Esophageal Cancer and Barrett's Esophagus

Targeted molecular profiling and long-term outcome following minimally invasive esophagectomy and endoscopic treatment

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Table of content

Abbreviations

AC	Adenocarcinoma
SCC	Squamous cell carcinoma
EC	Esophageal cancer
CRT	Chemoradiotherapy
GERD	Gastrointestinal reflux disease
EAC	Esophageal adenocarcinoma
ESCC	Esophageal squamous cell carcinoma
BE	Barrett´s esophagus
LGD	Low-grade dysplasia
HGD	High-grade dysplasia
HMIE	Hybrid minimally invasive esophagectomy
TMIE	Totally minimally invasive esophagectomy
HRQL	Health-Related Quality of Life
UICC	Union for International Cancer Control
MMR	Mismatch repair system
MSI	Microsatellite instability
ddNTP	Dideoxynucleotides triphosphate
DNMT	DNA methyltransferase
MSP	Methylation-specific PCR
qMSP	Quantitative methylation-specific PCR
ROS	Reactive oxygen species
REK	Regional etisk komite
OS	Overall survival
DFS	Disease-free survival
MIE	Minimally invasive esophagectomy
OTE	Open transthoracic esophagectomy
CE-N	Complete eradication of neoplasia (HGD, EAC)
CE-D	Complete eradication of dysplasia
CE-IM	Complete eradication of intestinal metaplasia
RAMIE	Robotic minimally invasive esophagectomy
UPSS	The Utrecht Pneumonia Scoring System
i.q.r	Interquartile range

Thesis summary

Esophageal cancer (EC) is the 10. most commonly diagnosed cancer worldwide. Histologically, there are to main types – adenocarcinoma (AC) and squamous cell carcinoma (SCC), of which the latter accounts for approximately 90% of all cases. However, in Northern and Western Europe the situation is inversely related – more than 90% of all patients has adenocarcinoma (AC).

It is thought that esophageal AC (EAC) arises from a premalign condition known has Barrett´s esophagus (BE). This condition requires regular surveillance (upper endoscopy) in order to detect any dysplastic or neoplastic transformation. In case of dysplastic BE or superficial EAC, the treatment is endoscopic resection and/or ablation of the BE mucosa. If more advanced cancer, the standard curative is chemoradiotherapy (CRT) followed by esophagectomy.

The main aims of this thesis were to investigate the long-term outcome after hybrid minimally invasive esophagectomy (HMIE, paper 1), totally minimally invasive esophagectomy (TMIE, paper 3), endoscopic treatment for dysplastic BE/superficial EAC (paper 2) and to describe the prevalence of specific genetic and epigenetic alterations in patients with BE and EAC (paper 4). In the literature, there are limited data on long-term outcome, especially health-related quality of life (HRQL), following these treatment modalities as well as the prevalence of the specific genetic and epigenetic alterations in patients with BE and EAC.

In paper 1 we found a 5-year overall survival following HMIE of 49% and 53% for those with microscopically free resection margins (R0). The anastomotic leakage rate was 5%. Twelve percent had delayed surgery, more than 4 months after the esophagectomy, primarily due to local recurrence. After more than 5 years, the three main symptoms reducing HRQL were reflux (44%), fatigue (32%) and anxiety (32%). In paper 3 we looked at the same variables as in paper 1, but in a series of patients operated with TMIE. The 5-year overall survival was 53% and 57% for the R0 resected. The anastomotic leakage rate was 14% and 11% had delayed surgery, none for local recurrence. Anxiety, cough and insomnia were the three most common symptoms reducing HRQL after 5 years in 35%, 32% and 27% of the patients, respectively.

In paper 2 the patients were treated with EMR and/or RFA for dysplastic BE or superficial esophageal cancer. After a median follow-up time of almost two years, 78% of patients with LGD, 66% with HGD and 89% of those with T1a/T1b had complete

histologically remission or downstaging. Postprocedural, 2% experienced bleeding and 8% developed a stricture that needed treatment. Almost 2.5 years after the initial treatment, 88% of the patients experienced no dysphagia and 87% reported adequate (not reduced) HRQL.
In paper 4 we used tissue samples from the surgical specimen following esophagectomy as well as biopsies from 19 non-dysplastic BE patients. We examined the prevalence of specific genetic (*TP53* and MSI status) and epigenetic (DNA promotor hypermethylation of *APC*, *CDK2A*, *MGMT*, *TIMP3* and *MLH1)* alterations in this population of patients. We found that 28% of the patients with EAC had mutations in *TP53*, while 6% showed MSI. None of the BE patients had these alterations. The epigenetic alterations were frequently seen in both EAC (5-62%) and non-dysplastic BE (16-89%).

Papers in the thesis

Paper 1: Hauge T, Amdal CD, Falk RS, Johannessen HO, Johnson E. Long-term outcome in patients operated with hybrid esophagectomy for esophageal cancer - a cohort study. Acta Oncol. 2020 Jul;59(7):859-865.

Paper 2: Hauge T, Franco-Lie I, Løberg EM, Hauge Truls, Johnson E. Outcome after endoscopic treatment for dysplasia and superficial esophageal cancer - a cohort study. Scand J Gastroenterol. 2020 Sep;55(9):1132-1138.

Paper 3: Hauge T, Førland DT, Johannessen HO, Johnson E. Short- and long-term outcomes in patients operated with total minimally invasive esophagectomy for esophageal cancer. Dis Esophagus. 2021 Sep 7. Epub ahead of print.

Paper 4: Pinto R, Hauge T, Jeanmougin M, Pharo H D, Kresse S H, Honne H, Winge S B, Five M-B, Kumar T, Mala T, Hauge Truls, Johnson E, Lind, G E. Targeted genetic and epigenetic profiling of esophageal adenocarcinomas and non-dysplastic Barrett's esophagus. Clinical Epigenetics. 2022. In Press.

Introduction/background

Esophageal cancer in general

Esophageal cancer (EC) is the tenth most commonly diagnosed cancer worldwide and the sixth most common cause of cancer-related death [1]. The main symptoms of EC are persistent and increasing level of dysphagia, weight loss and painful swallowing (odynophagia).

Histologically, squamous cell carcinoma (SCC) and adenocarcinoma (AC) and are the two main types of EC. It has been estimated that alcohol consumption, smoking and diet low in fruit and vegetable accounts for 90% of all cases of all esophageal squamous cell carcinoma (ESCC) in the US [2]. In the high endemic regions going from Northern Iran to Central China ("esophageal cancer belt") less is known about the underlying risk factors for SCC, but it is thought to include poor nutritional status, low intake of fruit and vegetables and drinking beverages at a high temperature.

Most, if not all cases of esophageal adenocarcinoma (EAC) arise from a premalign condition known as Barrett´s esophagus (BE) in which the normal stratified squamous epithelia of the lower esophagus transform (metaplasia) into simple columnar epithelium due to prolonged tissue injury, typically by chronic gastrointestinal reflux disease (GERD). In addition to chronic GERD (> 5 years) main risk factors with regard to development of BE includes advanced age (> 50 years), being male, tobacco usage and Caucasian race [3]. About one third of the Norwegian adult population experience GERD and approximately 7% of patients with GERD have BE, of whom 14% have dysplasia [4-5]. Other known risk factors include smoking and obesity, each of which approximately doubles the risk compared to non-smokers and those with BMI < 25, respectively [6-7].

Patients with BE without dysplasia have a yearly incidence of 0.33% for progression into cancer, while BE with low-grade dysplasia (LGD) or high-grade dysplasia (HGD) have a yearly incidence of 0.4-13% and 6-19%, respectively [8]. Further, the length of the Barrett segment is associated with the risk of cancer progression, with a segment > 3 cm having a 7.7 times higher risk of malignant transformation as compared to a segment between 1-3 cm [9]. A segment > 10 cm without dysplasia has a risk of progression to malignancy as compared to that of LGD [10].

The precise molecular mechanism that governs this transformation is incompletely understood. A main theory is that GERD directly damages the DNA, causing transformation into Barrett's metaplasia and further EAC, by multiple changes to the DNA [11]. Other risk

factors, like obesity and smoking, may further increase the risk of malignant transformation by regulating the expression of genes [11].

