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Review

Achalasia and associated esophageal cancer risk: What lessons can we learn from the molecular analysis of Barrett's–associated adenocarcinoma?

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A B S T R A C T

Idiopathic achalasia and Barrett's esophagus (BE) are preneoplastic conditions of the esophagus. BE increases the risk of esophageal adenocarcinoma (EAC), while achalasia is associated with both EAC and esophageal squamous cell carcinoma (ESCC). However, while the molecular mechanisms underlying the transformation of esophageal epithelial cells in BE are relatively well characterized, less is known regarding these processes in achalasia. Nevertheless, both conditions are associated with chronic inflammation and BE can occur in achalasia patients, and it is likely that similar processes underlie cancer risk in both diseases. The present review will discuss possible lessons that we can learn from the molecular analysis of BE for the study of achalasia-associated cancer and contrast findings in BE with those in achalasia. First, we will describe cellular fate during development of BE, EAC, and ESCC, and consider the inflammatory status of the epithelial barrier in BE and achalasia in terms of its contribution to carcinogenesis. Next, we will summarize current data on genetic alterations and molecular pathways involved in these processes. Lastly, the plausible role of the microbiota in achalasia-associated carcinogenesis and its contribution to abnormal lower esophageal sphincter (LES) functioning, the maintenance of chronic inflammatory status and influence on the esophageal mucosa through carcinogenic by-products, will be discussed.

1. Introduction

Achalasia is an uncommon motility disorder of the esophagus characterized by impaired esophageal peristalsis and reduced lower esophageal sphincter (LES) relaxation [1]. The impeded flow of ingested food and secretions from the esophagus into the stomach leads to clinical symptoms as dysphagia, regurgitation of undigested food, weight loss and chest pain [2]. Current evidence suggests that an initial inflammation in the myenteric plexus, most likely caused by a viral disease or other environmental factors, leads to an autoimmune response in genetically susceptible individuals. This results in a degeneration of the myenteric ganglion neurons that control esophageal motility [1].

As achalasia is a disorder with poorly studied etiology, available treatments aim to alleviate symptoms by diminishing the LES pressure [1]. This can be achieved by endoscopic botulin toxin injection, surgical myotomy with or without a fundoplication, pneumatic dilatation or per-oral endoscopic myotomy (POEM) as a new minimally invasive treatment [3,4]. However, effective treatment of achalasia may prompt significant sphincter insufficiency, resulting in gastro-esophageal reflux disease (GERD) and its complications such as chronic inflammation of the esophagus (esophagitis) and Barret's esophagus (BE) [5]. BE is characterized by the replacement of normal squamous epithelium of the lower esophagus with metaplastic columnar epithelium. This transformation is called intestinal metaplasia, and poses a risk factor for the development of esophageal adenocarcinoma (EAC). The annual EAC incidence rate in BE cohorts varies from 0.12 to 3.55% in different studies [6] while the global EAC incidence rate is 0.7 per 100,000 but varies greatly across countries [7]. Treatment of achalasia is associated with an increased EAC risk, with an incidence of 21.23 (StDev31.6) cases per 100,000 patient-years at risk compared to 3.2 cases/100,000 patient-years in the general population in this study [8]. Prevalence for EAC in achalasia patients is 6 per 1000 after endoscopic cardia dilatation, and 7 cases per 1000 achalasia patients after myotomy in studies exclusively evaluating results after endoscopic cardia dilatation and myotomy respectively [8]. Fundoplication after laparoscopic myotomy decreases the incidence of postoperative GERD by preventing acid reflux into the esophagus [3].

On the other hand, in suboptimal treated or non-treated achalasia patients, bacterial overgrowth and chemical irritation from the ongoing decomposition of food and saliva can also lead to chronic hyperplastic esophagitis and malignant transformation of esophageal epithelial cells to esophageal squamous cell carcinoma (ESCC) [9]. A recent review and meta-analysis determined the risk of ESCC in achalasia patients to be 312.4 (StDev 429.16) cases per 100,000 patient-years at risk, compared to 4.3 cases/100,000 patient-years in the general population in this study [8]. Worldwide, ESCC accounts for around 90% of the 456,000 cases of esophageal cancers seen each year, with a global incidence rate

