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Microbiome and Potential Targets for Chemoprevention of Esophageal Adenocarcinoma

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Abstract

Esophageal cancer is one of the deadliest cancers, with a dismal prognosis. It is increasingly recognized that esophageal cancer is a heterogeneous disease. It can be subdivided into two distinct groups: squamous cell carcinoma and adenocarcinoma, based on histological appearance. In the Western world, the incidence of squamous cell carcinoma was considerably higher than esophageal adenocarcinoma (EA) until the 1990s when, due to a dramatic increase, the incidence of EA surpassed that of squamous cell carcinoma. EA typically follows a well-established stepwise evolution from chronic inflammation due to reflux esophagitis (RE) that progresses to metaplasia (Barrett's esophagus- BE) to dysplasia, which often culminates in EA. The pathophysiology of EA is complex and involves diverse factors, including gastroesophageal reflux, gastric acid secretion, dysfunction of the antireflux barrier, gastric emptying disturbances, and abnormalities in esophageal defense mechanisms. The current understanding of the etiology of EA is mainly derived from epidemiological studies of risk factors such as cigarette smoking, obesity, gastroesophageal reflux disorders (GERD), and low fruit and vegetable consumption. Numerous studies have been done but the factors that drive the dynamic increase in the incidence of EA remain elusive. The advent of widespread antibiotic use occurred in the 1950s, preceding the surge of EA. Based on this temporal sequence, it has been hypothesized that antibiotics alter the microbiome to which the esophagus is exposed in patients who have GERD and that chronic exposure to this abnormal microbiome (i.e., changes in species diversity or abundance) accounts

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for the increase in EA. If changes in the proposed factors alter the stepwise progression (RE-BE-dysplasia- EA), they may represent potential targets for chemoprevention. New discoveries will help improve our understanding of the biology and pathogenesis of these cancers, and aid in finding novel therapeutic targets.

Keywords

microbiome; innate immunity; chemoprevention; esophageal adenocarcinoma

Normal Human Microbiome

The human body can be viewed as a superorganism composed of an amalgam of both microbial and human cells.^{1, 2, 3} The term “microbiome” was initially coined by Joshua Lederberg to indicate “the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space.”^{4, 5} Although more recently “microbiota” has been used to refer to the actual microorganisms that are resident in the human body, reserving “microbiome” for the full complement of microbial genes, gene products, and genomes of the microbiota, the latter term continues to be used on occasion for the totality of the inhabiting organisms themselves.⁵ The composition of microbial species varies in relation to different anatomical sites of the body.⁶ In the orodigestive tract, there is a distinct but related microbiome that inhabits the mouth, the esophagus, the stomach, the small intestine and the colon. The colon has the largest and most multifarious microbiome in the body due to its extensive surface area (approximately 2,700 square feet). Its lumen contains the vast majority of these microbes with bacteria accounting for the bulk of the microbes. Of note, the colonization of the gut with microbes begins at birth, resulting in a microbiome that contains approximately one quadrillion cells, which is 10-fold greater than the number of human cells in the body.⁷ *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* phyla constitute the vast majority of the dominant human gut microbiome.^{8, 9, 10} The host relationship with these microorganisms can vary from mutualism to pathogenicity.^{11, 12} The bacteria that are assumed to be mutualists are beneficial to the human host by aiding digestion, supporting vitamin production, stimulating the development of the gut immune system, and serving as an inherent barrier to the invasion of pathogens. In exchange, the bacteria are afforded with vital nutrients by the human host which provides the bacteria with a secure environment when nutrients are scarce. To support this symbiotic relationship, equilibrium must be achieved. The obligatory role of the immune system is to balance permissive, tolerogenic responses to food antigens and commensal microbes with potentially damaging inflammatory responses to pathogens.^{11, 13}

Human Microbiome in Diseases

Alterations in the microbial ecosystem have been shown to be linked to a multitude of human diseases including inflammatory bowel disease (ulcerative colitis and Crohn’s disease), metabolic disorders (diet-induced obesity, insulin resistance, diabetes mellitus 2), cardiovascular disease, peptic ulcer disease and mucosa-associated lymphoid tissue (MALT) lymphoma (due to *Helicobacter pylori* infection).^{7, 8, 14} The mechanisms by which these

disorders are linked to disruptions in the microbiome are currently under investigation, but it has been suggested, for example, that the microbes colonizing the epithelial barrier surfaces interact with the host either directly or through released products, regulating local inflammation and immunity.^{15, 16} The host immune system and the gut microbiome appear to have a physiological interaction. This is central to preventing tissue-damaging inflammatory responses directed against commensals (such as different species of *Lactobacilli* and *Proteobacteria* in the small intestine, *Clostridia* and *Bacteroides* in the colon), while avoiding infection by pathogens (e.g., *Salmonella* and *Shigella* spp.) or the uncontrolled growth of indigenous pathobionts (e.g., *Clostridium difficile* and vancomycin-resistant enterococci).^{15, 17} Stability and resilience are hallmarks of most microbiomes, that is, they have the ability to restore themselves after perturbation.^{15, 18} However, when changes occur in the microbial composition that are beyond the resilience capacity of the microbiome, permanent alterations of the microbial ecosystem may occur. This altered microbiome is distinct from the microbiome of healthy individuals.¹⁵

Dysbiosis is a term that describes such microbial alterations that disrupt the symbiotic relationship between the host and the microbiome.^{15, 19} In dysbiosis, an uncontrolled growth of harmful microorganisms could lead to a dysregulated inflammatory or immune response against commensals. This dysregulation may ultimately lead to severe acute and chronic tissue damage causing the conditions mentioned previously (ulcerative colitis and Crohn's disease, diet-induced obesity, insulin resistance, type 2 diabetes mellitus 2, cardiovascular disease, peptic ulcer disease and MALT lymphoma, asthma and atopy).^{6, 7, 15, 20, 21, 22, 23, 24} However, establishing the true causal mechanisms responsible for the evolution of clinical dysbioses are complicated since a definitive biomarker of dysbiosis often cannot be attributed to a single species, or even a collection of species.⁷ Therefore, it is critical to expand our understanding of the processes by which alterations in microbiome composition promote disease states in order to improve our understanding of the underlying connection between the microbiome and disease.⁷

Human Carcinogenic Pathogens

Approximately 16% of human cancers are related to infectious agents or infection-associated chronic inflammation, with higher percentages in less developed countries (22.9%) than in more developed countries (7.4%).^{15, 25} Worldwide, approximately 1.9 million new cancer cases are diagnosed per year that are linked to four major infectious agents, namely, *H. pylori*, human papillomaviruses, and hepatitis B and hepatitis C viruses.²⁵ Studies of infectious cancers have mainly concentrated on single microorganisms.

