neoplasia (0.2 g/L of drinking water of ampicillin, neomycin, metronidazole; 0.1 g/L vancomycin; 2 weeks) (Wong et al. 2017). Remarkably, this outcome demonstrated, testing five patients, that CRC microbiota has a pro-cancer role.

Although the use of conventional mice has been universal and experimentally critical to validate the connection between susceptibility to chronic inflammation, dysbiosis, and CRC (Li et al. 2019; Menghini et al. 2017) and the notion that IL17 itself has a primary role in colon carcinogenesis and not necessarily the cell types that produce IL17 (Housseau et al. 2016), the presence and interaction between the "naturally occurring" resident mouse microbiota and the transplanted human microbiota within the mouse gut often benefit or requires validation in GF models. As pursued by Wong et al., GF mice have helped to determine with precision the inflammatory pathways and molecular mechanisms associated with cancer lesion induction and worsening as an exclusive consequence of human gut microbes (Wong et al. 2017). GF experimentation has determined that the gut microbiota from CRC patients induces in the mouse colon a higher induction of proliferating (Ki-67-positive) cells, T-helper 1 (Th1) and Th17 cells, and proinflammatory cytokines, including C-X-C motif chemokine receptor 1, C-X-C motif chemokine receptor 2, interleukin 17A (IL17A; 2.25 vs. 0.44%), IL22, and IL23A. Collectively, real-time polymerase chain reaction arrays revealed enhanced genes involved in cell proliferation, apoptosis, angiogenesis, invasiveness, stemness, and metastasis linked to CRC-derived fecal microbiota. Microbiome analysis revealed enrichment of *B. fragilis* at 32 weeks, with depletion of *Faecalibacterium prausnitzii*, *Clostridia*, and various *Lachnospiraceae* at 8 and 32 weeks after gavage.

Other examples of GF experimentation in CRC include the use of genetically modified mice (often related to the C57BL/6 J strain, used as the reference for the mouse genome) that are derived under GF conditions. Tomkovich et al. at Jobin's laboratory, University of Florida, have characterized the effect of CRC-derived microbiota on carcinogenesis using the mouse model $Apc^{Min/+}$ raised as GF and after exposure to various types of CRC-derived bacteria or microbiota. Studies have

also indicated that the microbiota and inflammation are critical to colon tumorigenesis. However, locoregional effects drive the severity of intestinal cancer pathologies with different intensity depending on mouse genetics, animal age, and organ location. In the context of IL10 deficiency, mice $Apc^{Min/+}$ were more prone to have significant colonic lesions compared to IL10-competent mice. In contrast, tumorigenesis in the small intestine was, however, pronounced in $Apc^{Min/+}$ mice as a function of age, regardless of the status of the IL10 genetic background (Tomkovich et al. 2017). In density terms, the tumor burden (more, larger tumors) in all cases was higher in GF mice exposed to CRC-patient microbiota compared to conventional mice, supporting the concept that commensal microbes increase the risk of cancer lesions. Others have repeatedly highlighted how the intestinal tracts of GF animal models are influenced when exposed to carcinogenic human microbiota (Zhan et al. 2013).

Germ-free studies have also facilitated the study of digestive biofilms when present in humans. Studying the severity of colon tumor formation in three mouse colon tumor models (GF $Apc^{Min\Delta 850/+;IL10-/-}$ or GF $Apc^{Min\Delta 850/+}$ and specific pathogen-free $Apc^{Min\Delta 716/+}$ mice), it has been demonstrated that mucous-invasive bacteria, present in gut biofilms of CRC patients and healthy controls undergoing colonoscopies when present, induce more colon inflammation and tumors compared to donors free of biofilm formation (Tomkovich et al. 2019).

Germ-free experimentation can enable the study of innate and unexpected novel mechanisms of disease modulation triggered by microbes, for further validation in humans, as well as to determine the epigenetic nature of DNA alterations induced by human microbes. In a recent study conducted by Sobhani et al., CRC-associated dysbiosis in humans caused unique alterations in the mouse DNA. Stool samples from CRC patients transferred into GF mice caused aberrant crypt foci, DNA alteration, and luminal microbiota alterations when animals were examined at 7–14 weeks post-colonization. Microbiota associated with CRC induced large number of hyper-methylated genes in GF mice (Sobhani et al. 2019). Several gene promoters including SFRP1,2,3, PENK, NPY, ALX4, SEPT9, and WIF1 promoters were hypermethylated in CRC. Mechanistically, the GF mouse model helped to determine that CRC microbiota from patients induces epithelial cell proliferation (K167 marker), which is augmented with AOM, and increased expression (1.7–19 fold) of transcription factors HES1, KLF4, and ELF3, but not of MATH1. Increased inflammation was reflected by higher IL1β, IL6, and MIP2α and lower IL10, IL23, and INFγ. In transplanted mice, *Fusobacteria*, *Parvimonas*, *Butyrivibrio*, *Gemella*, and *Akkermansia* were increased, while *Ruminococcus*, *Bifidobacterium*, *Eubacteria*, and *Lachnospira* were reduced. Such dysbiotic microbiota caused more incidences of mutations at global exon/intron level in the colonic tissue of GF mice compared to spleen and to healthy control microbiota. The findings in GF mice were then validated in humans.

14.5.3 The Human Microbiome Modulates Immunotherapies and Side Effects in GF Animals

Research on animals raised in GF environments are becoming an important biomedical tool to understand the role that human microbes may have on therapies, the induction of side effects, and the promotion or suppression as modulators on anticancer immune responses. For instance, Vannucci et al. at the Academy of Sciences of the Czech Republic demonstrated that GF rats developed fewer tumors than conventional rats following a similar protocol of CRC induction, with GF rats showing a better immune reaction against cancer lesions through B, CTL, NK, and NKT cell responses (Vannucci et al. 2008). Such study indicates that an effective absent antigenic challenge and the absent baseline state of "physiological inflammation" in GF animals (caused by commensal intestinal microbes) enhances the ability of GF models to mount more effective immune responses against cancer. In studies conducted at the University of Florida with *Campylobacter jejuni*, the most frequent bacterial cause of human gastroenteritis, GF preclinical $APC^{min/+}$ models (and 1% DSS) helped to determine that rapamycin diminish the tumorigenic capability of *C. jejuni* in susceptible hosts (He et al. 2019).

Among the variables that could contribute to interpatient heterogenous response to anticancer therapies is the differential composition of the patients' microbiome, which has been shown to affect antitumor immunity and immunotherapy efficacy in preclinical mouse models (Matson et al. 2018). A study conducted at the University of Chicago, trying to determine why anti-PD-1-based immunotherapy, which has had a major impact on cancer treatment, only benefits some patients, also suggested that the commensal microbiome modulated anticancer immune responses in cancer patients, studying feces from metastatic melanoma patients before treatment. Germ-free mice reconstituted with fecal gut microbiota obtained from patients that responded to therapy were enriched with *Bifidobacterium longum*, *Enterococcus faecium*, and *Collinsella aerofaciens*, which resulted in tumor control, enhanced T-cell response, and increased efficacy of anti-PD-1 therapy in mice (Matson et al. 2018; York 2018).

Germ-free mice are also fundamental to mechanistically understand the side effects of cancer treatments where excessive pain perception in patients occurs secondarily to therapy. As described by Shen et al. at Harvard Medical School, chemotherapy-induced pain is a dose-limiting condition that affects 30% of patients undergoing chemotherapy (Shen et al. 2017). By using GF mice and chemotherapeutic agent oxaliplatin, the authors demonstrated that the gut microbiota is critical for the induction of pain, being inducible in animals as mechanical hyperalgesia. Remarkably, their study revealed that GF mice had no pain compared to microbiota-carrying mice. Of clinical value, conventional mice receiving antibiotics also exhibited less pain, suggesting for the first time that the microbiota determines the severity of pain a cancer patient could feel during cancer treatment. After restoring the GF mice with microbiota, the GF protection to the chemotherapy-induced pain was abrogated. Mechanistically, comparative GF experiments allowed determining

that the administration of antibiotics did not alter the distribution of the drug. Most importantly, the "tunable" expression of TLR4 receptors on the surface of hematopoietic cells, e.g., macrophages, partly mediated the presence of such a phenotype of pain (Shen et al. 2017).

Targeting CTLA4 (negative regulator of T-cell activation) through specific antibodies (Ab) is a successful mechanism employed for protection against cancer. Elegant studies conducted by Vetizou et al. in a multi-institutional collaboration in France revealed that gut microbiota is essential for the antitumor properties of CTLA4 blocking through antibodies (Vétizou et al. 2015). Studies in human patients and mice demonstrated that distinct species of *Bacteroides* (e.g., *B. thetaiotaomicron* or *B. fragilis*) are crucial for the antitumor effects of CTLA4 blockade. Of relevance to modern GF study designs, the CTLA4 blockade had no effect on tumors in GF mice, but the colonization of the same GF mouse line with *B. fragilis* reestablished the expected antitumoral effect of the CTLA4 blockade effect on tumor cells. Of interest the effect was also rescued by immunization of mice with *B. fragilis* polysaccharides or by adoptive transfer of *B. fragilis*-specific T cells (Vétizou et al. 2015). Although antibiotic-treated mice were also unresponsive to the blockade, thanks to the GF model system authors were able to confidently establish that *Bacteroides* have a crucial role in the immune antitumoral modulatory effects of CTLA4 blockade (Vétizou et al. 2015).

14.5.4 The Variable Human Microbiota May Induce Inflammatory Bowel Disease in GF Models

One of the important unknowns in IBD is how the diet and the microbiome interact to modulate disease. As a modern influential example, a 2012 study conducted by Devkota el al., at Eugene B. Chang Laboratory, University of Chicago, showed employing GF mice deficient in IL10 that the consumption of a diet high in saturated milk-derived fat, but not polyunsaturated safflower oil fat, changed the microbiome assemblage promoting the abundance of sulfite-reducing pathobiont *Bilophila wadsworthia* (Devkota and Chang 2015; Devkota et al. 2012). Remarkably, mono-association infections in such GF mice could only occur with the consumption of the milk fat diet, promoting taurocholic acid, Th1 response, and the expected development of the IL10 colitis as an experimental surrogate model for human IBD.

Our Digestive Health Research Institute, at Case Western Reserve University, specializes in digestive and liver diseases and so herein this section provides some of the numerous examples in which GF animals have facilitated the study of small intestinal models relevant to IBD (Basson et al. 2019a, b; Menghini et al. 2019; Rodriguez-Palacios et al. 2018a, b; Rodriguez-Palacios et al. 2015a, b; Rodriguez-Palacios and Cominelli 2018a, b). Recently, GF mice were critical to characterize and confirm that the anti-inflammatory effect induced by an anti-IL1A antibody in a mouse line (SAMP1/YitFc) prone to spontaneous Crohn's disease-like ileitis

depends on the microbiome (Menghini et al. 2019) and the important discovery that the fecal microbiome of a person either normal or suffering IBD (Crohn's disease, ulcerative colitis) during periods of disease inactivity (remission) may unpredictably carry a microbiome that has the potential to induce inflammation in the small intestine or suppress it upon transfer to GF mice prone to Crohn's-like ileitis (Basson et al. 2019a, b). The latter discovery fuels a preclinical strategy to use GF mice to identify the best personalized anti-inflammatory microbiome for ulterior use by patients during periods of IBD flare-ups.

Of remarkable similarity to the study conducted by Vetizou et al. on CTL4 blockade in cancer models, our group has identified that the blockade of the pro-inflammatory cytokine IL-1α has no detectable anti-inflammatory effect in chronic intestinal inflammation in the GF SAMP Crohn's disease-like prone mouse model SAMP1/YitFc, as expected, in contrast to the anti-inflammatory effect observed in microbiota-colonized mice (Menghini et al. 2019), further supporting the role of the gut microbiome in modulating immune and biological therapies (Alderton 2016; Sivan et al. 2015; Snyder et al. 2015). With respect to the anti-IL1-alpha therapy, the antibody blockade induced significant changes in the gut microbiome compared to dexamethasone or control isotypes, with remarkable reduction in the *Proteobacteria* to *Bacteroidetes* ratio, *Helicobacter*, and increased *Mucispirillum schaedleri* and *Lactobacillus salivarius* (Menghini et al. 2019). With the recognition that the microbiota plays an important role, potential microbial indicators of a promising anti-inflammatory response due to the IL1-A blockade in humans are under investigation.

Other groups have used various genetic mouse models of IBD under GF conditions. At the University of Michigan Medical School, Gabriel Nunez's laboratory has demonstrated, for instance, that neonatal acquisition of *Clostridia*, but not *Bacteroidales*, protects GF mice against colonization by bacterial pathogens, including pathogenic *Citrobacter rodentium* and a strain of *Salmonella enterica* serovar *Typhimurium* deficient in the type III secretion system encoded by the pathogenicity island 2 (*DspiA*), which replicates normally in the intestine but is deficient in systemic spread (Kim et al. 2017).

14.5.5 Nutrients and Microbial Metabolites Enhance Therapeutic Efficacy of Immunomodulators

GF animals have also been relevant to determine the impact of nutrition in health independently from microbes (Luckey 1959, 1963; Luckey et al. 1955; Miyakawa and Luckey 1968). A recent study funded by the Sugar Research Foundation (SRF) demonstrated interesting findings linking fructose-containing sugar (sucrose) to cancer and hyperlipidemia. This study utilized GF rodents to prove their hypothesis. Further, the microbiota was shown to have a crucial role in hypertriglyceridemia induced by carbohydrates. Conventional rats fed with high-sugar diet were

compared to a group fed high-starch diet, and it was observed that sucrose consumption leads to elevated levels of beta-glucuronidase, which is known for having an increased association with bladder cancer in humans (Kearns et al. 2017).

Evidence is accumulating proposing a critical association of maternal obesity with enhanced risk of obesity and nonalcoholic fatty liver maladies in children. Soderborg et al. at the University of Colorado Anschutz Medical Campus led an exploration that compared GF mice exposed to fecal microbiota from 2-week-old infants born to obese mothers compared to normal-weight mothers. GF mice colonized with stool microbes of infants from obese mothers had increased expression of liver genes for endoplasmic reticulum stress pathways. Also, gene expressions for innate immunity combined with periportal inflammation histological signs were additionally elevated. These mice showed impaired macrophage function and enhanced intestinal permeability. Accelerated nonalcoholic fatty liver disease (NAFLD) and gain in weight were observed in mice with microbiota derived from obese mother infants when exposed to the western diet. Functional evidence supported the casual role of infant dysbiosis (through obese mothers) in child obesity and NAFLD (Soderborg et al. 2018). Cai et al. at Pennsylvania State University earlier contributed to our understanding of how the gut microbiota influences obesity and related metabolic disorders, by studying the antioxidant "tempol," which reduced weight gain in mice by modulating the microbiota (Cai et al. 2016). The oral antioxidant decreased cecal bacterial fermentation and increased fecal energy excretion in a dose-dependent manner testing three doses (1, 10, and 50 mg/kg, for 5 days). By using serum and liver (1-H NMR) metabolomics in conventional mice, the authors identified a dose-dependent decrease in glycogen and glucose, enhanced glucogenic and ketogenic activity (phenylalanine, tyrosine), and glycolysis pathway activation, all features of glucose catabolism, with upregulation of antioxidant metabolic gene networks (Pepck and G6pase activation and Fabp1, Hnf4a, ChREBP, and Cd36 mRNA reduction). Of interest, no significant changes in liver and serum profiles were observed in GF animals, defining a modulation role for the intestinal microbiota over the catabolic state induced by the antioxidant in conventional mice. Therein, results illustrate that therapeutic modification of metabolic pathways can be triggered via alterations in the gut microbes. GF mice are uniquely critical to identify medications that exert biological effects in a dose-/microbiota-dependent matter that significantly shift the host toward catabolic (body weight losing) states (Cai et al. 2016).

In a study conducted at the University of Alabama at Birmingham, Paul et al. used humanized GF mice with transplanted gut microbiota from breast cancer patients before and after chemotherapy to identify the effect of dietary "genistein" (a polyphenol, isoflavone found in soy) on the inhibition of tumor progression (Paul et al. 2017). Genistein induced in humanized GF mice differs in microbial composition compared to control diet (increase genera *Lactococcus* and *Eubacterium* and families *Verrucomicrobia*, *Lachnospiraceae*, and *Ruminococcaceae*) in GE-fed mice. In two of three patients, the post-chemotherapy microbiota yielded the disappearance of propionic acid conjugates (4-ethylphenol and 2-4-hydroxyphenol).

GE showed lower tumor burden and increased the latency of breast tumor reducing tumor growth (Paul et al. 2017).

The gut microbiota has a role in fermenting fiber in to short-chain fatty acids such as butyrate. Butyrate has been shown to have a crucial role in tumor suppressing properties in CRC cell lines. A study conducted by Donohoe et al. at the University of North Carolina Chapel Hill showed how a gnotobiotic mouse model was used to demonstrate that dietary fiber protects against colorectal tumorigenesis in a microbiota- and butyrate-dependent manner. Tested in vivo, using GF mice colonized with mutant and wild-type strains of butyrate-producing bacteria, the authors established that dietary fiber has a critical role in tumor suppression in a mechanistic fashion that is dependent on both the microbiota and the production of butyrate (Donohoe et al. 2014). Remarkably, the GF study showed that due to the Warburg effect, butyrate is metabolized less in tumors where it accumulates to act as a histone deacetylase inhibitor, which in turn promotes histone acetylation affecting cell proliferation and apoptosis. Validated in human cancer, Donohoe et al. demonstrated increased butyrate and histone acetylation in CRC lesions.

With respect to organic acids from the Krebs' cycle, capillary electrophoresis time-of-flight mass spectrometry metabolome analysis of cecal content has shown that the amounts of succinate are very low in GF mice and that only reconstitutions with certain bacteria (e.g., *Bacteroidales*) result in increased amounts in the mouse gut (Kim et al. 2017), a mechanism that is attuned to the ability of such specific phylum to produce large amounts of succinate as recently reviewed (Basson et al. 2016). Similar effects have been observed in GF mice colonized with complex microbiotas, with differences depending on age in neonates. Further, the oral administration of organic acids, for instance, succinate, but not acetate or lactate, promotes the colonization of *Clostridia* IV and XIVa clusters (4–5 logs), while in contrast, the administration inhibits *S. Typhimurium* (*DspiA*, by ~100-fold in GF mice receiving a *Clostridia* consortium). By unclear reasons, succinate also seems to reduce the concentration of oxygen in the intestine of GF mice (Kim et al. 2017).

14.5.6 Germ-Free Models Enable the Study of NAFLD and Oral and Lung Cancer

Nonalcoholic fatty liver disease is common among obese people and is frequent manifestation of metabolic syndrome; however, not all individuals with obesity develop NAFLD. Le Roy et al. in a study led by the Institut National de la Recherche Agronomique (INRA), in France, demonstrated that the gut microbiota plays a critical role in NAFLD using GF animals. Of mechanistic interest, the study showed that among obese C57BL/6 J mice, the mice that developed NAFLD (but not the obese with normal livers) had a very distinct microbiota capable of inducing the liver disease in other GF when feces were transplanted (Le Roy et al. 2013).

To date, no single oral microbial composition profile has been definitely linked to the risk of developing oral squamous cell carcinoma. Recent studies conducted by Stashenko et al. in a multi-institutional collaboration lead by the University of Florida College of Dentistry provided a better understanding of the oral microbiome's role in the oral lesions. Similar as in colon cancer, findings in GF mice revealed that different oral microbial populations enhance tumorigenesis and tumor burden. Metatranscriptomic analysis and 16S rRNA gene sequence analysis were applied to characterize the longitudinal alterations in profile and function of oral microbiome in 4-nitroquinolin-1-oxide (4-NQO)-induced model of OSCC in germ-free mice (Stashenko et al. 2019). Although promising, in context, it is important to highlight that in Japan, studies have shown that pathobionts and commensals could have comparable and innocuous systemic effects on GF mice, despite orally administered periodontitis-associated bacteria induce pathological changes in the liver and intestine which are relevant for periodontitis (Yamazaki et al. 2020). Gopalakrishnan et al. conducted a study to examine gut and oral microbiome of melanoma patients encountering anti PD 1 therapy. When responder patient's microbial profiles were compared with nonresponders, a notable difference was found in composition and diversity of microbiome. Increased antitumor activity was noted in responder patients. In GF-recipient mice, fecal microbiome transplantation (FMT) experiment was done to examine the relation between gut microbiome and reaction to the immune checkpoint inhibitor. Mice transplanted with anti-PD-1 therapy responder stool samples showed reduced tumor development when compared with mice with stool transplant of nonresponders (Gopalakrishnan et al. 2018).

Germ-free models are also becoming critical to study other organs known for having simpler microbial communities, compared to the gut. Rega et al. carried out a research to explain the antitumor effect of lipopolysaccharide (in dose-dependent manner) in mouse model of B16-F10-induced metastatic lung cancer. It was established from this study that in low-dose lipopolysaccharide-treated tumor-bearing mice, tumor burden was higher in the lung due to ablation of plasmacytoid dendritic cells that resulted in decreased immunosuppressive environment in the lung. On the other hand, such dendritic cells from the lungs of high-dose lipopolysaccharide-treated tumor-bearing mice facilitated Th1 and cytotoxic cells to arrest the tumor development (Rega et al. 2013).

In another immunological study conducted at the Massachusetts Institute of Technology, by Jin et al., revealed that in contrast to the gut microbiota, the lung microbiota (commensal microbiota) activate lung resident γδ T cells and thus provoke the lung adenocarcinoma-associated inflammation. The lung is exposed to microbial variety through inhalation. In this study, authors studied the interaction between host microbiota and lung cancer development by utilizing a genetically engineered mouse model. The model that mimics the histopathological and genetic features of human lung adenocarcinoma was developed by a Kras point mutation and p53 loss. Findings of this study revealed that GF mice were protected from development of lung cancer induced by p53 loss and Kras mutation. It was observed that local microbiota could promote cancer progression and provoke inflammation

via lung resident γδ T cells. Depleting the microbiota effectively suppressed the lung cancer development (Jin et al. 2019).

14.5.7 Modern Germ-Free Models Provide Insight on Muscle-Skeletal, Mental, and Brain Health

The gut microbiota also influences the skeletal muscle mass and function. Skeletal muscle is important not only for locomotion but also for regulating metabolism. Lahiri et al. studied the interactions between the gut microbiota and skeletal muscle in mice (Lahiri et al. 2019). They identified genes and signaling pathways that regulate skeletal muscle mass and function in response to the gut microbiota. Biochemical functional analysis revealed the microbiota alters the function of neuromuscular junctions. These findings open the door to a better understanding of the role of gut microbes in the mechanisms underlying loss of muscle mass (Lahiri et al. 2019). More recently, Li et al. at Emory University showed that bone formation dependent on the parathyroid hormone requires butyrate production by the gut microbiota (Li et al. 2020). Remarkably, butyrate was found to be required for parathyroid hormone PTH to increase the number of bone marrow Tregs, which in turn stimulated the production of the osteogenic Wnt ligand (Wnt10b) by bone marrow CD8+ T cells, which activated Wnt-dependent bone formation (Li et al. 2020).

GF mice have aided in the understanding of how the gut microbiome is linked to behavior and mental health. Clinically enduring, Tang et al. discovered in an unprecedented study that Toll-like receptor 4 (TLR4) in the vascular endothelium, together with the microbiome, determines the risk of suffering actual morphological lesions in the cerebrum (called "cerebral cavernous malformations" in humans), by studying GF mice (Tang et al. 2017). Cerebral cavernous malformations cause stroke and seizures for which no treatment exists. Such lesions result from the loss of an adaptor complex that downregulates MEKK3-KLF2/4 signaling in brain endothelial cells. Tang et al. discovered for the first time that endothelial TLR4 and the gut microbiome are critical upstream signaling stimulants for the formation of such cavernous lesions. They also showed that TLR4 activation by Gram-negative bacteria or lipopolysaccharide speeds lesion development, while genetic or pharmacologic blockade of TLR4 signaling prevents such lesions in GF animals. Validation in humans led scientists to identify polymorphisms that favor TLR4 gene expression (or that of its co-receptor CD14) in association with higher lesion burden in patients with cerebral cavernous malformations. Of utmost relevance, GF mice were protected from such pathology, and a single dose of antimicrobials altered the susceptibility in animals. These studies are critical to understand and identify strategies for disease treatment (Ridler 2017; Starke et al. 2017; Tang et al. 2017).