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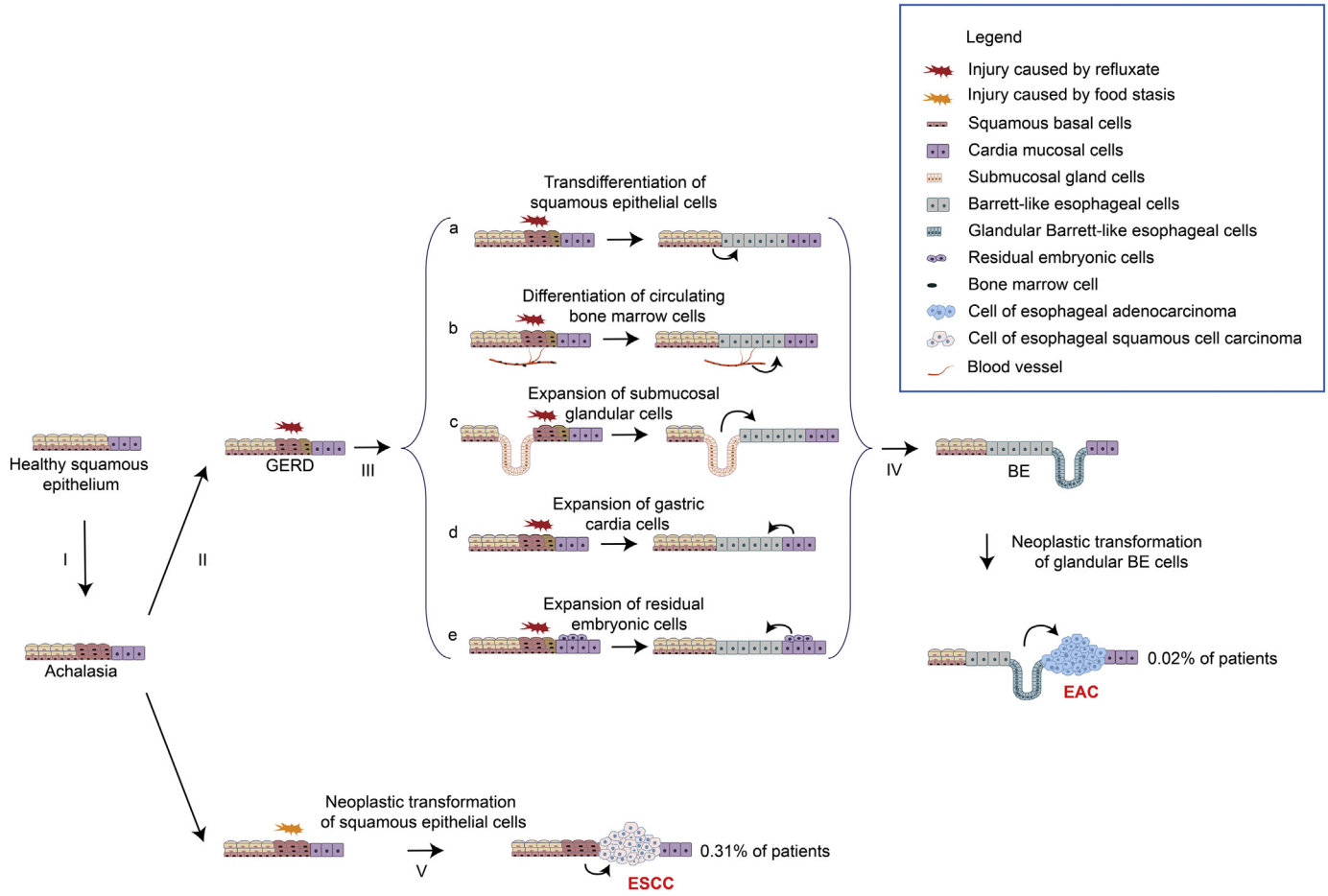

Fig. 1. Cells of origin of Barrett's esophagus (BE), esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC) in achalasia patients (extended from Jiang et al. 2017 [111]. I. The esophageal epithelium of achalasia patients is different from normal squamous epithelium: it is inflamed, has dilated intercellular spaces and increased infiltration of inflammatory cell [38]. II. Treatment of achalasia can lead to gastro-esophageal reflux disease (GERD) and BE (III). Several hypotheses are suggested to explain cells of origin of BE: a) BE epithelium arises through transdifferentiation of stratified squamous esophageal epithelium; b) circulating bone marrow cells transdifferentiate to BE epithelium; c) BE arises from expanded esophageal submucosal gland cells; d) BE originates from stem and progenitor cells (Lgr5+) in the cardia mucosa; e) BE originates from quiescent residual embryonic cells (REC) at the squamous-columnar junction. IV. BE can lead to EAC wich originates from glandular cells near the stomach (0.02% of achalasia patients/year). Sporadically, ESCC can develop from BE (not shown). V. ESCC is derived from squamous epithelial cells (0.31% of achalasia patients /year).

of 5.2 per 100,000 [7].

Also ESCC in BE patients can occur, although very rare [10]. Thus, development of esophageal cancer in achalasia patients may occur via two independent pathways: direct transformation of squamous epithelium to ESCC or to EAC after BE development.

Despite the fact that achalasia is associated with an increased risk of two different types of esophageal cancer, there are no generally accepted recommendations on follow-up evaluation for achalasia patients [11]. This may be explained by the fact that although the gastro-esophageal cancer risk in patients with longstanding achalasia is much higher than in the general population, the absolute risk is relatively low [12,13] as idiopathic achalasia is a relatively rare disease with a mean incidence of 0.3–1.63 per 100,000 people per year in adults. Nevertheless, the majority of patients with esophageal carcinoma have a poor prognosis as they are often diagnosed at advanced stages, no longer eligible for curative surgery. Delay in the diagnosis of esophageal carcinoma is related to difficulties in endoscopic surveillance of achalasia due to stasis of food and because the main symptoms of esophageal carcinoma are mistakenly attributed to achalasia. Therefore, approximately 80% of patients are inoperable at initial diagnosis [14]. Radiotherapy, chemotherapy or chemoradiotherapy in both EAC and ESCC are less efficient resulting in a 5-year survival rate of 19% for esophageal cancer and only 0.9% for advanced esophageal cancer [15].

A better insight into the molecular pathways governing esophageal cancer development in achalasia and BE may be of use to identify patients at risk, better inform patients on associated neoplastic progression risk after dilatation treatment aiming to improve surveillance and treatment strategies.

While the possible mechanisms determining the progression of BE to EAC are relatively well studied, EAC or ESCC in the context of achalasia are relatively underinvestigated. However, taking into account that both disorders are associated with chronic inflammation of the esophagus and development of BE in achalasia patients has been described [16], it is likely that similar molecular mechanisms contribute to cancer development. This review will consider possible lessons that can be learned from the molecular analysis of BE-associated adenocarcinoma for studying achalasia-associated cancer development and describe the probable role of chronic esophagitis, gene mutations and molecular pathways alterations and microbiota changes involved in the malignant transformation.