Seven oncogenic viruses are known to play a significant causal role in human cancer.^{15, 26} Herpesviruses account for two of these oncogenic viruses: (1) Epstein-Barr virus (EBV), known to be linked with Burkitt's lymphoma, nasopharyngeal carcinoma, and certain types of gastric carcinoma, and (2) Kaposi's sarcoma-associated herpesvirus/human herpesvirus type 8, which causes Kaposi's sarcoma, multicentric Castleman's disease and other pathologies in immunosuppressed individuals.^{15, 26} Two hepatitis viruses that cause chronic hepatitis, i.e. (3) hepatitis B virus and (4) hepatitis C virus (HBV and HCV) have been shown to be associated with hepatocellular carcinoma (HCC).^{15, 27} (5) The human

papillomaviruses (HPV), specifically the high-risk strains, are often associated with anogenital cancers, a subset of head and neck cancers and skin cancers.^{5, 15, 28} Even though HPVs have evident cell-transforming ability, other factors such as infection-associated genital tract inflammation and vaginal dysbiosis are considered important cofactors for cancer progression.^{15, 29, 30} The pathogenic determinant of T-cell lymphomas is the (6) human T-cell lymphoma virus which is prevalent in selected geographical areas.^{15, 31} Lastly, the (7) Merkel cell polyomaviruses are identified in a rare aggressive form of skin cancer, Merkel cell carcinoma in immunosuppressed individuals.¹⁵ Of note, with the exception of HCV, all the known human oncogenic viruses contain at least one oncogene in their genomes and may directly transform healthy cells to tumor-forming cells.¹⁵ It is worth noting that chronic liver infection may occur as a result of HBV and HCV, and such infections cause over 80% of HCC.¹⁵ The pathophysiology implicated in the development of chronic liver infection involves the immune response to the viral infection. In a subset of patients there is progression to fibrosis and cirrhosis and ultimately to HCC. Different mechanisms of immune evasion are utilized by the HBV and HCV viruses. HBV infection initially induces only a very limited innate immune response, whereas HCV uses different mechanisms for evading the innate response.¹⁵ The sequence of events by which HCV evades this innate response includes the inhibition of both type I IFN production as well as the response to type I IFN.¹⁵ Patients that are able to clear the infection produce a vigorous T-cell response whereas patients whose infection evolves into chronic infection and cirrhosis produce a delayed and transient T-cell response.¹⁵ While HBV induces the progression to HCC via direct cellular transformation, for both viruses the key issue for the progression of HCC is its dependence on immune-mediated inflammation. When HBV induces chronic liver disease, gut dysbiosis may occur as a result of this injury. This gut dysbiosis has been characterized by its increased population of several fungal species and a reduction in the amount and composition of *Bifidobacterial* species.¹⁵ The intestinal microbiome has been shown, specifically in experimental animals, to regulate the evolution of liver disease and hepatocellular carcinoma.^{15, 32} It has been speculated that the interaction among the potential direct viral cell-transforming effect and resultant inflammation-induced carcinogenesis could be identified for all oncogenic viruses.^{15, 33} A typical example of the interplay between the microbiome, inflammation and cancer progression is the Rous sarcoma virus which does not induce tumor formation in sterile embryos but does so in microbiome-containing chickens at sites of inflammation.¹⁵

Indigenous bacteria could potentially play a role in human and animal carcinogenesis. *H. pylori* is the only bacterial species that has been identified as a class I human carcinogen by the International Agency for Research on Cancer, by virtue of its definite association with gastric carcinoma and lymphoma.^{15, 34} *H. pylori* promotes gastric cancer by inciting an initial inflammatory response, which includes the production of IL-1 β by *H. pylori* infected cells, in addition to epithelial injury and atrophy, decreased acid secretory functions, and intestinal metaplasia.^{15, 35} This sequential series of events may span several decades. However, in patients diagnosed with advanced atrophic body gastritis, *Helicobacter* organisms are not detected in the stomach even when serology is positive, suggesting previous infection.¹⁵ These findings have culminated in an alternative model for *Helicobacter* promoted carcinogenesis. This alternative model suggests that long-term

interaction between the host and *Helicobacter* organisms leads to the deterioration of the gastric compartment, resulting in dysbiosis with the expansion of cancer promoting microorganisms.^{15, 36} In addition, the development of lymphoma has also been linked to other bacteria, namely, *Chlamydia psittaci* (lymphoma of ocular adnexal appendages), *Borrelia burgdorferi* (cutaneous MALT lymphoma), and *Campylobacter jejuni* (immunoproliferative small intestinal disease (IPSID)). Of note, the aforementioned conditions have only been demonstrated in humans (Table 1).³⁷

Microbiome in Cancer

Until the development of next generation sequencing methods in the 1990's, researchers focused solely on the role of single microorganisms in the pathogenesis of single cancer types. This narrow focus was largely due to the limitations of culture-dependent methods which by nature are selective for only a small proportion of the microbial community. While the cell culture methods led to isolation and understanding of hundreds of bacterial species, single recent technical advances in high throughput sequencing and bioinformatics have made it possible to scrutinize the entire microbial community in the human body.³⁸ Initially, the role of the microbiome in carcinogenesis was implied in several mouse models of inflammatory bowel disease where cancer developed in mice hosted in a conventional environment but not in mice raised under germ-free housing conditions.³⁹ Newer studies suggest that the colonic microbiome could contribute to carcinogenesis through activation or production of carcinogens and/or initiation and maintenance of inflammation.³⁶

