Title: Insights into the Pathophysiology of Esophageal Adenocarcinoma

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Abstract

Although researchers have identified genetic alterations that contribute to development of esophageal adenocarcinoma, we know little about features of patients or environmental factors that mediate progression of chronic acid biliary reflux to Barrett’s esophagus and cancer. Increasing our understanding of the mechanisms by which normal squamous epithelium progresses to early-stage invasive cancer will help formulate rational surveillance guidelines and allow us to divest resources away from patients at low risk of malignancy. We review the cellular and genetic alterations that occur during progression of Barrett’s esophagus, based on findings from clinical studies and mouse models of disease. We review the features of the luminal and mucosal microenvironment of Barrett’s esophagus that promote, in a small proportion of patients, development of esophageal adenocarcinoma. Markers of clonal evolution might be used to determine patient risk for cancer and set surveillance intervals.

Keywords: EAC, BE, metaplasia, biomarker
Although esophageal adenocarcinoma (EAC) is rare in many parts of the world, its incidence has increased 4%–10% each year in developed countries since the 1970s. Median survival times are short, chiefly because most patients are diagnosed with advanced-stage disease that is not curable. EAC prevalence therefore closely follows its incidence. Barrett’s esophagus (BE), defined as the replacement of the squamous epithelial lining native to the esophagus by metaplastic columnar epithelium, is a well-established risk factor for EAC. Acid biliary reflux from the stomach leads to development of BE, the initial step in progression to EAC, which can be tracked by histologic and genetic changes.

The increase in EAC incidence appears to be a result of the increased prevalence of BE. The exact population prevalence of BE is difficult to establish, because this disease does not produce symptoms and because of the weak association between reflux complaints and BE. Furthermore the operative definition of BE is not consistent among expert guidelines, so the true prevalence is uncertain. Notwithstanding these caveats, population estimates of BE range from 1% to 5%. Retrospective population-based studies found that rates of progression from BE to cancer range from 0.10% to 0.13% per year, indicating that BE is mostly a long-term benign condition. Many patients in surveillance programs are therefore needlessly exposed to the risks and negative effects of routine screening. The flip side to this argument is that BE is an under-diagnosed condition, because more than 85% of patients with newly diagnosed EAC have no history of either BE or heartburn complaints. For this reason, the clinical return (and cost-benefit) of BE surveillance programs are subject to scrutiny. Trials such as the British Barrett’s Oesophagus Surveillance Study and the BarrettNET registry (in Munich, Germany), comprising more than 5000 patients to be followed for more than 10 years, aim to compare all-cause and disease-specific mortality between patients who have been randomly assigned to groups that will undergo endoscopic surveillance every 2 years vs patients with a more conservative clinical follow up. Hopefully findings from these studies will provide some guidance on this issue. The sample size and length of follow up of these trials are a clear indication of the complexity and magnitude of this endeavor.

In BE, the inflammatory microenvironment and somatic genomic alterations in stem cell populations are believed to mediate progression to EAC. We review the mechanisms by which these factors promote carcinogenesis based on findings from clinical studies and
Progression From Reflux Esophagitis to BE, Based on Histology

The seemingly straightforward histopathologic definition of BE as a metaplastic condition whereby the native squamous epithelium of the distal esophagus is replaced with columnar epithelium belies an altogether far more complex microscopic process. Biopsies from patients contain a range of columnar phenotypes. Despite emphasis on the cellular composition of the epithelium, the BE segment is organized, like all mucosal layers of the gastrointestinal tract, into a quasi-repetitive arrangement of glands. Every gland is maintained by a unique population of stem cells and can therefore be thought of as a singularly evolving unit within the mucosal sheet. These metaplastic glandular units have a variety of appearances. However, morphologies of the various types of glandular units do not vary within or among patients—they often resemble gland types found elsewhere in the gastrointestinal tract, either in healthy individuals or patients with gastrointestinal disease.

The gland phenotype most commonly associated with BE has a mixed epithelial lining comprising scattered goblet cells against a background of columnar cells with properties that are indistinguishable from gastric foveolar cells (Figure 1A and B). This dual pattern of epithelial differentiation is reflected in its mucin core peptide and expression pattern of trefoil factor (TFF), with goblet cells producing the intestinal type mucin (mucin 2, oligomeric mucus/gel-forming, MUC2) as well as TFF3, whereas foveolar cells produce the gastric type mucin (MUC5AC) and TFF1\textsuperscript{22}. These MUC proteins contain abundant oligosaccharide side-chains, which allow these proteins to bind copious amounts of water after secretion into the gut lumen. These MUC proteins further self-aggregate, which creates a visco-elastic gel that coats the underlying epithelium\textsuperscript{23}. This peculiar pattern of mixed gastric and intestinal lineage differentiation has been widely described as specialized epithelium or specialized metaplasia, or, simply, intestinal metaplasia. Older publications referred to this as type II or type III incomplete intestinal metaplasia—terms that are now obsolete\textsuperscript{24}.