1.1. Cellular fate in BE, EAC and ESSC

The normal esophageal mucosa consists of a nonkeratinizing, stratified squamous epithelium, lamina propria, and muscularis mucosae [17]. Gastroesophageal reflux is a normal physiological process in

humans, occurring after a meal. In addition to gastric acid, the refluxate contains pepsin, bile, pancreatic enzymes, ingested foods and their metabolites [18]. Anti-reflux and tissue resistance mechanisms are in place to protect the esophageal mucosa against these abrasive fluids [19].

However, these physiological defense mechanisms may no longer be sufficient when poor closure of the LES occurs and a subsequently increased frequency of gastroesophageal reflux causes GERD [19]. Esophageal epithelial barrier function is disrupted in GERD, with decreased expression of tight junction proteins resulting in increased barrier permeability compared with healthy subjects [20]. GERD, itself characterized by squamous hyperplasia, elevated presence of intraepithelial inflammatory infiltrate, epithelial cell necrosis and lack of surface maturation, is a precursor to BE. The metaplastic columnar epithelium of BE appears to be more resistant to reflux-induced injury than the native squamous cells [21] making it tempting to speculate that selection pressure contributes to the development of BE. It is composed of mucinous columnar epithelial cells arranged in surface and crypt epithelia, and contains a variable number of scattered goblet cells, enterocytes, Paneth cells, endocrine cells, and cells with combined gastric/intestinal or intestinal/squamous-cell features [22].

There is a lot of debate regarding the cell of origin of BE. So far, six hypotheses have been suggested [23]: 1) transdifferentiation of esophageal squamous epithelial cells, 2) expansion of submucosal glandular epithelium, 3) expansion of gastric cardia cells, 4) differentiation of circulating bone marrow cells, 5) expansion of residual embryonic cells located at the squamous-columnar junction, 6) p63 + KRT5 + KRT7 + basal cells in a transitional zone between the epithelium of the esophagus and cardia (Fig. 1).

The suggestion is made that whatever the cell of origin, this is also the origin of the subsequent progression to EAC. However, while it is clear that EAC originates from glandular cells near the stomach, BE consists of many cell types, and thus it remains uncertain which cells are the main drivers of EAC. In contrast, ESCC is derived from squamous epithelial cells and appears to be driven by carcinogenic environmental influences, but may also go through dysplastic precursor lesions (Fig. 1) [24]. Cell types present in the esophageal mucosa in described esophageal disorders are summarized in Table 1.

2. The possible role of chronic inflammation in the progression of BE and achalasia toward esophageal cancer

2.1. Inflammation and BE

Chronic inflammation is associated with an increased risk of malignant disease. Around 20% of human cancers are related to chronic inflammation caused by infections, exposure to irritants or autoimmune disease [25]. Inflammation may contribute to cancer development through numerous mechanisms, including DNA damage, angiogenesis, promotion of cellular proliferation, and inhibition of apoptosis [26]. Indeed, inflammatory conditions of the esophagus, specifically reflux esophagitis and BE, have been implicated in the development of esophageal adenocarcinoma [27]. Metaplasia can be accompanied by acute and chronic inflammation of the lower esophagus resulting in increased release of proinflammatory mediators [28]. Key mediators connecting inflammation and BE carcinogenesis include ROS, NFκB pathway activation, inflammatory cytokines, prostaglandins, and immune modulatory microRNAs [28]. For instance, IL-1β, a pleiotropic pro-inflammatory cytokine upstream of inflammatory IL-6 and TNF-α signaling cascades, is overexpressed in BE. Clinical studies have suggested that polymorphisms in the *IL-1β* gene cluster are associated with BE, suggesting that genetic factors predisposing for altered immune regulation contribute to BE susceptibility [29]. In addition, inflammation markers, particularly C-reactive protein and IL-6, were proposed as potential markers for patients with a higher risk of progression to EAC [30]. Furthermore, expression of TNF-α as well as its receptor TNF-R1 are progressively increased from normal squamous mucosa to BE and EAC [31]. The inflammatory link with esophageal adenocarcinoma is further strengthened by the observation that regular use of nonsteroidal anti-inflammatory drugs and aspirin is correlated with decreased risk of cancer development [32].

2.2. Inflammation and achalasia

To what extent inflammation plays a role in achalasia and its progression to ESCC or BE-EAC is less clear. Systemic inflammation has been shown in achalasia patients as compared to healthy controls [33], and corresponded with increased Th17 and Th2 (INF-γ) cytokine levels and decreased Th1 levels [34], although these findings were not substantiated in another study [35]. Achalasia is in itself thought to be an auto-immune condition, in which the mesenteric plexus neurons and ganglions are surrounded by infiltrating $CD8^+$ cytotoxic T-cells, eosinophils, B-cells and mast cells and anti-neuronal antibodies are being produced [36]. However, different subtypes of achalasia may be distinguished, with type I devoid of esophageal motility and minimal pressurization, type II showing a simultaneous contraction and esophageal pressurization and type III characterized by spastic contractions. Different achalasia types show differences in the number of infiltrating immune cells as well as the number of ganglions. It is as yet unclear whether these subtypes are different disease entities, or whether they are progressive stages of disease, but it has been proposed that while type III is caused by a different etiology, type II is an early form of achalasia showing active inflammatory response against neurons and type I represents a late form of achalasia where the loss of immune-provoking ganglions results in loss of inflammation [37]. However, histological analysis of the full-layer mucosa in early and advanced achalasia showed that inflammation was present in early achalasia, but histological esophagitis with findings of increased inflammatory cell infiltration and dilated intercellular spaces were also observed in patients with late achalasia [38]. Furthermore, in patients with end-stage achalasia, the squamous mucosa is consistently altered compared with control specimens and closely resembles that seen in GERD with

Table 1
Different cell types present in esophageal lining of the esophagus in health and diseases.