14.5.8 Sex-dependent Microbiome-driven Vascular, Immune Cell Biology, and Disease Gender Bias

To further complement the array of possibilities with GF models, studies could be conducted to determine to what extent the genetic sex of any given species, i.e., mice, determine basic mechanisms of functional biology that varies markedly between males and females as a function of age (Edwards et al. 2020; Scott et al. 2020), osteoporosis (Locantore et al. 2020), serotonin (Lyte et al. 2020; Walsh et al. 2020), or how the microbiome positively influence healing (neointimal hyperplasia) after vascular injury (Chen et al. 2020). As an specific example, a study of vascular resistance, recently conducted by Edwards et al. at the University of Toledo, showed that male and female GF mice presented a decrease in contraction of resistance arteries compared to conventional mice, with more pronounced changes in GF males, which have increased vascular stiffness (inferred from leftward shift in the stress–strain vessels curve data) and inward hypotrophic remodelling as a feature of a derived chronic reduction in blood flow (Edwards et al. 2020). Studying oxidative stress, the same investigators showed that the generation of reactive oxygen species (ROS) from bone marrow-derived neutrophils is augmented in GF male mice (Edwards et al. 2020), suggesting that immune cells could have different fundamental microbiome-dependent differences in response to comparable stimuli (Locantore et al. 2020), which could explain in part why numerous diseases have sex-bias in severity and complications as in IBD (Greuter et al. 2020), spondyloarthritis (Rusman et al. 2018), or in more recently relevant COVID-19 (Jin et al. 2020; Palaiodimos et al. 2020).

14.5.9 Single Bacterial Genes Modulate the Intestinal Phenotype in GF Models

Despite the gain in knowledge, these types of studies, where entire microbial communities or samples are used to examine the effect on the host biology, do not reflect the impact of individual bacteria or their interactions with their host. To elucidate such interactions and identify more specific target therapeutics, the use of GF can be uniquely advantageous over in vitro systems based on a few cells functioning ex vivo (e.g., organoids on a chip).

Some of the earlier studies deciphering the role of Wnt signaling and bacterial genes in IBD originated from the studies on *E. coli* and cultured epithelial cells by R. Balfour Sartor, University of North Carolina at Chapel Hill, and Jun Sun, University of Rochester (Liu et al. 2012). As *Wnt2* inhibits enteric bacterial-induced inflammation (IL-8) in intestinal epithelial cells, interestingly the protein AvrA (an anti-inflammatory bacterial molecule) from *E. coli* and Salmonella normalized the expression of *Wnt2* in vivo. In GF, the *E. coli* strain F18 expressing AvrA promoted and changed the distribution of *Wnt2* expression by the intestine (Liu et al.

2012). Subsequent studies have helped decipher the role of vitamins, yersiniabactin and long adhesin A from *E. coli*, and iron on the modulation of intestinal pathogens and fibrosis (Ellermann et al. 2019, 2020; Schmitz et al. 2019; Sun 2018). More recently, gnotobiotic studies, where GF are colonized with single or well-known simple microbial communities, helped to reveal that while *Fusobacterium nucleatum* isolates with FadA and Fap2 adhesins failed to induce inflammation and tumorigenesis, *pks* + *Escherichia coli* promoted tumorigenesis in the *ApcMin/+*; *Il10−/−* model in a colibactin-dependent manner, suggesting that bacterial genes, specifically colibactin, in the apc min model are drivers of carcinogenesis (Tomkovich et al. 2017).

Another example of bacteria genes mediating CRC in preclinical GF models, *C. jejuni*81–176 was shown to increase the tumor burden compared with uninfected GF APC min/+ mice. Mechanistically, *C. jejuni* with a mutated *cdtB* subunit, a cytolethal distending toxin, attenuated the severity of tumors induced by the human-derived strain of *C. jejuni* in vivo (and decreased DNA damage in cell/enteroids in vitro). Out of several upregulated colonic genes, 22 depended on the presence of the c*dtB gene*. The gene mutation also influenced the microbial gene expression (metatranscriptomic) profile and the accompanying (microbiome screened) communities in the infected mice (He et al. 2019). In France, an elegant study showed the complex interaction between the *Listeria monocytogenes* bacteriocin Lmo2776 and the gut microbiome. Of interest, bacteriocin targets the intestinal commensal *Prevotella copri* and modulates intestinal infection and inflammation in mice in a microbiome-independent fashion (Claus 2019; Rolhion et al. 2019).

Clostridioides difficile infections (CDI) in humans, the most frequent and lethal nosocomial gastrointestinal pathogen in hospitals and the community, have cost the life of countless patients affected with debilitating conditions including cancer and immunosuppressive conditions and IBD. Such infections known to be facilitated by the consumption of antimicrobials and gut microbiome disruptions (dysbiosis) have also been studied in GF models. One of the latest studies functionally determined that the GF mice transplanted with fecal samples from patients with dysbiosis at Mayo Clinic showed increased gut amino acid concentrations and greater susceptibility to CDI (Battaglioli et al. 2018). At the same time, a mutant *C. difficile* strain unable to use proline as energy source failed to colonize mice regardless the dysbiosis state of the patients studied. Diets low in proline and protein prevented CDI, suggesting that amino acids are driven by the microbiome and essential for CDI. Because diet can promote dysbiosis depending on the genetic background and disease susceptibility of the host (Rodriguez-Palacios et al. 2018a, b), because genetics determine the pattern of structural damage in the gut (Rodriguez-Palacios et al. 2015a, b), because foods may harbor toxigenic *C. difficile* (Hoover and Rodriguez-Palacios 2013; Rodriguez-Palacios et al. 2006, 2007, 2009, 2013, 2020a; Rodriguez-Palacios and Lejeune 2011), because diet may influence the colonization of certain commensals, including *Enterococcus faecalis* which may inhibit other gut microbes (Rodriguez-Palacios et al. 2018a, b), studies in GF models are now important to further identify risk factors linked to microbiome dysbiosis and CDI among the most susceptible individuals (Battaglioli et al. 2018).

14.5.10 Human Enteroviral Infections Induce Microbiome Changes in Humanized GF Models

Thus far, the studies mentioned have been conducted in laboratory rodents; however, the relevance of other gnotobiotic systems and also pressurized housing systems like GF calves and piglets has provided tremendous insights into the biology of human viral infections, including norovirus and other enteroviruses like rotavirus, which may be difficult to culture in the laboratory. Important examples include Linda J Saif's group at the Ohio State University (Vlasova et al. 2019; Ward et al. 1996; Yuan et al. 1996) and Lijuan Yuan at the Virginia-Maryland College of Veterinary Medicine (Lei et al. 2019; Liu et al. 2013; Yuan et al. 2017). Earlier studies, for instance, demonstrated that probiotic LGG mono-association modifies and suppresses virus-induced autophagy in the ileum of gnotobiotic pigs (Liu et al. 2013; Wu et al. 2013). In a recent study, 16S rRNA gene sequencing demonstrated that human norovirus infection (genotype GII.4) is markedly altered by the intestinal human microbiota in GF pigs. Enhanced viral infection was observed due to the presence of the human gut microbiota, at the same time that the infection altered the gut microbiota. Alterations occurred at the phylum level for *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* and at the genus level for *Bifidobacterium*, *Enterococcus*, *Clostridium*, *Anaerococcus*, *Ruminococcus*, *Lactobacillus*, and *Bacteroides* (Lei et al. 2019).

14.5.11 GF Animals as Models to Study the Biology and Filtration Materials Against COVID-19

With the recent emergence of the COVID-19 respiratory pandemic, numerous in vitro studies were either cited from past efforts or reformulated to determine to what extent materials proposed for medical or nonmedical masks were effective at controlling the dispersion of aerosolized particles or liquid microdroplets. As a more functional experimental based test, in vivo, we proposed the use of germ-free mice, and the principles of multilayer filtration to support the in vivo testing and biological study of microorganisms dispersed in microdroplets to help understand and communicate the ability of two-layer filtration systems to protect the environment and mice from bacteria carried in microdroplets (Rodriguez-Palacios et al. 2020b, c). The study consisted in covering GF Nestiso cages with GF mice sprayed with and without filtration materials and determine if animals were colonized over time under various conditions. The study showed remarkably 100% protection by a textile material suitable for community-based mask fabrication.

14.6 Historic Evolution of Germ-Free Housing Technologies

Today, all available housing options to raise GF mice continue to use the same pressurized technologies that were pioneered in the 1940s (Smith 1949; Vowles et al. 2015), which have been extensively documented since the 1960s (Heneghan 1973; Luckey 1959, 1963; Luckey et al. 1955).

As recently illustrated by Betty Theriault at the University of Chicago at the 2019 Annual Midwest DDRCC Alliance Meeting in Chicago (with permission), in the 1960s, the interest in re-deriving mice as GF using flexible film isolator technology was critical to producing new mouse colonies absent of adventitious pathogens prevalent at the time. Newly re-derived mouse colonies produced by commercial vendors were then inaugurated as SPF (specific pathogen-free) colonies thanks to the colonization of GF animals with the so-called Schaedler flora and later "altered Schaedler flora." The latter flora is a defined community of eight bacterial species believed to be fully commensal (four oxygen-sensitive (EOS) *Fusobacterium* species, two Lactobacilli, one spiral bacteria of the *Flexistipes* genus, and one *Parabacteroides; AYGZ00000000.1, AQFT00000000.1, AYJP00000000.1, AQFU00000000.1, AQFV00000000.1*). An additional advantage to the invention of plastic and design of the flexible film isolator was that it allowed the production of flexible film isolator prototypes. These proved to be less cost prohibitive for purchase and operation than its stainless steel predecessors. Reduction in equipment and operational costs as well as the sharing of flexible film isolator technology within the laboratory animal community facilitated the creation and implementation of GF units in new academic centers. Concurrently, molded plastic became available which together with polyester Reemay filters for passive ventilation of plastic shoe-size boxes improved mouse housing toward the mid-1990s.

To further protect animals from adventitious pathogens, housing based on ventilated cages, with docking systems providing HEPA-filtered ventilated air, became common in the late 1990s and early 2000s. The combination of barrier practices, ventilated housing, and the use of biosafety cabinets helped to control the dissemination of diseases. While genetically engineered mouse models with specific pathogen-free status accelerated in usage, the usage of gnotobiotic and GF mouse models declined. Toward the mid-2000s, only a few commercial GF centers remained, owing to the low demand of GF animals. With the emergence of microbiome science, the GF and gnotobiotic mouse model interest resurged. Initially, flexible film isolator housing was used; however, to accommodate increased demand on space and resources, hermetic-sealed, positive pressure-ventilated caging systems began to be marketed and used for GF housing.

14.7 Portable Emerging Non-pressurized Housing GF Technology

As a very competitive solution to traditional pressurized isolation systems (Fontaine et al. 2015; Vowles et al. 2015), which are anchored to electrical outlets and backup generator systems (in the event of power failure), a new strategy has been proposed and validated (Rodriguez-Palacios et al. 2018a, b) (Fig. 14.5). Referred to as "double cages" initially but formally on its first publication as Nested Isolation (NesTiso), this system of GF housing is based on nonelectric passive air filtration for efficient cage ventilation and effectiveness and solves several of the problems encountered with multi-cage or pressurized isolators. Using an innovative design based on the "chimney effect," where hot air around the animals floats and moves upward, Case Western Reserve University is prototyping a highly functional version of the patent-protected housing system for GF animals. As an advantage to existing systems, NesTiso complements the electricity-dependent, HEPA-filtered ventilation systems available today. This environmentally friendly, green technology also reduces cost of energy and maintenance of electrical equipment.

As a differentiation engineering advancement, such recent technology, Nested Isolation, has helped scientists at Case Western Reserve University to conduct recently published GF and Fecal matter transplantation experiments in Garious germ-free models (Basson et al. 2019a, b; Menghini et al. 2019). Supported with more than 150,000 mouse days' worth of published data (Rodriguez-Palacios et al. 2018a, b), there are ongoing efforts to make such a portable technology accessible to a broader range of scientists, including those that have had no previous access to GF research. The ability to mass-produce such a caging GF system will enable large numbers of scientists to breed and use GF animals. Although testing and validation would be required for every particular scenario and isolation facility and regulations,

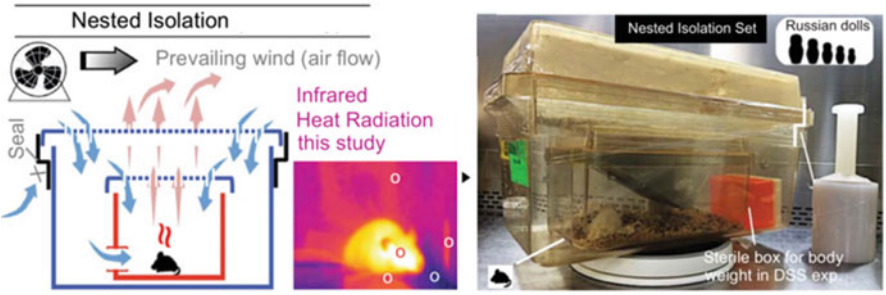

Fig. 14.5 New germ-free housing system based on multilayer passive filtration and Nested Isolation ("NesTiso"). With the simple principle of one cage nested inside a larger cage functioning as a "bubble barrier," this housing system is portable and scalable and allows the rearing/maintenance of mice GF without the need of pressurized ventilation. Commercial prototyping and validation studies are patent-pending and under way at Rodriguez-P. laboratory, Case Western Reserve University. Images unmodified from Rodriguez-Palacios et al. (2018a, b), Creative Commons Attribution 4.0 International License http://creativecommons.org/licenses/by/4.0/

the use of the portable system could also enable the study of BSL-3 pathogens under the same room as is not permissible under modern biosafety and biosecurity regulations, because this multilayer isolation system prevents the movement of microbes in both directions, in and out of the compartment housing the animal host or the microbe of interest.

14.8 Conclusion

In conclusion, the above investigations support the notion that GF animals and specifically GF mice played an essential role in understanding the molecular mechanisms of cancer development and the cancer response to immunotherapy. With the increase of publications indexed in the Public Library of Medicine and the need to understand with precision the role of the microbes in human disease, it is expected to see a reemergence of GF facilities throughout the globe to help scientists better characterize host-pathogen interactions. Despite the progress, most studies have used C56BL7 GF-derived mice. With the expansion of GF system technologies and the portability of passive ventilation strategies, it is expected that more genes or genetic line models will be used in the future. With over 400 papers publish in "germ-free" in 2019, the present chapter only seeks to highlight a sample of several notable studies to centralize the listing of an array of experimental possibilities that can be explored using GF biology to advance our understanding of human, animal, and even plant diseases, given that microbes are adapted to the gut environment and the diet we consumed which is in great proportion plant matter.

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Chapter 15
Machine Learning in Identification of Disease-Associated Microbiota

Derek Reiman, Ulises Sosa, and Yang Dai

Abstract Metagenomic studies of the microbiome community have revealed associations of the microbiome community to host disease state. The detection of these associations can rely on statistical analyses identifying differentially abundant taxa between diseased and healthy populations. Accurate prediction of the host phenotype from a metagenomic sample and identification of the associated microbial markers are important in understanding potential host-microbiome interactions related to disease initiation and progression. However, associations of individual microbes to a particular disease have shown contradictory results in past studies, possibly due to dynamic and complex natures of different microbes. To handle the complex nature of the microbiome, machine learning methods have begun being employed. Machine learning algorithms are a set of methods in which a model learns intrinsic patterns in data and use them to predict labels of data. In this chapter, we introduce the commonly used machine learning methods in metagenomic studies. We show readers how to use the currently available tools found in Python libraries. Our purpose is to demonstrate the proper training and analysis of machine learning models for microbiome researchers, who may not have experience in machine learning or Python programming.

Keywords Microbiome · Machine learning · Python · Jupyter

15.1 Introduction

Machine learning (ML) models have become increasingly applied to modeling the human microbiome for identification of microbial biomarkers and assisting the diagnosis of many diseases (Knights et al. 2011; LaPierre et al. 2019; Pasolli et al. 2016; Vangay et al. 2019). The access to many ML tools has made possible for

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researchers to explore the potential of these models. However, training and testing a ML model require following the adequate protocol, an aspect that may not be familiar to researchers without data science background. In this chapter, we will use open-source tools to obtain the predictive models in a step-by-step fashion for microbiome researchers. Jupyter notebook is an open-source service for interactive computing and mark down documentation using Python (https://jupyter.org/). It can be installed through a conda environment manager such as Anaconda (https://www.anaconda.com/). We will then employ Python's *scikit-learn* package in order to build and train machine learning models (Pedregosa et al. 2011).

15.2 Materials

15.2.1 Software

The Anaconda environment manager can be installed following the instruction at the Anaconda website. We recommend the use of the Python 3 version for this analysis. We will use Anaconda to set up an environment that we will work out of, and we will name it *tutorial*. We will then install Jupyter Notebook and the *scikit-learn* package through conda. Conda is an open-source package and environment management system that runs on Windows, macOS, and Linux operating systems. It quickly installs, runs, and updates packages and their dependencies. In addition, we will also install the *pandas* library which provides the functionality of constructing data frame structures in Python (McKinney 2011).

```
$ conda create --name tutorial
Collecting package metadata (repodata.json): done
Solving environment: done

## Package Plan ##

 environment location: /anaconda3/envs/tutorial

Proceed ([y]/n)? y

Preparing transaction: done
Verifying transaction: done
Executing transaction: done
#
# To activate this environment, use
#
# $ conda activate tutorial
#
```

(continued)

```
# To deactivate an active environment, use
#
# $ conda deactivate

$ conda activate tutorial
(tutorial) $ conda install jupyter scikit-learn pandas
Collecting package metadata (repodata.json): done
Solving environment: done

## Package Plan ##

 environment location: /anaconda3/envs/tutorial

 added / updated specs:
 - jupyter
 - pandas
 - scikit-learn
```

Next, we use the terminal to navigate to the folder where the dataset of interest is located and start a local Jupyter Notebook server using the command *jupyter notebook*. Your browser should open a new tab showing the Jupyter layout.

```
(tutorial) Springer Book Chapter $ jupyter notebook
[I 19:40:57.406 NotebookApp] The Jupyter Notebook is running at:
[I 19:40:57.406 NotebookApp] http://localhost:8888/?
token=22f02f588ad3a4cf65ce44d382f1555c631c81e96397dda8
[I 19:40:57.406 NotebookApp] or http://127.0.0.1:8888/?
token=22f02f588ad3a4cf65ce44d382f1555c631c81e96397dda8
[I 19:40:57.407 NotebookApp] Use Control-C to stop this server and
shut down all kernels (twice to skip confirmation).
[C 19:40:57.423 NotebookApp]

 To access the notebook, open this file in a browser:
 file:///Users/derek.reiman/Library/Jupyter/runtime/nbserver-
84962-open.html
 Or copy and paste one of these URLs:
 http://localhost:8889/?
token=22f02f588ad3a4cf65ce44d382f1555c631c81e96397dda8
 or http://127.0.0.1:8889/?
token=22f02f588ad3a4cf65ce44d382f1555c631c81e96397dda8
```

From the Jupyter layout, we can create a new notebook for our analysis by selecting from the top right of the window New → Python 3 Notebook. This will open a new notebook file from which we will perform our analyses.

15.2.2 Datasets

For our analyses, we will focus on a dataset of 57 patients with invasive breast cancer and 21 healthy patients from Wang et al. (2017). The microbiome samples were taken from three separate sites: breast tissue, oral cavity, and urine. For each site, 16S rRNA genes were amplified and sequenced. The sequencing data were processed using the QIIME pipeline (Bolyen et al. 2019; Caporaso et al. 2010).

To run the tutorials, each dataset should contain two files: an abundance table and metadata. In our analyses, we will structure the data in tab-delimited files such that each row in the abundance table represents an operational taxonomic unit (OTU) or microbial feature and each column is a sample. For simplicity, our metadata will just contain the disease status of the patient that the microbiome sample came from, whereas the order of the rows in this file matches the order of the columns of the abundance table with respect to the samples. The datasets were obtained from Wan et al. (2017). This includes 67, 269, and 135 OTUs in breast tissue, oral cavity, and urine, respectively (Table 15.1).

Each of the three datasets is treated as a dataset with binary classes. In the following tutorials, we will consider patients with cancer to be of class 1 and healthy patients to be of class 0. We can do this by checking if a value in the list is equal to "Cancer Patient," resulting in a value of *True*. All other values, which here would be "Healthy Patient," result in *False*. We then cast them as integers, converting *True* values to 1 and *False* values to 0 by typing:

```
breast_labels = (beast_labels == "Cancer Patient").astype(int)

oral_labels = (oral_labels == "Cancer Patient").astype(int)

urine_labels = (urine_labels == "Cancer Patient").astype(int)
```

Table 15.1 Abundance table and metadata

(tutorial) BC_Oral $ head -n 2 abundance.tsv

Other genus (class Actinobacteria)	0.0	4.23e-06	4.49e-05	0.0	0.0	0.0	1.49e-05	3.98e-05	0.0	0.0	
0.0	0.0	0.0	0.0	5.39e-06	0.0	0.0	0.0	7.67e-06	0.0	3.66e-05	0.0
0.0	0.0	0.0	2.02e-05	0.0	0.0	5.62e-06	0.000622996		0.0	0.0	0.0
0.0	0.0	2.09e-05	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.0	0.0	0.0	9.08e-06	3.71e-05	0.0	0.0	0.0	0.0	0.0	2.54e-05	6e-06
0.0	1.7800000000000002e-05		0.0	0.0	0.0	0.0	0.0	7.18e-06	0.0	0.0	0.0
0.000195981	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		
Other genus (order Actinomycetales)		0.0	4.23e-06	0.0	1.21e-05	5.87e-06	0.0	0.0	4.42e-06	0.0	
0.0	0.0	0.0	0.0	0.0	0.0	5.94e-06	0.0	1.28e-05	0.0	0.0	5.23e-
06	0.0	6.29e-06	0.0	0.0	0.0	0.0	8.950000000000001e-05		0.0	0.0	6.99e-
06	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.07e-06	0.0	0.0
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.0	1.2e-05	0.0	0.0	7.87e-06	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.0	0.0	4.67e-06	0.0	0.0	0.0	0.0	0.0	0.0			

(tutorial) BC_Oral $ head labels.txt

Cancer Patient
Cancer Patient
Cancer Patient
Cancer Patient
Cancer Patient
Cancer Patient
Cancer Patient
Cancer Patient
Cancer Patient
Cancer Patient

15.3 Methods

15.3.1 Data Import

In this tutorial, we will use Python's *pandas* library to import our data files. To use the library's functionality, first import it using the *import* command. The abbreviation *pd* is set as the reference to call the library's functions. Then we can import our data using the *read_csv* function. This function requires that the first parameter is the filename. In addition, we can specify that the file is tab-delimited with *sep="\t"* and that the first column should be our data frame index using *index_col=0*, and since these files have no headers, we use *header=None* as the following:

```
import pandas as pd
breast_abundance = pd.read_csv("BC_Tissue/abundance.tsv", sep = "\t",
index_col = 0, header = None)
breast_labels = pd.read_csv("BC_Tissue/labels.txt", sep = "\t",
header = None)
oral_abundance = pd.read_csv("BC_Oral/abundance.tsv", sep = "\t" ,
index_col = 0, header = None)
urine_abundance = pd.read_csv("BC_Urine/abundance.tsv", index_col =
0, sep = "\t", header = None)
urine_labels = pd.read_csv("BC_Urine/labels.txt", sep = "\t", header =
None)
breast_abundance.head(5)
```

	1	2	3	4	5	6	7	8	9	10	...	54	55	56	57	58	
Actinomyces	0.000000	0.000000	0.00813	0.0	0.000000	0.008403	0.0	0.0	0.000000	0.012195	...	0.047181	0.012821	0.010526	0.010204	0.006211	0.00
Corynebacterium	0.013196	0.000000	0.00813	0.0	0.014493	0.000000	0.0	0.0	0.000000	0.012195	...	0.009206	0.003205	0.052632	0.061224	0.006211	0.00
Micrococcus	0.000000	0.000000	0.000000	0.0	0.000000	0.000000	0.0	0.0	0.025210	0.000000	...	0.000000	0.000000	0.000000	0.000000	0.000000	0.00
Rothia	0.000000	0.000000	0.000000	0.0	0.000000	0.000000	0.0	0.0	0.008403	0.36585	...	0.018412	0.006410	0.000000	0.000000	0.000000	0.00
Mycobacterium	0.000000	0.061728	0.000000	0.0	0.000000	0.016807	0.0	0.0	0.000000	0.000000	...	0.000000	0.000000	0.000000	0.000000	0.000000	0.00

5 rows × 63 columns

15.3.2 Data Preprocessing

Before training machine learning models, it is important to preprocess the data. This includes removing features (OTUs in this case) that are rarely found in samples as well as making sure that the features are of similar scale. We will do this using the *preprocessing* module of the *scikit-learn* library in two steps.

```
import numpy as np
from sklearn.preprocessing import StandardScaler

# Filter rare OTUs
breast_num_samples = len(breast_abundance.columns) # Number of
Samples

breast_otu_num_pos = (breast_abundance > 0).astype(int).sum(axis = 1)
# Number of non-zeros in each OTU

breast_otu_prop_pos = breast_otu_num_pos/breast_num_samples
#Proportion of non-zeros in each OTU

filt_breast_abundance = breast_abundance.loc[breast_otu_prop_pos >
0.1] # Keep all OTUs present in over 10%

# Log Normalize Data
log_breast_abundance = np.log2(filt_breast_abundance +1) #Log2
transform data
norm_breast_abundance = StandardScaler().fit_transform
(log_breast_abundance.transpose()) # Z-Norm transformed data
# Restructure Data Frame
norm_breast_abundance = pd.DataFrame(data = norm_breast_abundance,
columns = log_breast_abundance.index.values)

norm_breast_abundance.head(5)
```

Step 1: We remove the features that are only found in at least certain percent of the samples

Here we choose 10% as the threshold. In order to do this, we first determine the number of samples in the dataset by calculating length (*len* function) of the list of columns in the abundance table. Since the columns represent the samples, this gives the total number of samples. Next, we count the number of nonzero values in each feature. To do this, we set all positive values to 1 and all zero values to 0 using *(breast_abundance>0).astype(int)* and then add the values in each row by

	Actinomyces	*Corynebacterium*	*Micrococcus*	*Rothia*	*Mycobacterium*	*Propoionibacterium*	*Yaniella*	*Bifidobacterium*	Unknown genus (family Coriobacteriaceae)	*Atopobium*	...
0	−0.537039	−0.208871	−0.300905	−0.336011	−0.407567	0.537855	−0.278197	−0.400898	−0.440463	−0.439117	...
1	−0.537039	0.352635	−0.300905	−0.336011	2.259231	0.296964	−0.278197	−0.422898	−0.440463	−0.439117	...
2	0.129606	−0.263842	−0.300905	−0.336011	−0.407567	−0.411375	−0.278197	−0.422898	−0.440463	−0.439117	...
3	−0.537039	−0.352625	−0.300905	−0.336011	−0.407567	0.396549	−0.278197	−0.422898	−0.440463	−0.439117	...
4	−0.537039	−0.194850	−0.300905	−0.336011	−0.407567	−0.207478	2.121494	−0.422898	−0.440463	−0.439117	...