In crypts in the small intestine and colon, stem cells reside strictly at the base of the gland and move up along the crypt (and villus) as they differentiate and mature. In BE glands, alternatively, the stem cell compartment is located about one-third up the height of
the gland; mature cell lineages show bidirectional flow from this stem cell compartment towards the lumen as well as towards the base of the gland. This was demonstrated in a study of patients with EAC scheduled for esophageal resection. Patients were given an infusion of a thymidine analogue at different timepoints before surgery. Tracing the distribution of this indelible label in daughter cell populations confirmed bidirectional migration within BE glands and showed that cell migration toward the glandular base compartment of the gland occurred much more slowly than toward the superficial crypt compartment of the gland. The label had been all but lost from the superficial crypt population in little over a week, whereas non-dividing cells that contained the thymidine analogue were detected for as long as 10 weeks after label infusion in the gland base population.

The mucous base of the BE gland is lined by a population of columnar cells that express MUC6 and also secrete bicarbonate (HCO$_3^-$). This buffers the caustic refluxate and, together with the mucinous gel that covers the metaplastic mucosa, protects the lining of the distal esophagus. It is important to be aware of this functional compartmentalization to understand the unique functional properties of the BE gland.

Expression of LGR5, a marker of stem cells, about one-third up the height of the gland provides support for this location of the stem cell niche. LGR5 mRNA is detected at the junction of the MUC5AC+ and TFF1+ cells and the MUC6+ and TFF2+ cells, the origin of the bidirectional cell flux and site of maximum proliferative activity (shown by immunohistochemical analysis for Ki-67, see Figure 1). These observations help us to understand the cells and their functions in the BE gland. It is important to note that this bi-directional compartmentalization is not unique to the metaplastic esophagus—it resembles the basic architecture of the pyloric gland in the normal gastric antrum.

There are a small numbers of other gland types, which together constitute the metaplastic mosaic of the columnar esophagus (see Figure 2). Best studied of these is the cardiac gland (also transitional gland or non-goblet columnar gland). In essence, the epithelial lining and bidirectional architecture of the cardiac-type gland are identical to those of the BE gland, except for an absence of goblet cells in these glands. This makes the cardiac gland the simplest, in terms of differentiated epithelial cell types, of all BE gland types. The cardiac gland contains only MUC5AC+ and TFF1+ foveolar cells along the superficial crypt compartment and MUC6+ and TFF2+ cells along the mucous base. These glands have been
studied extensively, because they are the type most commonly found in biopsies of patients with short-segment BE and in biopsies from patients with columnar metaplasia of the neo-distal esophagus following esophageal resection. This gland type may be the earliest detected during development of BE. It is also indistinguishable, in terms of glandular architecture and cell composition, from reparative glands (known as pseudo-pyloric metaplasia) found in, for example, terminal ileitis in patients with Crohn’s disease.

The remaining gland types are variations on a theme. The cardiac type gland may show oxyntic differentiation in the form of scattered parietal cells, at which point the gland is essentially comparable to similar glands found in the transitional mucosa of the gastric incisura or gastric pylorus (see Figure 2). Contrary to common belief, parietal cells are not restricted to corpus mucosa and are abundant in normal pyloric mucosa. Mature chief cells are also found in these glands, which contain the complete complement of cell types normally found in the gastric body and fundus, although the irregular packing of these glands indicates that this is non-native, post-inflammatory mucosa. In many patients, these glands are found in the context of anatomic features of the esophagus such as submucosal gland complexes, so these fundic-type glands develop as part of the metaplastic mosaic. It is important to determine whether these metaplastic oxyntic glands develop from cardiac glands and, if so, whether they are true stem-cell derived metaplasias or a manifestation of varying levels of oxyntic gland differentiation. Varying levels of differentiation of these archetypal gastric glands have important implications for the temporal dynamics of glandular metaplasia in the atrophic stomach.

Some glands have mature intestinal differentiation, with Paneth cells at the base and enterocytes along the superficial crypt compartment (Figure 2). These are the only glands that completely lack gastric mucin core proteins; they are also the most rare of gland types described. However, tissues from some patients have high levels of Paneth cell differentiation.