Worldwide SCC accounts for approximately 90% of all EC and more than 80% of all SCC located to the esophagus (ESCC) are found in Central and South-East Asia [12-13]. In Northern and Western Europe as well as in the US, 90% of all EC are AC [12]. Since the 1970s the incidence of EAC has gradually increased in the Western populations, while the incidence of ESCC has decreased. The increase in EAC can partly be explained by an increase in BMI, while the decrease in ESCC may be related to a decrease in alcohol consumption and smoking [14].

In 2020 a total of 388 (291 men and 97 female) patients were diagnosed with EC in Norway making it the 16. (men) and 24. (female) most common cause of cancer [15]. For both men and women, the incidents rates have increased the last 40 years with about 1.5, the 5-year relative survival rates with a 2-fold in women (14,4%-31.7%) and a 5-fold in men (5%-22.9%), while the mortality rates are relative stable [16].
Due to comorbidity and/or too advanced disease at the time of diagnosis, the fraction of patients undergoing curative surgery lies stable around 30% [17].

Assessment of Barrett´s esophagus and esophageal cancer

In case of Barrett's esophagus, the lesion should endoscopically be classified according to the Prague Classification by measuring the circumferential (C) segment and the maximum length of any Barret´s tongue (M) [18]. Using a high definition endoscope biopsies are taken according to the Seattle protocol –at least four-quadrant biopsies every 2 cm in the normal looking Barrett's segment [19]. Additionally, targeted biopsies are taken from any endoscopic abnormalities. The lesion is histologically characterized as non-dysplastic, indefinite of dysplasia (marked epithelial changes, but not sufficient for the diagnosis of dysplasia) or dysplastic, where the latter lesions is subclassified as LGD and HGD. Due to the increased cancer risk, patients with dysplasia or long Barrett´s (> 10 cm) should be referred to a Barrett expert center for further treatment and follow-up [10]. The histology should be confirmed by a gastrointestinal (GI) expert pathologist. Patients with LGD or indefinite dysplasia should be observed for six months on optimal anti-reflux medication (proton pump inhibitor, PPI), since up to 30% of patients diagnosed with LGD at the first examination, will not have it on a consecutive second examination [10].

BE without dysplasia are followed up by a renewed gastroscopy with biopsies, every 5 years if < 3 cm and every 3 years for lesions between 3-10 cm until the age of 75 [10].

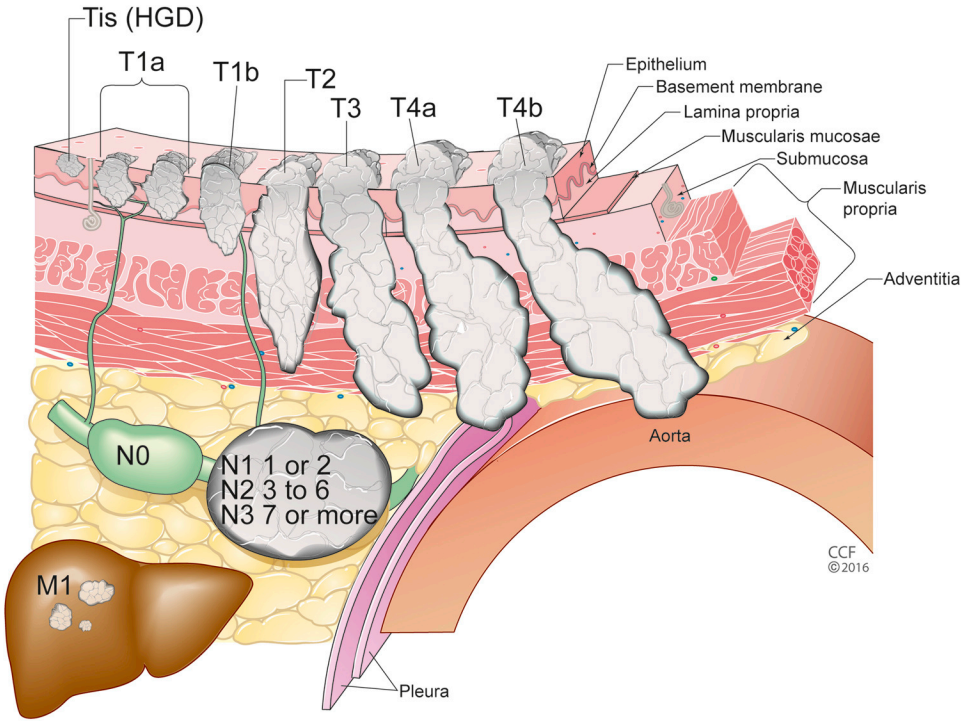

Figure 1: Classification of EC based on TNM8. This figure was published in [20], Copyright: Elsevier (2017).

The initial evaluation of any esophageal tumor is based upon an upper endoscopy with biopsies of the tumor and a CT of the neck, chest, abdomen and pelvis to further diagnose its extent and the presence of any metastasis. Endoscopic ultrasound (EUS) and PET-CT are used in selected cases [21]. The diagnosis of cancer is made when there is infiltration of cancer cells through the basement membrane of the esophageal wall (Fig. 1). The tumor is graded according to Union for International Cancer Control (UICC) TNM stage, where T classifies the tumor, N the level of lymph node metastasis and M the present of any distant metastasis. The tumor stage is classified as T1a (tumor invades lamina propria or muscularis mucosa), T1b (submucosa), T2 (muscularis propria), T3 (adventitia), T4a (surrounding structures that can be removed – i.e. pleura, pericardium and the diaphragmatic crura) or T4b (invades structures that cannot be removed). T1b tumors are further subdivided according to the Japanese Classification of Esophageal Cancer into sm1 (tumor invades the upper third of the submucosa), sm2 (the middle the third of the submucosa) or sm3 (the lower third) [22]. The more invasive the tumor is, the higher risk of lymph node metastasis, being 1-2% in HGD/T1a cancers, 6% in T1b EAC sm1, 23% in sm2 and 58% in T1bsm3 [23-24] .

After the diagnostic evaluation is completed, all patients with potential curative EC should be referred to a esophageal center and discussed at a multidisciplinary team meeting (MDT), consisting of surgeons, oncologists, gastroenterologists, radiologists and sometime pathologists, in order to find the optimal treatment strategy [21].

Treatment of locally advanced esophageal cancer

The standard treatment of locally advanced esophageal cancer (cT2-4a or the presence of regional lymph node metastasis) is neoadjuvant chemoradiotherapy (CRT) followed by surgery using the Ivor-Lewis esophagectomy, tri-incisional (McKeown) or the transhiatal approach [21].

Ivor Lewis published in 1946 a novel technique for tumors located in the lower third of the esophagus: the stomach was first mobilized, a right-sided thoracotomy with esophagectomy was performed before the continuum of the alimentary tract was reestablished by pulling the stomach up through a dilated hiatus and an end-to-side anastomosis with the remainder of the esophagus was made (Fig. 2) [25]. Advantages of this technique includes full exposure to the esophagus for lymphadenectomy, while it is less suited for more proximal tumors and the anastomosis is less surgically accessible in the chest cavity in case of complications. In addition to the traditionally thoracolaparotomy the use of minimally invasive technique in one compartment – hybrid minimally invasive esophagectomy (HMIE) or two compartments - totally minimally invasive esophagectomy (TMIE) and robotic minimally invasive esophagectomy (RAMIE) can be utilized.