5 rows × 63 columns

appending *sum(axis=1)*, resulting in the number of positive values in each row or feature. Then, we divide these values by the total number of samples to get the proportion of positive values. Lastly, we only keep the rows in which the proportion of positive values is greater than 0.1 using *breast_abundance.loc [breast_otu_prop_pos>0.1]*.

Step 2: First we log-normalize the data by adding 1 to every value and then taking the $\log_2$ transformation

The value 1 is added as a pseudocount. Then, we use the *StandardScaler* from *scikit-learn* to normalize all features with 0 mean and unit variance. Notice that we transpose the data before the normalization. This is because the scaler will normalize each column across rows.

We will keep this orientation for the data since the following machine learning methods will require that each row represents a sample and each column represents a feature. Lastly, we reconstruct a data frame using the normalized data, specifying that the names of the columns are the OTU features from the previous data frame. In the examples below, we use the breast tissue dataset to demonstrate the procedure of training and testing.

15.3.3 Random Forest

Random forest (RF) is ML models based on learning an ensemble of decision trees (Breiman 2001; Tin Kam Ho 1998). Given a set of samples = $\{x_1, x_2, \ldots, x_n\}$ with k classes, the model trains a set of decision trees and takes the average of the trees to give a single robust decision tree. Each tree is trained using a bootstrapped subset of the training data. While growing each tree, a decision rule is made at each node by selecting the best feature from a random subset of features that best splits the data into two subsets. Decision rules are evaluated using entropy or the Gini impurity metric. In our tutorial, we will use the Gini impurity for making decisions. For a set of samples with k classes, let p_i be the proportion of samples of class i for $i \in \{1 \ldots k\}$. The Gini impurity of the set is calculated as

$$I_G(p) = 1 - \sum_{i=1}^{k} p_i^2 \tag{15.1}$$

Once a RF model is trained, features can then be evaluated using the mean decrease impurity. For each node, an importance score for the feature being split upon is calculated as the decrease in the Gini impurity from before and after the split

weighted by the proportion of total samples that were split. A feature's overall importance is then calculated by averaging the weighted scores of that feature over all the decision trees in the ensemble by typing:

```
from sklearn.ensemble import RandomForestClassifier
clf = RandomForestClassifier(n_estimator = 500)
clf.fit = ( norm_breast_abundance, breast_labels)
feature_scores = pd.DataFrame(data =clf.feature_importance_, index =
norm_breast_abundance.columns.values, columns = [" Scores"])
```

	Score
Corynebacterium	0.072384
Methylobacterium	0.046329
Unknown genus (family Alcaligenaceae)	0.045717
Atopobioum	0.040944
Lactobacillus	0.039113
Prevotella	0.030204
Unknown genus (family Enterobacteriaceae)	0.029979
Actinomyces	0.028474
Lysinibacillus	0.027738
Peptoniphilus	0.027178

We train the RF model using the *RandomForestClassifier* in the *ensemble* module of *scikit-learn*. In this example, we will set the number of decision trees (*n_estimators*) in the ensemble to be 500. We then fit the model using the *fit* function, passing it both the dataset and the class labels. Once the model is trained, we obtain the feature scores with *featrure_importances_* and collect them into a data frame for easy viewing. These scores represent how well a feature can separate the data. However, the RF model does not tell us which class a feature is more important in.

Another characteristic of the RF models that we will look at is to determine how generalizable the model is. Since each tree is trained with a bootstrapped set of the training data, we will have a subset of samples that were not used for building the tree. These samples are called the out-of-bag (OOB) samples, and they can be used to evaluate the accuracy of their respective tree, giving an OOB score. By adding the parameter *oob_score=True* to our *RandomForestClassifier*, we tell our model to keep track of this score for each decision tree, and we can retrieve the average OOB score after training our classifier with *oob_score_*. This score tells us how well each decision trees predicted its OOB samples and can give us a sense of how generalizable the model is. This is achieved by typing:

```
clf = RandomForestClassifier(n_estimator = 500, oob_score = True)

clf.fit(norm_breast_abundance, breast_labels)

print("OOB Accuracy: %.3f" % clf.oob_score_)

OOB Accuracy: 0.635
```

15.3.4 Support Vector Machine

Support vector machines (SVMs) are ML models by learning the best hyperplane separating data into classes (Cortes and Vapnik 1995). The orientation and position of this hyperplane are determined by a subset of data points, called support vectors, which lie close to the hyperplane. The hyperplane will be determined by a set of weights (w) and an intercept (b) through model training. The class of a microbiome sample x_i represented by m features can then be predicted as

$$\widehat{y}_i = \text{sign}\left(w^T x_i + b\right) \tag{15.2}$$

Note that each weight w_j represents the importance of a feature j in determining the class label.

In this tutorial, we will use the linear kernel, which considers the distance between two points as the inner product, $K(x_i, x_j) = \langle x_i, x_j \rangle = {x_i}^T x_j$. However, SVMs can also use nonlinear kernels to separate data (Hastie et al. 2009). We restrict this tutorial to the linear kernel since nonlinear kernels are less interpretable by typing:

```
from sklearn.svm imort SVC
from sklearn.model_selection imort GridSearchCV
parameters = { 'kernel' : ['linear'] , 'C' : [0.01, 0.1, 1, 10, 100]}
clf = GridSearchCV(SVC() , parameters, cv = 5 )
clf.fit(norm_breast_abundance, breast_labels)
feature_scores = pd.DataFrame(data = clf.best_estimator_.coef_.
reshape(-1) , index = norm_breast_abundance.colums.values, columns =
[" Scores"])
```

	Score		Score
Unknown genus (family Alcaligenaceae)	0.103387	*Corynebacterium*	−0.163195
Sphingomonas	0.099258	*Atopobium*	−0.142976
Stenotrophomonas	0.089988	*Acinetobacter*	−0.099129

(continued)

	Score		Score
Lactobacillus	0.084507	*Actinomyces*	−0.078393
Pesudomonas	0.083216	Unknown genus (class TM7-3)	−0.074532
Unknown genus (family Enterobacteriaceae)	0.081090	*Catonella*	−0.073821
Peptoniphilus	0.077492	*Ochrobactrum*	−0.072328
Unknown genus (family Oxalobacteraceae)	0.076875	*Sporocarcinia*	−0.070317
Burkholderia	0.074705	*Rhizobium*	−0.058156
Unknown genus (order Streptophyta)	0.063486	*Veillonela*	−0.053397

We train the SVM model using *SVC* from the *svm* module of *scikit-learn*. Because SVM models can be trained with different kernels, where each kernel has different sets of hyper-parameters, we use a grid search strategy to find the best values for the hyper-parameters. In the linear kernel, there is one hyper-parameter *C*, which helps regularize (i.e., control the size of) the model by balancing the tasks of maximizing the margin and minimizing the misclassification rate. For each selected value of *C*, we train the model using the *fit* function on both the dataset and the class labels in a cross-validated fashion. This means that we split the data into partitions of equal size and use all but one partition to train a model. We then use the left-out partition to evaluate the model. This is repeated such that each partition is held out once, resulting in multiple models. In our example we split the data into five partitions by specifying $cv = 5$. The default evaluation of scoring for *SVC* is accuracy; however, different metrics can be specified using the *score* parameter when constructing the *SVC* object. The best hyper-parameters are chosen based on the average score over the cross-validated models over the set of hyper-parameters. The best set of hyper-parameters is then used on the full set of data to train the final SVM model.

Once the full model is trained, we obtain the best model using *best_estimator_* and then extract the weights *w* from that model with *coef_*, collecting them into a data frame for easy viewing. Using these weights, we can look at how impactful each OTU feature was in the prediction. The OTUs with the more negative weights are more predictive of the negative class (here healthy patients). Likewise, the OTUs with the more positive weights are more predictive of the positive class (here cancer patients).

15.3.5 Logistic Regression

Logistic regression is a ML model that uses a logistic function to model a binary-dependent variable (Sperandei 2014). Given a set of samples $X = \{x_1, x_2, \ldots, x_n\}$, a logistic regression model predicts the class of a sample by using a threshold value (e.g., 0.5) on the value

$$\widehat{y}_i = \frac{1}{1 + e^{-(\beta_0 + \beta x_i)}} \tag{15.3}$$

Here β_0 is a bias value, and β represents the vector of weights to be multiplied by the vector of features. During training, we can penalize these weights in order to regularize the model, helping to prevent overfitting. The two most common forms of regularization are the L_1 and L_2 regularizations,

$$L_1(\beta) = \sum_j |\beta_j| \tag{15.4}$$

$$L_2(\beta) = \sum_j \beta_j^2 \tag{15.5}$$

The L_1 regularization technique will penalize the weights in such a way that many weights will become 0, effectively removing the respective feature from the predictive model. On the other hand, the L_2 regularization technique will penalize the weights in order to prevent any large weights, which could lead to unstable predictions. These regularizations are used in other linear models as well. Least absolute shrinkage and selection operator (LASSO) regression models use least squares regression in conjunction with L_1 regularization (Tibshirani 1996). Additionally, ridge regression is a least squares regression model that uses L_2 regularization (Hoerl and Kennard 1970). We type:

```
from sklearn.linear_model import LogisticRegression
from sklearn.model_selection import GridSearchCV
parameters = {'C' : [0.01, 0.1, 1, 10, 100]}
clf = GridSearchCV(LogisticRegression(penalty = "l2"), parameters, cv
= 5)
clf.fit(norm_breast_abundance, breast_labels)
feature_scores = pd.DataFrame( data = clf.best_estimator_.coef_.
reshape(-1), index = norm_breast_abundance.columns.values, columns =
["Score"])
```

	Score		Score
Pseudomonas	0.822503	*Corynebacterium*	−0.751704
Unknown genus (family Alcaligenaceae)	0.818542	*Atopobium*	−0.732263
Unknown genuse (family Oxalobacteraceae)	0.788776	*Ochrobactrum*	−0.661831
Other genus (family Phyllobacteriaceae)	0.608472	*Rhizobium*	−0.660642
Dialister	0.580910	*Bacillus*	−0.522599
Stenotrophomonas	0.576248	*Actinomyces*	−0.507828
Other genus (family Bacillaceae)	0.565256	*Acinetobacter*	−0.435300
Unknown genus (family Enterobacteriaceae)	0.538462	*Catonella*	−0.382062
Capnocytophaga	0.484344	*Lysinibacillus*	−0.336528
Peptoniphilus	0.453498	*Sporocarcinia*	−0.252696

We train the logistic regression model using *LogisticRegression* from the *linear_model* module of *scikit-learn*. Similar to training an SVM model, logistic regression has a hyper-parameter (C) that needs to be tuned. We create a list of potential C values stored in a variable, *parameters.* The *GridSearchCV* object will perform model selection using during *fit* and return the model using the best value. More specifically, it will perform cross-validation similar to the hyper-parameter selection in SVM models. The default value for cross-validation is fivefold cross-validation, meaning that the data is partitioned into five sets. Each value of C is evaluated using the average accuracy of the five cross-validated models and the C with the best score is used to fit the entire dataset. In addition, we specify the use of L_2 regularization with *penalty="l2"*; however, you could pass the value *"l1"* here to use L_1 regularization.

In order to extract the features from the logistic regression model, we consider positive weights to be applied to OTUs predictive of the positive class and negative weights to be applied to OTUs predictive of the negative class (cancer patients and healthy patients respectively). Similar to the case for SVM, we obtain the best model using *best_estimator_* and then extract the weights with *coef_*.

15.3.6 Multi-layer Perceptron Neural Network

The value of a perceptron is a linear combination of the values from the previous layer that is then passed to a nonlinear activation function (Dreyfus 1990; Haykin 1994). This allows neural network models to uncover nonlinear relationships within data. More explicitly, the values of the lth hidden layer h_l is calculated as

$$h_l = \Psi\left(W_l^T h_{l-1} + b_l\right) \tag{15.6}$$

Here h_{l_1} are the values from the previous hidden layer. W_l are the weights connecting h_{l_1} to h_l and b_l is a bias value. Lastly, Ψ is a nonlinear activation function applied to the perceptrons. This nonlinear transformation can be applied over multiple hidden layers.

We train the neural network using the *MLPClassifier* from the *neural_network* module of *scikit-learn.* We construct a model containing a single hidden layer of 100 perceptrons (*hidden_layer_sizes*). In addition, we choose to use the limited-memory BFGS (L-BFGS) solver since we are working with a smaller dataset. In application, neural networks contain many hyper-parameters that should be tuned, including number of hidden layers, hidden layer sizes, activation functions, learning rate, and regularization penalties. This leads to an enormous amount of combinations for hyper-parameters, and there are various methods designed to handle this optimization (Bergstra et al. 2011; Olden et al. 2004). However, we will not be discussing that in this tutorial. Instead, we use the default settings and fit the model, giving a model with a single hidden layer of 100 nodes and one output node. The sigmoid activation is applied to this output node to squish all numbers between 0 and 1 by typing:

```
from sklearn.neural_network import MLPClassifier
clf = MLPCassifier(hidden_layer_sizes = (100, ), solver = "lbfgs")
clf.fit (norm_breast_abundance, breast_labels)

scores = np.matmul(clf.coefs_[0], clf.coefs_[1] )

feature_scores = pd.DataFrame(data = scores.reshape(-1), index =
norm_breast_abundance.columns.values, columns = ["Scores"])
```

	Score		Score
Unknown genus (family Oxalobacteraceae)	5.073471	*Corynebacterium*	−4.284834
Unknown genus (family Alcaligenaceae)	3.798394	*Atopobium*	−3.745294
Other genus (family Phyllobacteriaceae)	3.603175	*Ochrobactrum*	−3.681576
Pseudomonas	3.546305	*Acinetobacter*	−2.938242
Peptoniphilus	2.842468	*Rhizobiyum*	−2.731692
Porphyromonas	2.820974	*Lysinibacillus*	−2.072285
Unkonwn genus (family Enterobacteriaceae)	2.641882	*Catonella*	−1.982853
Sphingomonas	2.518721	*Actinomyces*	−1.644902
Lactopbacillus	2.227284	*Bacillus*	−1.520352
Capnocytophaga	2.046131	*Sporocarcinia*	−1.454132

We then evaluate each feature by looking at its cumulative impact on prediction. This is done by multiplying the weight matrices of the first and last layer, resulting in a vector of scores with the same length as the number of features (Olden et al. 2004). We consider that positive values increase the value of the output node toward 1 (cancer patients) and negative values decrease the value of the output node toward 0 (healthy patients).

15.3.7 Model Evaluation

When training machine learning models, it is important to evaluate how robust the model is. That is, we want to make sure that the model is not overfitting the data. We can do this by evaluating our model in a cross-validated manner. Specifically, we partition the data into multiple sets, training the model on all but one of the partitions and then testing the model on the held-out partition.

To train the machine learning models in a cross-validated fashion, we will use *StartifiedKFold* from the *model_selection* module of *scikit-learn*. This will partition our data stratified by class in order to keep the class proportions similar in each partition. This will require us to specify the number of splits to partition the data into (*n_splits*), and in this case we choose a value of 5 in order to perform fivefold cross-validation. Before training, we will also set up a data frame in order to store evaluation metrics. Here, we will evaluate models based on the area under the receiver operating characteristic curve (AUC-ROC). The ROC curve is a way of

measuring a model's diagnostic capabilities through plotting the true-positive rate (TPR) against the false-positive rate (FPR) at various thresholds where

$$\text{TPR} = \frac{TP}{P} \tag{15.7}$$

$$\text{FPR} = \frac{FP}{N} \tag{15.8}$$

Here TP represents the number of the positive samples that were correctly predicted, P represents the total number of true-positive samples, FP represents the number of the samples that were incorrectly predicted as positive, and N represents the number of the negative samples. In addition, we evaluate models using recall, precision, and $F1$ score. These metrics are defined as

$$\text{Recall} = \text{TPR} = \frac{TP}{P} \tag{15.9}$$

$$\text{Precision} = \frac{TP}{TP + FP} \tag{15.10}$$

$$F1\ \text{Score} = 2 * \frac{\text{Recall} * \text{Precision}}{\text{Recall} + \text{Precision}} \tag{15.11}$$

We also construct lists to store the true-positive rates for different machine learning models, which will be used for visualizing AUC-ROC plots later in order to help view the difference in model performance. We type:

```
from sklearn.model_selection import StratifiedKFold
from sklearn.metrics imports roc_auc_scores, f1_score,
precision_score, recall_score, roc_curve
from scipy import interp

# Data frame to store evaluation metrics
metrics = pd.DataFrame(index = "AUC", "Precision", "Recall", "F1"],
columns = ["RF", "SVM", "Logistics", "MLPNN"]).fillna(0.0)

# Model parameter search space for SVM and Logistics Regression
svm_parameters = {'kernel' : ['linear'], 'C' : [0.01, 0.1, 1, 10, 100]}
logistic_parameters = {'C' : [0.01, 0.1, 1, 10, 100]}
alphas = np.logspace(-10, 1, 400)

# Feature importance vectors
rf_feature_scores = []
svm_feature_scores = []
logistic_feature_scores = []
mlpnn_feature_scores= []

# Stratified-K-fold splitting
num_splits = 5
skf = StratifiedKFold(n_splits= num_splits)
```

```
# Lists for AUC-ROC visualization
rf_tprs = []
svm_tprs = []
logistic_tprs = []
mlpnn_tprs =[]
mean_fprs = np.linspace(0, 1, 101)
x = breast_abundance.transpose( ).values
y = breast_labels.values
```

Next we will use the *split* function on our *StratifiedKFold* object, passing both the dataset and the class labels. We will iterate over the training and testing partitions using a *for* loop. For each iteration, we subset the data and labels using the training and testing indices. We then perform the log-transformation and standard normalization in the same manner as in the *Data Preprocessing* section. Note that the normalization function is only fit to the training set in order to prevent any bias from the testing set. We then use the training set to train the ML models and store their predictions of class probabilities using the *predict_proba* function, which takes just the probability of being a positive class. This way a value of 0 indicates the negative class, and a value of 1 indicates a positive class. Note that in order to use this function for the SVM model, we must set *probability=True* as a model parameter. We type:

```
for train_index, test_index in skf.split(x,y):

# Set up train and test sets
x_train, x_test = x[train_index] , x[test_index]
y_train, y_test = y[train_index] , y[test_index]

# Log transform data
x_train = np.log2(x_train +1)
x_test = np.log2(x_test+1)

# Train a scaler on the training set ONLY, then transform train and test
set using scaler
scaler = StandardScaler().fit(x_train)
x_train = scaler.transform(x_train)
x_test = scaler.transform(x_test)

# Fit machine learning models
rf = RandomForestClassifier(n_estimator = 500).fit(x_train, y_train)
svm= GridSearchCV (SVC(probability = True) , svm_parameterm, cv=5).fit
(x_train, y_train)
logistic =GridSearchCV(LogisticRegression(penalty = "12"),
logistic_parameters, cv =5).fit(x_train, y_train)
mlpnn = MLPClassifier(hidden_layer_sizes =(100, ), solver = "lbfgs").fit
(x_train, y_train)

# Model test predictions
rf_pred = rf.predict_proba(x_test)[: , 1]
svm_pred = svm.predict_proba(x_test) [: , 1]
```

```
logistic_pred = logistic.predict_proba(x_test) [: , 1]
mlpnn_pred = mlpnn.predict_proba(x_test)[: ,1]

# Model feature scores
rf_feature_scores.append(rf.feature_importances_)
svm_feature_scores.append(svm.best_estimator_.coef_.reshape(-1))
logistic_feature_scores.append(logistic.best_estimator_.coef_.
reshape(-1))
mlpnn_feature_scores.append(np.matmul(mlpnn.coefs_[0], mlpnn.
coefs_[1]))
```

Next, we calculate various evaluation metrics for the models using this testing partition and store them in the data frame. All of these functions come from the *metrics* module from *scikit-learn*. The function *roc_auc_score* uses the true class labels and the probabilities of the positive class to calculate the AUC-ROC. The other functions, *precision_score*, *recall_score*, and *f1_score* require the true class labels and predicted class labels. Therefore, we round the probabilities such that values greater than 0.5 become the positive class label and values lower become the negative class label. We type:

```
# Store metrics
metrics.loc["AUC"]["RF"] += roc_auc_score(y_test, rf_pred)
metrics.loc[" Precision"]["RF"] += precision_score(y_test, np.round
(rf_pred))
metrics.loc["Recall"]["RF"] += recall_score(y_test, np.round
(rf_pred))
metrics.loc["F1"]["RF"] += f1_score(y_test, np.round(rf_pred))

metrics.loc["AUC"]["SVM"] += roc_auc_score(y_test, svm_pred)
metrics.loc[" Precision"]["SVM"] += precision_score(y_test, np.round
(svm_pred))
metrics.loc["Recall"]["SVM"] += recall_score(y_test, np.round
(svm_pred))
metrics.loc["F1"]["SVM"] += f1_score(y_test, np.round(svm_pred))

metrics.loc["AUC"]["Logistic"] += roc_auc_score(y_test,
logistic_pred)
metrics.loc[" Precision"]["Logistic"] += precision_score(y_test, np.
round(logistic_pred))
metrics.loc["Recall"]["Logistic"] += recall_score(y_test, np.round
(logistic_pred))
metrics.loc["F1"][" Logistic"] += f1_score(y_test, np.round
(logistic_pred))

metrics.loc["AUC"]["MLPNN"] += roc_auc_score(y_test, mlpnn_pred)
metrics.loc[" Precision"][" MLPNN"] += precision_score(y_test, np.
round(mlpnn_pred))
metrics.loc["Recall"][" MLPNN"] += recall_score(y_test, np.round
(mlpnn_pred))
metrics.loc["F1"][" MLPNN"] += f1_score(y_test, np.round
(mlpnn_pred))
```