Remarkably, the distribution of these gland phenotypes is not random—they appear in recurrent temporal and spatial patterns along the BE segment. Intestinal phenotypes are found more proximally at the squamo-columnar junction, whereas cardiac and oxyntocardiac gland phenotypes are proportionally more common around the gastroesophageal junction. For example, Harrison et al found that intestinal differentiation was almost twice as common in proximal biopsies taken near the squamo-columnar junction.
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comparing the biopsies collected from the gastro-esophageal junction, with a clear stepwise gradient in between\textsuperscript{29}. The density of glands containing intestinal differentiation correlates with the pH gradient along the BE segment; the less acidic the local average pH (closer to the squamo-columnar junction) the higher the proportion of glands with goblet cell differentiation\textsuperscript{31}.

The functional significance of this spatial distribution of gland phenotypes is unclear. We proposed that this distribution in gland types could result from local selection for cells that can survive in the harsh environment of the acid biliary refluxate at the distal esophagus\textsuperscript{26}. The soluble component of bile acids, which acts as a detergent and solubilizes lipid cell membranes through micelle formation, could be responsible for this environment. Studies of bile salt solubility found it to be greatest at intermediate luminal pH ranges seen most proximally, whereas bile acids are insoluble and therefore incapable of forming micelles at lower pH ranges in the distal esophagus\textsuperscript{31, 32}. In vitro studies have shown that solubilized duodenal bile salts are a strong inducer of CDX2 expression and goblet cell differentiation\textsuperscript{33-35}. The pH gradient along the BE segment could therefore create a proximal–distal gradient of bile salt solubility, which could determine the relative proportion of specific gland phenotypes along the length of the esophagus.

Temporal analysis of gland phenotype distribution is complicated because, in most patients, the BE segment is static over time and does not expand (or contract) despite years, or in some cases decades, of endoscopic follow-up evaluations\textsuperscript{36}—even in patients with continued esophageal exposure to acid biliary reflux\textsuperscript{37}. However, patients that have undergone cardia-esophagectomy because of esophageal cancer lose normal sphincter function, which provokes severe gastro-esophageal reflux. Consequently, about half of these patients develop columnar mucosa in the remnant distal esophagus de novo.

Longitudinal studies found that the length of columnar mucosa increases over time and histopathologic analyses demonstrated that the glandular phenotype changes from purely cardiac-type mucosa to BE glands, with intestinal differentiation\textsuperscript{38-40}. These observations indicate that the mucous cardiac-type gland is the earliest gland phenotype that develops in the reflux-damaged distal esophagus; it might change to either an intestinal (goblet cells and Paneth cells) or gastric (parietal and chief cells) lineage differentiation (Figure 2). In support of this model, some studies have shown cardiac type glands to undergo early intestinalization, with submaximal levels of villin and CDX2 expression\textsuperscript{41, 42}. 
Studies have shown clonal ancestry of canonical specialized BE glands and non-intestinalized cardiac-type glands—these various phenotypes do not develop independently, but arise via phenotypic variation within glands derived from a common ancestor.\(^{18}\)

Progression from cardiac-type glands to intestinalized epithelium was demonstrated in a mouse model of BE (EBV-L2-IL1B mice). These mice overexpress interleukin 1 beta (IL1B) in the esophageal and squamous forestomach epithelium, and develop spontaneous esophagitis that progresses to metaplasia at the gastro-esophageal junction and adenocarcinoma with older age.\(^{20}\) Addition of bile acids (0.2% deoxycholic acid) to their drinking water accelerates onset of intestinal metaplasia and tumorigenesis. Metaplastic esophageal tissues from these mice have increased levels of TFF2, CCKBR, MUC5AC, CDX2, and K19, compared to esophageal tissues of control mice. In EBV-L2-IL1B mice, bile acids lead to demethylation of gene promotor regions, leading to increased expression of IL6, CDX2, and Notch, promoting commitment to the intestinal cell lineage. The earliest morphologic manifestation of glandular differentiation in the distal esophagus therefore appears to be the simple cardiac-type gland, which can evolve with time into either an intestinal or gastric cell glandular phenotype.