Since the launch of the NIH Human Microbiome Project in 2008, the relationship between the microbiome and cancer has received heightened attention.^{4, 5} This heightened attention comes as new discoveries have demonstrated that inflammation and intestinal metaplasia of the distal esophagus are associated with alterations in the microbiome microorganisms that may play a causal role in the development of cancer.^{1,4,5} *H. pylori*, which has a well-established association with gastric cancer and lymphoma, has also been recently associated with pancreatic cancer owing to the observation that persons with gastric ulcers secondary to *H. pylori* have increased risk of pancreatic cancer development.^{40, 41,42} While this has sparked much new interest, the data are currently sparse and inconsistent, whereas more convincing evidence connecting bacterial periodontal disease and pancreatic cancer may exist.^{42, 43} Briefly, the association between periodontal disease and pancreatic cancer may be secondary to systemic inflammation promoted by various oral microbes, especially, *Porphyromonas gingivalis*.^{42, 43} Interestingly, another bacterium that has been associated with periodontal disease and has been speculatively linked to colorectal cancer is *Fusobacterium*.^{44, 45} Microbes that also have been demonstrated in colorectal cancer samples in humans and have speculatively been associated with colorectal cancer are *Bacteroides fragilis* and *Streptococcus bovis*, the latter particularly in patients who develop infective endocarditis.^{44, 46, 47, 48, 49} Furthermore, *S. bovis* has also been associated with hematopoietic malignancy.^{37, 50} It should be emphasized that despite the fact that oral cavity cancer, specifically squamous cell carcinoma, has been linked to numerous potential bacteria (examples include: *Veillonella*, *Fusobacterium*, *Prevotella*, *Porphyromonas*, *Actinomyces*, *Clostridium*, *Haemophilus*, *Enterobacteriaceae*, *Streptococcus spp.*, and *Gemella spp.*), no pathognomonic bacteria have yet been identified.⁵¹ In addition, squamous cell carcinoma

has been linked to changes in abundance of the oral microbiome such as those reported with *Firmicutes*, particularly *Streptococcus*, and *Actinobacteria* (especially *Rothia*), which were substantially decreased in relation to normal tissue. This, however, was not the case with bladder cancer where recent research showed markedly increased *Streptococcus* in urine samples in patients with cancer but not in normal subjects (Table 2).^{52, 53}

Studies have indicated several potential mechanisms through which the microbiome participates in carcinogenesis. 1) Innate immunity induced by the microbiome can result in chronic inflammation which may promote carcinogenesis.³⁸ A disequilibrium between the microbiome and host immunity leads to quantitative and/or qualitative changes of the microbiome and hence alters the composition of the essential bacterial molecules at the specific body site, forming microorganism-associated molecular patterns (MAMPs). Alterations in MAMPs trigger pattern receptors of innate immunity, including Toll-like receptors (TLR) and/or nucleotide-binding-oligomerization-domain (NOD)-like receptors.^{37, 54} Stimulating the receptors activates the NF- κ B pathway and causes expression of cytokines and interleukins.

2) Bacteria have been shown to generate genotoxins that may induce genomic damage. As an example, Gram-negative bacteria produce a toxin known as cytolethal distending toxin which causes double stranded DNA damage and genomic instability.^{55, 56} Colibactin cytotoxic necrotizing factor 1, *B. fragilis* toxin, hydrogen sulphide, and superoxide radicals are additional examples of genotoxins.^{56, 57, 58} 3) Bacteria produce virulent factors that may have tumor promoting effects, for example, cytotoxin-associated gene A (CagA) and vacuolating toxin A (VacA) produced by *H. pylori* stimulate the development of gastric cancer while virulence factor Fad A of *Fusobacterium nucleatum* through interaction with E-cadherin, activates β -catenin signaling, and promotes colorectal cancer development.^{36, 59, 60} 4) Bacteria may also activate procarcinogens by metabolizing carcinogenic chemicals. The progression to cancer can be promoted by the oral and intestinal microbiome by regulation of obesity-induced inflammation, metabolic activation and generation of carcinogens (e.g. nitrosamine and acetaldehyde), activation of dietary phytochemicals, metabolism of hormones, xenobiotics, and modification of tumor promoting bile acids.⁶¹

Risk Factors and the Changing Trend of the Esophageal Adenocarcinoma

One of the mysteries in modern medicine is the drastic increase in the incidence of EA, which has increased over 600 percent since the 1970s.^{62, 63} The driving force behind the striking change in incidence remains elusive despite extensive studies of conventional risk factors such as diet, obesity, smoking, *Helicobacter pylori* infection and perhaps the use of antibiotics.⁶⁴

The role of diet and obesity as risk factors for EA has been corroborated in multiple studies, and there seems to be a linear association between increased body mass index (BMI) and EA.⁶⁴ Additionally, evidence from multivariable analyses indicates that the correlation between the risk of developing EA and general or central adiposity continues regardless of the inclusion of probable confounders such as smoking, symptomatic reflux, physical

activity and the intake of fruit, vegetables, red meat and total caloric intake.⁶⁵ Several potential mechanisms by which diet and obesity may result in an increased risk for EA have been proposed. One such mechanism is the increased intra-abdominal pressure that may occur secondary to the accumulation of abdominal adipose tissue. This increased intra-abdominal pressure may directly result in the development of gastro-esophageal reflux which is a confirmed risk factor for the development of BE and EA.⁶⁶ Adipose tissue is metabolically active, as such, it secretes pro-inflammatory cytokines and adipokines. These intercellular mediators can stimulate cellular proliferation, reduce apoptosis and initiate neoplastic transformation in the esophageal epithelium. This adipose tissue-based mechanism thus provides an additional link by which diet and obesity may serve as a risk factor for EA.⁶⁶ Dietary elements have also demonstrated the potential to promote obesity and relaxation of the lower esophageal sphincter, conceivably inciting gastro-esophageal reflux and subsequently leading to the stepwise progression to EA. This concept is illustrated by chocolate. Chocolate has been shown to induce the opening of the lower esophageal sphincter, which may result in increased reflux.⁶⁷ A multitude of additional dietary components exist that could promote the evolution of EA either directly or indirectly. As such, there is evidence that diets high in meat and low in fruits and vegetables are associated with the development of EA.⁶⁷ Currently, however, there is no convincing evidence that identifies a distinct molecular dietary mediator.