Next we calculate the true-positive and false-positive rates for each model and different thresholds using the *roc_curve* function from the *metrics* model from *scikit-learn*. We then interpolate the true-positive rates across the range (0,1), making sure that the first value is 0 and the last value is 1. The lists of true-positive rates are stored for each model separately. We type:

```
fpr, tpr, _ = roc_curve(y_test, rf_pred)
rf_tpr = interp(mean_fprs, fpr, tpr)
rf_tpr[0] = 0.0
rf_tprs.append(rf_tpr)

fpr, tpr, _ = roc_curve(y_test, svm_pred)
svm_tpr = interp(mean_fprs, fpr, tpr)
svm_tpr[0] = 0.0
svm_tprs.append(svm_tpr)

fpr, tpr, _ = roc_curve(y_test, logistic_pred)
logistic_tpr = interp(mean_fprs, fpr, tpr)
logistic_tpr[0] = 0.0
logistic_tprs.append(logistic_tpr)

fpr, tpr, _ = roc_curve(y_test, mlpnn_pred)
mlpnn _tpr = interp(mean_fprs, fpr, tpr)
mlpnn _tpr[0] = 0.0
mlpnn _tprs.append(mlpnn _tpr)
```

After training, we can look at the average statistics by dividing the data frame by the number of splits used by typing:

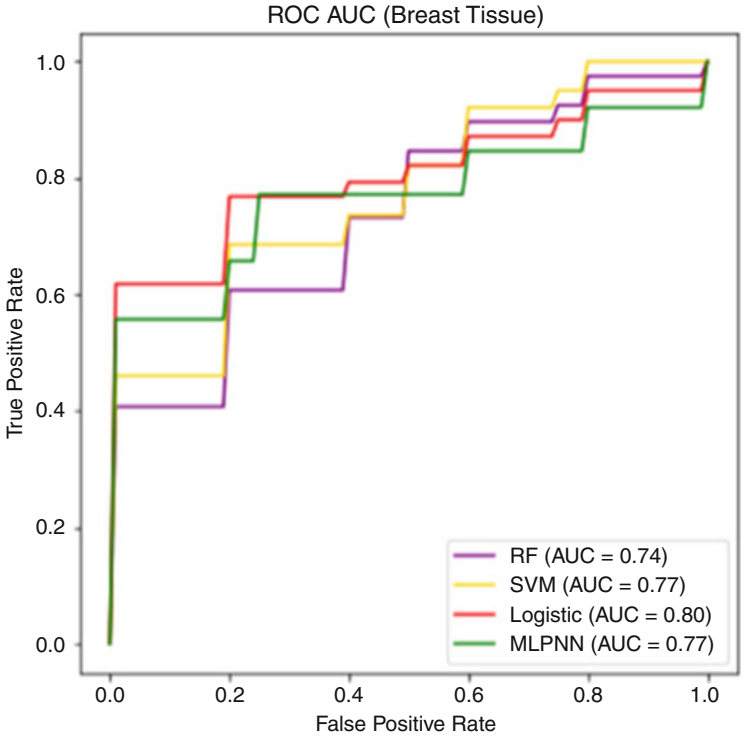
```
breast_metrics = metrics/num_splits
breast_metrics
```

	RF	SVM	Logistic	MLPNN
AUC	0.736429	0.770714	0.804286	0.767857
Precision	0.709848	0.772338	0.793651	0.789286
Recall	0.850000	0.821429	0.671429	0.696429
*F*1	0.760255	0.779919	0.701279	0.728333

We can visualize the ROC for the different models using the stored true-positive rates. To do so, we take the mean of the true-positive rates within each machine learning model using the *mean* function where *axis=0* indicates that we are taking the mean across the first dimension of the matrix. Then, we can use the *pyplot* module from the *matplotlib* Python library (which we will denote as *plt*) for visualization. We begin by setting up a figure that is 8 × 8. We then plot the false-positive rates as the *x*-axis and the true-positive rates as the *y*-axis in order to generate the ROC for that machine learning model. For each plot, we specify a different color and label, which will be displayed in our legend. Lastly, we annotate the plot's title and axes and then show the figure by typing:

```
import matplotlib.pyplot as plt
breast_mean_rf_tprs = np.array(rf_tprs).mean(axis = 0)
breast_mean_svm_tprs = np.array(svm_tprs).mean(axis = 0)
breast_mean_logistic_tprs = np.array(logistic_tprs).mean(axis=0)
breast_mean_mlpnn_tprs = np.array(mlpnn_tprs).mean(axis = 0)

plt.figure(figsize = (6, 6))
plt.plot(mean_fprs, breast_mean_rf_tprs, color = 'purple', label = "RF
(AUC = % .2f )" % breast_metrics.loc["AUC"]["RF"])
plt.plot(mean_fprs, breast_mean_svm_tprs, color = 'gold',label="SVM
(AUC = % .2f )" % breast_metrics.loc["AUC"]["SVM"])
plt.plot(mean_fprs, breast_mean_logistics_tprs, color = 'red', label =
"Logistic (AUC = % .2f )" % breast_metrics.loc["AUC"]["Logistic"])
plt.plot(mean_fprs, breast_mean_mlpnn_tprs, color = 'green', label =
"MLPNN (AUC = % .2f )" % breast_metrics.loc["AUC"]["MLPNN"])
plt.legend( loc = " lower right")
plt.xlabel("False Positive Rate")
plt.ylabel("True Positive Rate")
plt.title("ROC AUC (Breast Tissue)")
plt.show( )
```



In addition, we can aggregate the feature scores across all models. To do this, we take the average feature score within each machine learning model across all cross-validation partitions. We will then store these scores into a data frame for later analysis. We type:

```
feature_score_df = pd.DataFrame (index = breast_abundance.index.
values, columns = ["RF", "SVM" , "Logistic", "MLPNN"]).fillna(0.0)

feature_score_df["RF"] = np.array(rf_feature_scores).mean(axis=0)
feature_score_df["SVM"] = np.array(svm_feature_scores).mean(axis=0)
feature_score_df["Logistic"] = np.array(logistic_feature_scores).
mean(axis=0)
feature_score_df["MLPNN"] = np.array(mlpnn_feature_scores).mean
(axis=0)
feature_score_df.to_csv("BC_Tissue/feature_scores.tsv", sep = "\t")
feature_score_df.head(10)
```

	RF	SVM	Logistic	MLPNN
Actinomyces	0.025336	−0.070658	−0.368613	−2.061236
Corynebacterium	0.075407	−0.130724	−0.336228	−3.415127
Micrococcus	0.009830	0.021898	−0.192237	−0.119093
Rothia	0.013275	0.035389	0.286142	0.819886
Mycobacterium	0.009282	0.020795	−0.037772	0.062156
Propionibacterium	0.020436	0.009804	−0.129734	0.364447
Yaniella	0.008952	0.041797	0.249778	1.571067
Bifidobacterium	0.011247	0.020922	−0.127679	0.581296
Unknown genus (family Coriobacteriaceae)	0.006055	0.002321	−0.149301	−0.041508
Atopobium	0.037604	−0.120209	−0.206812	−3.250947

Next, we take the scores from each model and create a data frame where each column is the ranked list based on the feature importance. Since SVM, logistic regression, and MLPNN models have negative values, which can indicate important features, we first take the absolute values of the scores. We then sort the features based on the descending order of the absolute values within each ML model and store the ordered row names as a column in a new data frame by typing:

```
feature_ranks = feature_score_df.abs().rank(ascending = False)
breast_feature_ranking_df["RF"] = feature_ranks["RF"].sort_values
().index.values
breast_feature_ranking_df["SVM"] = feature_ranks["SVM"].sort_values
().index.values
breast_feature_ranking_df["Logistic"] = feature_ranks["Logistic"].
sort_values().index.values
breast_feature_ranking_df["MLPNN"] = feature_ranks["MLPNN"].
sort_values().index.values

breast_feature_ranking_df.head(10)
```

	RF	SVM	Logistic	MLPNIN
0	*Corynebacterium*	*Corynebacterium*	Unknown genus (family Alcaligenaceae)	Unknown genus (family Oxaiobacteraceae)
1	*Methylobacterium*	*Atopobium*	*Pseudomonas*	*Corybacterium*
2	Unknown genus (family Alcaligenaceae)	*Acinetobacter*	*Ochrobactrum*	*Atopobium*
3	*Lactobacillus*	Unknown genus (family Alcaligenaceae)	Unknown genus (family Oxaiobacteraceae)	Unknown genus (family Alcaligenaceae)
4	*Atopobium*	*Sphingomonas*	*Actinomyces*	*Pseudomonas*
5	*Prevotella*	Unknown genus (family Enteraobacteriaceae)	Other genus (family Bacillaceae)	*Ochrobactrum*
6	*Lysinibacillus*	*Actinomyces*	*Corynebacterium*	Other genus (family Phyllobacteriaveae)
7	Unknown genus (family Enterobacteriaceae)	*Pseudomonas*	*Acinetobacter*	*Acinetobacter*
8	*Sphingomonas*	*Lactobacillus*	*Bacillus*	*Peptoniphilus*
9	*Actinomyces*	*Peptoniphillus*	Unknown genus (family Enterobacteriaceae)	Unknown genus (family Enterobacteiaceae)

15.3.8 Feature Aggregation

As shown above we can obtain average feature scores for each ML model using the model parameters. However, since each set of scores is on different scaled, it is not as appropriate to combine the scores across ML models. In order to obtain a single unified list in this tutorial, we will use a rank aggregation technique, which creates a rank list that minimizes the dis-concordance with the individual rank lists. For this, we will use the *RankAggreg* package from R (Pihur et al. 2009).

To use the *RankAggreg* package, we first save the feature ranking data frame using the *to_csv* function from *pandas*. Then, after making sure *RankAggreg* is installed in R, which can be done using *install.packages*, we load the package using *library("RankAggreg")*. We type:

```
feature_ranking_df.to.csv("BC_Tissue/ranked_feature.tsv", sep =
"\t" )
```

```
> install.packages("RankAggreg", dependencies = TRUE, repos = "http://
cran.us.r-project.org")
> library("RankAggreg")
> setwd("/Users/derek.reiman/Desktop/Springer Book Chapter/")
```

```
> ranked_features <- read.table("BC_Tissue/ranked_features.tsv",
sep="\t", header=T,
colClasses="character", row.names=1)
> top_10_features <- RankAggreg(t(as.matrix(ranked_features)),
10, method="CE",
distance="Spearman", verbose=F)$top.list

> print(top_10_features)
[1] "Corynebacterium"
[2] "Atopobium"
[3] "Unknown genus (family Alcaligenaceae)"
[4] "Methylobacterium"
[5] "Actinomyces"
[6] "Unkonwn genus (family Enterobacteriaceae)"
[7] "Pseudomonas"
[8] "Acinetobacter"
[9] "Lactobacillus"
[10] "Rhizobium"
```

Next, we have to switch to the directory where our datasets are using the *setwd* function. From here, we can load the data frame using *read.table*, making sure to specify that we are using tab-delimited tables and that the table contains a header. We can then pass this table to the function *RankAggreg*; however, we need to make sure to cast it as a matrix using *as.matrix* and to transpose this matrix using *t*. The next parameter specifies how many features we want in our top list, which here we choose a value of 10. This function allows the user to choose between a cross-entropy Monte Carlo algorithm and a genetic algorithm for selecting the top ranked features. We will use the cross-entropy algorithm by specifying *method="CE"*. We then specify that we want to minimize the Spearman correlation between our proposed top features and the ranked lists our machine learning models found by specifying *distance="Spearman"*. Lastly we store the top ranked aggregated features by adding *$top.list* at the end of the function and print out our aggregated list.

Additionally, we have developed a tool Meta-Signer that will perform the machine learning model training and evaluation as well as feature aggregation, providing a summarized report of the evaluations and feature rankings. The tool uses an expanded architecture for MLPNN models using the *TensorFlow* Python libraries and can be found at https://github.com/YDaiLab/Meta-Signer.

15.4 Results

Finally, we briefly provide results for the three datasets using the methods outlined in this tutorial. Users can use our script and datasets provided at https://github.com/YDaiLab/Book-Chapter-Tutorial to generate the results.

First, we report the mean AUC-ROC, precision, recall, and $F1$ score as evaluation metrics and generate the ROC plots over tenfold cross-validation. We observed that different models had different predictive performance considering different metrics.

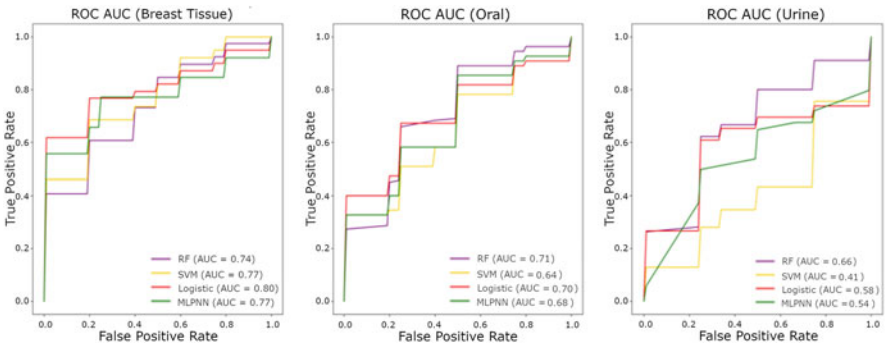


Fig. 15.1 ROC plots over tenfold cross-validation

Table 15.2 AUC, precision, recall, and *F*1 results for breast, oral, and urine

		RF	SVM	Logistic	MLPNN
Breast	AUC	0.736	0.770	**0.804**	0.768
	Precision	0.710	0.772	**0.794**	0.789
	Recall	**0.850**	0.821	0.671	0.696
	*F*1	0.760	**0.780**	0.701	0.728
Oral	AUC	**0.711**	0.639	0.703	0.675
	Precision	0.742	0.735	**0.811**	0.778
	Recall	**0.982**	0.909	0.709	0.800
	*F*1	**0.844**	0.812	0.754	0.782
Urine	AUC	**0.659**	0.411	0.584	0.540
	Precision	0.723	0.704	**0.762**	0.701
	Recall	0.911	**0.933**	0.611	0.631
	*F*1	**0.805**	0.801	0.666	0.654

The number in bold indicates the highest value in a row

For example, logistic regression models had higher precision but lower recall among the machine learning methods. However, no single method consistently outperforms other models, suggesting the need of evaluating multiple models for prediction of host phenotype. In addition, the aggregated list of features may help us identify a robust assessment on their association to disease (Fig. 15.1) (Table 15.2).

In addition, we generated the top 10 features generated through rank aggregation for the three datasets considering the average feature scores of the four machine learning methods discussed in this tutorial (Table 15.3).

15.5 Summary

In this tutorial, we demonstrated the training of several a machine learning models and an approach to feature evaluation based on the learned models in microbial datasets using Python and R packages. Both Python and R are open-source scripting

Table 15.3 Top ranked OTUs

	Breast tissue	Oral	Urine
1	*Corynebacterium*	Unknown genus (family Actinomycetaceae)	Unknown genus (family Coriobacteriaceae)
2	*Atopobium*	Other genus (family Gemellaceae)	*Gardnerella*
3	Unknown genus (family Alcaligenaceae)	*Kingella*	*Ruminococcus*
4	*Methylobacterium*	Unknown genus (family F16)	*Shuttleworthia*
5	*Acinetobacter*	*Fusobacterium*	*Agrobacterium*
6	Unknown genus (family Enterobacteriaceae)	*Streptococcus*	*Lactobacillus*
7	*Actinomyces*	*Butyrivibrio*	*Trueperella*
8	*Pseudomonas*	*Leptotrichia*	*Peptoniphilus*
9	*Lactobacillus*	*Propionibacterium*	*Haemophilus*
10	*Peptoniphilus*	*Rothia*	*Finegoldia*

tools that provide powerful statistical and machine learning libraries. We outlined a workflow on how to train four common machine learning models (RF, SVM, logistic regression, and MLPNN) as well as how to evaluate and rank features using each model's parameters. Lastly, we aggregated the ranked lists into a single ranked list using the *RankAggreg* package in R. We performed this workflow on datasets representing the breast, oral, and urinary microbiome of patients with breast cancer versus healthy patients from a study by Wang et al. (2017).

Machine learning approaches allow the exploration of datasets in a nonlinear approach, allowing for the identification of more complex combinations of features associated to health status. By aggregating multiple ranked lists across machine learning models, we believe that one can obtain a more robust feature ranking. In addition, statistical methods such as ANCOM (Mandal et al. 2015) can be used to generate ranked lists that can be incorporated into the aggregation as well. However, the approach based on machine learning models is limited in the fact that although it allows for features to be ranked and aggregated, it is difficult to determine the significance of these features or how to determine a cutoff for the top ranked features.

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Chapter 16
Mediation Analysis of Microbiome Data and Detection of Causality in Microbiome Studies

Yinglin Xia

Abstract Microbiome research has basically focused on three factors: environment, microbiome, and host. The interactions among these three factors are dynamic and complicated. Three general hypotheses have been developed to detect these interactions. Among these hypotheses, testing the mediated effects of environmental factors and host mediated by microbiome is the most often designed, because mediation analysis of the human microbiome in these dynamic and very complicated relationships could potentially provide insights into the role of the microbiome in health and the etiology of disease and, more importantly, lead to novel clinical interventions by modulating the microbiome.

However, microbiome data are high-dimensional, structured as phylogenetic tree, sparse, non-normally distributed, and are often characterized by the presence of a large portion of zero values and hence are skewed to the right and heteroscedastic. Thus, the suitable methods for mediation analysis of microbiome data are rare. Several methods for mediation analysis of microbiome data were just developed in most current years. In this book chapter, we first introduce traditional mediation models and mediation models in omics studies as backgrounds and then focus on describing and reviewing specifically designed mediation models in microbiome studies. Traditional mediation models include two broad types of frameworks for mediation analysis: one is structural equation modeling (SEM)-based mediation analysis, which covers "product method" or "product of coefficients method" and "difference of coefficients method", respectively. Another is counterfactual-based mediation analysis, which uses "potential outcomes" or "counterfactual outcomes" method.

The data features and statistical issues of microbiome studies are more similar to those in other omics studies, such as high dimensionality and sparsity; thus the mediation models from omics studies provide more insights and motivations to develop the mediation models in microbiome studies, such as how to test multiple putative mediators simultaneously using permutation (MultiMed), how to reduce

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high-dimensional mediators through regularization or penalization (HIMA), and how to transform high-dimensional mediators into low-dimensional and uncorrelated mediators using the spectral decomposition (CausalMM). This book chapter mainly aims to introduce seven specifically designed mediation models in microbiome studies. They are (1) distance-based omnibus test of mediation effect (MedTest), (2) multivariate omnibus distance mediation analysis (MODIMA), (3) causal compositional mediation model (CCMM), (4) isometric log-ratio transformation for microbiome mediation (IsometricLRTMM), (5) sparse microbial causal mediation model (SparseMCMM), (6) mediation analysis for zero-inflated mediators (MedZIM), and (7) nonparametric entropy mediation (NPEM). All these models were developed to target specific data structure and features of microbiome data (e.g., dimensionality, compositionality, sparsity, zero-inflated) through either SEM-based or counterfactual-based mediation frameworks. We complete this chapter with comments on current mediation models for microbiome data analysis and how to understand establishing causality in microbiome studies.

Keywords Mediation analysis · Features of microbiome data · Omics studies · SEM-based mediation analysis · Counterfactual-based mediation analysis · Entropy mediation analysis · Statistical mediation models for microbiome data

16.1 Introduction

Microbiome research basically focuses on three factors: environment, microbiome, and host. The interactions among environment (or genetic), microbiome, and host are dynamic and very complicated. To detect the dynamic interactions among these three factors, three general hypotheses are generally developed. Among these three hypotheses, testing the mediated effects of environmental factors and host mediated by microbiome is often designed. Mediation analysis of the human microbiome is very important because it can address more central hypothesized linkage and the mechanisms among these three dynamic and very complicated factors, which could potentially provide insights into the role of the microbiome in health and the etiology of disease and, more importantly, lead to novel clinical interventions by modulating the microbiome.

The relative abundance of microbiome often have several distinct features (Xia et al. 2018a, b, c, d): (1) structured as a phylogenetic tree, (2) high-dimensional and underdetermined, (3) compositional, (4) often sparse along the presence of a large portion of zero values, (5) right skewed and heteroscedastic, and thus (6) over-dispersed and (7) non-normally distributed. All these features of microbiome data pose major challenges for developing methods for mediation analysis of microbiome data (Xia et al. 2018a, b, c, d). Several methods for mediation analysis of microbiome data were just developed in most current years.

Five motivating applications are considered in this book chapter to describe the use of these models. In such a study, the tri-variate associations among environment,

microbiome, and host are measured especially to test whether the independent variables predict the outcome variable is mediated by the microbiome. The first example is a cross-section analysis of 98 healthy volunteers on diet, BMI, and gut microbiome composition (Wu et al. 2011). In this testing, the associations between diet and BMI, between the gut microbiota and BMI, and between diet and the gut microbiota are all statistically significant. The mediation analysis was to test whether the association between diet and BMI is mediated by the gut microbiota. The second example is antibiotic use data (Cho et al. 2012). The data have a total of 96 samples (50 cecal and 46 fecal) across 50 animals with four antibiotic uses and one control. The mediation analysis was to test whether the association between antibiotic use and percent fat is mediated by the microbial composition. The third example is a longitudinal analysis of 67 pregnant women to investigate arsenic exposure in pregnant women (environmental factor) impacts the health of their children (Farzan et al. 2013). In the third example, the mediation analysis was to examine whether maternal arsenic exposure (total in utero arsenic level) impacts treated cough effect of their children mediated by gut microbiome of infants (Nadeau et al. 2014). There is a presence of a large portion (54%) of zero values in this microbiome data. The fourth example consists of totally 24 mice with 10 treated with a commercially available probiotic cocktail and other 14 as control (Arthur et al. 2013). This example was used to test whether the effects of treatment probiotic cocktail on outcome variable dysplasia score (abnormality of cell growth) are mediated by the relative abundance of each microbiome OTU (Li et al. 2019). The fifth example is also an antibiotic treatment (Schulfer et al. 2019). This murine microbiome experiment was to investigate whether sub-therapeutic antibiotic treatment would alter gut microbiome composition and whether this change would influence the body weight gain later in life. The data have a total of 58 mice (21 females, 12 antibiotic treatments and 9 controls, and 37 males, 24 antibiotic treatments and 13 controls) at day 21 and 28. The mediation analysis was to test whether the role of sub-therapeutic antibiotic treatment in body weight gain is mediated by the gut microbiome.

The remaining of this chapter is organized this way. We will first review two traditional mediation models including SEM-based and counterfactual-based mediation analyses in Sect. 16.2, which we hope will provide a solid knowledge background for understanding the development of mediation models in microbiome studies. Then, we present more current developed mediation models in omics studies in Sect. 16.3. Due to similar data structures and features, the mediation models in omics studies provide more insight and motivations to develop the mediation models in microbiome studies. In Sect. 16.4, we will introduce and describe seven mediation models in microbiome studies including distance-based omnibus test of mediation effect (MedTest), multivariate omnibus distance mediation analysis of microbiome (MODIMA), causal compositional mediation model (CCMM), isometric log-ratio transformation for microbiome mediation (IsometricLRTMM), sparse microbial causal mediation model (SparseMCMM), mediation analysis for zero-inflated mediators (MedZIM), and nonparametric entropy mediation (NPEM), respectively. At the end of this section, we will comment on current mediation models for

microbiome data analysis. In Sect. 16.5, we will discuss detecting causality and how to understand establishing causality in microbiome studies from philosophic ontology (metaphysics), methodology, and specifically from a statistical theory of probability.

16.2 Traditional Mediation Models

As a background for mediation analysis of microbiome data, in this section, we will introduce the basic notions and frameworks for two broad types of traditional mediation analysis: (1) using structural equation modeling (SEM), which covers "product method" or "product of coefficients method" and "difference of coefficients method", respectively, and (2) through "potential outcomes" or "counterfactual outcomes" method.