What promotes columnar transformation of the distal esophagus when acid biliary reflux first hits the naïve squamous mucosa of the distal esophagus? This is a question of great contention and one on which opinion is sharply divided. Several models have been proposed, but 2 models that have (arguably) been studied most. The transdifferentiation model proposes that squamous stem cells with chronic exposure to the corrosive effects of acid-biliary reflux slowly change their differentiation lineage, downregulating the native squamous expression program and upregulating a columnar cell expression program via upregulation of lineage-determining factors such as SOX9.\(^{34, 45, 46}\) Support for this model comes from a trial of patients with reflux successfully treated with proton pump inhibitors (PPIs) who discontinued acid suppression for 2 weeks.\(^{47}\) In this relatively short time period, all patients had progressive symptoms—some with severe erosive (Los Angeles Grade C) reflux esophagitis. Biopsies from non-eroded areas were infiltrated by large numbers of lymphocytes, so inflammatory cells might contribute to pathogenesis.

In the chronic wounding model, continuous micro-trauma, due to caustic reflux, erodes small patches of squamous epithelium, which are repaired by the wound-healing process (see Figure 3). This response activates proliferation of nearby epithelial progenitors
to cover the epithelial defect. Wounding at the squamo-columnar junction elicits proliferation of squamous and columnar progenitors on either side of the epithelial defect. This is recognizable as a thin layer of undifferentiated epithelial cells covering a fresh wound bed (Figure 3A). Although this is essentially a stereotypical wound healing response, exposure to reflux promotes selection for phenotypes best adapted to the harsh environment, such as mucin-producing columnar progenitors. With recurrent bouts of reflux and ulceration, the columnar epithelium expands, progressively replacing the distal esophageal squamous epithelium (Figure 3B-F).

Lineage tracing experiments in mouse models of BE indicate that metaplastic lesions originate from stem cells in the gastric cardia\textsuperscript{20, 28, 48}, which over time expand proximally into the squamous esophagus, replacing squamous epithelia (Figure 4). This progression associates with development of dysplasia in the mice. Expansion seems to occur first in the cardia, presumably in response to inflammatory cytokines, in contrast to esophageal injury without reflux, leading to squamous lineage regeneration through Ker15-negative cells\textsuperscript{49}. The esophageal microenvironment and signaling pathways regulate tissue regeneration and cell fate decisions. In summary, a pool of stem cells in a niche at the gastro-esophageal junction (labeled by LGR5\textsuperscript{19}, CCK2R\textsuperscript{50}, and CAR4\textsuperscript{21} in mice) expands and, with the development of genomic instability, enters a phase of clonal evolution that proceeds over a period of years and, in some patients, resulting in EAC.

It is not clear whether cyclic wounding and expansion occurs within a short time period or progresses over many months or years. Progressive widening of the lower esophageal sphincter, due to slowly increasing abdominal pressure and increased reflux, might progressively erode the distal esophageal epithelium over many years. There is some (indirect) evidence for this from studies of patients\textsuperscript{51}. For example, in carefully executed studies analyzing esophageal reflux by detailed pH and manometry measurements across the lower esophageal sphincter, McColl et al found that, in patients with a large waist circumference, acid reflux extends more proximally into the lower esophageal sphincter. Importantly, this correlated with a longer zone of cardiac-type columnar mucosa at the gastro-esophageal junction\textsuperscript{52}. Furthermore, in some patients, the histologic squamocolumnar junction can actually move distally, covering the gastric cardia. Derakhshan et al observed this phenomenon in a patient with long-standing atrophic gastritis and pernicious anemia who underwent gastrectomy because of an incipient gastric cancer. The
resection specimen clearly showed a collar of squamous epithelium, which occupied the anatomic cardia\textsuperscript{53}. Given the atrophic gastritis, the most parsimonious explanation is that in this case, due to continuous erosion of the friable gastric mucosa, the squamous epithelium outcompeted native glands in the proximal stomach.

**A Permissive Environment for Esophageal Carcinogenesis**

Identification of environmental factor that contribute to carcinogenesis might facilitate patient risk stratification for surveillance programs (see \textsuperscript{54} for review)—especially because reasons for the rapid increase in incidence of BE and EAC are unclear\textsuperscript{55}. Environmental factors might partially explain the increased incidence,\textsuperscript{56} given that BE is rare in children and adolescents\textsuperscript{57} and life style changes appear to mediate the increase in BE incidence in the Far East\textsuperscript{58}. Risk factors for GERD, BE, and EAC overlap, and include obesity, tobacco use\textsuperscript{59, 60}, alcohol consumption\textsuperscript{61}, and stress levels\textsuperscript{62, 63}. Male sex and older age are consistently identified as covariates and this association persists irrespective of ethnic background and nationality\textsuperscript{64, 65}. The most proximate risk factor for BE and EAC likely is chronic reflux and associated conditions such as hiatal hernia or esophagitis\textsuperscript{66}. The effects of chronic reflux are likely to vary among patients, provoking in some individuals an inflammatory microenvironment promotes cancer development.