Cigarette smoking has been implicated as a culprit in various forms of cancer. As for esophageal cancer, it is well established that cigarette smoking is a key risk factor for squamous cell carcinoma, yet early studies suggested that smoking serves a significant although limited risk factor for EA.⁶⁶ Furthermore, the risk among ever-smokers was substantiated in an extensive pooled analysis based in the Barrett's and Esophageal Adenocarcinoma Consortium (BEACON) and further population-based case-control and cohort studies which found that the risk for ever-smokers was roughly doubled, exhibiting a convincing and significant dose-response relationship with cumulative exposure.⁶⁸

Current studies suggest that a high cumulative smoking exposure increases the risk of the sequential neoplastic transformation from BE to EA. It is also possible that smoking may play a causal role in the development of BE in addition to the evolution of BE to EA. Despite these associations, the specific mechanism has not been elucidated. Hardikar et al have suggested that BE development and progression may be due to an amalgamation of exposure to various chemicals, namely N-nitrosamines, the potentiation of reflux secondary to relaxation of the lower esophageal sphincter by tobacco smoke, and the persistent inflammatory effects of smoking that may induce cellular proliferation.⁶⁶ Despite these findings, the more than 50% decline in the prevalence of cigarette smoking since 1965 has been paradoxically coincident with the drastic increase in the incidence of EA.⁶⁹

Another risk factor that has been implicated in the development of EA is infection with *Helicobacter* organisms. *H. pylori* often colonizes the gastric mucosa during childhood, and it has been demonstrated that infection with this bacterium could potentially decrease the risk of EA by approximately 50%.⁶⁴ A possible explanation for the inverse relationship is the fact that this infection often results in gastric atrophy, which results in a decrease in both the acidity and volume of gastric juices, culminating in reduced tissue damage in gastro-

esophageal reflux. This hypothesis is supported by a case-control study and meta-analysis that found an inverse association between pepsinogen I, a serum marker for gastric atrophy, and EA.⁶⁴ Since the prevalence of infection with *H. pylori* has decreased in Western populations, a reduction in the incidence of peptic ulcer disease and gastric cancer has also been noted.⁶⁴ It has been postulated that this reduction in the prevalence of *H. pylori* infection may in part account for the increasing incidence of EA. Other studies suggest that *H. pylori* may act as a significant protective factor against EA via a mechanism involving ghrelin, a hormone that stimulates appetite and contributes to the development of obesity. Studies suggest that eradication of *H. pylori* may result in increased serum levels of ghrelin, which may in turn lead to obesity.⁷⁰ As mentioned previously, obesity may then prompt relaxation of the lower esophageal sphincter leading to gastro-esophageal reflux which could then initiate the cascade of events giving rise to BE and EA. There is also evidence that suggests that *H. pylori* stimulates apoptosis in Barrett's derived EA cells via the Fas-caspase cascade, providing yet another potential mechanism that may account for the possible protective effects of *H. pylori*.⁷⁰ Of note, the decreased prevalence of *H. pylori* infection in Western populations during the 20th century parallels the increased rate of gastro-esophageal reflux disease, BE and EA.⁷¹ On the other hand, in regions of the world where *H. pylori* is still fairly widespread, human populations appear to be safeguarded from acid-related esophageal diseases.⁷¹

The widespread use of antibiotics since the 1950s could have contributed to the drastic increase in the incidence of EA. In 1935, the first antibiotic sulfonamide was discovered, followed by penicillin in 1942. Nearly all major classes of antibiotics were discovered between 1940 and 1970. The widespread use of antibiotics in humans started in the 1950s, nearly two decades prior to the takeoff of EA. Exposure to antibiotics may occur during treatment for infectious diseases or through consumption of food or use of hygiene products containing antibiotics. There is no direct evidence to support antibiotic use as the cause of the recent surge of EA, but it might have affected several major risk factors of EA. First, antibiotic treatment at that time (1970s), although not directed at *H. pylori*, could have eradicated *H. pylori* and caused the observed decrease in the incidence of gastric cancer and the increase in EA. Second, in mice, sub-therapeutic antibiotics consistently increase the relative abundance of *Firmicutes* at the cost of decreasing the abundance of *Bacteroidetes*.⁷² In humans, *Firmicutes* and *Bacteroidetes* are the two main phyla in the colonic microbiome. The ratio of *Firmicutes* to *Bacteroidetes* is consistently higher in countries with a higher prevalence of obesity such as in North America and Europe but lower in countries like Asia and Africa.⁷³ Overweight people have more *Firmicutes* but fewer *Bacteroidetes* than lean controls.⁶ Weight loss and diet therapy enriches *Bacteroidetes* and depletes *Firmicutes*. In infants, exposure to antibiotics during the first 6 months of life is associated with a consistent increase in body mass from 10 to 38 months and a similar effect has been observed in adults.^{74, 75} These data suggest that exposure to antibiotics changes the colonic microbiome in favor of gaining body weight, which could cause obesity, increase intragastric pressure, and induce gastroesophageal reflux. Third, antibiotic use could also have altered the microbiome in the distal esophagus. The altered microbiome might have played a more direct role in the initiation and progression of reflux disorders than *H. pylori*

or obesity by induction of chronic inflammation and/or activation of carcinogens as discussed in detail below.

Microbiome in Gastroesophageal Reflux Disorders

One of the first studies of the esophageal microflora was in the field of surgery, where bacteria may play a role in postoperative infection.⁷⁶ This work was hampered by the belief that no intrinsic bacteria populated the esophagus. Bacteria were not reliably shown to be present by means of cultivating luminal washes. Rare microbes were isolated by conventional culture and it was postulated that they represented contamination either by swallowing contents from the oropharynx or by stomach reflux.⁷⁶ Merely a few bacterial species were identified, which was much less than the 280 bacterial species isolated from the oral cavity through culture, implying that the technique of cultivation is of limited sensitivity.⁷⁶ Prior to 2004, changes in the esophageal microbiome had not been fully investigated, partly because of the complexity and difficulties in culture and analysis. This is exemplified by the number of attempts to date that have studied the esophageal microbiome with only limited success.⁷⁶ More powerful techniques that are independent of cultivation became available in the early 2000s. Using broad-range 16S rRNA gene PCR, coupled with cloning and Sanger sequencing, Pei et al examined mucosal biopsies from the distal esophagus of 4 normal human adults.⁷⁷ They found that the normal esophageal microbiome is composed of 6 major phyla (*Firmicutes*, *Bacteroides*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *TM7*) and is dominated by the genus *Streptococcus*. An esophageal microbiome is invariably present in all samples from the esophagus.⁷⁸ It resembles the upstream oral microbiome in composition but in-situ staining with tissue Gram stain and fluorescent in situ hybridization (FISH) demonstrated the physical presence of bacteria adherent to the esophageal mucosa.^{77, 79} These observations made it feasible to study the role of the microbiome in esophageal diseases.