Researchers may have different definitions of mediation analysis in their fields; however, mediation analysis generally is referred to as a set of techniques that assess direct and indirect effects (i.e., mediated effects) to explain the relationship among tri-variates: independent variable(s), mediator(s), and outcome(s). Distinguishing from typical regression approaches, the power of mediation analysis lies in its capability to estimate and test the mediated effects. In the research fields, several sets of variables have been used to describe the three-variable relationship, such as independent variable (predictor, treatment, initial variable, antecedent variable, causal ancestor, program exposure)-mediating variable (mediated, intervening or intermediate variable, process variable, mediator, surrogate or intermediate endpoints in medical literature, intermediate endpoint, proximal measure)-dependent variable (criterion variable, outcome variable, consequent variable, causal descent, ultimate endpoint, distant measure). In this book chapter, we use their combinations exchangeably. However, to keep consistent, we mostly use independent variable (exposure)-mediator-outcome (response) to label the three-variable relationship. In the case, treatment is differentiated from the independent variable, we also use the variable treatment. Other variables or terms that are often used in literatures are moderator (interaction variable, effect modifier, effect measure modifier), confounder, and covariate.

16.2.1 Typical Features of SEM-Based Mediation Framework

Modern mediation analysis began with Wright's theory and method of path coefficients (Wright 1920, 1921, 1923, 1934). In path analysis, Wright quantified mediation mechanisms and described the path analysis methods for mediating processes among other variables in system of relations. Wright's path analysis opened the

approach of modern SEM mediation analysis. Now path analysis of mediation model is reviewed as a special case of SEM mediation approach.

SEM is referred as to a system of regression-type models linked together a conceptual model, path diagram to analyze complex, and dynamic relationships among observed and unobserved variables. A typical SEM model consists of a measurement model and a structure model.

Although similar in appearance, SEM is fundamentally different from traditional regression. For example, in traditional regression variables are mandated to clearly distinct whether are dependent (effect-receiving) or independent (effect-imparting), whereas in SEM models, one key feature is that one variable could be independent and dependent variables. To avoid confusion, in SEM literatures, *endogenous* and *exogenous* variables are often used instead. In the SEM equations or matrices, exogenous variables always represent independent variables, endogenous variables serve as dependent variables, and at least one endogenous variable is needed for specifying SEM. In SEM models, mediators could be both *endogenous* and *exogenous* variables. It is precisely this type of reciprocal role a variable plays that enables SEM to infer causal relationships (Xia et al. 2012a).

16.2.1.1 Product of Coefficients Method

The framework of Barron and Kenny approach had antecedents such as in psychology (Woodworth 1928), in cognitive dissonance (Brehm and Cohen 1962), in industrial and organizational psychology (James and Brett 1984), and in mediational hypothesis testing (Judd and Kenny 1981a, b; Fiske et al. 1982; Sobel 1982; James and Brett 1984). However, the framework of product of coefficients method for mediation analysis was clearly depicted in Baron and Kenny's landmark paper (1986), which deserves a first introduction in this beginning stage of SEM mediation framework coverage. It is the work of Baron and Kenny landmark paper (Baron and Kenny 1986) that makes mediation analysis very popular first in psychology and social sciences and then extended to epidemiology, biomedicine, and other fields.

This paper has contributed toward the development of SEM mediation framework in several ways. Among them, the following four notions and techniques are very important.

First, this paper describes causal pathways of a three-variable system (independent variable, mediator, and outcome variable) via a diagram of path analysis. The framework of Baron and Kenny's mediation analysis is based on the single-mediator model (SMM). The role of a mediator variable is conceptualized in the causal path diagram in Fig. 16.1.

SMM is a three-variable system depicting three paths. Path a is from the independent variable to the mediator (α_{xm}). Paths b and c are two causal paths: Path b is the effect of the mediator on the outcome variable (β_{my}), and Path c is the direct effect of the independent variable on the outcome variable (γ_{xy}). Based on these two causal pathways, we can write a SEM mediation model for testing the causal relationship in terms of two linear regressions:

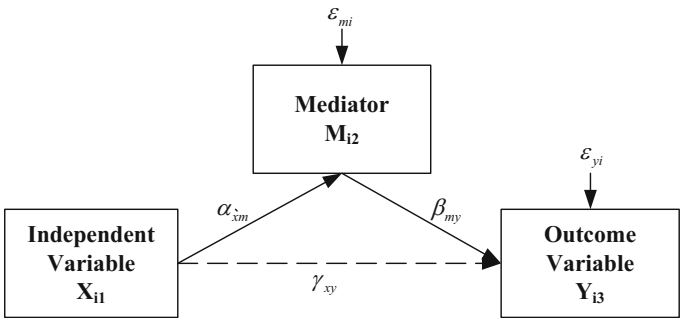

Fig. 16.1 Path diagram for the SEM mediation model

$$M_{i2} = \alpha_0 + \alpha_{xm} X_{i1} + \varepsilon_{mi} \tag{16.1}$$

$$Y_{i3} = \gamma_0 + \gamma_{xy} X_{i1} + \beta_{my} M_{i2} + \varepsilon_{yi} \tag{16.2}$$

Equation (16.1) describes the effect of the independent variable on the mediator; Eq. (16.2) describes the effect of both the independent and mediator variables on the outcome. Combining (16.1) and (16.2) represents a mediation model that describes an underlying mediation relation of the independent variable (X_{i1}) to the mediator (M_{i2}) to outcome (Y_{i3}), where α_0 and γ_0 are the interceptors and ε_{mi} and ε_{yi} are error terms.

Second, it is in this paper that a hypothesis testing mediation is proposed. Baron and Kenny adopted Sobel's hypothesis testing method for the indirect effect (Sobel 1982) and specifically modified the formula of standard error for estimating mediated effect by production of coefficients α_{xm} and $\beta_{my}(\alpha_{xm}\beta_{my})$. This method for estimating mediation is named as "product method" or "product of coefficients method."

The standard error of product $\alpha_{xm}\beta_{my}$ based on Sobel (1982) can be written as:

$$S_{\widehat{\alpha_{xm}}\widehat{\beta_{my}}} = \sqrt{\widehat{\alpha}^2_{xm} S^2_{\widehat{\beta}_{my}} + \widehat{\beta}^2_{my} S^2_{\widehat{\alpha}_{xm}}} \tag{16.3}$$

Baron and Kenny (1986) use the exact standard error of product $\alpha_{xm}\beta_{my}$:

$$S_{\widehat{\alpha_{xm}}\widehat{\beta_{my}}} = \sqrt{\widehat{\alpha}^2_{xm} S^2_{\widehat{\beta}_{my}} + \widehat{\beta}^2_{my} S^2_{\widehat{\alpha}_{xm}} + S^2_{\widehat{\alpha}_{xm}} S^2_{\widehat{\beta}_{my}}} \tag{16.4}$$

But the added term $S^2_{\widehat{\alpha}_{xm}} S^2_{\widehat{\beta}_{my}}$ is negligible, so most commonly used covariance structure software such as EGS, Mplus, and LISREL still use Sobel's formula.

The hypothesis testing of mediated effect or indirect effect is called product of coefficients method for testing mediation or "product of coefficients tests"

(MacKinnon 2008). We can perform the hypothesis testing of the mediated effect using confidence intervals, which is based on the estimated standard error such as from a *t*-test. In the mediation model of Fig. 16.1, γ_{xy} represents the direct effect that describes the pathway from the independent variable X_{i1} to the outcome Y_{i3}, while controlling for the mediator M_{i2}. The indirect effect describes the pathway from the independent variable to the outcome through the mediator. The total effect is the sum of the direct and indirect effects of the independent variable on the outcome without considering other covariates.

Third, this paper comprehensively discussed the assumptions, qualifications of mediation, and steps to establish mediation. According to Baron and Kenny, two assumptions are required for the mediational model (Baron and Kenny 1986): (1) the mediator has no measurement error (otherwise the mediated effect tends to be underestimated while the direct effect tends to be overestimated when all coefficients are positive), and (2) the dependent variable does not cause the mediator (otherwise the feedback bias will occur in mediational chains). Baron and Kenny described the qualifications of mediation and steps to establish mediation in a series of causal tests: (1) the independent variable should be significantly related to the presumed mediator; (2) the presumed mediator should significantly affect the outcome when the independent variable is controlled; (3) the independent variable should significantly affect the outcome when only the independent variable is included; and (4) when controlling for the mediator, the direct effect from the independent variable to the outcome should be reduced, or nonsignificant, and with a completely mediated, the direct effect should be zero. The qualifications of (1) and (2) have generally been considered as important for accepting to establish mediation. However, the steps (3) and (4) are often criticized for not correct, or not accurate thus not necessary (MacKinnon 2008; VanderWeele 2015). Although the controversies exist in the literature regarding to qualifications of mediation and steps to establish mediation, the descriptions by Baron and Kenny have opened a wide discussion and enabled a path for later researches.

Fourth, it is in this paper that the moderator and mediator variables have been distinguished conceptually and strategically as well as statistically in social psychology. The moderator-mediator distinction provides an opportunity to develop methods for both mediation and interaction, particularly for interaction analysis, although the framework of Barron and Kenny is mainly single-mediator model (SMM). Both moderator and mediator variables are the functions of a third variable; the mediator "represents the generative mechanism through which the focal independent variable is able to influence the dependent variable of interest," whereas the moderator "partitions a focal independent variable into subgroups that establish its domains of maximal effectiveness in regard to a given dependent variable" (Baron and Kenny 1986). The antecedent for distinction of moderator and mediator and for testing mediation under the appearance of moderator may be back to James and Brett (1984). The method for estimating moderated mediation by using the product method and the approaches for hypothesis testing of moderated mediation were proposed by Preacher et al. (2007). Various topics about mediation and moderation were discussed in more details in (MacKinnon 2008; Hayes 2013). However, the

Preacher et al.'s mediation approach was criticized for lacking of decomposition property (the sum of direct and indirect effects equals to a total effect) (VanderWeele 2015).

In terms of which standard error and critical value are calculated, several variants of standard error of product of coefficients exist in literatures, including Goodman's unbiased standard error (Goodman 1960), Bobko and Rieck's standardized variables' regression coefficients and partial regression coefficient (Bobko and Rieck 1980), and MacKinnon and colleagues' three alternative methods (MacKinnon et al. 1998, 2002).

The causal-step methods have been reviewed having several limitations (MacKinnon et al. 2002) including (1) underpowered to detect small effects unless the effect or sample size is large, (2) do not have a joint test of all the three pathways, (3) cannot directly estimate the magnitude of the indirect effect of the independent variable on the outcome (Baron and Kenny 1986; Kenny et al. 1998), (4) not feasible to extend the causal step method to conduct multiple mediation analysis (West and Aiken 1997; MacKinnon et al. 2000), and (5) the requirement of significant relationship between the independent and outcome does not consider the situation of cancel-out total effects when indirect effect and direct effect have opposite directions as in "inconsistent" mediation models (MacKinnon et al. 2000).

16.2.1.2 Difference of Coefficients Method

Decomposing a total effect into direct effect and indirect effect is another most often used approach in mediation analysis. This method for testing mediation is called difference method or "difference in coefficients tests" (MacKinnon 2008). In Eqs. (16.1) and (16.2), summing the direct (γ_{xy}) and indirect ($\alpha_{xm}\beta_{my}$) effects is the total effect. To obtain the coefficient of total effect, we need to fit additional model, an outcome model with independent variable but without adjusting mediator:

$$Y_{i3} = \theta_0 + \theta_{xy} X_{i1} + \varepsilon_{xyi} \tag{16.5}$$

The mediated or indirect effect equals the difference of the independent variable coefficients ($\theta_{xy} - \gamma_{xy}$) in the two regression models (Alwin and Hauser 1975; Judd and Kenny 1981a, b; Mackinnon and Dwyer 1993).

The difference method has been used as early as 1970s (Susser 1973; Alwin and Hauser 1975) and the substantial discussion in (Mackinnon and Dwyer 1993; MacKinnon et al. 2002). The standard error of the difference method for determining mediation derived by McGuigan and Langholtz (1988) (McGuigan and Langholtz 1988; Mackinnon and Dwyer 1993; Mackinnon et al. 1995; MacKinnon 2008) is given as:

$$S_{\widehat{\theta}_{xy}-\widehat{\gamma}_{xy}} = \sqrt{S^2_{\widehat{\theta}_{xy}} + S^2_{\widehat{\gamma}_{xy}} - 2rS_{\widehat{\theta}_{xy}} S_{\widehat{\gamma}_{xy}}} \tag{16.6}$$

where the covariance between $\widehat{\theta}_{xy}$ and $\widehat{\gamma}_{xy}$, $rS_{\widehat{\theta}_{xy}} S_{\widehat{\gamma}_{xy}}$ is the mean square error (MSE) in Eq. (16.2) divided by the sample size times the variance of the independent variable ($\mathrm{MSE}/\left(N * S^2_{xi1}\right)$).

The simulation studies show that the difference method and the product method are closely related and yield identical estimates of mediated effect when the outcome is continuous, whereas when the outcome is a binary and a logistic regression is used, the standard error is inflated relative to the true standard error and has worse performance compared to product methods (Mackinnon and Dwyer 1993; Mackinnon et al. 1995).

The difference methods also have several variants differing in using regression or correlation coefficients, including Freedman and Schatzkin's difference between the adjusted and unadjusted regression coefficients (Freedman and Schatzkin 1992), Clogg et al.'s standard error of difference (Clogg et al. 1992), and Olkin and Finn's difference between the simple and partial correlation (Graf and Alf 1999).

Similar as the product method, the framework of the difference method has been reviewed having several limitations that (1) the underlying model does not appropriately use the non-directional correlations for testing the difference between simple and partial correlation, (2) does not provide a clear framework for generalizing hypothesis testing in models with multiple mediators (MacKinnon et al. 2002), and (3) the standard error is inflated when the outcome is a binary and a logistic regression is used.

16.2.1.3 Remarks

The approach of structural equation modeling was opened by Wright and rediscovered by Duncan (1966). Most statistical methods developed before 2008 were summarized and introduced in MacKinnon's mediation analysis book (MacKinnon 2008). This book is one of fundamental resources on the mediation analysis and especially for the mediation analysis from the SEM approach. A brief overview of SEM beginnings, historical development, statistical and philosophical (theoretical) controversies, and its applications in the social sciences has been conducted in the paper (Tarka 2018).

Although modern mediation analysis started with Wright, it was Barbara Burks who first explicitly represented a mediator with a diagram in 1926 and believed it was she who actually invented path diagrams independently of Sewall Wright. She was ahead of Wright and others in regard to mediation (Pearl and Mackenzie 2018) (p. 304). Blalock (1971) developed the more general methods in covariance structure modeling. The covariance structure modeling has been continually developed through the works of Jöreskog (1970, 1973), Keesling (1972), Wiley (1973), and

others. In the meanwhile various statistical methods in covariance structure modeling have been developed as well as including methods for non-normal data (Browne 1984); ordinal, limited, and discrete variables (Muthén 1983, 1984); model specifications (Bentler and Weeks 1982; McArdle and McDonald 1984); bootstrap estimation of direct and indirect effects (Bollen and Stine 1990); and comparisons of mediation testing (MacKinnon et al. 2002).

The SEM-based mediation analysis has many advantages.

First advantage lies on SEM's conceptual model and framework. Through a conceptual model, path diagram, and system of linked regression-style equations, SEM is able to analyze complex and dynamic relationships among observed and unobserved variables. Specifically, SEM is feasible to estimate measurement errors by using multiple indicator latent factors, test complex mediational mechanisms by decomposition of effects, and test moderation mechanisms through multiple group analysis or interaction terms. Thus, SEM has been reviewed as a second generation of multivariate statistical techniques (Fornell 1983). Theses statistical techniques provide an alternative approach to general linear modeling (GLM) such as the *t*-test, ANOVA, ANCOVA, MANOVA, MANCOVA, or multiple regression (Tarka 2018).

Second, the most appealing benefits of using SEM lie on its mediation framework. For example, SEM is designed to test mediation hypotheses in a single analysis, whereas in traditional regression, several linear models need to be specified. In SEM, multiple independent variables, mediators, or outcomes can be easily implemented.

Third, SEMs allow for estimating direct and indirect effects by modeling covariance and correlation matrices.

However, the SEM methodologies that were developed until recently are usually restricted in linear parametric settings. "No comparable methodology has been devised to extend its capabilities to models involving dichotomous variables or nonlinear dependencies" (Pearl 2010). It is not appropriate to use the methodologies developed in SEM to model with interactions or nonlinearities (Robins and Greenland 1992; Pearl 2001).

First, although decomposition of total effects into a direct and an indirect effect in linear models has been extended to nonlinear models [see the books Fosen et al. (2006), MacKinnon (2008), and Hayes (2013)], *the approach in SEM nonlinear and nonparametric models is not always justified*. This is because in SEM nonlinear models, (1) it is not clear and sometimes even flawed to use decomposition to precisely interpret association parameters as direct and indirect effects (Vansteelandt 2012). (2) The distinction between causal parameters and their regression interpretations can easily be conflated and produced distorted results since where the direct and indirect effects are defined in terms of structural or regression coefficients in nonlinear models (MacKinnon et al. 2007; Pearl 2010). (3) The association between the mediator and outcome could be confounded by another factor other than the independent variable. When a confounder exists, we will create spurious associations between the independent variable and outcome through the confounder even when there is no direct effect of the independent variable on the outcome (Pearl 1998, 2010; Cole and Hernán 2002). Thus, SEM methodologies are often criticized

for not adequately addressing issues of confounding/endogeneity in inferring causal relationships (VanderWeele 2015) (Chap. 2, p. 30).

Second, the SEM methodologies have not well-addressed mediation issues in nonparametric models. The limitations of Barron and Kenny approach include the following: (1) the framework of Barron and Kenny mainly is single-mediator model (SMM), and (2) no covariate can be included in the regression model.

Third, the SEM methodologies are difficult to accommodate zero-inflated mediators. This is because the linear model formulation does not work well with mediators that have two-part distributions under the traditional linear mediation framework.

Fourth, the product and difference methods have different performances when the outcome is binary. The differentiation between these two methods are reviewed as one limitation of SEM approach for testing mediated effect (VanderWeele 2015).

16.2.2 Counterfactual-Based Mediation Framework

16.2.2.1 Lewis' Counterfactual Model

Microbiome and host may have causative interactions. The microbiome research community now tries to understand the causal role of microbiota in the underlying molecular mechanisms (Xia and Sun 2017; Fischbach 2018). To understand the causal effects of microbiome on host, recently several mediation models for microbiome analysis have been developed under the counterfactual (contrary to fact) framework. In this subsection, we will review the development of counterfactual framework to provide some preliminaries to understand mediation models of microbiome data from the counterfactual perspective.

Causation analyses have become popular in the last 50 years, especially since the development in the 1970s of possible world semantics for counterfactuals. In metaphysics of causation, David Lewis is best known for his theory on counterfactual analysis of causation (Lewis 1973a, b, c) because he most thoroughly elaborated counterfactual theory of causation. Lewis employed possible world semantics for counterfactuals. His theory states truth conditions for counterfactuals in terms of similarity relations between possible worlds. It related to *comparative similarity* of worlds (Lewis 1973a, b): "One world is said to be *closer to actuality* than another if the first resembles the actual world more than the second does."

We do not know specifically whether or not the counterfactuals theory of possible world and actual world in metaphysics had impacted the development of counterfactuals theory in statistics. But it is clear that both counterfactual theories link the similarity and connection between two counterfactuals. In the counterfactual literature, the basic counterfactual model was recognized rooting in Lewis' "counterfactual model" (Robins and Greenland 1992).

16.2.2.2 Rubin's Counterfactual Framework

The causal framework in statistics was developed by Donald Rubin and Paul Holland in the 1970s (Rubin 1974, 2005; Holland 1986). The potential outcome idea was rooted in Neyman's work (1923). The causal framework was established through the concept called counterfactual. It is the counterfactual concept that makes it statistically or methodologically possible to estimate causal effects of treatments in nonrandomized data. Let $y(E)$ and $y(C)$ be the measured values that the unit received the experimental Treatment E and that the unit received the control Treatment C, respectively, and then, based on Rubin (1974), the causal effect of the E versus C treatment on Y for that trial is $y(E) - y(C)$. Since we can never observe both $y(E)$ and $y(C)$ for the same unit, measuring $y(E) - y(C)$ is impossible. Thus, it is reasonable to extend the measurements to multiple (M) trials and take the "typical" causal effect of the E versus C treatment for the M trials, i.e., to average the causal effects for the M trials:

$$\frac{1}{M}\sum_{j=1}^{M}\left[y_j(E) - y_j(C)\right], \tag{16.7}$$

where $y_j(E) - y_j(C)$ is the causal effect of the E versus C treatment for the jth trial.

Since 2000, a causal modeling era has started because in the early 2000s, a paradigmatic shift began to take from the Lewisian counterfactual theory and traditional statistical methodology to the approaches that sought to precisely counterfactual relationships within formal structural equations modeling.

16.2.2.3 Counterfactual-Based Mediation Framework

The interesting thing is that at the beginning, the proposers of counterfactual-based causal inference were not optimistic for mediation analysis (MacKinnon 2008; Pearl and Mackenzie 2018). For example, although Robins and Greenland (1992) proposed the concept of counterfactual-based mediation analysis, their negative options on mediation analysis have affected the literature until 2000. Rubin created the counterfactual causal inference framework, but he thought that mediation analysis is "deceptive" (Rubin 2004) although he dismissed this idea shortly in the next year (Rubin 2005). The substantial works of mediation methods and models from counterfactual perspective are from Pearl and later developments from VanderWeele (Pearl 2001; VanderWeele and Vansteelandt 2009, 2010; VanderWeele 2009).

The counterfactual-based mediation framework has been achieved through redefining mediation analysis, addressing the issues raised in SEM and generalizing the analytic approaches from linear parametric models to nonlinear and nonparametric models in counterfactual model settings. We summarize the main works of these developments as below.

Redefine Causality as a Statistical Methodology Rather than Philosophical Ontology

First of all, causal inference is a descriptive methodology about natural conditions of causal action rather than a theory on experimental conditions. Causal mediation is a method of the prescriptive and descriptive interpretations of direct effects and indirect effects (Pearl 2001). In other words, we should review causal mediation as a statistical methodology, and specifically as a statistical method on probability of causation. Based on Pearl if an effect comes from the descriptive perspective, then it is a natural effect, and if it comes from the prescriptive perspective, then it is the controlled effect.

Redefine Causal Direct and Causal Indirect Effects

SEM-based mediation analysis is often biased using controlling variables to estimate direct effects and in practice is difficult to estimate indirect effects in nonlinear models. To overcome the bias and difficulty, counterfactual-based mediation approach defines a more natural type of direct and indirect effects without controlling variables on the remaining paths. The concepts of natural direct and indirect effects were introduced in Robins and Greenland (1992) through numerical examples (Robins and Greenland 1992) and were deemed problematic (Pearl and Mackenzie 2018) (p. 401). Pearl (2001, 2009a, b) formalized natural direct and indirect effects, which leads to the mediation formula (Pearl and Mackenzie 2018) and generalized the SEM-based definitions of direct and indirect effects in the counterfactual-based settings.

The controlled direct effect of X of the transition from $X = x$ to $X = x'$ on Y can roughly be defined as:

$$\mathrm{CDE}_m \triangleq P(Y|X = x, M = m) - P(Y|X = x', M = m) \tag{16.8}$$

or equivalently using structural counterfactual notation:

$$\mathrm{CDE}_m \triangleq E(Y_{xm}) - E(|Y_{x'm}) \tag{16.9}$$

In the case M, take the specific values of 0 or 1, which may be easily understood by some readers; the formula of controlled direct effect can be written as:

$$\mathrm{CDE}(0) = P(Y = 1|X = 1, M = 0) - P(Y = 1, X = 0, M = 0) \tag{16.10}$$

where the outcome of Y is the change in X (say from $X = x$ to $X = x'$) while keeping all other accessible variables at their initial value. The "0" in CDE(0) indicates that the value of the mediator was forced to take zero. Similarly, when $M = 1$, we can write the formula as:

$$\mathrm{CDE}(1) = P(Y = 1|X = 1, M = 1) - P(Y = 1, X = 0, M = 1) \tag{16.11}$$

In above two versions of the controlled direct effect, we may be confused which one is correct and is chosen to report. To avoid the pitfalls of this overcontrolled experiment, Robins and Greenland used the concepts of "pure" direct and natural indirect (Robins and Greenland 1992), while Pearl used the concepts of "natural" direct and natural indirect. As Pearl stated in Pearl (2010):

> When the direct effect is sensitive to the levels at which we hold Z (here M), it is often more meaningful to define the direct effect relative to some 'natural' baseline level that may vary from individual to individual, and represents the level of Z (here M) just before the change in X.