Drugs such as PPIs, non-steroidal anti-inflammatory drugs (NSAIDs), and statins also affect risk of BE and progression to EAC\textsuperscript{67-69}. PPIs are used routinely to treat patients with reflux symptoms and promote esophagitis healing\textsuperscript{70}. Although epidemiologic studies have found PPIs to reduce the risk of BE progression\textsuperscript{71}, no randomized placebo-controlled trials have shown PPIs to have chemopreventive effect. There has been some inconsistency with regard to the effects of PPIs on risk of progression risk; some studies reported decreased risk\textsuperscript{72} and others reported increased risk of progression\textsuperscript{73, 74}. Reports of increased risk of esophageal cancer in patients taking PPIs could result from bias in reporting or indicate that micro-environmental changes cause competitive release of incipient neoplastic clones. A study in the EBV-LR-IL1B mouse model of BE found PPI-induced hypergastrinemia and genetic hypergastrinemia to increase development of columnar metaplasia of the esophagus and accelerate progression to dysplasia\textsuperscript{50}. In patients, PPIs also induce secondary hypergastrinemia\textsuperscript{75, 76}; when patients with BE were treated with PPIs and stratified according
to gastrin levels, those with the highest levels of gastrin were at increased risk of dysplasia or EAC\textsuperscript{77}.

The increasing incidence of BE and EAC have also been associated with improved sanitation and the consequent decrease in \textit{gastric Helicobacter pylori}, mainly in the West\textsuperscript{78, 79}. Although the inverse correlation between EAC incidence and \textit{H pylori} infection may relate strictly to decreased acid output (hypochlorhydria) and reflux\textsuperscript{80}, \textit{H pylori} colonization also significantly alters the native gastric flora and the esophageal flora. The bacterial community of the normal esophagus\textsuperscript{81} changes in patients with reflux-related disorders and BE\textsuperscript{82, 83} or patients receiving PPI therapy\textsuperscript{84}.

The common denominator of these environmental factors (constitutional, diet, and drug-related) is that they affect the local BE micro-environment. In some individuals, the combination of esophageal inflammation and genetic factors could promote BE progression and carcinogenesis.

**Inflammation Promotes Esophageal Carcinogenesis**

The tissue microenvironment promotes some of the earliest events in esophageal tumor development (Figure 4). Tissue-specific stem cells are believed to retain their undifferentiated state through physical proximity with a dedicated niche\textsuperscript{85, 86}. Stem cell proliferation and differentiation at the gastro-esophageal junction could be regulated by signaling pathways that are active throughout the mammalian gut, such as the Wnt, Notch, bone morphogenetic protein, and hedgehog pathways\textsuperscript{87}. We discussed how chronic wounding in a harsh environment can induce cephalad expansion of columnar epithelia into the esophagus, but little is known about the mechanisms of this process and subsequent tumorigenesis.

Inflammatory cells release signaling molecules that promote and accelerate tissue healing, but simultaneously establish a carcinogenic microenvironment, by increasing local tissue concentrations of mutagenic oxygen and nitrogen species\textsuperscript{88, 89}. Studies in mouse models have provided evidence for the interaction between the inflammatory microenvironment and tumor-initiating stem cells. Induction of IL1B and IL6 by bile acids leads to inflammation and activates gastric cardia stem cells, which promote columnar-like metaplasia of the distal esophagus and cell changes that result in dysplasia\textsuperscript{20}. Inflammation could also lead to progression of BE by altering the tissue-specific stem cell niche (Figure 4).
It will be a challenge to characterize the cells and signaling pathways involved in these processes, but increasing our understanding these mechanisms could lead to development of chemopreventive agents such as NSAIDs.

IL1b, IL6, and IL8, are upregulated in BE—particularly at the cardia. In mice, expression of IL1B induces chronic inflammation and dysplastic changes that require IL6. These cytokines are upregulated in different types of preneoplastic tissues and tumor microenvironments, in association with activation of nuclear factor (NF)-κB and are involved in development of BE and EAC. Inflammatory cytokines including interferon gamma (IFNG), IL1B, IL6, and IL8, are expressed by epithelial cells in response to acid and bile reflux and attract inflammatory cells including tumor-associated macrophages, neutrophil granulocytes, myeloid-derived suppressor cells, immature myeloid cells, mast cells, and adaptive immune T and B cells. Activation of a T-helper (Th) 1 cell response, characterized by production of IFNG, has been associated with acid reflux-induced esophagitis. This Th1 response found in esophagitis can change to a Th2 profile as BE is established. This shift is associated with an increase in IL4-producing Th2 cells and local increases in IL6. Finally, increases in inflammatory and anti-inflammatory cytokines, but fewer T cells, were found in EAC biopsies, compared with non-tumor tissues, indicating mixed inflammatory profile at this advanced disease of stage.