A comprehensive study of the distal esophageal microbiome in diseases using culture-independent techniques indicated that the intricacy of the esophageal microbiome is on par with the complexity of the microbiome found in the in mouth, stomach, colon, vagina, and skin.¹ The microbiome of the distal esophagus can be classified into two groups, type I and type II.¹ The type I microbiome is composed primarily of Gram-positive bacteria and is more closely associated with a normal esophagus whereas the type II microbiome is enriched with Gram-negative bacteria and is generally associated with an abnormal esophagus. *Streptococcus* is the most dominant genus in the esophageal microbiome and its relative abundance is significantly higher in the type I microbiome than in the type II microbiome. In the type II microbiome the decrease in the relative abundance of *Streptococcus* is compensated by an increase in the relative abundance of 24 other genera. Specifically, the most prominent increases involve *Veillonella*, *Prevotella*, *Haemophilus*, *Neisseria*, *Rothia*, *Granulicatella*, *Campylobacter*, *Porphyromonas*, *Fusobacterium*, and *Actinomyces*, many of which are Gram-negative anaerobes or microaerophiles and are putative pathogens for periodontal disease. Overall, Gram-negative bacteria comprise 53.4% of the type II microbiome but only 14.9% of the type I microbiome. Of note, these observations support the prior hypothesis that the esophageal microbiome is altered in reflux disorders.¹

A recent study in Japan used cloning and Sanger sequencing to assess the esophageal microbiome of 18 subjects that included 3 groups (normal esophagus, RE, and BE) with 6 subjects in each.⁷⁶ A novel approach was utilized to quantify total bacterial loads by means of quantitative 16S rRNA gene PCR. The result showed that the number of bacteria which is comparable to 10^6 to 10^7 colony-forming units per sample was similar in the three investigated groups. Hence, changes in the relative abundance of taxa, as opposed to absolute bacterial loads, are potentially more pertinent to esophageal diseases. The proportion of microorganisms were found to be *Veillonella* (19%), *Prevotella* (12%), *Neisseria* (4%), and *Fusobacterium* (9%), which were more prevalent in patients with RE and BE than in controls. These results confirm previous findings that the esophageal microbiome is altered in reflux disorders with an enrichment of Gram-negative bacteria.¹ In one study, bacterial quantification was performed using staining biopsies from esophageal diseases and subsequently found that bacteria were detected more often in BE than in non-BE. It was also discovered that metaplasia and dysplasia were both associated with increasing bacterial stain scores.⁸⁰ Remarkably, Macfarlane et al found that the esophagus in the majority of patients with BE was colonized with *Campylobacter* species, specifically *C. concisus* and *C. rectus*, whereas *Campylobacter* was not identified in any of the controls. Furthermore, this result was confirmed by FISH using specific 16S rRNA oligonucleotide probes which demonstrated that Barrett's esophageal mucosae is colonized by *Campylobacter* species.⁷⁹

The type II microbiome, with its larger content of gram-negative bacteria, may well engage innate immune functions of the epithelial cells in a different way than the type I microbiome. This would be due to the type II microbiome producing larger amounts of gram-negative microbial components, such as lipopolysaccharides (LPS), or endotoxin.^{76, 81} Physiological emptying of the stomach after a meal reduces the pressure and reduces the opportunity for reflux. In mice treated with LPS, it was found that nearly 80% of food intake remains in the stomach 4 hours after a meal, which is significantly delayed compared to untreated mice (25%).⁸² The delay in gastric emptying induced by endotoxin can be blocked by pretreatment with either the nonselective cyclooxygenase (COX) -1 and -2 inhibitor indomethacin or selective COX-2 inhibition. Thus, the Gram-negative enriched type II microbiome might contribute to the development of gastroesophageal reflux by an increase in the intragastric pressure via reducing gastric emptying. Another factor that determines the development of pathologic reflux is the muscle tone of the lower esophageal sphincter, which serves as a gatekeeper against reflux. In a mouse model, LPS caused a dose-dependent fall in the basal tone. Of note, the LPS effect can be prevented by pretreatment with the inducible nitric oxide synthase (iNOS) inhibitor L-canavanine.⁸³ Thus, the type II microbiome might cause abnormal relaxation of the lower esophageal sphincter and contribute to the etiology of gastroesophageal reflux and the carcinogenesis of EA.

The pathway involved in the induction of iNOS and COX-2 expression by Gram-negative bacteria might start with triggering Toll-like receptors (TLRs). TLRs are a class of membrane-spanning proteins that recognize structurally conserved molecules derived from microbes. Activation of TLRs induces nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B). This, in turn, leads to the upregulation of

expression of its downstream target genes involved in inflammation, including iNOS and COX-2, IL-1b and IL-8, innate immune responses, adaptive immune responses, apoptosis blocking, cell proliferation, and cell differentiation.⁸ TLRs1, 2, 3, 4, 5, 7, and 9 are expressed in human esophageal epithelial cells. Of particular interest is TLR4 whose natural ligand is LPS. In tissue biopsies from the esophagus, TLR4 expression is significantly increased in EA, BE, and RE compared to normal esophagus. LPS stimulation of ex vivo cultures of esophageal biopsies resulted in NF- κ B activation, and COX-2 expression increased significantly in BE biopsies but not in normal squamous esophagus biopsies.^{84, 85} An increase in activation of the NF- κ B pathway has been observed along the sequence of RE, BE and EA.^{86, 87, 88, 89} Interestingly, an elevation in expression of the NF- κ B pathway's downstream gene COX-2 occurs along the progression from low-grade dysplasia to high-grade dysplasia in BE and EA.⁹⁰ iNOS expression is also increased in BE and EA.⁸⁵ Proinflammatory cytokines regulated by the NF- κ B pathway, such as IL-1b and IL-8, are increased sequentially in the progression of RE, BE and EA.^{88, 91} In addition, it has been demonstrated that IL-4 and IL-6 are increased in RE and BE.^{89, 91} Overall, activation of the TLR4-NF- κ B pathway is evident in reflux disorders and may contribute to malignant transformation.