The reason that called the direct effect and indirect effect as "natural" is that in Pearl's counterfactual setting, the mediator is allowed for choosing its "natural" value: "let the mediator choose the value it would have, for individual, in the presence of treatment." The natural direct effect is defined as:

$$\mathrm{NDE}_{x,x'}(Y) = E(Y_{x',M_x}) - E(Y_x) \tag{16.12}$$

The specific case can be written as:

$$\mathrm{NDE} = P(Y_{M=M_0} = 1|X = 1)) - P(\mathrm{Y}_{M=M_0} = 1|X = 0), \tag{16.13}$$

where Y_{x',M_x} represents the value that Y would attain under the operation of setting $X = x'$ and, simultaneously, setting M to whatever value it would have obtained under the setting $X = x$.

Pearl (2001) showed under certain assumptions of "no confounding" the natural direct effect can be reduced to:

$$\mathrm{NDE}_{x,x'}(Y) = \sum_m [E(Y|x', m) - E(Y|x, m)]p(m|x) \tag{16.14}$$

The intuitive interpretation is that the natural direct effect is the weighted average of the controlled direct effect, using the causal effect $p(m|x)$ as a weighing function.

The natural indirect effect of the transition from x to x' is defined as:

$$\mathrm{NIE}_{x,x'}(Y) \triangleq E[(Y|x, m_{x'}) - E(Y_x)] \tag{16.15}$$

It is the expected change in Y affected by holding X constant, at $X = x$, and changing M to whatever value it would have attained had X been set to $X = x'$. The NIE in the specific case is defined as:

$$\mathrm{NIE} = P(Y_{M=M_1} = 1|X = 0) - P(Y_{M=M_0} = 1|X = 0) \tag{16.16}$$

Pearl (2001, 2010) showed that the total effect of a transition in general is $\mathrm{TE}_{x,x'}(Y) \triangleq E(Y_{x'} - Y_x) = \mathrm{DE}_{x,x'}(Y) - \mathrm{IE}_{x,x'}(Y)$, and in linear systems, the standard additive formula $\mathrm{TE}_{x,x'}(Y) = \mathrm{DE}_{x,x'}(Y) + \mathrm{IE}_{x,x'}(Y)$ can be obtained when reverse transitions amounts to negating the signs of their effects.

Generalize the Counterfactual Mediation Analysis

The general formula for mediation effects in the counterfactual-based settings can be written as: $\mathrm{NIE}_{x,x'}(Y) = \sum_m E(Y|x,m)[P(m|x') - P(m|x)]$, and the specific formula can be written as:

$$\begin{aligned}\mathrm{NIE} = &\sum\nolimits_m [P(M = m|X = 1) - P(M = m|X = 0)] \\ &\times P(Y = 1|X = 0, M = m).\end{aligned} \tag{16.17}$$

Owning to its generality and ubiquity, Pearl (2010) called this expression as the "mediation formula," which consists two formula: the natural direct effect and the natural indirect effect. This general formula for mediation effects is applicable to nonlinear and nonparametric models as well as any type of variables. First, it does not need to make any assumptions for X, Y, and M. So it can be used in nonlinear models. For example, when the outcome Y is binary, the ratio (1 − IE/TE) represents the fraction of direct effect and (1 − DE/TE) represents the fraction of mediated effect. Second, it can be extended to perform nonparametrical data (VanderWeele 2009; Pearl 2010).

Allow for the Presence of Independent Variable-Mediator Interactions

Mediation analysis in the presence of interaction is one of important topics in the SEM-based mediation literatures (Preacher et al. 2007; MacKinnon 2008; Hayes 2013). However, within the SEM-based mediation framework for a linear causal model, the formula of indirect effect equals to total effect minus direct effect cannot work out in the models that involve interaction or moderation (Pearl and Mackenzie 2018) (p. 322).

One of important developments in counterfactual framework is to extend the SEM-based mediation formulae to allow for the presence of independent variable or exposure-mediator interactions (VanderWeele and Vansteelandt 2009, 2010; VanderWeele 2010, 2013, 2014; Valeri and VanderWeele 2013). The works from the counterfactual framework are to develop the interaction methods and provide appropriate interpretations. The development is still within the decomposition setting: decomposing total effect into a direct and indirect effect. For example, VanderWeele and Vansteelandt (2009, 2010) derived methods for estimating direct and indirect effects in linear and logistic regressions with the independent variable

(exposure)-mediator interaction. These works extended causal mediation analysis for parametric models with interactions from Pearl's mediation formula (Pearl 2001) and generalized SEM-based regression approach proposed by Baron and Kenny (1986).

Add No-Confounding Assumptions to Ensure a Casual Interpretation

Robins and Greenland (1992) not only showed that the SEM-based method through adjusting for the mediator to estimate direct effects is biased but also demonstrated that further assumptions must need to obtain a valid estimate of the direct and the indirect effects.

The counterfactual framework adopted the sufficient cause framework in statistics, philosophy, and other sciences (Cole and Maxwell 2003). Based on the discussions in SEM-based mediation analysis (Judd and Kenny 1981a, b; James and Brett 1984; MacKinnon 2008), the researchers in counterfactual-based mediation analysis thought that no-confounding assumptions could ensure a casual interpretation for controlled direct effect and natural direct and natural indirect effects. The no-confounding assumptions are (1) no unmeasured confounding of the treatment-outcome relationship, (2) no unmeasured confounding of mediator-outcome relationship, (3) no unmeasured confounding of the treatment-mediator relationship, and (4) no mediator-outcome confounder that is affected by the treatment. The first two assumptions are needed to ensure identifiability of controlled direct effect, while the last two assumptions are for the identifiability of natural direct and indirect effects (Valeri and VanderWeele 2013). An extensive discussion of the assumptions necessary for causal inference can be found from Holland (1988), including randomization, linear effects, and the fully operated treatment effect (i.e., no partial intervening variable effect).

Final Check with a Sensitivity Analysis

When you are aware that unmeasured confounding may be an issue in your study, the researchers in counterfactual-based mediation analysis typically recommend you make a final check with the sensitivity analyses (Imai et al. 2010a, b; VanderWeele 2010, 2015).

16.2.2.4 The Linking of Counterfactual-Based and SEM-Based Mediation Analyses

Counterfactual-based mediation framework in its early development has begun adopting the formal interpretation and symbolic machinery for analyzing such counterfactual relationships from structural equation models (Pearl 2010). The researchers in the field of counterfactual or causal inference explicitly recognized

the connections between counterfactuals and structural equations (Simon and Rescher 1966; Balke and Pearl 1995; Pearl 2009a, b). Currently, the SEM framework and central issues including distinction of mediator and moderator and causal pathways have been widely adopted by counterfactual-based mediation analysis. The counterfactual-based researchers have focused on developing the tools to reduce confounding, emphasizing the assumptions and generalizing the approaches that developed in linear parametric models to nonlinear and nonparametric models.

First, methodological principles of causal inference have been developed using structural modeling approach. Under the structural theory, every causal relationships should be investigated following four structured steps (Pearl 2010): (1) *define* the target quantity, (2) *assume* causal relationships along with graphics to represent pathway structure, (3) *identify* the target quantity, and (4) *estimate* or *approximate* the target quantity. The first priority in SEM is to define the target quantity as "causal effect," "mediated effect," "effect on the treated," or "probability of causation." The second most important thing in SEM is to explicate causal assumptions, which is highly evaluated by counterfactual researchers as having removed the lingering difficulty for causal analysis (Pearl 2010).

Second, through defining controlled direct effects, natural direct and natural indirect effects, and their implements, counterfactual-based mediation analysis shifts the mediation analysis into causal mediation analysis under the counterfactual framework. For example, counterfactual-based mediation analysis emphasizes the importance of defining direct effect. Having been affected by the sufficient cause framework, the approach of counterfactual-based mediation analysis focuses on "holding the mediating variables fixed" to define the controlled direct effects. Through defining the controlled direct effect, the pathway from independent variable to outcome is directly linked regardless of whether confounders are present and whether the error terms are correlated or not (Pearl 2010).

Third, mediation in counterfactual-based mediation setting can be defined and implemented in nonlinear models and even can be performed in nonparametric estimation through a two-step regression. However, when multiple mediators appear, the mediated effects need to be estimated through a parametric approximation (VanderWeele 2009).

16.2.2.5 Typical Features of Counterfactual-Based Mediation Framework

The counterfactual theories of causation basically tell us that the meaning of causal claims can be explained in terms of counterfactual conditionals of the form "If A had not occurred, C would not have occurred."

In summary, counterfactual-based mediation analysis has following features:

1. Emphasize on the no-confounding assumptions and conceptual definitions of causal effects (VanderWeele 2015) (p. 30).
2. Use the counterfactual framework to translate the SEM approach.

3. Avoid simple linear models and allow for nonlinearities and interactions.
4. Create the notions of natural direct and the natural indirect effects and the decomposition property: a total effect = natural direct and the natural indirect effects.
5. Emphasize the importance and advantages of decomposition property: under the counterfactual mediation framework, a total effect can be decomposed into a direct effect and an indirect effect even when under the context of interactions and nonlinearities. For example, when an independent variable-mediator interaction is presented and when the outcome is binary (VanderWeele 2015) (p. 34), the decomposition property is still valid.
6. Emphasize sensitivity analysis for mediation.

16.3 Mediation Models in Omics Studies

Recently, with the advent of high-throughput biomedical data generated by new technologies, such as microarrays, next-generation sequencing, and high-throughput metabolomics, a few mediation models have been proposed to analyze multiple mediators in high-dimensional data setting. We briefly review them as below (Table 16.1).

16.3.1 Test Multiple Putative Mediators Simultaneously Based on Permutation (MultiMed)

Boca et al. (2014) proposed a permutation-based approach to test multiple putative mediators between a known risk factor and a disease in omics studies while controlling the family wise error rate. The permutation tests start with testing for a single association and then extend to test multiple associations based on either the coefficient of independent variable or the correlation coefficients between the independent variable and outcome. Both permutation methods for testing a single mediator and the extensions for testing multiple mediators within the SEM-based mediation framework are described.

Basically the testing framework states that for a significant effect of the independent variable on the outcome to exist, the correlation between the two has to be nonzero, $\rho(X, Y) > 0$. Furthermore, if the relationship is in fact mediated by M, both the correlation between the independent variable and the mediator and the conditional correlation of the mediator and the outcome, given the independent variable, should be nonzero, $\rho(X, M) > 0$ and $\rho(rM|X, rY|M) > 0$, respectively. Here, $rM|X$ and $rY|X$ denote the residuals of the conditional correlation on regression of X on M and X on Y, respectively. Some strategies of extension to non-normally distributed models are also discussed such as using the counterfactual-based approach proposed in (VanderWeele and Vansteelandt 2010) for binary outcome.

Table 16.1 Main techniques used in omics mediation models

Model/ study	Mediation framework	Mediator, exposure, and outcome	Method for testing mediation effect	Technique for reducing dimensionality
MultiMed (2014) (Boca et al. 2014)	SEM-based	Single and multiple (or high-dimensional), biological mediators (genes or metabolites); continuous or discrete exposure; and continuous and binary outcomes	Nonparametric permutation test	Correlation
HIMA (2016) (Zhang et al. 2016)	SEM-based	Multiple (or high-dimensional), biological (DNA methylation) mediators; single continuous exposure; and continuous outcome	Joint significance test	Sure independence screening and regularization/penalization (i.e., minimax concave penalty)
CausalMM (2016) (Huang and Pan 2016)	Counterfactual-based	Single and multiple (or high-dimensional), continuous biological mediators (genes); single continuous exposure (microRNA); and continuous and dichotomous outcomes	Monte Carlo testing procedure	Spectral decomposition

16.3.2 Reduce High Dimensionality of Mediators Through Regularization or Penalization (HIMA)

Zhang et al. (2016) extended the multiple mediator model to the high-dimensional setting, which was motivated by an epigenome-wide DNA methylation study. In this study the authors applied their developed model to study how the high-dimensional DNA methylation markers mediate the relationship between smoking and lung function. For high-dimensional data, one of the most important statistical challenges is how to deal with the large p and small n (the number of variables larger than the sample size) problem. The proposed method takes three key steps to ensure that the mediation model is appropriate for modeling the high-dimensional omics data.

First, the proposed method employs the sure independence screening (SIS) (Fan and Lv 2008) to identify those mediators with large absolute effect and reduce the very large pool of potential mediators to the number of less than the sample size so that the dimensionality of the mediators is reduced and the mediators can be modeled. Second, the proposed method conducts the variable selection through

regularization or penalization with the minimax concave penalty (MCP) (Zhang 2010) to most likely identify important mediators. Third, it carries out joint significance testing for mediation effects based on the MCP-penalized estimate in Step 2 to increase the power of hypothesis testing.

16.3.3 Transform High-Dimensional Mediators into Low-Dimensional and Uncorrelated Mediators Using the Spectral Decomposition (CausalMM)

Except through regularization or penalization to reduce high dimensionality of mediators as above Zhang et al.'s approach, another way of reducing high-dimensional continuous mediators is to transform the correlated high-dimensional mediators into a series of causal mediation models with single continuous mediator. For example, Huang and Pan (2016) used spectral decomposition to transform high-dimensional gene expression mediators into low-dimensional and uncorrelated mediators. Different from the approach proposed by Zhang et al. (2016), which was developed based on SEM framework, this approach was developed under a causal mediation model, and the mediation effect is defined with counterfactual notation (such as, natural indirect effect). Thus CausalMM can include the interaction of independent variables and mediators. To ensure to appropriately identify the estimates of mediation effects, CausalMM also discussed and proposed the sufficient model assumptions.

16.3.4 Remarks

All above three models were proposed to deal with multiple mediators, and the last two were also to target reducing high dimensionality. They may be appropriate to address some features of omics data such as high-dimensional and under-sampled issues. Because the proposed methods were developed for high-dimensional omics data, they could inspire the researchers in the field of microbiome to develop their own methods. For example, the proposed methods could provide the insights for microbiome researchers into how to model multiple mediators, how to reduce dimensionality, and how to use counterfactual-based framework to include the interaction of independent variable and mediator. However, there exist limitations in the proposed methods because the data features and structures between microbiome and omics are not completely the same: except for high dimensionality, microbiome data are also compositional and over-dispersed. Thus, it is still difficult to generalize the applications of the methods developed in omics to microbiome data. Additionally, almost all the mediation approaches in omics studies were developed within linear regression-based framework. Thus, they describe the

relationship between the independent variable (X), mediator (M), and outcome (Y) in terms of linear regressions. However, microbiome data are structured as a phylogenetic tree, high-dimensional, compositional, and highly skewed and often with many zeros, which violates the basic assumptions (e.g., normality or linearity) of existing methods. Thus a linear-based mediation model is not appropriate for microbiome data.

Specifically, both Boca et al.'s and Zhang et al.' approaches are restricted to the SEM-based mediation setting. The normal assumptions among independent variable, mediator, and outcome are challenging in omics studies. Like other SEM-based mediation approaches and counterfactual-based causal mediation approach, the implementation of Boca et al.'s approach needs to assume that the casual paths exist, while no unmeasured confounders exist. An R package called MultiMed (with the function name medTest) is included in online supplementary material. The R package called HIMA for implementing the approach by Zhang et al. (2016) is available at https://github.com/YinanZheng/HIMA. The R code called MedTest for implementing the approach by Huang and Pan (2016) is available from the supplement materials of the paper. Other mediation models were also proposed in omics studies, such as Bayesian regularized mediation analysis with multiple exposures (Wang et al. 2019) and high-dimensional mediation analysis, to identify causal genes (Zhang 2019).

16.4 Specifically Designed Mediation Models in Microbiome Studies

Statistically, both traditional mediation model and related mediation models in other omics studies provide the frameworks, concepts, procedures, and notations for newly proposed mediation models of microbiome data. However, neither traditional mediation models nor related mediation models in other omics studies can fit the unique features of microbiome data, as indicated in the motivating examples. In current years, several mediation models have been specifically designed for analysis of microbiome data. We will focus on introducing the frameworks and methods of mediation analysis of microbiome data throughout the rest of the review book chapter below (Table 16.2).

16.4.1 Distance-Based Omnibus Test of Mediation Effect (MedTest)

16.4.1.1 MedTest Method

Chen and his colleagues (Zhang et al. 2018) proposed a distance-based approach for testing the mediation effect of the human microbiome. The method was developed to

Table 16.2 Main techniques used in microbiome mediation models

Model/study	Mediation framework	Mediator, exposure and outcome	Method for testing mediation effect	Technique for reducing dimensionality
MedTest (2018) (Zhang et al. 2018)	SEM-based	Multiple (or high-dimensional), distance-based microbiome mediators (either continuous or binary); single continuous exposure; and single continuous outcome	Permutation test of the overall mediation effect	Sample-wise multiple distance metrics (phylogenetic tree-based and non-tree-based distances)
MODIMA (2019) (Hamidi et al. 2019)	SEM-based	Single mediator and multiple (or high-dimensional), multivariate distance-based microbiome mediators; multivariate continuous and binary exposures, and multivariate continuous outcome	Permutation test	Pearson distance correlation and partial distance correlation
CCMM (2019) (Sohn and Li 2019)	Counterfactual-based	Multiple (or high-dimensional) and compositional microbial mediators; single continuous or binary treatment (exposure); and single continuous outcome	An extension of the Sobel test and a bootstrap test	Log contrast compositional regression
IsometricLRTMM (2019) (Zhang et al. 2019)	SEM-based	High-dimensional and compositional, specific isometric log-ratio-transformed mediator; single continuous exposure and continuous outcome	Joint significance test of the exposure and on the mediator, and the mediator on the outcome	Isometric log-ratio-based transformation and de-biased Lasso technique

(continued)

Table 16.2 (continued)

Model/study	Mediation framework	Mediator, exposure and outcome	Method for testing mediation effect	Technique for reducing dimensionality
SparseMCMM (2020) (Wang et al. 2020)	Counterfactual-based	Multiple (or high-dimensional) and compositional microbial mediators; single binary treatment, and single continuous outcome	Permutation tests and, in particular, linear log contrast regression model and Dirichlet regression model are used to estimate the causal direct effect of treatment and the causal mediation effects of microbiome at both the community and individual taxon levels	Regularization techniques
MedZIM (2020) (Li et al. 2020)	Counterfactual-based	Single mediator (microbial taxon); single binary exposure; and single continuous outcome	Delta method, Riemann-Stieljes integration, and bootstrapping	Not available
NPEM (2020) (Carter et al. 2020)	SEM-based	Multiple (or high-dimensional) mediators (microbial taxa); multiple continuous exposures and single dichotomous outcome	Iterative one-sided Extreme Studentized Deviate test and the chi-square test	Feature reduction techniques of information theory and Mahalanobis distance

analyze the biological and high-dimensional mediation effects. An R package called MedTest was developed for fitting the proposed distance-based omnibus test of mediation effect model. Hereinafter, we refer to this method as the MedTest method. The MedTest method uses the sample-wise distance matrices (both the phylogenetic tree-based and non-tree-based) instead of working with the original OTU data. The purposes of using distance matrices as input data are for dimension reduction and for implicitly capturing the (nonlinear) transformation of the OTU abundances. This method also uses permutation test for adjusting the potential non-normally distributed test statistic and thus can properly control the type I error.

16.4.1.2 Using Distance Metrics to Reduce High Dimensionality

In this approach, the mediator M is microbiome feature vector defined as $f_M^{(l)} = (l = 1, \ldots, L)$ or, more generally, defined as a scalar function of the original OTU abundance vector $f_M^{(l)} : \mathbb{R}^m \to \mathbb{R}$ where microbiome feature presents the abundance or prevalence of a taxonomic group, the weighted average of several functionally related OTUs, or even the richness of the entire microbial community. The mediation model can be written as:

$$\begin{aligned} Y &= X\gamma^* + \varepsilon \\ f_M^{(l)} &= X\alpha_l + \varepsilon_l'(l = 1 \ldots L) \\ Y &= \sum\nolimits_l^L f_M^{(l)}\beta_l + X\gamma + \varepsilon'' \end{aligned} \tag{16.18}$$

with the null hypothesis $H_0 : \alpha_l\beta_l = 0$ for $\forall f_M^{(l)}$ where the coefficient vectors γ^* and γ represent the total effect and the direct effect of the independent variable X on the outcome Y, respectively, and ε, ε_l', and ε'' are random error terms that are independent of X and the scalar function $f_M^{(l)}$. Potential confounders can be included, as in general multivariate model given a predictor vector X. There are two distinctive features of this method: (1) it jointly analyzes all OTUs based on community-level analysis and uses principal coordinate analysis (PCoA) on a distance matrix to incorporate the tree structure information of microbiome data. Thus, this approach not only captures the variation of evolutionarily related OTUs but also avoids multiple testing issues. (2) The mediation effects are tested through a distance-based test statistic and a nonparametric permutation test, which accommodates the non-normally distributed microbiome data.

16.4.1.3 Remarks

MedTest highlights the importance of omnibus test based on multiple distance measures such as unweighted, weighted, and generalized UniFrac distances and Jaccard and Bray-Curtis distances over a specific distance measure. Given the multivariate, non-normality, sparsity features of microbiome data and mediating microbiome taxa are unknown a priori, the proposed distance-based omnibus test of mediation effect model is an appropriate alternative approach to fit mediation effects of microbiome data. However, the model also has disadvantages such as (1) this mediation model only teats microbiome features (taxa) as mediators but does not fit the mediation analysis that treats microbiome as independent variables or outcomes and other factors (environmental, genetic, or disease) as mediators. (2) This model is unable to differentiate direct effect from indirect effect because it does not directly test mediation effects for individual taxa. The package MedTest is freely available at https://github.com/jchen1981/MedTest. The paper on MedTest

method was cited in these studies (Koh 2018; Hamidi et al. 2019; Leong 2019; Zhang et al. 2019; Zitnik et al. 2019).

16.4.2 Multivariate Omnibus Distance Mediation Analysis (MODIMA)

16.4.2.1 MODIMA Method

The framework of MODIMA (Hamidi et al. 2019) is also distance-based multivariate omnibus test, which incorporates an entire omics assay as a mediator. In contrast to the approach of distance-based omnibus test of mediation effect model (MedTest), which uses the sample-wise distance matrices (PCoA) to reduce dimensions and to capture the nonlinearity of OTUs, MODIMA analyzes the multivariate exposure-mediator-response triples through partial distance correlation.

MODIMA method consists of two components: MODIMA test statistic and permutation test. The MODIMA approach is under the framework of Pearson correlations and partial correlations, but it uses distance correlation and partial distance correlation statistics to ensure the correlation between vector-valued random variables instead of between random variables. The idea is similar to MedTest method, which uses a sample-wise distance matrix to reduce dimensionality. By using a distance metric (such as Euclidean distance or specialized distance for microbiome data), MODIMA develops a test statistic to test the dependences among independent variables (X), mediator variables (M), and outcome variables (Y) within each random vector. The test statistic is written as:

$$S_d(d_X(X), d_M(M), d_Y(Y)) = d\mathrm{Cor}(d_X(X), d_M(M))pd\mathrm{Cor}(d_Y(Y), d_M(M)|d_X(X)), \tag{16.19}$$

where $d(.)$ present appropriate pairwise distance matrices computed from the potentially multivariate observations of independent variables (X), mediator variables (M), and outcome variables (Y). Equation (16.19) indicates that distance matrices for independent variables $d_X(X)$ mediator variables $d_M(M)$, and outcome variables $d_Y(Y)$ are the input data for both distance correlation between the independent variables and mediator variables and partial correlation between the mediator variables and the outcome variables.

16.4.2.2 Permutation Testing of Mediation Effects

Following from Boca et al. (2014), MODIMA method takes the permutation testing approach. The test statistic is:

$$P = \frac{1}{q}\sum_{i=1}^{q} 1(S_d \leq S_d(i)), \tag{16.20}$$

where S_d is the MODIMA test statistic under the null hypothesis; P is the p-value of the observed S_d; $S_d(i)$ is the re-computed test statistic when the correlation between the independent and mediator variables is smaller than the partial correlation between the outcome and the mediator variables; and q is the number of permutations.

16.4.2.3 Remarks

The mediation approach of MODIMA is built on that of Boca et al. (2014) and extends it to the high-dimensional data through correlating the distance matrices between the independent and mediator variables and partially correlating the distance matrices between the mediator and the outcome variables given the matrices of independent variables. Compared to traditional approaches and distance-based omnibus test of mediation effect, MODIMA allows for multivariate exposures and responses. It was shown that MODIMA methods are robust and sensitive and increasing empirical power compared to the methods of Boca et al.'s permutation-based testing and Zhang et al.'s sample-wise distance matrices (Hamidi et al. 2019).