Progression from ulcerative esophagitis to BE might therefore be accompanied by a shift in cytokine expression patterns. Myeloid and dendritic cells are recruited during esophageal progression of metaplasia to dysplasia and carcinoma. In the mouse model of BE, IL1B induces columnar metaplasia, in part, through recruitment of immature myeloid cells. Furthermore, a CDX2-dependent reduction in a subpopulation of immature myeloid cells with immune suppressor properties prevents dysplasia. Epithelial CDX2 might therefore protect against disease progression by limiting the production of the immature myeloid cells. Cardia and esophageal tissues from EBV-L2-IL1B mice given bile acids to accelerate BE development have a shift in myeloid phenotype towards granulocytic differentiation. Mixed acute (granulocytic) and chronic (IL1B) inflammatory responses might therefore accelerate carcinogenesis. In mice, a myeloid subtype of the CD11b+Ly6G+ granulocyte lineage, tumor-associated neutrophils, support tumor growth by producing nitric oxide, angiogenic factors, and matrix-degrading enzymes.
The tumor stroma contains mesenchymal cells, which might increase proliferation of nearby epithelial cells or recruit and polarize cells of the adaptive and innate immune system into those that promote tumorigenesis\textsuperscript{98,99}. There is evidence from preclinical and clinical studies that mesenchymal cells contribute to the development of gastrointestinal cancers\textsuperscript{100-102} and are associated with gene expression patterns that increase inflammation\textsuperscript{103}.

The composition of the human microbiome changes with and affects many human diseases, including cancer development\textsuperscript{104}. The esophagus contains a complex but conserved population of resident microbes, with an estimated 140 bacterial species—95 of which have been identified\textsuperscript{81}. The esophageal microbiome changes in patients with reflux-related disorders or BE, compared to healthy individuals \textsuperscript{82}. Esophageal microbial diversity decreases and community composition is altered in patients with EAC, including decreased Gram-negative (\textit{Veillonella}, \textit{Megasphaera}, and \textit{Campylobacter}) and Gram-positive taxa (\textit{Granulicatella}, \textit{Atopobium}, \textit{Actinomyces}, and \textit{Solobacterium}) and increased \textit{Lactobacillus fermentum}. However, researchers found no significant differences in the microbiomes of samples of BE or EAC compared to healthy esophageal tissue\textsuperscript{105}. There is no clear evidence that 1 particular species contributes to progression of BE. However, given the differences in species composition between patients with vs without EAC, a small community of microbial species might contribute to carcinogenesis, or could simply result from it. In either case, distinct microbial ecotypes be biomarkers of patients at risk.

The World Health Organization has classified \textit{H pylori} as a class I carcinogen because of its role in gastric adenocarcinoma development. It is estimated that over half of the world’s population is infected with \textit{H pylori}\textsuperscript{106}. There are however well-documented disparities in global \textit{H pylori} prevalence and the inverse correlation between regional gastric cancer risk and EAC risk\textsuperscript{6}. Given that \textit{H pylori} infection provokes chronic atrophic gastritis and hypochlorhydria, it is thought that \textit{H pylori} can decrease the effects of environmental risk factors, such as chronic reflux, to decrease EAC risk.

Patients with BE who are infected with \textit{H pylori} have slower rates of aneuploidy and a non-significant trend towards lower incidence of EAC\textsuperscript{107}. The composition of the gastric microbiome might therefore affect carcinogenesis in the distal esophagus. In patients with reflux esophagitis, the microbiomes of the esophagus and the stomach, particularly the pyloric antrum, overlap in microbial composition\textsuperscript{107}. \textit{Streptococcus} and \textit{Prevotella} species
dominate the upper gastrointestinal tract, but the ratio of these species has also been associated with waist-to-hip ratio and hiatal hernia length. There could be interactions among modifiable risk factors. Importantly, in addition to a possible direct genotoxic effect on the junctional epithelium, an esophageal barrier defect can lead to translocation of non-pathogenic bacteria, which affects immune homeostasis by shifting the balance towards tumor-promoting immune responses, similar to the proposed effects of bile acids.