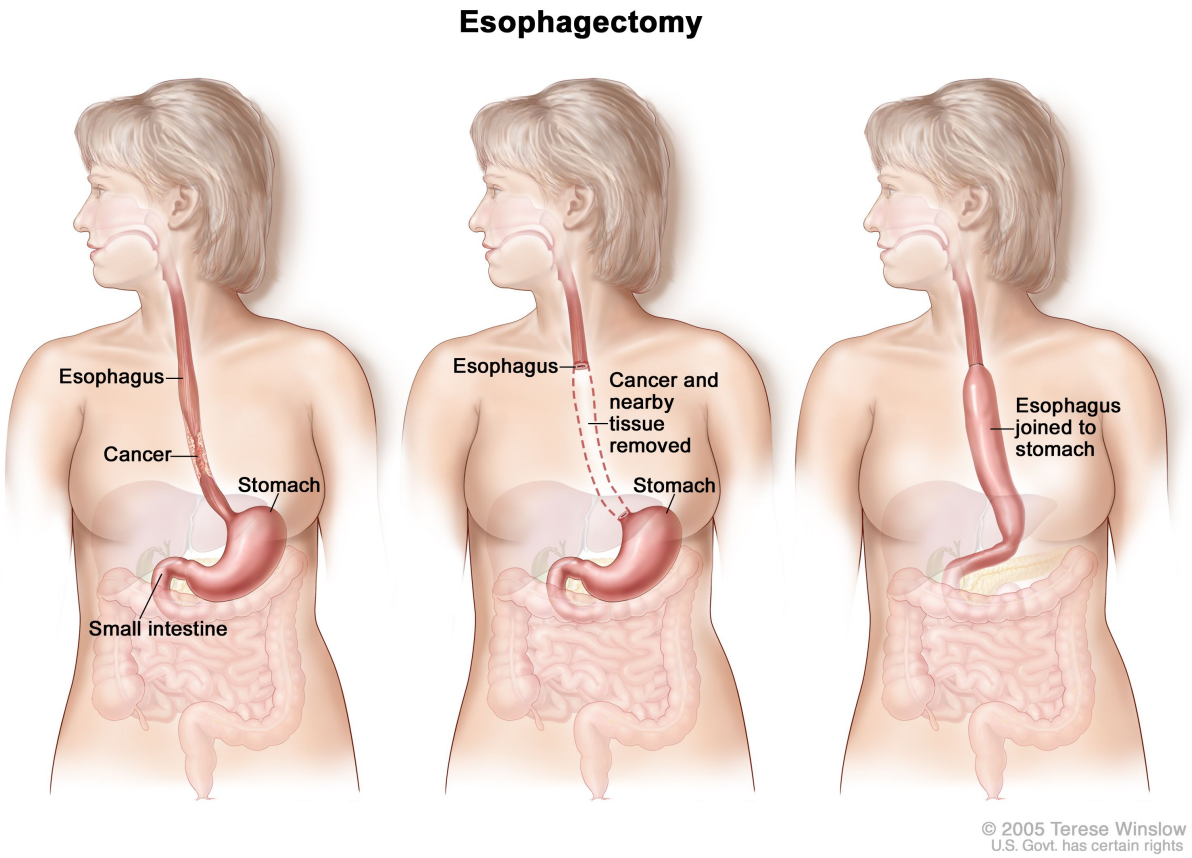

Figure 2: Schematic illustration of the Ivor-Lewis esophagectomy. © 2005 Terese Winslow LLC, U.S. Govt. has certain rights.

The tri-incisional technique, first presented by McKeown in 1969, is traditionally carried out in three stages: during the abdominal part the stomach is mobilized, the esophagus is excised through a right-sided thoracotomy, before the anastomosis is conducted with a right-sided cervical incision [26]. The main advantage of this technique includes full access to the entire esophagus, thus, and in comparison to the Ivor-Lewis esophagectomy, suited for more proximal tumors and easier access to the cervical anastomosis in case of complications. The drawbacks of McKeown TMIE includes a higher anastomotic leakage rate, 90-days mortality and more postoperative morbidity compared to Ivor Lewis TMIE [27].

With the transhiatal approach the esophagus is bluntly dissected from the abdominal part, typically the chest is not opened, thus suited for patients with significant comorbidity. The main disadvantage is the inability to perform at full thoracic lymphadenectomy.

In Norway esophagectomy is conducted at four university hospitals - located in Oslo, Bergen, Trondheim and Tromsø with yearly approximately 60, 40, 30 and 20 cases, respectively [17].

During the last 15 years there have been a gradual shift from open esophagectomy (laparotomy and/or thoracotomy) towards minimally invasive surgery (laparoscopy and/or thoracoscopy). I 2020 52% of the esophagectomies in Norway were conducted using thoracolaparoscopy (totally minimally invasive surgery), 34% with hybrid resection (open surgery in either the chest or abdomen, minimally invasive in the other compartment), while the remaining 13% had open surgery [17]. Further, there is a great diversity among the different hospitals regarding surgical technique. In 2020 Oslo operated 96% of all cases with thoracolaparoscopy, the corresponding numbers for Bergen, Trondheim and Tromsø were 3%, 57% and 8%, respectively [17]. In Bergen 76% of the patients underwent hybrid resection, 14% in Trondheim and 92% in Tromsø [17].

In patients with non-resectable tumors, due to surgical or medical contraindications, CRT with up to 50Gy can be used as definitive treatment for EC. Especially, SCC is highly sensitive for RT and can be used as curative treatment with well comparable 5-year survival rate as surgery alone [28]. For AC the long-term results are considerable worse compared to trimodality treatment (CRT and surgery), especially in high-risk patients (male, N+, poor histology) [29].

In Norway there are no organized follow-up after esophagectomy with regard to local or metastatic recurrence [21].

Treatment of dysplasia and superficial esophageal cancer

In Barrett´s patients with superficial esophageal cancer (T1aN0M0) or dysplasia (LGD, HGD) all visible lesions should be resected for proper histological diagnosis followed by ablation of all remaining Barrett´s epithelium (Fig. 3) [10].

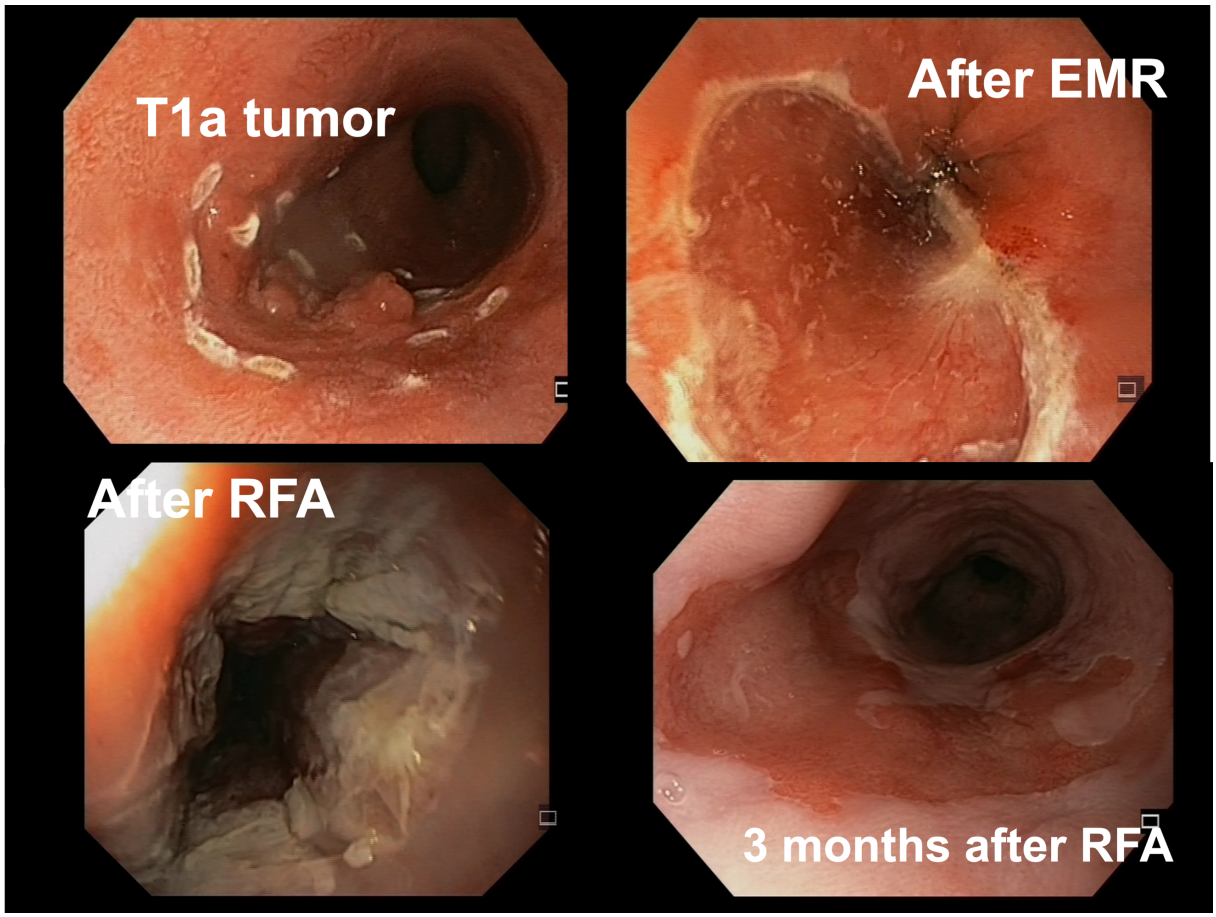

Figure 3: T1a tumor marked with diathermia (upper left) resected using EMR (upper right). The result directly after RFA (bottom left) and after three months (lower right). Copyright Truls Hauge, Dept of Gastroenterology, Oslo University Hospital, Ullevål.