Currently, mediation models for microbiome data are still rare; MODIMA is an alternative method that works on a specific distance metric. However, MODIMA methods also have some limitations. For example, (1) the development of MODIMA was based on distance correlation and partial distance correlation methods. Further research is necessary to conclusively validate on their appropriateness of application to a given microbiome dataset. (2) The comparisons of MODIMA versus MultiMed and MedTest were conducted in the single-mediator model, and the comparisons of MODIMA versus MedTest were conducted in the multiple mediator model. However, the data that were used to compare the different models may not be appropriate or at least not optimal because the data simulated by MODIMA assuming normal distribution and linear correlations for independent, mediator, and outcome variables. MODIMA also set the mediator parameters based on Dirichlet-multinomial distribution and a mixture of two datasets (saliva and tonsils) from the Human Microbiome Project to model over-dispersion of microbiome data. Microbiome data are typically not normal, not linear, and the dissimilarities between taxa are not Euclidean distances. Thus, the assumptions of normal distributions, linearity, and Euclidean distance do not typically present the features of microbiome data. (3) The comparisons between MODIMA and MedTest conducted by MODIMA were based on the Euclidean distance and three dissimilarity matrices: Bray-Curtis, Jensen-Shannon divergence (JSD), and UniFrac. The performances may not be comparable because the matrices used in MODIMA are not exactly same as those used in MedTest. And (4) compared to MedTest, one more limitation of MODIMA is that it has works on a specific distance metric rather than pooling analyses from multiple metrics (Hamidi et al. 2019). The R package "energy" can be used for

calculating the distance correlation of the independent-mediator variables and partial distance correlation of mediator-outcome variables (Székely and Rizzo 2018). The R package MODIMA test is available at https://github.com/Alekseyenko/MODIMA.

16.4.3 Causal Compositional Mediation Model (CCMM)

16.4.3.1 CCMM Method

CMM (Sohn and Li 2019) presents compositional mediation model. The R package developed for CMM is called "ccmm" presenting causal compositional mediation model. The model takes the compositional approach to estimate the causal direct and indirect effects when mediators are high-dimensional and compositional. CMM consists of two components (Sohn and Li 2019): (1) a sparse compositional mediation model aiming to estimate the causal direct and indirect (or mediation) effects in the simplex space and (2) tests of total and component-wise mediation effects using bootstrap. The framework of CMM was developed using two components of techniques: compositional estimation methods (Aitchison 1982; Billheimer et al. 2001) and the linear log contrast regression (Lin et al. 2014; Shi et al. 2016). The techniques of composition and linear log contrast ensure the compositional data "legally" in Aitchison's simplex space and have desirable properties. The compositional mediation model is written as:

$$M_i = \left(m_0 \bigoplus a^{T_i} \bigoplus h^{X_i}\right) \bigoplus U_{1i}, \tag{16.21}$$

$$Y_i = c_0 + cT_i + (\log M_i)^T b + gX_i + U_{2i}, \text{ subject to } b^T 1_k = 0, \tag{16.22}$$

where m_0 and c_0 are the baseline composition for M_i and Y_i, respectively; a, b and c are path coefficients; h and g are nuisance coefficients corresponding to the covariate X; and 1_k is a vector of k ones. The distribution of U_{1i} is not specified, while the distribution of U_{2i} is assumed as normally distributed with $U_{2i} \sim N(0, \sigma^2)$.

With the baseline composition m_0, composition parameter a, and nuisance h, the formulations in Eq. (16.21) are to express how a treatment perturbs a composition from the baseline composition. The main goal of Eq. (16.21) is thus to ensure all the calculations within the simplex space and therefore have an intuitive interpretation. Equation (16.22) is a typical regression model which links a treatment and a composition to an outcome in this case. The distinctive feature is the imposed linear constraint, $b^T 1_k = 0$, which plays a key role for ensuring the estimated regression coefficients have desirable properties for compositional data.

16.4.3.2 Hypothesis Testing of Mediation Effects

Through formulating the equations of (16.21) and (16.22) under the mediation framework, the model decomposes the total effect of T_i on Y_i into the direct effect, c, and the total indirect effect, $(\log a)^T b$. CMM is developed for the compositional data. It has the compositional and additive properties: the total indirect effect equals to the sum of the component-wise indirect effects. We can write the null hypothesis of no total compositional mediation effect as below:

$$H_0 : (\log a)^T b = 0 \tag{16.23}$$

The null hypothesis of no component-wise mediation effect can be written:

$$H_0 : \log\left(ka_j\right) b_j = 0, \forall_j \in \{1, 2, \cdots, k\}. \tag{16.24}$$

because the different mediators may have positive or negative mediation effects, which results in the total mediation effect not correctly presenting the actual mediation effect. The authors of this model suggest testing both hypotheses to avoid a misleading conclusion about the mediation effect.

The null hypotheses (16.23) and (16.24) are tested by an extension of the Sobel test (Sobel 1982) and a bootstrap approach with the later to avoid imposing an assumption of normality for the indirect effect (Sohn and Li 2019). With a sensitivity analysis, a causal total indirect effect is developed. However, we have no intention to introduce the development here. The interested readers can reference the original paper for the details.

16.4.3.3 Remarks

CCMM method has been the first to introduce compositional techniques in mediation analysis and may effectively avoid large p and small n problem (i.e., the parameter space larger than the sample size). Furthermore, CCMM may have higher power in detecting mediating taxa when no zero counts exist (Carter et al. 2020). However, CCMM method has been reviewed having limitations. For example, (1) as a common strategy in compositional data analysis, CCMM replaces zero counts by arbitrary error term of 0.5. This is a limitation, which fails to address the zero-inflated data structure (Xia et al. 2018a, b, c, d; Li et al. 2019). Actually, CCMM could fail to converge when the proportion of zeros is high and number of taxonomic units is large (Carter et al. 2020). (2) CCMM could result in much higher false-positive rates to detect the associations between exposure variables and taxonomic abundance because this method does not correct for correlation between exposure variables (Carter et al. 2020). Moreover, CCMM method only considers univariate (i.e., a single) exposure variable (Zhang 2019; Carter et al. 2020) and is proposed for continuous response, though theoretically it could handle a binary response via a

logit link function (Carter et al. 2020). The R package "ccmm" is available at https://rdrr.io/cran/ccmm/. Examples of using CCMM method are available in this study (Tang et al. 2019). Papers of citing the CCMM method can be found from these publications (Li 2019; Srinivasan et al. 2019; Wang et al. 2019; Zhang et al. 2019; Zitnik et al. 2019; Walter et al. 2020).

16.4.4 Isometric Log-Ratio Transformation for Microbiome Mediation (IsometricLRTMM)

16.4.4.1 IsometricLRTMM Method

Zhang et al. (2019) proposed a mediation method for microbiome data using isometric log-ratio transformation. The motivation of this development is that current mediation methods are incapable of inferencing for a specific mediator in the presence of high-dimensional nuisance confounders. The proposed method has two main steps: first, uses the isometric log-ratio transformation (Egozcue et al. 2003) to transform the relative abundance (RA) of microbiome data, and then uses ilr-transformed variables as the mediator variables (compositional mediators). Second, uses the de-biased Lasso technique (Zhang and Zhang 2014) to estimate and test the mediation effect of a mediator of interest among a large number of mediators. The proposed method was developed under the SEM-based mediation framework.

16.4.4.2 Inference on the Ilr-Transformed Mediation Effect

Since the proposed method was developed within the SEM-based mediation framework, it uses the product coefficient ($\alpha_k\beta_k$) method to inference the mediated effects. Where α_k represents the relation between independent variable (X) and mediators (M), β_k is the regression coefficient vector representing the relation between mediators (M) and outcome (Y) adjusting for the effects of X and the covariates (Z). The null hypothesis of testing for mediation effect can be written as below:

$$H_0 : \alpha_1\beta_1 = 0 \text{ vs.} H_1 : \alpha_1\beta_1 \neq 0. \tag{16.25}$$

In above hypothesis testing, the effects of X on M (α_1) can be estimated via ordinary least squares (OLS) estimator; however, one big challenge of analyzing microbiome data is the issue of high dimensionality, which results in large p and small n problem (in this case, the number of mediators p is larger than the sample size n). To solve this problem, the proposed method utilizes the de-biased Lasso technique to estimate β_1. Next, the mediation effect $\alpha_1\beta_1$ is tested through the joint significance test developed by the same authors (Zhang et al. 2016).

16.4.4.3 Remarks

Zhang et al. (2019) differentiate their ilr-transformation method from CCMM method in three aspects: first, the framework of CCMM method is established directly in the simplex space, while the high-dimensional mediation model of ilr-transformation method is constructed in the Euclidean space via using the ilr-transformed mediators. Second, the techniques that these two methods used to transform mediators (M) are different: CCMM method uses the additive log-ratio (alr) transformation, while the ilr-transformation method utilizes the isometric log-ratio transformation. Because these two approaches use different transformations, their mediation models have different capabilities: CCMM method can be used to detect the mediation effect of individual composition, whereas ilr-transformation method can be used to find the relative mediation effect of a specific composition versus the rest of compositions. Third, their statistical methods for testing compositional mediators are different: CCMM method uses the Sobel test (Sobel 1982), whereas the ilr-transformation method employs the joint significance test (Zhang et al. 2016).

The proposed ilr-transformation method targets two features of microbiome data: compositionality and high dimensionality. This presents one direction of newly developed statistical methods for analyzing microbiome data. However, the proposed method has some limitations: first, the ilr-transformation is an orthonormal isometry, which addresses certain difficulties of additive log-ratio (alr) and centered log-ratio (clr) transformations, but its interpretability is subject to the selection of its basis (Xia et al. 2018a, b, c, d) (p. 338). For example, the relative mediation effect is difficult to interpret biologically, which may limit its application in microbiome data. Second, similar to other compositional data analyses, the proposed method replaces zero counts by the maximum rounding error 0.5. Microbiome data are sparse, which is mainly due to zeros and small values in read counts. Replacing zero values with small nonzero counts cannot solve sparsity problem; rather it makes the sparsity problem even more complicated (Xia et al. 2018a, b, c, d) (p. 389). Third, as Zhang et al. (2019) stated, the joint significance test method of medication effects is not a robust inference method because the p-values calculated for the exposure and ilr-transformed mediators and the outcome variable and ilr-transformed mediators are both based on the normal distribution assumptions. The proposed method paper was cited in this publication (Srinivasan et al. 2019).

16.4.5 Sparse Microbial Causal Mediation Model (SparseMCMM)

16.4.5.1 Casual Mediation Model

Similar to CCMM, SparseMCMM (Wang et al. 2020) was specifically designed for analyzing causal microbiome effects and identifying the specific microbial agents in

the high-dimensional and compositional microbiome data. However, the focuses of these two approaches have some differences. In CCMM (Sohn and Li 2019), the package that implements the CCMM method is named as "ccmm" presenting "causal compositional mediation model," and the sufficient causal assumptions for detecting causal effects are discussed; however, the model is called as "compositional mediation model (CMM)," in which "causal mediation" is only implicated.

In contrast, the SparseMCMM (Wang et al. 2020) was named as "microbial causal mediation model" and explicitly stated that the method follows VanderWeele's causal counterfactual mediation framework.

The SparseMCMM was designed to detect the association among three factors: treatment (T, binary variable), microbiome (M, a vector of compositional microbial mediators), and outcome (Y, continuous variable). The log-ratio analysis (Aitchison 1982) and Dirichlet regression (Hijazi and Jernigan 2009) are the two available methods to analyze compositional (relative abundance) data. The distinctive feature of SparseMCMM lies on its combining these two compositional methods together and then assessing the causal mediation effect of microbiome on the outcome under the counterfactual mediation framework (VanderWeele and Vansteelandt 2009, 2010; VanderWeele 2016). SparseMCMM consists of two basic regression models: compositional or log-ratio analysis model and Dirichlet regression.

Compositional (Log-Ratio Analysis) Model

The compositional (log-ratio analysis) model is written as:

$$\begin{aligned} Y_i &= \alpha_0 + \alpha_T T_i + \alpha_M^T[\log(M_i)] + \alpha_C^T[\log(M_i)]T_i + \alpha_X^T X_i \\ &\quad + \varepsilon_i;\ \text{subject to } \alpha_M^T 1 \\ &= 0,\ \text{and } \alpha_C^T 1 = 0, \end{aligned} \tag{16.26}$$

where α_0 is the intercept, α_T is the coefficient of treatment, respectively; $\alpha_M = \left(\alpha_{M_1}, \ldots, \alpha_{M_p}\right)^T$ and $\alpha_C = \left(\alpha_{C_1}, \ldots, \alpha_{C_p}\right)^T$ are the vectors of coefficients of microbial mediators and interactions between treatment and mediators, respectively; $\alpha_X = \left(\alpha_{X_1}, \ldots, \alpha_{X_q}\right)^T$ is the vector of coefficients of covariates; and $\varepsilon_i \sim N(0, \sigma^2)$ is the error term. With this formulation, this model is able to model the outcome that is determined by the treatment, compositional mediators, interactions between the treatment, and mediators and covariates. The two constraints terms $\alpha_M^T 1 = 0$ and $\alpha_C^T 1 = 0$ ensure that the analysis within the compositional domain.

Dirichlet Regression

The Dirichlet regression in the generalized linear model fashion is written as below:

$$E\left[M_{ij}\right] = \frac{\gamma_j(T_i, X_i)}{\sum_{m=1}^{p} \gamma_m(T_i, X_i)},$$
$$\log\left\{\gamma_j(T_i, X_i)\right\} = \beta_{0j} + \beta_{Tj} T_i + \beta_{Xj}^T X_i \tag{16.27}$$

where β_{0j} is the intercept and β_{Tj} and β_{Xj} are the coefficients of treatment and covariates for the jth taxon, respectively. The Dirichlet regression has two specific assumptions: (1) given treatment and covariates, mediators are distributed as Dirichlet regression; and (2) their microbial relative means are linked by treatment and covariates. Given above formulation, this model is able to model the microbial relative abundance as a function of treatment and covariates.

16.4.5.2 Hypothesis Testing of Microbiome Mediation Effects

To determine the direct and mediation effects, three additional equations are formulated under the counterfactual framework:

$$\mathrm{DE} = \alpha_T + \alpha_C^T E[\log(M)]T = 0, X], \tag{16.28}$$

$$\begin{aligned}\mathrm{ME} &= \left(\alpha_M^T + \alpha_C^T\right)\{E[\log(M)|T = 1, X] - E[\log(M)|T = 0, X]\} \\ &= \sum_{j=1}^{p} \left(\alpha_{M_j} + \alpha_{C_j}\right)\left\{E\left[\log\left(M_j\right)|T = 1, X\right] - E\left[\log\left(M_j\right)|T = 0, X\right]\right\} \\ &= \sum_{j=1}^{p} \mathrm{ME}_j\end{aligned} \tag{16.29}$$

Summing DE and ME gives the total effect of the treatment on the outcome:

$$\mathrm{TE} = \mathrm{DE} + \mathrm{ME} = E\left[Y_{T=1,M(T=1)} - Y_{T=0,M(T=0)}|X\right]. \tag{16.30}$$

where DE is the expected difference of the outcome between the treatment and without treatment when the mediators take the value under without treatment. ME presents the mediation effect (summation of the individual mediation effects from each taxon), which is the expected difference of the outcome between the mediators (microbiome composition) under treatment given covariates and the mediators under without treatment given covariates.

The term ME_j is the product of two coefficients $\left(\alpha_{M_j} + \alpha_{C_j}\right)$: the jth main effect's microbial coefficient and the interaction effect coefficient of this taxon and the treatment on the outcome; and $\{E[\log(M_j)|\, T = 1, X] - E[\log(M_j)|\, T = 0, X]\}$ represents the treatment effect on the jth taxon.

The hypothesis tests for microbiome mediation effects are developed at both community and taxon levels. The null hypothesis of no overall mediation effect (OME) at the community level $H_0 : \mathrm{ME} = 0$ is defined as:

$$\begin{aligned} \mathrm{OME} &= \left(\widehat{\alpha}_M^T + \widehat{\alpha}_C^T\right)\left\{\widehat{E}[\log(M)|T=1,X] - \widehat{E}[\log(M)|T=0,X]\right\} \\ &\equiv f\left(\widehat{\alpha},\widehat{\beta}\right), \end{aligned} \tag{16.31}$$

and the null hypothesis of taxon levels is to test whether at least one component-wise ME_j is significantly nonzero: $H_0 : \mathrm{ME}_j = 0, \forall\, j \in \{1,\ldots,p\}$.

By considering the high dimensionality of the mediators, it is defined as:

$$\mathrm{CME} = \sum_{j=1}^{p} \mathrm{ME}_j^2 \equiv g\left(\widehat{\alpha},\widehat{\beta}\right). \tag{16.32}$$

It is challenge to estimate the parameters. To obtain parameter estimation, several strategies were proposed, including estimating log-likelihood function from the models with the nonlinearity and constraints, high dimensionality of microbiome data, linear log contrast, and Dirichlet regressions. How to derive the asymptotic distributions of the mediation test statistics was also discussed. The interested readers can reference the original paper for details (Wang et al. 2020).

16.4.5.3 Remarks

SparseMCMM has the advantages: (1) compared to other microbiome medication models, it has capability of estimating microbiome mediation effects at both the community and taxon levels through rigorous statistical modeling framework. (2) It provides an approach to deal with the issues of compositional and high-dimensional mediators through log contrast regression and regularization techniques. (3) It is explicitly instrumented with covariates; therefore, this model is more feasible to be used for hypothesis testing of advanced and complicated microbiome mediations.

Especially, to compare SparseMCMM and MedTest, the different focuses and strengths have been noticed: MedTest focuses on detecting an overall mediation effect by using an ensemble of distance measures, whereas SparseMCMM has the capability of testing mediation effects at both community and taxon levels, and in the situation of both positive and negative component-wise mediation effects being present, its test of component-wise mediation effects (individual mediation effects from each taxon) is more powerful. SparseMCMM and CCMM share two general features: (1) they both were developed under the causal mediation framework with assuming that the mediators are compositional and high-dimensional. (2) Both methods emphasize that performing hypothesis testing at both total community- and component-wise mediation effects is important and are capable of conducting

hypothesis testing of total compositional and component-wise mediation effects. CCMM also provides a clear interpretation of component-wise indirect effects (Sohn and Li 2019). By considering that the opposite directions of different component-wise mediation effects could disguise the actual total mediation effect, CCMM also suggests conducting both total and component-wise mediation effects testing to avoid misleading results of medication effects. However, these two methods differentiate them from each other in several essential ways (Wang et al. 2020), for example, (1) either using Dirichlet regression (SparseMCMM) or using the algebraic structure of a composition under the simplex space to characterize the relationship between treatment and microbiome composition; (2) whether it is flexible (SparseMCMM) or not flexible (CCMM) to handle the interaction between treatment and microbiome to address potential concerns regarding the bias due to neglecting the presence of interaction effects; and (3) either selecting casual taxa using regularization techniques (SparseMCMM) or identifying the key taxa using confidence interval estimates (CCMM). The R package SparseMCMM has been developed for this proposed causal mediation models. It is available from https://sites.google.com/site/huilinli09/software and https://github.com/chanw0/SparseMCMM.

16.4.6 Mediation Analysis for Zero-Inflated Mediators (MedZIM)

16.4.6.1 MedZIM Method

MedZIM (Li et al. 2020) was developed under the potential outcomes (PO) framework to address the challenges of modeling mediation effects of the zero-inflated microbiome data structure. The goals of this method are to (1) disentangle the confusion of mediation effect due to zeros and (2) identify the observed zero-valued data to differentiate structural zeros (true zeros) from false zeros. The MedZIM method takes the approach of modeling the zero-inflated data as two-part distributions to decompose the mediation effect of a zero-inflated mediator into two components: a unit change of positive counts or continuous values and a discrete jump from zero to a positive value. Thus, based on this method, the total mediation effect is obtained by summing each of the estimated and tested mediation effects from these two components.

MedZIM consists of two equations and is written as:

$$Y = \beta_0 + \beta_1 M + \beta_2 1_{(M>0)} + \beta_3 X + \beta_4 X 1_{(M>0)} + \beta_5 XM + \varepsilon 1(.), \tag{16.33}$$

$$T(\theta) = \upsilon_0 + \upsilon_1 X, \tag{16.34}$$

where Eq. (16.33) presents a general form of linear parametric zero-inflated distributions, such as zero-inflated beta (ZIB), zero-inflated log-normal (ZILoN), and

zero-inflated Poisson (ZIP). The term of 1(.) is an indicator function, and ε is the random error term assuming normally distributed. The βs are regression coefficients. This model is able to model exposure-mediator interactions owning to including the two interaction terms: $X1(M > 0)$ and XM in Eq. (16.33). The function $T(\theta)$ in Eq. (16.34) models the association between mediator (M) and independent variable (X) (i.e., treatment or risk factor) through the parameter θ of the mediator M and two K-dimensional regression coefficients υ_0 (intercept vector) and υ_1 (slope vector).

16.4.6.2 Mediation Effect and Direct Effect Under the Counterfactual-Based Framework

The mediation analysis for zero-inflated mediator is implemented through a crucial Limit of Detection (LOD) mechanism for observing zero value of the mediator called as "LOD mechanism." LOD mechanism describes two types of zeros in the observed abundance data: true abundance of zero (i.e., absence) and abundance that is reported as zero as a consequence of the measurement procedure.

$$P(M^* = 0|M, L) = 1_{(ML<1)} \tag{16.35}$$

where M^* denotes the observed value of the mediator M. L is the library size (i.e., sequencing depth), and the product ML presents the sample absolute abundance (SAA) of the taxon in a sample. Under the LOD mechanism, a value of zero is observed when all SAA below 1: LOD.

The full formulation of direct and mediation effect or indirect effects is complicated. Briefly they are formulated under counterfactual-based framework using Riemann-Stieljes integration (ter Horst 1986). The model defines three equations: NIE (natural indirect effect), NDE (natural direct effect), and CDE (controlled direct effect). Summing NIE and NDE equals to the total effect:

$$\text{NIE} = E(Y_{x_2Mx_2} - Y_{x_2Mx_1}), \tag{16.36}$$

$$\text{NDE} = E(Y_{x_2Mx_1} - Y_{x_1Mx_1}), \tag{16.37}$$

$$\text{CDE} = E(Y_{x_2m} - Y_{x_1m}), \tag{16.38}$$

where NIE, NDE, and CDE are average values from changing x_1 to x_2. M_x is the value of M when the independent variable $X = x$, and Y_{xm} is the value of Y when $(X, M) = (x, m)$.

By plugging Eqs. (16.33) and (16.34) into the above definitions of NIE, NDE, and CDE and using Riemann-Stieljes integration, Li et al. (2020) showed that the natural indirect effect is determined by the mediation effect due to the change of the

mediator on its numeric scale (NIE_1) and the mediation effect due to the discrete binary change of the mediator from zero to a nonzero status (NIE_2). The mediation effect and direct effect and their confidence intervals (CI) for ZIB are obtained using the delta method and an alternative approach bootstrapping (Efron and Tibshirani 1986).

16.4.6.3 Remarks

MedZIM model has the advantages: (1) taking into account the zero-inflated structure of the mediator and (2) considering the mechanism for observing false zero values of the mediator (Li et al. 2020). It was shown that the proposed model outperforms the causal mediation analysis approach which was developed under a PO framework and implemented in R package "mediation" (Imai et al. 2010a, b; Tingley et al. 2017) with simulated data for the mediators of zero-inflated beta (ZIB), zero-inflated log-normal (ZILoN) and zero-inflated Poisson (ZIP), and two real datasets with ZIB. However, the MedZIM model also has some limitations. First, the proposed approach was only illustrated through ZIB, ZILoN, and ZIP. More evidences from other zero-inflated distributions are needed to prove the appropriateness of the proposed approach because the literatures have shown that zero-inflated negative binomial (ZINB), zero-hurdle negative binomial (ZHNB), and even negative binomial (Zitnik et al. 2019) are all better than ZIP in modeling zero-inflated and over-dispersed data including microbiome data (Xia et al. 2012b, 2018a, b, c, d; Xu et al. 2015; Xia and Sun 2017). Second, the proposed method takes a single-mediator approach analyzing each individual mediator one by one and then adjusting for multiple testing using the FDR method. This approach is not an effective approach for high-dimensional mediation analysis. Third, the approach of MedZIM analyzing each taxon as a mediator one by one cannot handle high dimensionality and cannot adjust the correlation due to hierarchical structure of phylogenetic tree (Li et al. 2020). Fourth, a sensitivity analysis is needed to check and validate the model robustness and assumptions (Li et al. 2019).