Heath/disease	Cell types	Disease manifestation
Healthy mucosa	Squamous epithelial cells	–
GERD	Squamous epithelial cells	Immune infiltrate; barrier defect
BE	Mucinous columnar epithelial cells; enterocytes; Paneth cells; endocrine cells; cells with combined gastric/intestinal or intestinal/squamous cell features	Immune infiltrate; spatial mislocalisation of intestinal barrier cells
Achalasia	Squamous epithelial cells	Immune infiltrate
EAC	Derives from glandular cells near the stomach	Gland-forming tumor with variable grade of differentiation (as defined by gland formation or mucinous differentiation)
ESCC	Derives from squamous epithelial cells	Squamous cell hyperproliferation with variable degree of differentiation (as defined by keratinization)

GERD: gastro-esophageal reflux disease, BE: Barret's esophagus, EAC: esophageal adenocarcinoma, ESCC: esophageal squamous cell carcinoma.

different grades of esophagitis [39]. Thus, chronic esophagitis is present in achalasia patients, and as this is the main risk factor for ESCC development, it may also contribute to the increased risk of ESCC in patients with achalasia [40]. However, as the diagnostic technique to distinguish different achalasia subtypes (high-resolution esophageal pressure topography) is relatively novel [41], it is as yet unclear whether these carry different risk of cancer development.

The occurrence of chronic esophageal inflammation in achalasia patients can be partially explained by the LES-lowering therapy, which may enhance gastro-esophageal reflux and predispose to BE development. Indeed, GERD was diagnosed in up to 31.5% of achalasia patients following myotomy and in up to 33% following pneumatic dilatation [3,42]. BE develops in 8.4% of achalasia patients, while post-treatment LES pressures were lower in patients with BE than in those without [16]. Besides this systematic study, only a limited number of patients have been described with a combination of achalasia and BE. This includes one patient who developed BE after pneumatic dilatation [43], 31 patients after surgical myotomy [33,43,44] and interestingly, eight patients who were diagnosed with BE without being treated for their achalasia [33,43,45]. Several hypotheses might explain this phenomenon, the first being that the development of BE had occurred before the onset of achalasia [46]. Secondly, a combination of transient LES relaxations (TLESRs) and impaired esophageal clearance may co-occur in achalasia patients thereby causing both diseases independently [47]. The third explanation is that the esophagitis in untreated achalasia patients can be the consequence of fermentation and bacterial overgrowth through food stasis, especially when prominent dilatation of the organ is seen (see below) [45]. Smart et al. [48] showed a low pH in the esophagus of untreated patients without episodes of gastroesophageal reflux; in addition, increased lactic acid and a lower esophageal pH were present in patients with achalasia who had retained food, as compared with those who did not. As stasis of food is common in achalasia patients, even after dilatation treatment, it is difficult to distinguish between inflammation caused by acid reflux or food stasis. These various possibilities are probably not exclusive of each other; it is more likely that different mechanisms operate in different patients [46,49]. Hence, these findings indicate that there is a direct pathological connection between achalasia and the development of Barrett's segment. Chronic inflammation might be a link between these conditions and their progression to cancer and it seems reasonable to assume that the esophageal epithelium may be affected by similar processes during progression from GERD to BE and EAC and from achalasia to BE and EAC or to ESCC.

3. Genetic alterations and molecular pathways involved in cancer development in BE

While inflammatory processes are thought to contribute to carcinogenesis through the induction of DNA damage and growth advantage-conferring mutations, the molecular and genetic relationship between EAC, ESCC, and achalasia as their precursor lesion, is poorly understood. However, in order to identify possible therapeutic targets for prevention and treatment of esophageal carcinoma, the molecular pathways involved in the malignant progression from BE to EAC have received vast attention [6]. Improvements in high-throughput genomic technologies have led to a better understanding of the molecular basis underlying the development of EAC and ESCC [50].

Analysis of gene mutations revealed that in EAC, 26 genes are frequently and significantly mutated. Among these genes are tumor suppressors such as *TP53* (72% of cases) and *p16/CDKN2A* (12% of cases) as well as bacterial recognition receptor *TLR4* mutations (6% of cases) [51]. Interestingly, BE tissue appears to be highly mutated even prior to the occurrence of dysplasia, with a mutation rate superior to many other tumors at an advanced stage of development (6.76 mutations/Mb) [52]. Thus, an accumulation of mutations appears to underlie the BE-to-EAC sequence, which is already initiated at early BE stages. This is seen for instance for *TP53* mutations, which are scarce in BE, but accumulate in EAC [53]. However, while a shared mutational context suggests that the same mutational trigger underlies both BE and EAC, it has also been shown that the mutations in BE are clonal, and the specific mutations observed in different clones do not overlap greatly with those found in EAC (for example, mutation of *EYS*, *ARID1A,* and *ABCB1* genes was only shared in 28% of paired Barrett's and EAC samples) [52]. Furthermore, while *TP53* and *P16* mutations are homogeneously present within EACs, and appear to represent early events during carcinogenesis, clonality within EAC also exists, with loss of heterozygosity of *SMAD4* and *APC* not evenly distributed within the tumor [54]. Longitudinal genetic analysis of BE patients suggests that the number and diversity of clones within BE segments changes little over time [55], however, patients who progress to EAC during their lifetimes ($< 5\%$) develop signs of chromosome instability with gene losses and gains, genomic heterogeneity, selection of somatic chromosome abnormalities and catastrophic genome doublings [56]. Esophageal cancer development is also associated with a clear increase in copy number alterations (CNAs), which are much less frequent in BE. Some of these molecular abnormalities can be used to predict the neoplastic progression risk of BE [57]. For instance, high clonal diversity was associated with increased progression risk of BE [55].