PPI should be given to all patients with BE in order to control reflux symptoms, but in addition might reduce the risk of neoplastic transformation [3]. Resection should be done using endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD), while radiofrequency ablation (RFA) is the recommended ablation technique [10]. With EMR the esophageal lesion is marked using argon plasma coagulation, suctioned into a cap placed at the tip of the endoscope, a rubber band is placed at the base of the lesion creating a pseudopolyp prior to resection using a snare and electrocurrency (Fig. 4).

Figure 4: The Boston Captivator™ EMR used for the resection. Material provided courtesy of Boston Scientific. Copyright 2022 © Boston Scientific Corporation or its affiliates. All rights reserved.

Using ESD the lesion to be removed is initially marked, the submucosa layer is expanded using fluid injection and the mucosa is incised before it is removed from the deeper structures by submucosal dissection using an ESD knife.
Strictures and bleeding are the most common complications after EMR and ESD, being higher in the latter group ranging from 1-9% and 0-60%, respectively [30]. Further, a significant higher degree of R0 resection is archived after ESD compared to EMR, however this does not seem to have effect on the need for rescue surgery nor the degree of complete remission of neoplasia [30]. Additionally, ESD is more time consuming and technically more challenging.

RFA uses heat to destroy (ablate) the dysplastic epithelium of the esophagus (Fig. 5). It is used as the only treatment modality to destroy dysplastic BE without any visual abnormalities, often LGD and as adjuvant after endoscopic resection of a tumor to destroy the surrounding Barrett´s mucosa. After an average of 1-3.4 rounds with RFA, complete

eradication of dysplasia (CE-D) and intestinal metaplasia (CE-IM) is archived in 78% (95% CI, 70-86%) and 91% (95% CI, 87-95%), respectively [31].

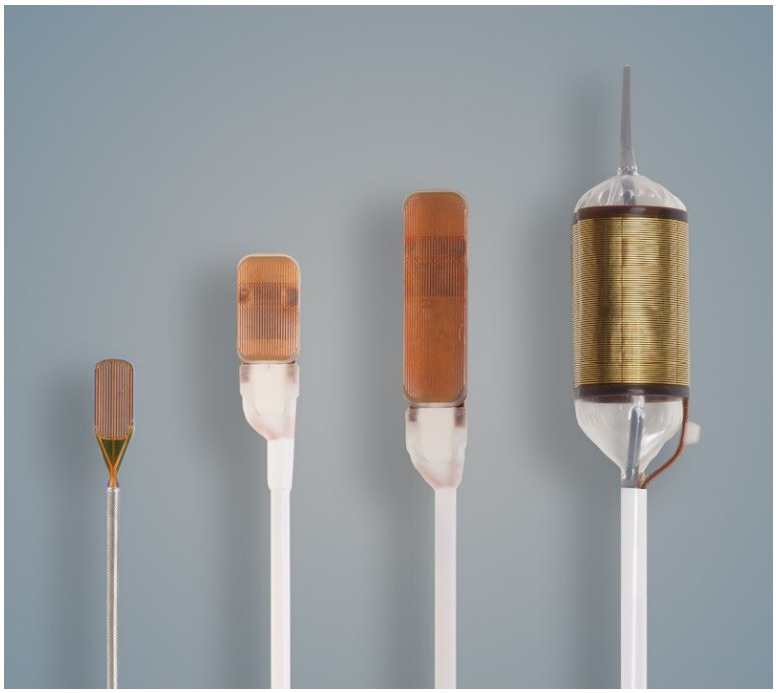

Figure 5: The probes used for RFA. At OUH Ullevål we used the Barrx^{TM} focal catheter (most left) and Barrx^{TM} 360 Balloon Catheter (most right). Reprinted with the permission of Medtronic.

About 8.8% of the patients experience adverse effects after RFA, typically stricture formation, bleeding and perforation being the most common complications in 5.6%, 1% and 0.6% of the patients, respectively [32].
EMR and RFA are conducted under (deep) sedation, ESD under general anesthesia with endotracheal intubation or propofol sedation. The patient goes home the same day.

In patients with Barrett´s dysplasia or T1a all remaining intestinal metaplasia should be removed in order to prevent recurrence [3]. After achieving complete eradication of all intestinal metaplasia (CE-IM), patients should be followed with regularly gastroscopies, every 3 months the first year, then annually, typically for 5 years unless dysplasia recur [21]. In case of recurrence, treatment and follow-up are restarted. During the follow-ups, targeted biopsies are taken from all endoscopic abnormalities, which may be guided by narrow-band imaging (NBI) and staining with 1.5-2% vinegar.
Due to a comprehensive follow-up protocol, patients with BE with or without dysplasia will require multiple gastroscopies even though only a minority of the patients will ever develop cancer. A standard gastroscopy is considered very safe with adverse event rates ranging from

0.01%-0.5%, though most patients find this examination rather uncomfortable [33]. Depending on the extent of the lesion and how many biopsies are to be taken, each examination takes from 5-30 minutes. Until now, no biomarker (for instance a blood test) is found suitable to substitute some of these gastroscopy check-ups. Further, there are limited data on long-term follow-up, especially QoL, after endoscopic treatment for Barrett´s dysplasia and T1a cancer. In paper 2 we try to give more insight into these questions.

Neoadjuvant or perioperative CRT

The majority of patients undergoing esophagectomy receives neoadjuvant or peroperative CRT or chemotherapy. There has been a significant and continues development in treatment regimens during the last 15 years. The MAGIC study published in 2006 showed that perioperative chemotherapy (ECF - Epirubincin, Cisplatin and Flurouracil) for resectable gastric or distal EC, significantly increased the 5-year survival rate when compared to surgery alone (36% vs 23%) [34]. In 2012 the CROSS trial revealed that five cycles of Carboplatin and intravenous Paclitaxel with concurrent 23 fractions of 1.8 Gy prior to surgery increased the median survival with 25 months, compared to surgery alone [35]. The 5-year overall survival in the surgical group was 34% compared to 47% in the chemotherapy-surgery group, being higher among patients with SCC. The German FLOT4 trial published in 2019 showed that patients with gastric and gastroesophageal AC who received perioperative FLOT (Flurouracil, Leucovorin, Oxaliplatin and Docetaxel) lived significantly longer than those receiving perioperative ECF/ECX (5-year survival being 50 months vs 35 months, respectively) [36]. The 5-year overall survival was 45% in the FLOT group, compared to 36% in the ECF/ECX group [36]. The ongoing ESOPEC-trial [37] is comparing perioperative chemotherapy (according to the FLOT-trial) with neoadjuvant radiochemotherapy according to the CROSS-trial.

Postoperative complications

The Clavien-Dindo system first introduced in 2004 is a widely used universal classification system for surgical complications, going from 1 (any deviations from the normal postoperative course without the need of specific interventions, e.g. fever or nausea) to 5 (death) [38]. We have used this system to classify the surgical complications in paper 1 and 3.