Bacterial products are sensed by receptors such as the toll-like receptors and NOD-like receptors. Activation of these pathways leads to the production of chemokines, inflammatory cytokines, and anti-microbial peptides. Wnt activation leads to barrier defects that cause aberrant expression and mislocalization of tight junction proteins, including occludin and claudins in epithelial cells, and downregulate production of protective mucins, which occurs during esophageal carcinogenesis. Bacterial invasion of the esophagus and gut in general could induce an inflammatory response that involves upregulation of cytokines such as IL17 and IL23, which promote tumor development.

**Progression of BE to EAC**

The cell intrinsic and extrinsic microenvironments of BE inevitably contribute to clonal evolution in BE tissues that sometimes leads to the development of EAC. Studying BE is a good way to study carcinogenesis in general, because biopsies are collected from patients over long time periods and provide records of changes that occur during tumorigenesis (see Figure 5A-C).

Analyses of genomic heterogeneity in BE have provided support for the clonal origin of the metaplasia. Genotype analyses of biopsies from the Seattle Barrett’s Esophagus cohort have found the BE segments to contain clonal alterations at the CDKN2A (chr 9p) locus (encodes p16). Genome-wide copy number analyses indicated that large-scale somatic mutations at fragile sites also tended to be clonal across the segment. Although these large clones could have been formed by expansion of late-arising mutant clones, through an already established BE esophagus segment, a more parsimonious explanation is that the founder BE cells had already acquired these genomic changes.

Except for the few founder lesions, BE tissues that do not progress to cancer acquire few alterations to genome structure (copy-number alterations or loss of heterozygosity events). By contrast, BE that progresses to EAC during surveillance frequently develop
genomic instability\textsuperscript{113}, often mediated by \textit{TP53} inactivation, leading to genome doubling\textsuperscript{15} followed by the rapid development of EAC. Single nucleotide alterations (SNAs), on the other hand, appear to accrue continually in benign and dysplastic tissue; except for the \textit{TP53} and \textit{SMAD4} genes, genic SNAs are not stage specific\textsuperscript{114}. Moreover, apart from \textit{TP53} and \textit{SMAD4} genes, there is no compelling evidence that cells with SNAs have a selective advantage, and consequently the BE segment is clonally mosaic, formed of a patchwork of genetically distinct lineages\textsuperscript{15, 115}.

BE appears to arise from a clone with a stable genome, and thereafter, cells with genome structural alterations (large-scale losses, gains, and rearrangements in the genome) but not SNAs, are selected. In most patients, there is no evidence of ongoing important evolution, so BE can be thought of as an evolutionarily indolent condition (Figure 5 D and E). It is plausible that the initial BE lesion is well-adapted to the microenvironment, such that slight alterations to the genotype or phenotype of BE cells (or glands)—those changes mediated by most SNAs—are not sufficiently advantageous to expand. In contrast, alterations in chromosome structure could cause changes in cell phenotypes—they are associated with dysplasia after all\textsuperscript{15, 113} —and be selected. Consistent with the concept that few clones undergo strong positive selection, a longitudinal study of 195 patients with BE found that the level of clonal diversity across the entire BE segment (as measured by single-cell genetic analysis of endoscopic brush specimens) to be typically fairly constant over time—no single clone came to dominate the segment over an average of almost 4 years of follow up\textsuperscript{116}.

These evolutionary dynamics have important ramifications for the development of biomarkers to predict EAC development. Changes in genome structure are more likely to identify patients at risk for progression than SNAs alone. More importantly, the level of clonal diversity across a BE segment could be used as a marker of risk for EAC, with higher diversity associated with increased risk\textsuperscript{117}. Because the level of clonal diversity does not change over time, the baseline level of diversity indicates a similar level of cancer risk to measurements made years later\textsuperscript{116}. Moreover, the typical time it takes for a new clone to form and grow to a detectable size could be used to set surveillance intervals.

\textbf{Future Directions}
A method to identify patients with BE most likely to progress to EAC would allow us to allocate resources on those at risk and avoid unnecessary procedures for those at low risk. Increasing our understanding of the pathogenesis of BE, including its clonal origin and early stages of progression to EAC, could lead to identification of biomarkers of risk. There are multiple competing models for the early development of BE and each of these has gained significant traction in recent years. This is an exciting field of research and an integrated approach will allow us to advance more rapidly. Evidence from studies of patients and mouse models indicates a sequence of changes in gland phenotypes, first initiated through a wounding and competitive replacement scenario. Lineage tracing data from a mouse model of BE corroborates this sequence of events. Genetic screens and new mouse models are needed to determine the features of patients, the immune response, and the luminal microenvironment that determine risk of BE development and subsequent carcinogenesis.\textsuperscript{49}
Figure legends

**Figure 1 The Specialized BE Gland**
A) Standard H&E-stained section of BE metaplasia showing goblet cells (arrowheads) along the superficial crypt compartment of the gland and mucous cells (arrows) lining the base of the gland. B) Cartoon of the glands shown in A. The goblet and foveolar cells are shown in pink, whilst the mucous base cells are shown in azure, and the gland’s stem cells are shown in magenta.