The protective role of *H. pylori* gastritis in EA exemplified a complex relationship between indigenous pathogens and the human host. A study from Stanford University School of Medicine analyzed the records of approximately 130,000 patients who had been followed since the 1960s, among whom 52 had developed EA. Blood-sampling data from the individuals who had esophageal cancer and 551 individuals who had not developed cancer were examined and compared for evidence of *H. pylori* infection. Patients who were infected with *H. pylori* had a risk of developing EA that was around 70% lower than the uninfected group. Of note, these findings were not altered by adjustment for other risk factors such as age, gender, obesity, or history of smoking.⁹² The finding that *H. pylori* infections may play a role in preventing the development of EA is consistent with many other studies.^{93, 94, 95} This protective mechanism is in part secondary to colonization by a particular strain of *H. pylori* that possesses the Cag pathogenicity island which is associated with a decreased risk for GERD and its complications.⁷⁶ Despite controversial findings among studies in different geographic regions and racial/ethnic groups, several studies have shown that the prevalence of *H. pylori* infection is lower in patients with RE and/or BE, suggesting that *H. pylori* may confer its protective activity by reducing the risk for early stage disease in the spectrum of RE-BE-EA.^{96, 97} Detailed analyses suggest that *H. pylori* eradication increases the risk for development of RE in patients who have existing risks for BE and EA, such as male gender and hiatal hernia.^{98, 99} There are two hypotheses explaining the underlying mechanism whereby *H. pylori* infection protects the esophagus. First, *H. pylori* infection may interfere and induce inflammation within the acid-secreting gastric corpus-related gastritis causing hypoacidity and less tissue damage in the esophagus in the setting of gastroesophageal reflux.^{100, 101} Second, *H. pylori* infection reduces ghrelin synthesis inducing early satiety, thereby preventing obesity, and promoting rapid gastric emptying, both effects reducing the likelihood of gastroesophageal reflux.^{102, 103}

Potential targets for chemoprevention of esophageal adenocarcinoma

The annual incidence of esophageal cancer in the general population of patients with BE is approximately 0.1 – 0.5% per year, which warrants periodic endoscopic screening for dysplasia in patients with BE.^{104, 105} The American Gastroenterological Association (AGA) recommends an endoscopic surveillance program as the standard of practice for patients with BE based on the unproven assumption that early detection will reduce deaths from EA and thereby prolong survival.¹⁰⁴ In patients who have confirmed Barrett's with high grade dysplasia, endoscopic eradication rather than surveillance is recommended. Radiofrequency ablation (RFA), with or without endoscopic mucosal resection (EMR), is also recommended for select individuals with nondysplastic BE who are judged to be at increased risk for further neoplastic progression.^{104, 106, 107} To complement the early detection and mucosal ablation/resection in management of patients with BE, a need exists for chemopreventive agents. Three classes of medications are potentially beneficial in reducing the cancer risk of BE: antacids, anti-inflammatory drugs, and antibiotics, of which antacids are the only medication routinely used in clinical practice in the treatment of reflux disorders.

Acid-suppressive medications, represented by proton pump inhibitors (PPIs), which are one of the most commonly used medications in the management of GERD, have been considered as a potential chemopreventive agent in EA. Gastric acid is a relevant component in the consideration of chemoprevention of EA because (i) in gastroesophageal reflux, it causes esophageal tissue damage which is repaired by intestinal metaplasia/dysplasia, (ii) acid and bile exposure have also been shown to upregulate COX-2 expression in BE, which has been implicated in esophageal carcinogenesis, and (iii) acidic gastric juice might convert the type I microbiome into the abnormal type II microbiome by killing acid-sensitive bacteria in the esophagus.^{107, 108, 109, 110} The suggested regimen would include complete acid suppression over a period of 6 months as measured by 24 hour pH monitoring. This approach has been shown to decrease markers of epithelial proliferation and increase cell differentiation markers in patients with BE.¹¹⁰ PPIs also have anti-inflammatory properties. These properties, which are independent of their acid-suppressive effects, may also contribute to their chemopreventive effect against EA.^{107, 108, 109, 110} In patients with BE, the formation of esophageal squamous islands may be induced by long-term aggressive PPI therapy and may decrease the length of the BE segment.^{107, 108, 109, 110} However, clinical studies have been inconclusive as to whether PPIs cause regression of established dysplasia in patients with BE. These studies have been inconclusive because of a short duration of PPI therapy (<6 months), small numbers of patients with dysplasia, and short-term follow-up for disease progression.^{107, 108, 109, 110}

Notably, to further complicate this matter, there has been a theoretical concern that prolonged acid suppressive therapy with PPIs can induce hypergastrinemia, which may induce proliferation and COX-2 upregulation, potentiating esophageal carcinogenesis.¹¹⁰ In contrast, while gastrin enhances epithelial restitution in Barrett's mucosa, other studies have demonstrated that it does not promote proliferation and expansion of Barrett's segments during long-term PPI treatment.¹¹⁰ Hence, there is no evidence from preclinical and clinical studies that prolonged therapy with PPIs promotes esophageal carcinogenesis.¹¹⁰ Additionally, some studies have suggested that lansoprazole (LPZ), a PPI, suppresses

adhesion molecule binding on malignant cells, thereby inhibiting a molecular interaction that is associated with metastasis.^{111, 112, 113} Indeed, PPIs may be utilized as a resource to control metastasis by virtue of their inhibitory effects on adhesion molecules. However, the AGA strongly recommends against the use of PPIs to prevent EA because evidence in support of this practice is indirect and does not include a long-term controlled clinical trial.