The most common serious complications following esophagectomy are pulmonary, arrhythmia and anastomotic leaks. Pulmonary complications, primarily pneumonia, is a

frequent complication, affecting 20-60% of all patients and is associated with an increased mortality rate of 5-10% [39]. Postoperative atrial fibrillation is reported in 16.5% (95% CI, 15.4-17.2%) of the patients and is associated with increased risk of mortality, pneumonia and anastomotic leakage [40]. Anastomotic leak is a dreaded complication associated with significant morbidity and a mortality rate ranging from 10-15% [41]. In case of leakage, there are three main treatment strategies (that may be combined): conservative (iv-antibiotics, nill per month, gastric drainage and percutaneous drainage of fluid collections), endoscopically (endoscopic vacuum-assisted closure (eVAC) and stents) or surgically with success rates varying from 77-100% in the non-surgical groups and 50% for the surgical group [41]. However, patients in the latter group are typically severe ill and not suited for non-operative management. Until now no evidence-based treatment strategy for esophageal anastomotic leaks exists and all three modalities are being utilized [41]. Following an anastomotic leak, the majority of patients experience significantly more difficulties with eating and more painful swallowing (odynophagia). Six months after an intrathoracic anastomosis leak the risks are fourfold and twofold increased, respectively compared to patients without a leak.

Quality of life and Patient-reported outcome measures

The World Health Organization (WHO) defines Quality of Life (QoL) as the "individual´s perception of their position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards and concerns" [42].
The term Health-Related Quality of Life (HRQL) is in the literature often used indistinguishably with QoL, which reflect the fact that none of the terms have a clear and universal definition [43].

Patient-reported outcome (PRO) is any report coming directly from the patient, without interpretation from others and gives us information on how they function and feel with regard to their health and any therapy given [44]. Patient-reported outcome measurements (PROM) are tools for assessment of PROs, typically consisting of validated questionnaires, like the Short Form Survey (SF-36) and the European Organization For Research And Treatment of Cancer (EORTC) QLQ-C30 and QLQ-OG25. They all provide information about the patients HRQL.

Data regarding long-term HRQL after HMIE, endoscopic treatment for Barrett´s dysplasia/T1a and TMIE are generally lacking in the literature. Paper 1, 2 and 3 attempts to bring insight into these questions.

Biobank

There is need for more research on esophageal cancer, especially on blood- or tissue-based tests that may predict if a given patient will response to a given treatment (predictive biomarkers) or to estimate treatment outcome (prognostic biomarkers).

A biobank is a collection of human biological material, that can be used for research ("research biobank") or treatment ("treatment biobank") [45].

As an example: For research on esophageal or gastric cancer, Oslo University Hospital established in 2013 a research biobank with tissue and blood samples from all included patients operated for these conditions at our department. Prior to inclusion, patients were fully informed, both orally and in written about the purpose of the Biobank as well as any pros and cons with participation and a written consent had to be signed. After consent, the biological material could be used for the accepted research, for instance to evaluate the prevalence of selected genetic and epigenetic markers in patients with EAC and BE without dysplasia (paper 4).

In order to ensure ethical standards, the establishment of a biobank in Norway must be approved by a regional ethical committee (REK). Further, the collection, storage, processing and ultimately destruction of data are regulated by Norwegian law, including mandatory registration of all Biobanks in a public register (Biobankregisteret) [46].

Molecular biology in cancer

Both genetic and epigenetic changes are thought to contribute to the malignant transformation from BE to EAC [11]. It has been shown that EAC contains a significant number of mutations including one of the highest numbers of copy-number alterations - a DNA fragment that is copied or deleted ones or more in a cell [47]. Chromosome instability (CIN) is another hallmark of most solid cancers, resulting in loss or gain of a whole (numerical CIN) or a fragment of a chromosome (structural CIN) during cell division [48].

Epigenetics (-epi from Greek = "above") is the study of hereditable changes in gene expression that do not alter the underlaying DNA sequence [49]. The main epigenetics change involves: DNA methylation, histone alterations and regulation by non-coding RNA [11]. DNA methylation occurs by transferring a methyl group to the 5′-position of a cytosine by a group of enzymes named DNMT (DNA methyltransferase). The methylated cytosine is typically followed by a guanine (CpG) and DNA regions with a high frequency of CpG (CpG islands) may be prone to methylation. Different degree of methylation will change the

expression of genes, specifically promotor hypermethylation are associated with loss of gene expression. Both hypermethylated CpG islands as well as hypomethylated DNA regions outside CpG islands are important factors in the pathogenesis of Barrett´s metaplasia, Barrett´s dysplasia and EAC [11].

As previously mentioned, chronic GERD is the main risk factor for development of EAC. Hydrochloric acid (HCl), bile salts (both components of GERD) and chronic inflammation have been shown to induce DNA damage resulting in development of EAC, either directly (DNA breaks) and indirectly (reactive oxygen species (ROS) – highly reactive components formed from oxygen) [11].

To prohibit amplification of damaged DNA and thus cancer formation, cells have several enzymes to detect and repair damage, one of them being the mismatch repair system (MMR). This highly conserved system corrects base-mismatches and short insertion/deletions after DNA replication and is thus important to prevent mutations. An impaired MMR system will result in a phenotype named microsatellite instability (MSI), i.e. multiple insertions/deletions located in repetitive short DNA fragments of 10-60 bp spread throughout the DNA that contains multiple repeats of 1-5 bp (microsatellites) [50]. The defect in the MMR system can be either inherited (e.g. Lynch syndrome) or sporadic and associated with multiple cancer types, most frequently colorectal, endometrial and gastric adenocarcinoma. Sporadic MSI is associated with inactivation of MLH1 due to hypermethylation of its promotor [51]. To set the diagnosis of MSI, tumor cells are compared with five distinctive microsatellite markers. If there is a difference in 2 or more of these markers it is classified as MSI-High (MSI-H), if only one differs, MSI-Low (MSI-L) [52]. MSI is infrequent in EAC, approximately 5-10% of all cases [53].

In order to conduct DNA repair, the damaged cell must be paused in cell cycle and repair enzymes must be activated. *TP53* ("the guardian of the genome") is one of the proteins that contributes to this task. It is the most commonly mutated gene across cancer types and it is mutated in > 50% of all human cancers [54]. In addition to inducing cell cycle arrest, *TP53* may activate cell death (apoptosis) in heavily injured cells and induce senescence, i.e. a permanent cell-cycle arrest as response to various stimuli. Both mechanisms preventing cancer formation [54]. Frequently mutated tumor suppressor gene in EC, includes among others: *APC*, *CDKN2A* and *MGMT* which, in addition to *TP53*, are analysed in paper 4.

DNA sequencing and detection of methylation patterns

DNA sequencing is the process of determining the sequence of nucleotides in a DNA sample. Several methods do exist and collectively they are divided into two main groups: First- and next-generation sequencing techniques. First generation sequencing emerged in the 1970s and Sanger sequencing, named after the two-time Nobel Prize winner and British biochemist Frederic Sanger (1918-2013), was the most common sequencing techniques for several decades and is still in use today [55].

The Sanger sequencing is based on the "chain termination method". The DNA fragment to be sequenced (template) is added with dNTP (nucleoside triphosphates containing deoxyribose; the building blocks for DNA) from all four bases, but in addition a significant lower number of dideoxynucleotides triphosphate (ddNTP) are added. ddNTP lacks a OH-group at position 3 in the deoxyribose, thus when added to the DNA chain further elongation terminates. In Sanger sequencing ddNTP will be incorporated randomly, thus stopping DNA polymerase and producing multiple oligonucleotides with different lengths. The fragments are then separated by electrophoresis and the sequence can be read from the 5´-> 3´position by reading from the bottom of the electrophoresis gel towards the top. This classic Sanger sequencing can be made automatically by adding fluorescent labeled dNTP and ddNTP and by having a computer reading the emitted fluorescent light directly.

As the need for more efficient sequencing technique emerged, second-generation (next generation) sequencing developed in the 21st century making in possible to sequence not one DNA fragment at the time, but simultaneous millions of fragments. This made it possible to sequence the whole human genome in just some days.

The detection of methylation patterns can be accomplished using prefabricated chips with hundreds to thousands of DNA probes that will bind to a florescent labeled fragment if present in the sample to be examined (methylation arrays).