In addition to gene mutations, altered gene transcription patterns are observed in BE and EAC. Based on this pattern, prediction models for progression have been developed with a 90-gene signature showing promise as a biomarker for low grade dysplasia in BE [58]. Within this signature, one third of genes was regulated by the proto-oncogene c-MYC, with other candidates HNF1-α, SP-1, NF-Y, E2F1, TP53, ESR1 and HIF1A following suit [58]. A recent review and meta-analysis confirmed the use of p53 immunohistochemical staining to improve risk stratification in BE surveillance [59].

3.1. Markers for progression of achalasia to cancer

Interestingly, esophagectomy specimens from achalasia patients also display a heightened frequency of p53 immunoreactivity, indicative of early changes related to ESCC risk [60]. Aberrant expression of the p53 protein correlated with grade of inflammation in idiopathic achalasia [61], and increased with progressive grades of dysplasia. A recent study further showed that patients with achalasia and retention esophagitis have higher positive rates of p53 and p16 expression (a key regulator at the G1-S checkpoint in the cell cycle often deregulated in cancers) than those from achalasia patients without retention esophagitis and control groups [62]. These data suggest that achalasia-associated chronic inflammation may mediate clonal evolution by generating a mutagenic pressure or providing a selective advantage to those clones able to survive an inflammatory insult [63]. However, aside from the above mentioned *TP53* mutations, the mutation burden in the mucosa of patients with achalasia is relatively uncharacterized and the exact genetic evolution from achalasia to esophageal squamous dysplasia and ESCC remains unknown. ESCC itself is characterized by aneuploidy of chromosomes 7, 11, and 17 as well as *TP53* gene deletion [64]. Aneuploidy was also reported to be present in achalasia and chagasic megaesophagus patients, with chromosome 7 monosomy or trisomy and chromosome 17 monosomy or trisomy being the most frequently occurring aneuploidies [64], suggestive of an achalasia-to-ESCC carcinogenic sequence. Mutation of the *PIK3CA* gene was reported to be associated with Chagas disease and ESCC, but there is no evidence regarding its association with idiopathic achalasia [65].

As for EAC and BE, clonal expansion of ESCC and its premalignant lesion esophageal squamous dysplasia are implicated by their highly heterogeneous and polyclonal nature [66]. Dysplasia is heavily mutated and harbors most of the driver events reported in ESCC, with *TP53* mutations a prerequisite for progression to ESCC. However, unlike BE to EAC progression, copy number alterations are already common in dysplastic stages and persist during the ESCC progression. Whether

Table 2
Dysregulated genes observed in Barrett's esophagus (BE), esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). When known, frequencies are reported (adapted from: [54, 71–74]).

Gene	BE (%)	EAC (%)	ESCC (%)
Receptor tyrosine kinases			
ERBB2	01–13	32	3
EGFR	0–4	15	19
KRAS	present, frequency not reported	14	7
PIK3CA	0–4	3	13
Cell cycle regulators			
CDKN2A	30–42	76	76
CCND1	present, frequency not reported	15	57
CCNE1	present, frequency not reported	14	4
RB	0–8	0	9
Proliferation and differentiation			
MYC	present, frequency not reported	32	23
SMAD4	0	24	8
GATA4	present, frequency not reported	19	1
GATA6	present, frequency not reported	21	3
TP63 OR SOX2		11	48
Chromatin remodeling			
KMT2D	4–13	1	14
Cell death			
TP53	2.5–72	75	69
Cell adhesion, migration, cytoskeleton organization			
TTN		55	34
MUC16		31	14
SYNE1	3–4	30	11
Other mutated genes			
TLR4	13	5	1
LRP1B	0–4	25	11

Table 3
Genes associated with achalasia. Adapted from [74–79]. When known, frequencies of mutation in esophageal cancer are reported (adapted from: [54, 71–74]).

Gene	Role in achalasia	EAC (%)	ESCC (%)
Cell death			
TP53	Increased expression in achalasia	75	69
Cell cycle regulators			
CDKN2A	Increased expression in achalasia	76	76
Immunology			
HLA	Variants confer susceptibility to idiopathic achalasia		0.62–2.17
Nos1	Mouse knockout model for achalasia		3
LTA/TNFα	Likely involved in onset of disease		
IL10	Likely involved in onset of disease	0	
IL23	Likely involved in onset of disease		
DNA methylation and repair			
Rassf1a	Mouse knockout model for achalasia		
Receptor tyrosine kinase signaling			
Kit	Mouse knockout model for achalasia	1	2
RET	Likely involved in onset of disease		3
Spry2	Mouse knockout model for achalasia		
Neurological processes			
GDNF	Likely involved in onset of disease		
VIPR1	Likely involved in onset of disease		
MYO5B	Likely involved in onset of disease		2
ADRB2	Likely involved in onset of disease		
Other			
AAAS	Likely involved in onset of disease		0
GUCY1A1	Associated with moyamoya disease 6 with achalasia		2
GMPPA	Associated with alacrima, achalasia and mental retardation syndrome		

EAC: esophageal adenocarcinoma.
ESCC: esophageal squamous cell carcinoma.

these copy number alterations are already present at achalasia stages remains unanswered.

Despite the presence of common denominators, including the aforementioned *TP53* point mutations, studies have indicated that ESCC is genetically more similar to other squamous cancers, such as head and neck, than to EAC [51,67]. Risk factors for ESCC include tobacco and alcohol consumption and this tumor is more common in the upper and mid-esophagus, whereas the EAC predominates in the lower esophagus and is associated with obesity and GERD [68]. A comparison of copy number alterations as well as DNA methylation, mRNA and microRNA expression patterns between 90 ESCCs and 72 EACs revealed a clear separation between these types of esophageal cancer [69]. Although both diseases share similarly high frequencies of overall and clinically relevant genomic alterations, different genetic mutations associated with specific cellular pathways, such as cell cycle, apoptosis, DNA repair mechanisms, growth factor receptors, have been identified in esophageal squamous cell cancers (see Tables 2, 3) [67]. When achalasia progresses to EAC, the oncogenic events are likely to be different compared to the progression to ESCC. Again, the mutational sequence from achalasia to BE remains unknown, and it would be of interest to compare mutational burden in achalasia-associated EAC to EAC that is not associated with achalasia.