16.4.7 Nonparametric Entropy Mediation (NPEM)

16.4.7.1 NPEM Method

Currently, An and her colleagues (Carter et al. 2020) proposed a nonparametric framework called nonparametric entropy mediation (NPEM) utilizing information theory for mediation analysis of high-dimensional metagenomic data. As reviewed in Sect. 16.2, the SEM-based mediation methods require standard regression assumptions, including linearity, additivity, no collinearity, and sample size larger than parameter space. while the counterfactual-based mediation methods often limit to either a single exposure variable and a linear relationship between parameters or

binary exposures or continuous responses. The counterfactual-based mediation approach also requires no-confounding assumptions to ensure a casual interpretation and to confirm unmeasured confounders via a sensitivity analysis.

However, many of these assumptions are often violated when counts data are used in the context of genomics and metagenomics studies. The aim of NPEM is to utilize information theory to detect significant mediation effects with high-dimensional exposures and mediators and varying data types while avoiding standard regression assumptions (Carter et al. 2020).

16.4.7.2 Hypothesis Testing of Mediation Using Mutual Information

NPEM model is constructed based on a multivariate stochastic process and the mutual information (MI) theory. NPEM is a nonparametric framework using feature reduction techniques of information theory to construct tri-variate mediation model on the SEM structure.

First, NPEM describes the mediation model as a multivariate stochastic process.

The mediation model assumes that the set of exposure variables (X = a vector of exposures), the set of microbial taxa (M = a vector of mediators), and a clinical outcome Y are generated from a multivariate stochastic process. And the relationship between variables from the stochastic processes can be examined using the mutual information.

Second, NPEM uses feature reduction techniques of information theory to define the contributed information to capture the unique mutual information from a variable X.

Information theory is a theory of communication that defines definite, unbreachable limits on precisely how much information can be communicated between any two components of any system, *regardless of whether* this system is man-made or natural (Shannon 1948; Shannon and Weaver 1949; Reza 1994; Stone 2015).

Information can be measured by Shannon entropy and mutual information (MI) (Shannon 1949). Shannon entropy is defined as:

$$H(X) = -\sum\nolimits_{x \in X} p(x) \log p(x), \tag{16.39}$$

where $p(x)$ represents the probability of observing $X = x$.

The joint Shannon entropy is defined as:

$$H(X, Y) = -\sum\nolimits_{x \in X} \sum\nolimits_{y \in Y} p(x, y) \log p(x, y), \tag{16.40}$$

which presents Shannon entropy of a multivariate process between two variables X and Y where $p(x, y)$ represents the probability of observing $X = x$ and $Y =$ y. Mutual information (MI) is defined as:

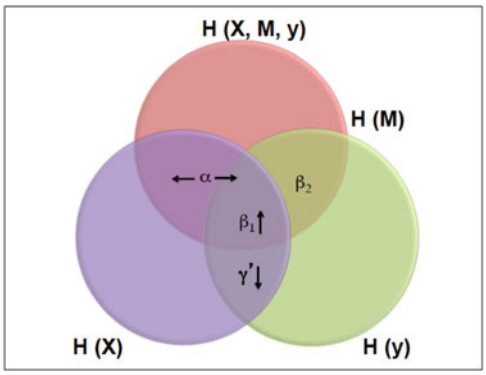

Fig. 16.2 Venn diagram representing information content and mediation effects within SEM-framework. In the diagram, the areas represent the model effects, for example, α (i.e., the intersection of blue circle and red circle) represents the relationship between the exposure and mediator, and β_1 (i.e., the intersection of three circles) represents the relationship between all three variables, while β_2 (i.e., the area of intersection of red and yellow circles, but not in blue) represents the relationship between the mediator and response excluding the exposure, and γ' (i.e., the overlap of blue and yellow circles) represents the total effect

$$\begin{aligned} \mathrm{MI}(X,Y) &= H(Y) + H(X) - H(X,Y) \\ &= \sum\nolimits_{x\in X}\sum\nolimits_{y\in Y} p(x,y)\log\frac{p(x,y)}{p(x)p(y)}, \end{aligned} \tag{16.41}$$

which presents the overlap of information produced by multiple stochastic processes. In NPEM, Carter et al. (2020) applied information theory to compare joint distributions of exposure, mediator, and outcome variables with the marginal distributions of subsets to measure association between these three variables. They assumed that exposure, mediator, and outcome variables are generated in a multivariate stochastic process and used mutual information to measure the dependency between these three variables. If the two variables are independent, then the information metric is zero. To further capture the unique mutual information from a variable X, the contributed information is additionally defined as:

$$C(X,Y,W) = \mathrm{MI}(X,Y) - \sum\nolimits_{w\in W}\frac{\mathrm{MI}(X,w)}{\|W\|^2}, \tag{16.42}$$

where W presents a set of measured variables.

Third, NPEM uses mutual information to represent the tri-relationships between the exposure and mediator and outcome.

The NPEM model is constructed based on SEM structure in Fig. 16.1. The NPEM model can be visualized in Fig. 16.2 through Venn diagram to represent information content and model effects.

In above Venn diagram, $\beta(=\beta_1 + \beta_2)$ represents the overlap in information contained by M and Y, β_1 represents the overlap of α and β, and β_2 represents the

unique information from M. The existence of mediation effects can be captured by measuring α and β_2. The two relationships α and β_2 can be expressed in terms of mutual information as MI(X, M) and MI(M, Y), respectively as shown in Fig. 16.2. The mediation effects exist only when $\beta_2 \neq 0$. Because when both $\beta_1 = 0$ and $\beta_2 = 0$, then M does not offer any information about Y, and there is no mediation effect, when $\beta_1 \neq 0$ and $\beta_2 = 0$; all information M provides about Y is also contained in X; this scenario is not considered as a mediation effect due to perfect collinearity between exposure X and mediator M. Thus, this Venn diagram shows that the overlap of all variables is not sufficient to define a mediation effect and any scenario where $\beta_2 = 0$ would not be considered a mediation effect (Carter et al. 2020).

NPEM model uses two approaches to test mediation using mutual information.

Univariate Entropy Measure

A univariate entropy measure (i.e., do not separate the zero and nonzeros counts for each taxon) is the single kernel approach, in which a single Gaussian kernel is used to estimate the distribution of OTU abundance and to calculate the contributed information.

Since microbiome data have a large number of taxa and generally only some of them play mediating effect, in other words, a vast majority of the signals observed are due to the bias effect. Thus, a true relationship between variables should be substantially higher than the expected bias. NPEM model is developed under this very general assumption. For each taxon j, the null hypothesis of no mediation effect is written as:

$$H_0 : C\left(X_i, M_j, S\right) \leq \varphi_{\alpha j}, \forall i \in \{1, \ldots, I\} \quad \text{or} \quad C\left(M_j, Y, T\right) \leq \varphi_{\beta_2}. \tag{16.43}$$

The alternative hypothesis is written as:

$$H_a : \exists i \in \{1, \ldots, I\} : C\left(X_i, M_j, S\right) > \varphi_{\alpha j} \text{ and } C\left(M_j, Y, T\right) > \varphi_{\beta_2}. \tag{16.44}$$

where $\varphi_{\alpha,\, j}$ and φ_{β_2} represent the expected bias for contributed information with a fixed taxon j and Y, which are conservatively estimated as the mean contributed information scores for taxon j, respectively:

$$\varphi_{\alpha j} = \sum\nolimits_{X_i \in (X-S)} \frac{C\left(X_i, M_j, S\right)}{|(X-S)|}, \tag{16.45}$$

$$\varphi_{\beta_2} = \sum\nolimits_{M_j \in (M-T)} \frac{C\left(M_j, Y, T\right)}{|(M-T)|}, \tag{16.46}$$

where $X - \mathrm{S}$ represents the set of exposure variables which are currently unselected and $M - T$ represents the set of OTUs which are currently unselected. A greedy

search algorithm for univariate test of mediation effects is developed using an iterative one-sided Extreme Studentized Deviate (ESD) test (Grubbs 1950), which was developed for unusually high value detection.

Based on these hypotheses of mediation effect, (1) for a particular taxon (j) to be a mediating taxon, there must be significant relationships from at least one exposure from the set of X variables, through this taxon (j) to the response Y; and (2) the mediation effect for each mediator must be evaluated across all exposures simultaneously within each fixed taxon j. Under this mediation paradigm, a FDR corrected p-value $p_{\alpha,\ j}$ is obtained by the ESD test of the α relationship between the full exposure set X and an individual microbial taxon (Mj); and a FDR corrected p-value $p_{\beta,\ j}$ is obtained by the ESD test of the β_2 relationships between the set of all microbial taxa M and the clinical response (Y). The final p-value for testing the mediation effect of taxon j is conservatively composited as $p_j = \max(p_{\alpha,\ j}, p_{\beta,\ j})$.

Bivariate Entropy Measure

A bivariate entropy measure is two-kernel approach to separately calculate contributed information metrics for both presence-absence and nonzero counts. In bivariate approach, an Aitchison-Aitken kernel is used to estimate presence-absence data, and a Gaussian kernel is used to estimate the distribution of nonzero counts of OTU abundance. Finally both contributed information scores are leveraged.

A general hypothesis testing whether a relationship is significant or not is proposed as:

$$H_0 : \| \vec{C} \| \leq \varphi \text{ vs. } H_a : \| \vec{C} \| > \varphi, \tag{16.47}$$

where $\| \vec{C} \|$ represents any norm or distance metric for the vector of two contributed information metrics and $\vec{C}$ represents from zero and nonzero counts. Mahalanobis distance (Mahalanobis 1936) is used to measure the difference in scale and correlation between presence-absence and nonzero counts.

$$\mathrm{MD}\left(\vec{C}\right) = \sqrt{\left(\vec{C} - \vec{\mu}\right)' \sum{}^{-1} \left(\vec{C} - \vec{\mu}\right)}, \tag{16.48}$$

where $\vec{\mu}$ represents the vector of means for $\vec{C}$ and $\sum$ represents the covariance of the two contributed information scores in $\vec{C}$. Mahalanobis distance normalizes each axis to a mean value of zero and variance of 1; a correlation between scores does not need to be conducted. The general hypothesis can be rewritten using the distance from expected bias:

$$H_0 : \mathrm{MD}\left(\vec{C}\right) \leq \varphi \;\; vs. \;\; H_a : \mathrm{MD}\left(\vec{C}\right) > \varphi. \tag{16.49}$$

For a particular taxon (j) to be a mediating taxon, similar to the univariate entropy measure, a significant mediation structure must bridge from at least one exposure through the taxon to the clinical response; and within each fixed taxon j, all exposures must be simultaneously evaluated.

For each fixed taxon j, the null and alternative hypotheses terms of Mahalanobis distance can be written as follows:

$$H_0 : \mathrm{MD}\left(\rightharpoonup C_{\alpha,i,j}\right) \leq \varphi_{\alpha,j}, \forall i \in \{1, \ldots, I\} \text{ or } \mathrm{MD}\left(\rightharpoonup C_{\beta_2,j}\right) \leq \varphi_{\beta_2}, \tag{16.50}$$

$$H_a : \exists i \in \{1, \ldots, I\} : \mathrm{MD}\left(\rightharpoonup C_{\alpha,i,j}\right) > \varphi_{\alpha,j} \text{ and } \mathrm{MD}\left(\rightharpoonup C_{\beta_2,j}\right) > \varphi_{\beta_2}. \tag{16.51}$$

A chi-square test is used to compare the two dimensions (i.e., for zero and nonzero parts) of Mahalanobis distance with 2 degrees of freedom to identify unusually high contributed information values (De Maesschalck et al. 2000).

A greedy search algorithm for bivariate test of mediation effects is developed same as the univariate test by evaluating the contributed information twice, once for the presence-absence data and once for nonzero counts data. And the final p-values are obtained also same to the univariate test approach.

16.4.7.3 Remarks

NPEM model mainly has two benefits, owing to using information-based methods: (1) does not need the assumptions of underlying distributions of data types of genomic or metagenomic data and response variable (e.g., clinical outcome) and (2) is flexible and capable in handling nonlinear or nonadditive relationships between variables. NPEM can handle any data types of variables such as continuous, discrete, or mixed. It was also showed that NPEM outperforms the nonparametric test (MultiMed) (Boca et al. 2014) and count-based regression model (iGWAS) (Huang et al. 2015) in terms of power and type I error (Carter et al. 2020). Thus, NPEM could be an alternative to the existing mediation analyses for integrating multiple omics datasets. However, the NPEM model has some limitations: (1) the performance of NPEM depends on the data characteristics and selected test statistic (Carter et al. 2020). For example, the optimal use of the univariate and bivariate tests is mainly determined by the signal strength in the data. The optimal use of a singular test and a sequential test is also affected by the proportion of zeros in the data. In practice, all these are hard for the users to choose the test methods to use if there is no procedure to test the signal size. (2) The singular Grubb's test is designed to select singular outliers, whether or not it is appropriate or optimal for testing multidimensional microbial taxa needs further researches to validate. (3) Mediation effects of microbial taxa could be positive or negative. However, Mahalanobis distance metric does not consider directionality; thus, not only the unusually low

signals may also be selected (Carter et al. 2020), but also the bidirectional mediation effects may be missed using the Mahalanobis distance metric. (4) The Gaussian kernel assumes normal continuous properties of the data. Although this assumption appears reasonable in many studies, including the one considered here, there may have examples in which they are not met. Unfortunately, any model assumptions are rarely checked in practice; and in fact, it would be extremely difficult to check them.

16.4.8 Some Comments About Current Mediation Models for Microbiome Data Analysis

16.4.8.1 Direction of Mediation Methods in Microbiome Studies

Currently, the direction of proposed mediation methods for microbiome data is correct because all these methods try to address some issues of microbiome data structure and features from their perspectives. However, as we reviewed in 2018 that mediation methods for microbiome data were in infant stage (Xia et al. 2018a, b, c, d), now it still in the developing period because some main issues have not been solved by these proposed methods, for example, detecting causality and generalizing the methods to all kinds of microbiome data. There is still lack of mediation models that treat environmental or host factors as mediation effects. Microbiome taxa have different levels. The phylogenetic tree can provide information of the taxonomical and evolutionary relationships among taxa and thus is another causal pathway to interpret mediation effects among treatment, microbiome, and outcome. Although currently MedTest includes the phylogenetic tree in computing dissimilarity matrix, and SparseMCMM adds an option to use the phylogenetic tree information, however, it still is a challenge to clearly clarify the causal path based on phylogenetic tree. Thus, the current medication models either do not have phylogenetic tree information or not directly use it. For example, MedTest uses phylogenetic tree-based distances, and SparseMCMM defers its choice of phylogenetic tree option.

16.4.8.2 Who Are Mediators: Microbial Taxa, Host, or Environment Factors?

Currently, microbiome mediation models all focus on microbiome as mediators. Actually, in real microbiome data, host or environment factors also could be mediators. For example, gut microbiome is increasingly recognized as an environmental factor that can shape the brain through the microbiota-gut-brain axis, and the host's metabolism mediates the causal role of dysbiosis of the gut microbiome on depression (Zheng et al. 2016).

16.4.8.3 Modeling Mediation Effects of Microbiome Data Is a Real Challenge

Generally, different mediation models only address partial issues or challenges of modeling mediation effects of microbiome data. Thus, it is challenge to confirm the true mediation effects of microbial taxa based on different mediation models. Typically, when we apply the different proposed models to the same dataset, we often obtain controversial results about mediation effects. For example, in above reviewed seven models, four of them used diet, BMI, and gut microbiome composition data (Wu et al. 2011) to illustrate their proposed methods. However, only MedTest obtains omnibus testing significant mediation effect, both MODIMA and CCMM fail to detect mediation effects. The ilr-transformation method identified three significant taxa without adjusting for multiple testing; however, after adjusting by FDR, none of taxa is significant.

16.4.8.4 Developing Longitudinal Mediation Models for Microbiome Data Analysis Is Difficult

It is a big challenge to develop mediation models for microbiome data analysis under the framework of longitudinal data setting. Thus, currently it still lacks of a longitudinal mediation model for microbiome data. The association among environmental factors, microbiome, and host is dynamic and very complicated. The more real and true microbiome data should have a temporal dimension, and thus mediational analysis of microbiome data should be performed within longitudinal data setting. However, it is a real challenge to accommodate the high-dimensional and compositional microbiome data into longitudinal and causal mediation model (Xia et al. 2018a, b, c, d).

16.4.8.5 Multicollinearity Especially Challenges the Mediation Analysis of Microbiome Data

Multicollinearity problems are common in multivariate analysis. However, multivariate taxa in microbiome data are compositional, which further complicates the issue of multicollinearity in microbiome research and especially in mediation analysis of microbiome data.

16.4.8.6 Model Fitting Assumptions and Modeling Issues Need to be Considered

When developing mediation methods for microbiome data, we need consider not only the specific features of microbiome data but also the common assumptions of

model fitting and issues of modeling mediation analyses. For example, in regression-based mediation model, the inferential assumptions include temporal precedence, measure timing, normality of independent variable, mediator and outcome, normality of product of coefficients, omitted inference, causal inference, and theoretical vs. empirical mediator.

16.4.8.7 Incorporating Multilevel SEM Modeling into Mediation Methods

Currently, mediation methods for microbiome data have not incorporated multilevel SEM modeling techniques. Microbial taxa have multiple ranks, to secure consistently estimating standard errors, and to test statistics due to dependence within the clusters, a mediation model for microbiome data may need to incorporate these different ranks of taxa in multilevel SEM modeling. Thus, an alternative approach to develop mediation methods for microbiome data is to incorporate multilevel SEM modeling techniques. In SEM, the development of latent growth curve modeling (LGCM) is the last milestone. The longitudinal microbiome data can be modeled in LGCM. In LGCM the analysis is based on repeated measures, in which the changes of microbiome are conceptualized as latent variables and the dependence of the repeated measures on these unobservable changes of microbiome are represented and interpreted by the factor loadings. LGCM is often used in the social sciences. Of course, using LGCM to analyze microbiome is more challenge, and imbedding a mediation model in LGCM to model microbiome data is even more difficult.

16.4.8.8 Mediation Analysis Is Not Causation Analysis Yet

Mediation analysis is not fully equal to causation analysis. In above reviewed mediation models, some are called "causal mediation" models. Actually we should note that mediation analysis and causal analysis are not completely overlapped. Because any statistical mediation models still subject to the same rules as association, that does not prove causality. To prove its causality, the mediation model depends on many assumptions such as no unmeasured confounders. For example, distance mediation does not suggest causal relationship (Zhang et al. 2018), and CCMM need meet several model assumptions to identify the causal direct and indirect effects (Sohn and Li 2019).

16.5 Detecting Causality in Microbiome Studies

Causality is a concept of both philosophy and statistics. Causality is a philosophic ontology or metaphysics and also a methodology. Causality is philosophic theory that meets scientific practice (Illari and Russ 2014).

16.5.1 Causality as a Philosophic Ontology or Metaphysics

First of all, causality is a philosophic ontology or metaphysics. Early in the third-century BC, Aristotle discussed how a thing comes about in his *Metaphysics* on efficient cause. This book was thought as the beginning of mediation analysis. He stated that "Plainly we are seeking the cause. And this is the essence (to speak abstractly), which in some cases is the end, e.g., perhaps in the case of a house or a bed, and in some cases is the first mover; for this also is a cause. But while the efficient cause is sought in the case of genesis and destruction, the final cause is sought in the case of being also" (Book Z, 17) (translated by W. D. Ross).

Inferring causality is really challenging from philosophic theory. Thus, skeptical about causality always exists since beginning of creating this concept until nowadays (Pearson 1900; Russell 1912; Norton 2003; Lipton and Ødegaard 2005; Briggs 2012). As a skeptical empiricist, Hume adopted the tradition of skepticism on causality. Thus, he argued persons are able to identify causes but can observe the regularity of events. His regularity definition of causation was given in Section VII of his book *An Enquiry Concerning Human Understanding in* 1748 when he talked about "the idea of necessary connection." Where he wrote: "We may define a cause to be *an object followed by another, and where all the objects, similar to the first, are followed by objects similar to the second*." Surprisingly it was also Hume who first gave the explicit definition of causation in terms of counterfactuals (Menzies 2014) immediately following the first definition. Where he wrote: "Or, in other words, *where, if the first object had not been, the second never had existed*." These two definitions of cause are very different.

In contrast, Kant argued that causation is a mere sensibility itself; but its nature is unknowable (see his major work *Critique of Pure Reason* in 1781) (Kant 1781). In this work, he took a synthesized way over traditional rationalists and empiricists attempting to explain the relationship between reason and human experience. Kant thus moved beyond the traditional metaphysics on causation. His theory of causation is totally different from Hume's skepticism.

Starting with J. S. Mill (1843: *A System of Logic*) (Menzies 2014), the counterfactuals were analyzed "metalinguistically" in terms of implication relations between statements. However, as empiricists, both Hume and Mill among other empiricists had no interest and hence did not try to explain causation via counterfactuals. Thus, the true conditions of counterfactuals were not clear until the development of possible world semantics in the early 1970s (Menzies 2014), although there were rigorous counterfactual analyses of causation in the late 1960s. The most popular approaches to causation in the 1960s generally focused on formulating causation in terms of necessity and sufficiency of causes for their effects, which treat causation as a natural law (Bernstein 2019). Such "sufficient cause" approach of causation first appeared in philosophy (Hempel 1965; Mackie 1965) and then impacted and extended to other fields such as in epidemiology (MacMahon and Pugh 1970; Rothman 1976), law (Wright 1988), and psychology (Cheng 1997; Novick and Cheng 2004) until early 2000s. The idea behind these approaches is the determinism.

Although in 1960s, there existed argues regarding whether or not successful causal claims require involvement of lawful regularity and broad natural laws (Scriven 1962), however, the paradigm shift of the causation theories occurred until early 1970s. In 1973, Lewis believed that the logical connection between cause and effect should be something besides necessity, sufficiency, or lawful regularity. His theories of causation became the seminal statement of the counterfactual approach (Bernstein 2019).

16.5.2 Causality as a Methodology and Specifically a Statistical Theory of Probability

Second, causality is also a methodology and specifically a statistical theory of probability.

Although there could be various differences on causation between SEM and counterfactual mediation approaches, the largest common feature they share is that both approaches focus on how to model causation. In other words, they treat causation as a statistical method.

SEM approach of modeling causation has been popular since Baron and Kenny's work; its root may be back to Wright's path analysis method (1921, Correlation and Causation) (Wright 1921). Counterfactual causation theory began at Rubin's early 1970s work. Although Rubin himself emphasized that it rooted from or inspired by Mill, Neyman, Fisher's works, actually Rubin's counterfactual causation theory was also developed under the context of Lewis' philosophy of causation and sufficient and necessary theory. The development from association to causality (Freedman 1999) has taken a long time, and there exist many arguments; however, casual inference has showed that it is possible to establish causality statistically for both randomized and non-randomized studies (Rubin 1974). Actually, the approach of casual inference has been continuing on statistical method. For example, counterfactual mediation analysis provides the opportunity to adjust covariates to reduce the confounding effects based on or cooperated SEM's strengths: path and equation and the modeling techniques. Counterfactual mediation analysis emphasizes the no-confounding assumption because it has also absorbed the sufficient and necessary idea of "necessary approach." Counterfactual mediation analysis further emphasizes that it can overcome the SEM's two limitations in modeling interactions and non-linearities. In summary, all these efforts of counterfactual mediation analysis have focused on methodology of causality.

16.5.3 How to Understand Establishing Causality in Microbiome Studies

The question is how to understand establishing causality in microbiome studies.

First, it is necessary and possible to re-establish causality in statistics for microbiome studies. Although causality has been always skeptical in philosophic ontology and never gained the status of a "law" or "principle" in physics (D'Ariano 2018), we need to re-establish causality in statistics. In statistics, causality is meaning in terms of probability, not like the determination stated by Max Planck, the founding father of quantum theory: "An event is causally determined if it can be predicted with certainty" (Planck 1941). As a theory of probability, the causal counterfactuals generally do two things to ensure the causal pathway works: one is sensitive analysis, and another is through assumptions of the models.

Second, although counterfactual approach has opened a way to re-establish causality in statistics, it is still a challenge to establish causality in microbiome studies due to very complicated microbiome data features. For example, how to correctly specify and test the causal models and rely on sensitivity analysis from both SEM and counterfactual approaches is arguable.

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