Another technique, which enables us to obtain whole genome epigenetic information is bisulfite sequencing. With this technique DNA is first denatured and treated with sodium bisulfite, which will convert cytosine to uracil, but cytosine that is methylated will not be converted. After PCR amplification, uracil will be converted to thymine, while the methylated cytosine will remain unchanged. By reading whether (and to what extent) cytosine is present (using DNA sequencing) the degree of methylation can be decided.

A third option is Methylation-specific PCR (MSP) which utilizes bisulfite treated DNA and two sets of primers, used separately and in two parallel reactions – one primer for the methylated version of a given gene the other for the unmethylated one [56]. The product is

amplified by PCR, separated by gel electrophoresis and the result is determined based on the presence or absence of bands in the two reactions.

In order to quantitative measurements, MSP has further been modified into Quantitative methylation-specific PCR (qMSP) [56]. For the gene of interest, specific fluorescence labeled probes (TaqMan) and primers are added to the DNA and if methylated, the probe will bind to it. During PCR-amplification, the DNA-bound probe will be cleaved, released and start to emit fluorescence. If the probe is unbound (i.e. unmethylated gene) florescence will not be emitted. The amount of fluorescence emitted is used to quantify the degree of methylation. For each gene a methylation threshold, expressed as percentage of methylated reference (PMR), is used to classify a sample as methylated or not. PMR is calculated by dividing the normalized amount of methylation for a given gene by the normalized amount of methylation in a positive control.

In paper 4 we used some of these techniques (Sanger Sequencing and qMSP) to analyse genetic and epigenetic changes in a series of patients with BE and EAC. The frequencies of these markers are highly aberrantly reported in the literature and this paper tries to improve the knowledge on their presence.

Thesis aims

The overall aim of the thesis was to evaluate the clinical outcome in patients endoscopically or surgically treated for Barret´s esophagus or esophageal cancer and to describe the prevalence of given molecular alteration in a large series of patients. More specifically, the aims were:

Paper 1:

(1) To get insight into the long-term outcome including survival and postoperative HRQL in patients operated with HMIE for esophageal cancer.

Paper 2:

(1) To evaluate the outcome, including effectiveness and post-procedural HRQL in patients endoscopically treated for dysplasia or superficial esophageal cancer".

Paper 3:

(1) To get insight into the long-term outcome including survival and postoperative HRQL in patients operated with TMIE for esophageal cancer.

Paper 4:

(1) To describe the prevalence of specific genetic and epigenetic alterations in patients with Barrett's esophagus and esophageal cancer

Materials and methods

Study design

All four papers included in this thesis are cross-sectional studies, where the general aim is to measure the prevalence of one or more given variables at a given time in a given population. More specific, paper 1, 2 and 3 aimed at measuring the short- and long-term outcome, including long-term postoperative HRQL following HMIE, endoscopic treatment for dysplastic BE, T1a, T1b (only R0) and TMIE, respectively. Paper 4 focused on the prevalence of two known genetic and five epigenetic markers in a series of 145 patients operated for EAC.

Paper 1-3 were written using the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) Criteria, which is a checklist of 22 items, published in 2007 of what to include and how to outline an observational study (cohort study, cross-sectional study and case-control study) in order to increase the quality of the individual papers [57].

Data source and collection:

HMIE and TMIE (paper 1 and 3):

All patients were operated at OUH Ullevål for EC. The tumor was located between the level of carina (mid esophagus) and with epicenter less than 2 cm bellow the gastroesophageal junction (Siewert II, see Fig. 6).

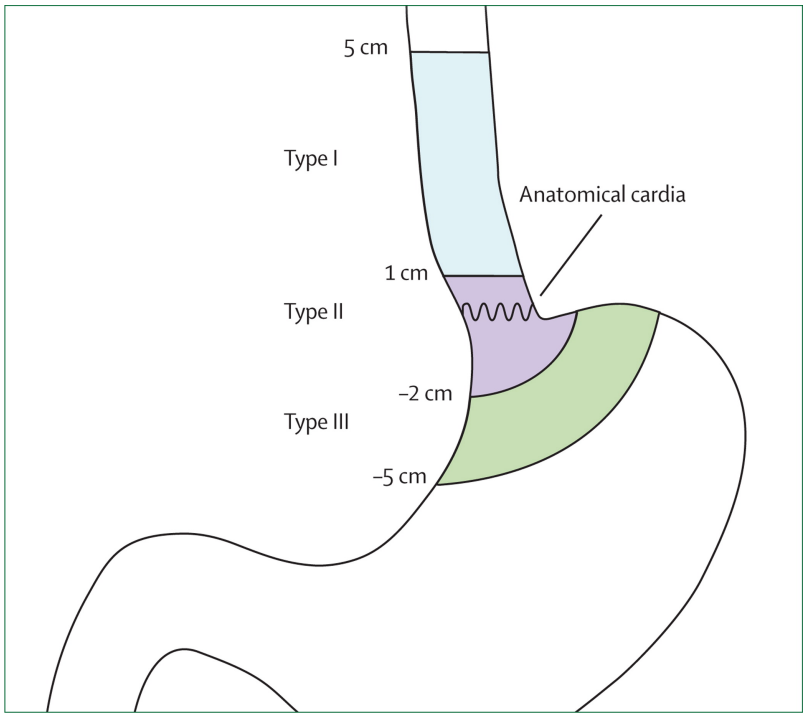

Figure 6: The Siewert classification. This figure was published in [58], Copyright: Elsevier (2011).

Paper 1 included all patients operated with HMIE from November 2007 to June 2013, while paper 3 included all patients operated with TMIE from June 2013 to January 2016.

According to Norwegian guidelines on esophageal cancer, no routine follow-up is recommended after esophagectomy [21]. However, based on a previous local guideline, most patients were followed up annually with a CT-scan for the first 5 years.

Using our hospitals patient administration system (PAS), and in cases of missing data, other hospitals´ PAS, outcome measurements were retrospectively registered in an Excel-created database. To address long-term postoperative HRQL, all patients alive were sent two validated questionnaires - EORTC QLQ-C30 version 3 and the gastroesophageal specific QLQ-OG25 as well as the Ogilvie dysphagia score [59-61]. All three questionnaires had to be answered in writing by the patient and returned to the study administration.
Additionally, survival data were verified using two national registries - The National Population Register and Norwegian Cause of Death Registry. The National Population Register ("Folkeregisteret") is used by the authorities and some private institutions to gain access into several crucial variables (e.g. time and place of birth, citizenship and time of death) about every person living (or previously lived) in Norway. To verify the cause of death we used the Norwegian Cause of Death Registry ("Dødsårsaksregisteret"). Access to both registers is highly restricted.

RFA and EMR (paper 2)

We used an SPSS-created database consisting of all patients endoscopically treated with RFA and/or EMR in the esophagus. The registry started in 2013, is continually and retrospectively updated and all treatments are conducted at the Department of gastroenterology OUH Ullevål. Outcome measurements were retrospectively registered using the hospital PAS. In cases where late follow-up took place at another hospital, the other hospital was contacted in order to get follow-up data. Additionally, HRQL was assessed using EORTC QLQ-C30, OG-25 and the Ogilvie dysphagia score.

Molecular analysis (paper 4)

The data source in paper 4 was our Biobank as well as 19 patients with BE and no current nor previous known history of dysplasia (control group). For Biobank-patients, tissue samples were taken during surgery (esophagectomy) at OUH Ullevål from September 2013 to May 2020. Tumor samples as well as samples from normal looking tissue at a distance of more than 10 cm from the tumor were biobanked directly after the surgery. Two biopsies (one from each location) were taken using a scissor and a forceps, but since August 2018 using a circular punch biopsy (diameter=5 mm). Biopsies from the 19 BE patients were taken after patient consent and during routine follow-up gastroscopy from November 2017 to February 2020. The biopsies were taken from the Barrett´s lesion as well as from macroscopically normal looking mucosa at a distance of more than 10 cm from the main lesion. Samples from both populations (surgical and endoscopic group) were temporarily stored in liquid nitrogen at -196 ºC, then in a deep freezer at -80 ºC prior to being transported to the Department of Molecular Oncology, Institute for Cancer Research, Oslo University Hospital – Norwegian Radium Hospital for further storage and genetic and epigenetic analysis.