3.2. Other molecular changes in achalasia patients

Except for investigation of the role of p53 and aneuploidy as predictive markers of neoplastic transformation, temporal information on the molecular pathways involved in malignant transformation of achalasia is limited. However, some studies have investigated the molecular events taking place during the presentation of achalasia itself.

A recent study found elevated miR-130a expression levels in esophageal mucosa specimens of achalasia patients [70]. miR-130 serves an important role in multiple types of tumors by targeting important phospho-protein modulators of cell survival and migration signaling [71]. Furthermore, functional classification of whole transcriptome analysis of achalasia esophageal biopsies revealed a significant overrepresentation of phosphorylation processes, intracellular signaling, cell communication, and development/differentiation processes, including cell death [72]. The major expression alterations contributing to this profile in achalasia patients were a downregulation of CYR61, CTGF, c-KIT, DUSP5, EGR1 and an upregulation of AKAP6 and INPP4B. One study investigating the onset and development of achalasia, although not its neoplastic transformation, revealed 1728 differentially expressed genes in achalasia mucosa [73]. These fell into 5 major classes of pathways: cell migration, cell signaling, neuron signaling, immune response, and actin stress fiber formation and regulation, suggesting that achalasia at early stages shows potential oncogenic alterations. Interestingly, upstream regulator analysis of these expression patterns revealed TLR4 and IL18 to be the most significant and activated regulators.

TLR4 is a transmembrane protein receptor that recognizes pathogen-associated molecular patterns released by viruses and bacteria [74]. The relationship between TLR4 signaling and tumorigenesis is complex with evidence showing that TLR4 signaling can enhance or suppress cancer development, depending on the model system [75]. TLR4 expression increases from normal esophageal epithelium to low-grade dysplasia – high-grade dysplasia -EAC and is associated with advanced stage and poor prognosis in EAC [76]. However, it was later shown that TLR pathway genes are frequently mutated in EAC and that these mutations decrease responsiveness to bacterial ligands [75]. TLR4 expression is also increased in ESSC [77], and correlates to tumor stage, differentiation grade (good, moderate and poor) and risk of metastasis [78], but mutations in *TLR4* gene are less frequent [79]. These data suggest a difference in how the bacterial composition in achalasia and

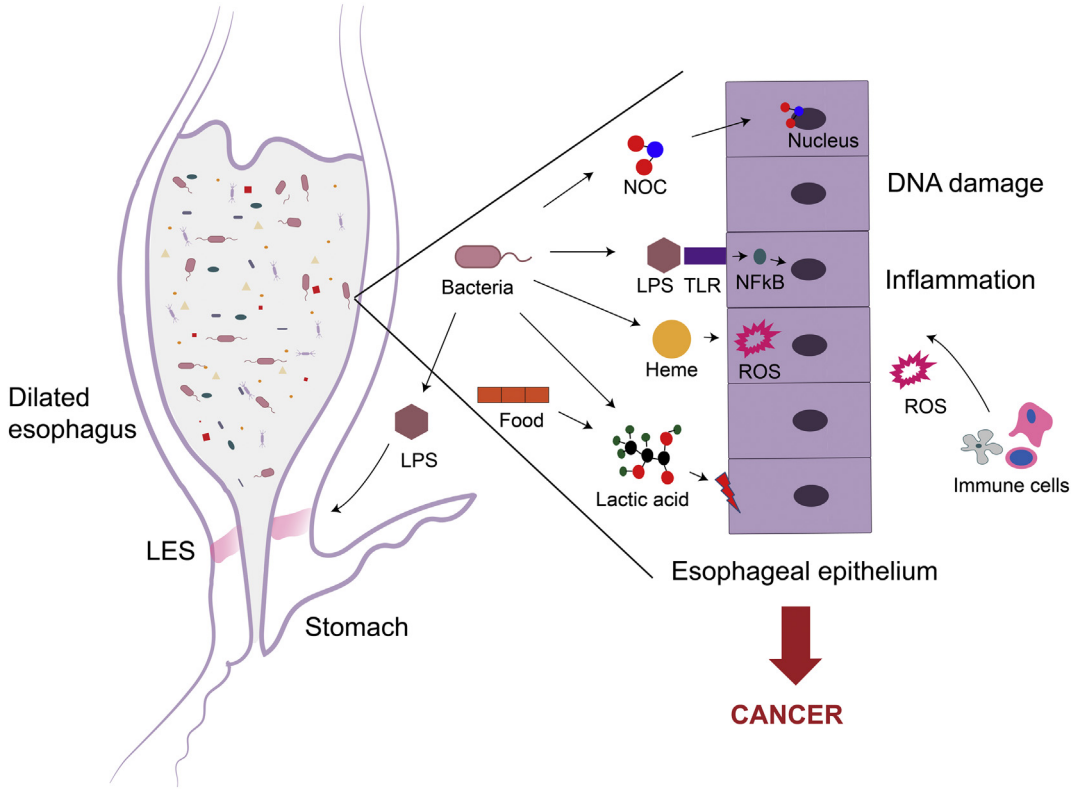

Fig. 2. In patients with achalasia, abnormal lower esophageal sphincter (LES) pressure leads to food accumulation and bacterial overgrowth in the dilated esophagus. Subsequently, DNA damage can occur through inflammation induced by N-nitrosocompounds (NOC), lipopolysaccharide (LPS) and lactic acid produced by bacteria, by lactic acid formed during the digestion of food, and by reactive oxygen species (ROS) produced by involved immune cells and during metabolism of bacterial heme. LPS can also contribute to LES relaxation and occurrence of BE. Altogether, it suggests possible role of microbiota in achalasia-associated cancer development. TLR: toll-like receptor.