Due to the evidence for activation of the TLR-NF- κ B pathway as well as the plausible hypothesis that the type II microbiome might serve as a trigger for the pathway in the spectrum of RE, BE, and EA, an anti-inflammatory strategy in chemoprevention of EA has been focused on inhibition of the pathway at various steps.^{1, 78} There are a few inhibitors, such as iNOS inhibitors, COX-2 inhibitors, and NF- κ B inhibitors, that could potentially stave the progression of EA.

NF- κ B inhibitors

NF- κ B is a major transcription factor that regulates genes responsible for both the host immune and inflammatory response, which is critical to the initial cellular response to chemical, bacterial, or viral stimuli. The NF- κ B pathway serves as a significant facilitator of many genes that are engaged in proliferation and apoptosis.⁸⁹ Amplification of the inflammatory response and the length of chronic inflammation may occur as a result of the interaction between cytokines (IL-1 and TNF- α) and the NF- κ B pathway. The cytokines upregulated by NF- κ B can also directly activate the NF- κ B pathway, which may promote the cascade of events culminating with EA.⁸⁹ The normal esophagus has no detectable activated NF- κ B, whereas NF- κ B activation was identified in patients with BE, with subsequent identification of high levels of active NF- κ B in EA.¹¹⁴ At least 750 NF- κ B inhibitors exist but only a few have been tested for chemoprevention of cancers.¹¹⁵ This group of compounds is comprised of antioxidants, peptides, small RNA/DNA molecules, microbial and viral proteins, small molecules, and engineered dominant-negative or constitutively active polypeptides. One such promising compound is resveratrol, which is found in the skin of the grape and red wine. In a rodent model of EA, resveratrol treatment caused a significant decrease in the severity of esophagitis but only a small diminution of the intestinal metaplasia and adenocarcinoma.¹¹⁶ No other NF- κ B inhibitors have been tested in animal models or humans.

COX-2 inhibitors

Cyclooxygenase-2 (COX-2) naturally occurs in the kidney and brain in physiologic conditions. It can also be expressed in multiple non-physiologic conditions, including RE, BE, and EA. As confirmed by *in vitro* studies, angiogenesis and increased invasiveness of malignant cells can be the result of COX-2 overexpression which also decreases the rate of apoptosis.^{85, 117} Further supporting the role of COX-2 inhibitors as a possible chemopreventive therapy are numerous animal and *in vitro* studies.¹¹⁷ One study demonstrated that when cells cultured from Barrett's epithelium are treated with a selective COX-2 inhibitor, there is a significant reduction in cell proliferation and COX-2 expression. As a matter of fact, in animal models, a reduced progression to adenocarcinoma was detected under COX-2-inhibiting therapy.^{117, 118} Aspirin, which is a non-steroidal anti-inflammatory drug (NSAID) is nonselective and inhibits both COX-1 and COX-2. Aspirin

has been proposed as a potential chemopreventive agent in controlling reflux and/or microbiome-induced esophageal inflammation, which dampens the subsequent risk of developing EA.¹¹⁹ Conversely, the “Chemoprevention for BE Trial”, a phase IIb multicenter randomized placebo controlled trial of celecoxib, a selective COX-2 inhibitor, in patients with BE and low- or high-grade dysplasia, showed that administration of 200 mg of celecoxib twice daily for 48 weeks does not appear to prevent progression of Barrett’s dysplasia to cancer.^{117, 120} To further evaluate the efficacy of aspirin, in 2009, the AsPECT trial began. This is the biggest, multicenter, randomized controlled clinical trial assessing the long-term chemopreventive effect of esomeprazole (PPI) with or without aspirin in minimizing the risk of progression to cancer in 5000 patients with BE; the results are much anticipated.^{121, 122, 123} Meanwhile, another study in the United Kingdom examined approximately 200 studies investigating the benefits and harms of taking aspirin - an area of continuing medical debate.¹²⁴ It was found that aspirin reduced not only the incidence but also the number of deaths from bowel, stomach and esophageal cancer by an estimated 30–40%. Other types of NSAIDs can potentially decrease the risk of EA and gastroesophageal junction adenocarcinoma (GEJAC).¹ In order to study the effects of non-aspirin NSAIDs and aspirin on esophageal cancers, the BEACON international consortium pooled 5 case–control studies and 1 cohort study to obtain 1140 GEJAC cases and 5314 controls.¹²⁵ Using a meta-analytic model, the authors arrived at a summary odds ratio (OR) for NSAID users relative to nonusers of 0.83 (95% CI: 0.66–1.03), whereas the OR associated with current use was 0.57 (95% CI: 0.39–0.83). When compared to overall NSAID use, aspirin was found to have analogous results. A dose–response relationship was identified between frequency and duration of use and disease risk for subjects with EA but not with GEJAC. In one of the individual studies included in this analysis aspirin use was shown to be associated with a more pronounced decrease in risk for EA when reflux symptoms were increased. As for GEJAC, no evidence of this interplay was identified.¹²⁶ In another prospective cohort study, no statistically significant association was identified between the use of aspirin or NSAIDs and risk of GEJAC.¹²⁵ The authors reported an OR associated with non-aspirin NSAID use of 0.80 (95% CI: 0.67–0.95) and an OR associated with aspirin use of 0.82 (0.65–1.04) in the accompanying meta-analysis. Altogether, the findings suggest a slight association between NSAID use and decreased risk of GEJAC. Conversely, a retrospective analysis found no significant difference in the risk of developing dysplasia or adenocarcinoma between patients taking aspirin and those not taking aspirin; however, a type II statistical error may have occurred although the data did not suggest a trend towards a chemoprotective effect of aspirin.¹²⁷

iNOS Inhibitors

Although the biological role of nitric oxide was identified in the mid-1980s, its therapeutic potential has not yet been fully elucidated for application to the gastrointestinal tract.¹²⁸ Nitric oxide, which can be made as a result of inducible nitric oxide synthase (iNOS), has been shown to be one of the key players in tumorigenesis since research has reported that iNOS serves as a vehicle for the progression of a large proportion of human and experimental tumors. Remarkably, it is well known that nitric oxide plays a fundamental role in mediating gastrointestinal mucosal defense; however, it has also been shown to be responsible for mucosal injury in specific situations.^{129, 130} Agents that are inhibitors of

nitric oxide synthesis and agents that are responsible for the release of nitric oxide have been valuable pharmacological tools in assessing the function of nitric oxide in gastrointestinal physiology and pathophysiology. The more recent inhibitors that are selective for one of the nitric oxide synthase isoforms may have even greater therapeutic potential, but currently there are no clinical trials to evaluate their role as chemopreventive agents in EA.¹²⁸ Likewise, agents that can capture nitric oxide or its metabolites such as peroxynitrite are encouraging as medications to avert nitric oxide-associated tissue injury. As a variety of human diseases, including cancer, may be linked to iNOS, iNOS selective inhibitors are emerging as an attractive chemopreventive drug class mainly due to their anti-inflammatory effects.¹³¹