Study population and sample size:

HMIE (paper 1)

The study population consisted of all 109 patients scheduled for Ivor Lewis HMIE, from the time the procedure was introduced at OUH in November 2007 and until June 2013, having at least five-year follow-up time for every patient. Criteria for inclusion was a surgical specimen with a T1-T4a (T-stage) tumor, with the possibility of regional lymph node metastasis, but without distant metastasis, significant comorbidity (advanced lung, heart or kidney disease) or

advanced age (primarily < 75 years of age). In eight patients the surgical specimen did not reveal any tumor cells and the T-stage was based upon preoperative examinations (CT, endoscopy, EUS).

RFA and EMR (paper 2)

All 83 patients, endoscopically treated with RFA and /or EMR for dysplastic BE, T1a and R0 resected T1b from June 2014 to December 2018, archiving at least one year follow-up time for every patient, were included. According to guidelines no pre- or perioperative (radio)chemotherapy were given.

TMIE (paper 3):

The 123 patients scheduled for Ivor Lewis TMIE, from the time the procedure was introduced at OUH in June 2013 and until January 2016 were include, having at least five-year follow-up time for every patient. The criteria for inclusion were the same as in paper 1.

Biobank (paper 4):

Patients included for molecular analysis were selected from the Biobank, at that time consisting of approximately 350 patients in total. Of the roughly 200 patients with EAC, 145 samples were randomly selected based upon T-stage, in order to best mimic the general European population of surgical esophageal cancer patients. Using the literature and at that time unpublished own data, the population was designed to include approximately 20% T1, 27% T2, 52% T3 and 4% T4 tumors [62]. Based on estimated time consume and financial costs an upper limit of 145 EAC samples was decided upon. Additionally, 19 non-dysplastic BE (endoscopic group) patients were included.

A total of 5 genes (markers), based on the presents of > 50% alterations and validated in at least three original articles, were selected for molecular analyses: *TP53, APC, CDKN2A, MGMT* and *TIMP3*. *TP53* was submitted to mutational analysis, the latter four to epigenetic analyses. Additionally, and due to scientific interest, *MLH1* was selected in order to explore MSI status, despite a low alteration frequency in EAC.

After targeted molecular profiling (genetic and epigenetic analysis) of all 145 EAC samples, 63 samples, including all without molecular alterations, were analysed by a pathologist in order to verify tumor cell content. Only samples with ≥ 5% tumor cells were included, thus excluding 37 samples. The remaining 108 samples were used for calculating all frequencies in paper 4.

Interventions:

HMIE, RFA/EMR and TMIE (paper 1, 2 and 3)

Outcome:

Multiple short- and long-term outcome measurements were retrospective registered, including patient demographics, survival, the use of (radio)chemotherapy, type of surgical and endoscopic procedures, histology and complications. For the surgical patients (paper 1 and 3) the Clavien-Dindo scoring system was used. It is a five-graded scoring system ranging from grade 1 and 2 (minor complications) to grade 3 (requiring surgical, radiological or endoscopic intervention), grad IV (organ dysfunction needing ICU-management) and grade V (death) [38]. Regression analyses were conducted (paper 1 and 3) in order to estimate the relationship between survival and several potential prognostic variables.

HRQL:

The EORTC QLQ-C30 version 3 (see appendix), QLQ-OG25 (see appendix) and Ogilvie dysphagia score was used to access HRQL. The QLQ-C30 is questionnaire used to access HRQL in a general population of patients with cancer. It consists of 30 questions where each patient has to answer question 1-28 with a grading scale going from 0 ("not at all") to 4 ("very much"). Question 29-30 are graded from 1 ("very poor") to 7 ("excellent").

To access the gastroesophageal specific HRQL, we used the EORTC QLQ-OG25. It consists of 25 questions where each patients grades each question from 1 ("not at all") to 4 ("very much").

In both questionaries, the individual scores are used to calculate six (QLQ-OG25) or nine (QLQ-C30) multi-items scales where each scale represents the sum of several questions. The nine multi-items scales in QLQ-C30 are: physical functioning, role functioning, emotional functioning, social functioning, cognitive functioning, pain, fatigue, nausea/vomiting, global QoL. The six multi-items in QLQ-OG25 are: Dysphagia, eating restrictions, reflux, odynophagia, pain and anxiety. The remaining ten (QLQ-OG25) and six (QLQ-C30) questions are not summarized but presented as a single items scale consisting of only one question. The individual scales are presented with a number from 0-100, where a high score represents a high degree of function or a high degree of side effects/problems.

The Ogilvie dysphagia score is a five-graded scale used to determinate the level of dysphagia, ranging from no problem eating a normal diet (score 0), normal diet avoiding certain solid foods (score 1), semisolid foods (score 2), liquids only (score 3) and complete dysphagia for even liquids (score 4). We defined poor dysphagia as a score > 1.

Molecular analysis (paper 4)

TP53 mutational analysis was conducted by sequencing the entire coding region (exons 2-11) followed by independent scoring of the results by two of the authors. Every detected mutation was confirmed by a new sequencing of that sample.

Bisulfite treated DNA was analysed for DNA promoter hypermethylation in *APC, CDKN2A, MGMT, MLH1* and *TIMP3* using qMSP. We calculated the threshold for scoring a sample as methylated or not, as the highest PMR value (for a given gene) among the normal mucosa samples in the 19 nondysplastic BE patients (reference population).

MSI analysis was conducted using the MSI Analysis System, Version 1.2 (Promega) [63]. It utilizes fluorescently labeled primers for a total five mononucleotide repeats (used for MSI status) and two pentanucleotide repeat markers (used for detection of contamination). After PCR-amplification the results were separated by electrophoresis prior to independent interpretation by two of the authors using the software GeneMapper. MSI-H (high) was defined using the Bethesda guidelines for colorectal cancer, in that two or more of the five MSI markers showed an aberrant peak [52]. MSI-L (low) when only one marker was aberrant, MSS (stable) when none.

Statistical Analyses

Statistical analyses were conducted using Graphpad prism 6 and SPSS version 25 (paper 1), SPSS version 26 and STATA SE version 16 (paper 2 and 3) and R software version 3.6.2 (paper 4). The analyses were conducted by Tobias Hauge (paper 1-3) with the assistance of statistician Ragnhild Sørum Falk (Oslo Centre for Biostatistics and Epidemiology, OUH). Demographic data were presented as frequencies and proportions (categorical data) and as mean, median and range (continues data). In paper 1 and 2 the overall survival was estimated using the Kaplan-Meier estimator and the Aalen-Johansen estimator was used to calculate the cause-specific mortality. In order to examine the relationship between overall survival and certain potential prognostic variables, uni- and multivariable cox proportion hazard analysis were conducted. Results were presented as hazard ratios (HR) with 95% confidence intervals (CIs). T-test was used for comparison of HRQL between the study population in article 2 and a non-cancerous reference population. In article 4 Fisher´s exact test was used to compare independent categorical data, the McNemar's for paired categorical data and the nonparametric Wilcoxon´s test for continues variable. P-values < 0.05 were considered significant.

Results/summary of the papers

<u>Paper 1:</u> Hauge T, Amdal CD, Falk RS, Johannessen HO, Johnson E. Long-term outcome in patients operated with hybrid esophagectomy for esophageal cancer - a cohort study. Acta Oncol. 2020 Jul;59(7):859-865.