BE contribute to the formation of ESSC and AEC, respectively.

4. The possible role of the microbiome in BE- and achalasia-associated cancer

One of the contributing factors to induce and maintain chronic inflammation is the microbiota. The classic germ theory of disease described an infectious disease as the direct result of a single pathogen which can become virulent and predispose the host to inflammation and disease, for example, *Helicobacter pylori* is a causative agent of gastric ulcers and gastric cancer and *C. difficile*, in causing diarrhea and colitis [80]. Nowadays, dysbiosis within the microbial community, defined as any change to the composition of resident commensal communities relative to the community found in healthy individuals is considered a potential cause of disease. Three types of dysbiosis have been described: 1) loss of beneficial microbial organisms, 2) expansion of overall pathobionts and 3) loss of overall microbial diversity [81]. Our body hosts 10^{13} bacteria, most of which reside in the intestine, requiring a complex interplay between the microbiota and mucosal epithelial cells, innate and adaptive immune responses [82]. Several diseases have now been associated with alterations in the microbiome, including gastrointestinal cancers [83]. Thus, it is tempting to speculate on a possible role of single microbial pathogens or dysbiosis in the development and progression of BE or esophageal achalasia toward cancer.

4.1. Microbiome in BE

Although the esophagus and stomach previously seemed uninhabitable, new molecular techniques allowing the detection of microbial DNA now indicate that a range of microbes is present in these organs. Sequencing of the universal bacterial 16S ribosomal RNA gene showed that Gram-positive bacteria are typical of the healthy esophagus [84]. Around 100 unique taxa are present in the esophagus, consisting of 6 major phyla: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and TM7, with 39% of isolates belonging to the Streptococcus genus, followed by *Prevotella* (17%) and *Veillonella* (14%). The healthy esophageal microbiome has been recently clustered into 3 functionally distinctive community types (esotypes) defined by diversity and composition [85]. The first esotype is characterized by an abundance of *Streptococcus* and *Prevotella* as well as increased levels of *Haemophilus* (*Haemophilus parainfluenzae*) and *Rothia* (*Rothia mucilaginosa*). Dominance of *Streptococcus* is typical for the second esoptype, whereas dominance of *Prevotella and Veillonella* is seen in the third group. Thus, the esophageal microbiota is a relatively stable and unique community and not merely composed of organisms in transit [86].

Yang et al. compared the microbiota of GERD and BE patients with healthy subjects and showed that the esophageal microbial community in GERD patients was associated with by a greater number of obligate anaerobes, such as Bacteroidetes phylotypes (e.g., 13% *Prevotella*) and Gram-negative Proteobacteria (e.g., 6% *Haemophilus*, 5% *Neisseria*). [87]. Generally, BE is also characterized by an increased relative abundance of Gram-negative bacteria, including *Fusobacterium*, *Neisseria*, *Campylobacter*, *Bacteroides*, Proteobacteria, and *Veillonella* taxa, and decreased levels of Gram-positive *Streptococcus*. Furthermore, the bacterial load in BE patients appears to be increased compared to non-BE patients [88]. Deshpande et al. have also described relevant microbial signatures and metabolic pathways associated with GERD and BE [85]. An enrichment of bacterial superpathway of hexitol degradation, microbial lactic acid production (homolactic fermentation) and an increase in heme biosynthesis from glycine or uroporphyrinogen was found in GERD, whereas heterolactic fermentation increased in BE.

Thus, GERD and BE are associated with distinct alterations in the esophageal microbial composition and their associated metabolic changes.

4.2. A role for the microbiome in achalasia and progression to EAC/ESSC?

To the best of our knowledge, the microbiome of patients with idiopathic achalasia is not characterized. However, this disease induces progressive dilatation of the organ, eventually causing megaesophagus, a clinical presentation which is also typical for patients with pseudoachalasia [89] and Chagas disease, a parasitic disease caused by *Trypanosoma cruzi* [90].

Culture-based studies have shown that the chagasic megaesophagus microbiome is dominated by anaerobic Gram-negative *Veillonella* and aerobic Gram-positive *Streptococcus* [91] and that bacterial load correlates with the degree of esophageal dilation. Our own studies suggest that achalasia is often accompanied by fungal growth (unpublished data) and it is to be expected that food stasis will induce local alterations of both the micro- and mycobiome in these patients.

Several theories have been discussed to explain how changes in microbiome could predispose to BE and EAC, and similar mechanisms of microbiota-associated cancer development could apply to achalasia-related cancer (Fig. 2).

First, abnormal LES function is incompletely understood, and might actually stem from esophageal dysbiosis. As mentioned above, it suggested that a Gram-negative bacteria-dominated esotype may play a causative role in GERD [87]. In animal studies, lipopolysaccharide (LPS), a product of Gram-negative bacteria, has been shown to relax the LES and delay gastric emptying [92]. Alternatively, this esotype might be secondary to the changes caused by gastric reflux [93]. Motility disturbances in BE patients such as weak LES pressure and poor contractility [94] may predispose to bacterial overgrowth in the distal esophagus or modification of the esophageal microbiome by selecting against acid-sensitive bacteria in the esophagus [93]. This can cause the further disturbance of the LES and increase of reflux.