Antibiotics

Given that the esophageal microbiome is altered in gastroesophageal disorders, antibiotics could potentially be used in the chemoprevention of EA through normalization of the esophageal microbiome (converting type II microbiome to type I microbiome) by increasing the relative abundance of *Streptococcus* which is depleted in the type II microbiome, as discussed previously. Notably, because the findings of microbiome alteration in GERD and BE is a very recent finding, the theoretical potential of using antibiotics to normalize the type II microbiome has not been tested in either animal models or human subjects. Nevertheless, erythromycin has been shown to significantly increase the lower esophageal sphincter (LES) pressure, without affecting the postdeglutition relaxation of LES.¹³² The effect of erythromycin on LES pressure was significantly more profound in patients with GERD than in healthy controls. The prominent effects of erythromycin on esophageal body motility and LES may be a result of the increased sensitivity to excitatory agents in patients with GERD. As a matter of fact, studies on experimental models of esophagitis in non-human subjects revealed increased irritability of the esophageal muscle, which has been attributed to the local release of inflammatory mediators.¹³³ The effect of erythromycin on LES has been mainly attributed to its motilin agonist property rather than the antibacterial activity. There have not been organized clinical trials testing the chemoprotective effect of motilin agonists for the risk of EA.

Conclusions

Esophageal microbiology is an understudied field, especially in EA. Data from the few available studies have established a convincing association between an altered microbiome and the various conditions such as reflux disorders that precede EA. The importance of microbiome changes in the human distal esophagus in reflux disorders is still under investigation; however, EA can no longer be seen as a disease in a sterile world. Further studies are needed to incorporate this relatively new concept into the already known prognostic factors. The technology is now available to conduct more sophisticated studies, improve our understanding of the biology, and aid in finding novel therapeutic targets in EAs. We are at the dawn of the era of truly personalized esophageal cancer care. Prevention of EA will and should be personalized and take the microbial factor into consideration. The choice of drugs for chemoprevention should target the pathway of LPS, TLRs, NF- κ B, COX-2, and iNOS (e.g., acid suppressive, anti-inflammatory and antibacterial drugs). These

drugs would offer a relatively safe, cost-effective means of preventing esophageal carcinogenesis and disease progression. Therefore, a complementary approach with an emphasis on risk reduction by chemoprevention, coupled with endoscopic surveillance programs for early detection and the recently developed mucosal ablation/resection therapies could potentially reverse the recent trend of increasing incidence of EA.

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Table 1

Carcinogenic microbes

Microbes	Body site	Malignancy	Pathogenes is	Prevention
Bacteria	Skin	Lymphoma ³⁷	Chronic inflammation	Antibiotics
	Small Intestine	Immunoproliferative small intestinal disease ³⁷		
	Eyes	Lymphomas ³⁷		
	Stomach	Carcinoma ^{15,34, 35} and lymphoma ³⁸		
Viruses	Skin	Merkel cell carcinoma ¹⁵	Chronic Inflammation Oncogenes	No cost effective prevention - low incidence
	Liver	Hepatocellular carcinoma ²⁷		Vaccination
		Hepatocellular carcinoma ²⁷ Lymphoma ³⁶		Vaccine in development
		Burkitt's Lymphoma ^{15, 26}		Vaccine in development
	Lymphoid organs	Nasopharyngeal carcinoma ^{15, 26,72}		No cost effective prevention - low incidence
	Oral cavity	Adult T-cell leukemia/lymphoma ⁵¹		Vaccination
	Oral cavity	Oropharyngeal carcinoma ^{15, 28, 29, 30}		No cost effective prevention - low incidence
		Anogenital carcinoma ^{15, 28, 29, 30}		Highly active antiretroviral therapy
		Kaposi sarcoma ^{15, 26}		Hygiene and anthelmintics
	Multiple organs	Lymphomas/Kaposi sarcoma ³⁶		
Parasites	Liver	Cholangiocarcinoma ^{a36}	Chronic inflammation	
	Urinary tract	Squamous cell carcinoma ³⁶		

Table 2

Microbiomes and cancer in humans

Cancer	Microbiome alteration	Pathogenesis
Colorectal cancer ^{38,39, 44}	Enriched microbes: <i>Bacteroides fragilis</i> , <i>Clostridium</i> , <i>Streptococcus bovis</i> , <i>Escherichia coli</i> , <i>usobacterium</i> , <i>Peptostreptococcaceae</i> , <i>Veillonellaceae</i> , and <i>Pasteurellaceae</i> Depleted microbes: <i>Lachnospiraceae</i> , <i>Ruminococcaceae</i> , <i>Lactobacillus acidophilus</i> , and <i>Bifidobacterium longum</i>	Dysbiosis Barrier malfunction Carcinogen metabolism Chronic inflammation Bacterial genotoxicity
Oral cancer ^{51, 52}	Enriched microbes: <i>Fusobacterium</i> and <i>Prevotella</i> Depleted microbes: <i>Streptococcus</i> and <i>Rothia</i>	Chronic inflammation Carcinogen metabolism
Bladder cancer ⁵³	Enriched microbe: <i>Streptococcus</i>	n/a
Pancreatic cancer ^{40, 41, 42, 43}	Enriched microbes: <i>Porphyromonas gingivalis</i> , <i>Neisseria elongate</i> , and <i>Streptococcus mitis</i> in oral cavity/saliva Association with <i>Helicobacter pylori</i> gastritis	Systemic chronic inflammation