**Figure 2. Gland Phenotypes in the Metaplastic Mosaic of the BE mucosa**
The top row shows photomicrographs of representative gland types in H&E-stained sections. The constituent cells of the various glands are indicated (details in the main text). The lower half of the panel shows cartoons of the various gland types with main cell types as shown below. The non-goblet columnar gland is the simple mucous gland of cardiac mucosa and it is the first gland of columnar metaplasia in the distal esophagus. Over time this pioneer gland may change and follow different lines of differentiation, along either gastric or intestinal pathways. Together these gland phenotypes constitute the metaplastic mosaic of BE mucosa.

**Figure 3 Models of Wounding and Competitive Replacement for Development of BE**
A) Low power overview (left) of the squamo-columnar junction in a BE segment. The columnar and squamous epithelia are indicated. Note the submucosal gland complexes, which confirm that this represents bona fide columnar metaplasia of the distal esophagus. High-power view of the squamocolumnar junction (right). The stroma shows granulation features indicative of recent epithelial denudation. The overlying epithelium is a single layer of proliferative, undifferentiated epithelium covering this defect. The squamous and columnar epithelium can be clearly identified on either end. This undifferentiated epithelium may derive from either squamous or columnar epithelial progenitors, but the corrosive acid-biliary reflux micro-environment will drive secondary selection for phenotypes best adapted to this harsh ecology thus favoring mucin-producing columnar progenitors. Lineage tracing experiments in mouse models (see also Figure 4) indicate that this undifferentiated layer of epithelium is clonally derived from the adjacent columnar epithelium. B) The squamocolumnar junction coincides with the anatomic junction of
stomach and esophagus. C) Chronic acid-biliary reflux damages and progressively erodes the native squamous epithelium leading to an ulcerative defect. D) This defect is covered with a single layer of undifferentiated epithelium as shown in A. E) Following selection, the epithelial progenitors mature as mucous glands. F) With time these mucous glands undergo various lines of differentiation, either intestinal (shown) or gastric, depending on the local micro-environment. With recurrent bouts of reflux and ulceration, the columnar epithelium expands, progressively replacing the distal esophageal squamous epithelium.

**Figure 4 Inflammation and the Luminal Niche Promote Expansion of Columnar Progenitors from Proximal Stomach**
A) The squamocolumnar junction coincides with the anatomic junction of stomach and esophagus. Stem cells within glands in the proximal stomach are indicated in orange B) Acid biliary reflux, possibly in conjunction with dysbiosis in the luminal niche, promote expansion of columnar progenitors. The reparative epithelium carries the clonal mark of glands within the proximal stomach. This situation mimics the lineage tracing data obtained from a mouse model of BE. Inflammation is indicated. C) With time this reparative epithelium matures as columnar glands through secondary selection for mucin-producing phenotypes.

**Figure 5 Dynamic Equilibrium and Clonal Stasis During Carcinogenesis**
A) A BE lesion shown in the distal esophagus. B) Endoscopic view of the BE segment (endoscopy picture courtesy of Rehan Haidry). C) The BE segment is a mosaic of glandular clones, which expand and contract over time. Subclones are visualized as colored patches within the BE segment. D) A diagram of the unfolded esophagus showing a BE segment (left). Every gland within the segment is maintained by a unique population of stem cells and can be thought of as a singularly evolving unit within the mucosal sheet. These gland units are shown as hexagons. Patches of colored hexagons denote subclones within the clonally derived BE segment. These clonal patches may contract or expand over time and the overall pattern is therefore consistent with dynamic equilibrium (middle). On rare occasions, some clones are transformed and contribute to tumorigenesis (right). E) Clonal abundance and diversity within the BE segment over time. The y-axis shows the clone size within the segment and the x-axis shows time. The parent clone to take hold within the segment has alterations at the *CDKN2A* locus and fragile-site alterations. From this parent clone,
subclones arise that can recede or expand over time. One clone sustains a biallelic mutation in \textit{TP53}, which promotes chromosomal instability and progression to EAC.
References


36. Moawad FJ, Young PE, Gaddam S, et al. Barrett's oesophagus length is established at the time of initial endoscopy and does not change over time: results from a large multicentre cohort. Gut 2015;64:1874-80.