The *second* possibility is that Gram-negative bacteria can promote chronic low-grade tissue inflammation, thereby contributing to the development of metaplasia and inflammation-induced carcinogenesis [95]. The esophageal mucosa faces a quite aggressive environment with permanent mechanical and chemical irritation: wear and tear, heating, or cooling after swallowing food boluses and contact with gastric and gastroduodenal acidic contents during reflux episodes. In inflammatory conditions such as GERD and achalasia [20], impairment of the esophageal barrier may, in theory, lead to direct contact of mucosal immune cells and microbiota. Thus, bacterial overgrowth and presence of Gram-negative bacteria in achalasia patients may lead to higher bacterial antigen loading on antigen presenting innate immune cells and subsequent triggering of adaptive immune responses conductive of neoplastic transformation. Bacterial products may also directly activate innate immune responses and promote tissue inflammation. For instance, LPS detection by TLR4 leads to activation of the pro-inflammatory transcription factor NF-κB, a signaling molecule that plays a pivotal role in many tissue inflammatory responses and expression levels of which are increased along the spectrum from BE to EAC [29].

In addition to general changes in the microbiome, particular species have also been associated with esophageal pathology [86]. While early culture-based studies of esophagectomy specimens from both EAC and ESCC samples did not note any difference in organisms isolated in benign versus malignant tissue [96], a more recent study using 16S sequencing found increased *Treponema denticola* and *Streptococcus* species in esophageal cancer patients compared to controls [97]. These *Streptococcus* strains were subsequently shown to induce pro-inflammatory cytokine production in esophageal cancer cell lines. Blackett et al. demonstrated significant enrichment of *Campylobacter* in GERD and BE, but not EAC, as compared controls and demonstrated that cytokines linked with carcinogenesis (eg IL-18) were increased in tissues colonized by *Campylobacter* [98]. This notion is consistent with the 'driver-passenger' theory, which states that carcinogenic bacteria may be displaced or outcompeted by non-carcinogenic bacteria due to changes in the local niche upon cellular transformation [99]. Another bacterial driver of esophageal cancer might be *Fusobacterium nucleatum*, a bacterium shown to directly promote colorectal carcinogenesis through activation of molecular pathways governing cell adhesion and migration (e.g. E-cadherin/b-catenin) [100], and the abundance of which has also been noted in BE [87,93].

Lastly, the *third* link from bacteria to carcinogenesis is through their metabolites (N-nitrosocompounds, lactic acid, heme). Gram-negative bacteria can reduce dietary nitrates to nitrites, which in turn are precursors of the carcinogenic N-nitrosocompounds formed in the acidic environment of the cardia and distal esophagus in reflux disease [101]. This hypothesis is supported by the fact that Macfarlane et al. identified bacterial species that produce nitrosamines in patients with BE. The presence of bacteria that promote nitrosamine production correlated with consequent tissue metaplasia and dysplasia [102]. Patients with megaesophagus also possess bacteria in the esophageal lumen with the capacity to metabolize nitrates [91]. Thus, increased concentrations of microbial metabolites within the esophagus and stasis of dietary carcinogens can be the reason for the increased prevalence of ESCC in patients with achalasia and megaesophagus [103]. In addition, it is shown that heme can damage colon epithelium and induce its hyperproliferation by causing oxidative stress; its production by bacteria is also increased in GERD [85,104].

Thus, while data characterizing the microbiome of achalasia patients is lacking, it is likely that some of the bacteria related to cancer development may also contribute to cancer risk in this group of patients. For ESCC, the microbiome is also relatively poorly characterized [105]. Nevertheless, Yu et al. observed a negative correlation between esophageal microbial richness and esophageal squamous dysplasia (the precursor lesion of esophageal squamous cell carcinoma) [106] and other studies showed that *Clostridiale, Erysipelotrichales* and *F. nucleatum* are enriched in patients with ESCC [107,108]. In addition, severity and prognosis of disease correlate with the presence of *Porphyromonas gingivalis* [109]. Thus, an increased bacterial load, presence of antigens, persistent mechanical and chemical irritation of epithelium and direct and indirect mediation of bacterial-induced inflammation may all contribute to carcinogenesis. Besides a role in neoplastic transformation, the microbiome can also contribute to some of the comorbidities of megaesophagus, such as recurrent aspiration pneumonia and chronic pulmonary infections, as well as infectious complications related to esophageal perforation during surgical or endoscopic procedures [110].

5. Concluding remarks

Achalasia is a rare esophageal disease that increases the risk of development of two types of cancer. Achalasia can progress to ESCC or BE and then to EAC. Both precursor lesions achalasia and BE are associated with chronic inflammation that can contribute to neoplastic transformation, however, BE is relatively well characterized in terms of genetic alterations, molecular pathways and microbiota changes. Analysis of genomic and transcriptomic changes in the esophageal mucosa of achalasia patients that do and do not progress to esophageal cancer might reveal novel targets for therapeutic approach and predictive markers. The studying of evolutionary dynamics of achalasia might be important both for understanding the fundamental process of neoplastic progression and for the clinical management of the disease. It might help to determine high risk patients for regular surveillance which is under discussion for achalasia patients and the spatiotemporal dynamics of clonal evolution occurring in achalasia might be potentially useful for optimization of endoscopic surveillance intervals in this group. BE is characterized by distinct alterations in the esophageal microbial content. Microbiota might play an even more important role in the transformation of epithelium in achalasia patients due to continuing food stack and bacteria overgrowth. It might contribute to

abnormal LES functioning, to the maintenance of chronic inflammatory status and influence esophageal mucosa with carcinogenic by-products. Moreover, the difference in responsiveness to bacterial ligands via TLR4 could be determinative to what type of cancer achalasia will progress.

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