The purpose with paper 1 was to get a better insight into the long-term outcome following HMIE for EC, especially on survival, delayed surgery and HRQL.
A total of 109 patients were included, out of which 98% had a distal EC or a tumor located at the gastroesophageal junction (Siewert II). Eighty percent were EAC and 59% had received neoadjuvant (radio)chemotherapy.
After a median follow-up time of 55 (range: 2-135) months we found a 5-year survival among the R0 and R0-R2 resected of 53% and 49%, respectively. None of the patients with R1-R2 resection survived for 5 years. The risk of dying from EC during the first 5 years following surgery was 45% (i.e. cumulative mortality). Using multivariable cox regression analysis we discovered that R0 patients with pTNM (6^{th} edition) stage IIB or III (i.e. lymph node metastases and/or T3/T4 tumor) had significantly reduced survival. On the other hand, chemo(radio)therapy significantly improved survival. Twelve percent of the patients underwent delayed surgery (i.e. more than 4 months postoperatively) in which the most common indication was cancer recurrence in 46% of the cases. Median 65.5 (range: 63-123) months postoperatively, 82% of the patients reported preserved function and low symptom burden, assessed using the EORTC QLQ-C30 and QLQ-OG25. The most common symptoms reducing long-term HRQL were reflux, fatigue and anxiety in 44%, 32% and 32%, respectively. Ninety percent of the patients experienced no dysphagia (Ogilvie score 0). The anastomotic leak rate was 5%.

In conclusion, 53% of the R0 resected patients were alive after 5 years and at that time 82% reported adequate (not reduced) HRQL. 12% underwent delayed surgery, in which recurrence of cancer was the most common indication.

<u>Paper 2</u>: Hauge T, Franco-Lie I, Løberg EM, Hauge Truls, Johnson E. Outcome after endoscopic treatment for dysplasia and superficial esophageal cancer - a cohort study. Scand J Gastroenterol. 2020 Sep;55(9):1132-1138.

The purpose with this paper was to study the postprocedural outcome, including HRQL, following RFA and/or EMR for dysplastic BE and T1a EC.

The population consisted of 86 patients, in which 26% had LGD, 51% HGD, 13% T1a and 7% T1b. Ninety-five percent of the patients in the two latter groups had EAC. After a median follow-up time of 23 months tumor regression or downstaging was achieved in 78% with LGD, 66% with HGD and 89% of patients with T1a/T1b. More specific, 92% of patients with T1a had complete remission.

Ten percent (n=9) experienced progression under treatment and 7% (n=6) had an initial T1b. Out of those 15 patients, two had progression into HGD. Five of the remaining 13 patients underwent esophagectomy out of which the surgical specimen revealed a tumor > T1aN0 in only one patient, thus actually needing surgery. The 8 remaining patients were not suitable for surgery primarily due to comorbidity.

Sixty-nine percent of the patients, median 28 (range: 8-65) months postprocedural, fulfilled the EORTC QLQ-C30, QLQ-OG25 and the Ogilvie dysphagia score questionnaires. Eighty-seven percent reported preserved function and low symptom burden. When comparing the EORTC QLQ-C30 scores to a European non-cancerous population with equivalent demographics there were no significant difference in 11 out of the 15 variables. Eighty-eight percent experienced no dysphagia (Ogilvie score 0). There were relatively few complications, with bleeding needing blood transfusions in 2% and strictures needing balloon dilatation in 8%.

In conclusion, RFA and EMR for treatment of dysplastic BE and T1a EC are safe and efficient with few complications. Median 28 months postprocedural 87% of the patients reported adequate (not reduced) HRQL. Eighty-eight percent experienced no dysphagia.

<u>Paper 3</u>: Hauge T, Førland DT, Johannessen HO, Johnson E. Short- and long-term outcomes in patients operated with total minimally invasive esophagectomy for esophageal cancer. Dis Esophagus. 2021 Sep 7 (Epub ahead of print).

The purpose with paper 3 was to study the short- and long-term outcome following TMIE for EC, including the need for delayed surgery and long-term HRQL.
These outcomes are limitedly reported in the literature, especially long-term HRQL.
A total of 123 patients were included, out of which 98% had a distal EC or a tumor located at the gastroesophageal junction (Siewert II). Eighty-five percent had EAC and 80% received neoadjuvant (radio)chemotherapy. After a median follow-up time of 58 months (range: 1-88) the 5-year overall survival for all patients was 53% (R0-2) and 57% for the R0 resected. The 5-year cumulative mortality for the R0 resected was 36%, meaning that this group had a 36% risk of dying from EC during the first 5 years. From multivariable cox regression analysis patients with pTNM (6^{th} edition) stage IIb and III (lymph node metastases and/or T3/T4 tumor) had significantly reduced survival. Perhaps somewhat surprisingly we did not find that the use of (radio)chemotherapy influenced survival, which might be related to the low number of patients that did not receive neoadjuvant treatment (20%).
The most common complications following TMIE were pneumonia in 37% and arrhythmia in 14%. Fourteen percent developed anastomotic leakage, which all were treated non-operatively.
Eleven percent (n=13) had delayed surgery median 26.5 (range: 5-67) following TMIE, in which symptomatic diagrammatic hernia was the main indication in 46%.
Median 60 (range: 49-80) months postoperatively, approximately 80% of the patients answered the EORTC QLQ-C30, QLQ-OG25 and the Ogilvie dysphagia score. Eighty-four percent reported preserved function and low symptom burden, 84% could eat a normal diet (Ogilvie score 0) while 16% had minor difficulties (Ogilvie score 1). The most common symptoms reducing HRQL were anxiety, cough insomnia and reflux in 35%, 32%, 27% and 24%, respectively.

In conclusion, patients undergoing TMIE experienced a 5-year survival of 57% (R0). Eighty-four percent reported adequate (not reduced) long-term HRQL. Thirteen percent had delayed surgery in which the main indication was symptomatic diaphragmatic hernia.

<u>Paper 4:</u> Pinto R, Hauge T, Jeanmougin M, Pharo H D, Kresse S H, Honne H, Winge S B, Five M-B, Kumar T, Mala T, Hauge Truls, Johnson E, Lind, G E. Targeted genetic and epigenetic profiling of esophageal adenocarcinomas and non-dysplastic Barrett's esophagus. Clinical Epigenetics. 2022. In Press.

The purpose with paper 4 was to describe the prevalence of specific genetic and epigenetic alterations in a series of patients with BE and EAC.
Based on a literature review, 2 genetic (*TP53* and MSI status) and 5 epigenetic (DNA promotor hypermethylation of *APC*, *CDK2A*, *MGMT*, *TIMP3* and *MLH1*) alterations were selected and analysed in a series of 145 EAC and 19 BE samples. To our knowledge, the prevalence of these markers has previously not been described in such a large series of patients.

Twenty-eight percent of the EAC samples harbored mutations in *TP53* and its presence was associated with increasing age, while the use of neoadjuvant treatment was associated with decreasing prevalence. The association between mutations in *TP53* and the use of neoadjuvant treatment was not found when adjusting for age. None of the BE samples had *TP53* mutations.
Among patients with EAC, 6% showed MSI, while none in the BE group had this trait. Promotor hypermethylation were frequently seen in both EAC (5-62%) and BE (16-89%) and in up to 12% of the normal mucosa samples located adjacent to EAC. In BE patients an association between *APC* hypermethylation and male gender was found. In patients with EAC an association between promotor hypermethylation of *CDKN2A*, *MGMT*, *TIMP3* and tumor location (Siewert I or Siewert II) as well as between hypermethylation of *TIMP3* and age or tumor stage were found. Additionally, an association between neoadjuvant treatment and the absence of promotor hypermethylation in *CDKN2A* and *TIMP3* was found in EAC, though this association did not remain significant when adjusting for age. For all genes, besides *MGMT,* promotor hypermethylation were more frequently observed in patients receiving neoadjuvant treatment compared to those that did not.

In conclusion, the frequencies of known genetic and epigenetic alterations have been described in a large series of patients with BE and EAC.

Discussion of main findings

In paper 1 our main aim was to "get insight into the long-term outcome including survival and postoperative HRQL in patients operated with HMIE for esophageal cancer". After a median follow-up time of 55 months (R0-R2) the 5-year overall survival was 49% (R0-R2) and 53% (R0), while the 90-days mortality and anastomotic leakage rate was 2% and 5 %, respectively. In a previous study, reporting the short-term outcome in the same series of patients, 33% had postoperative pneumonia, 13% arrhythmia and the median in-hospital stay was 16 days (9-88 days) [64]. Eighty percent of the patients received neoadjuvant (radio)chemotherapy. Six patients (6%) were median 26 months after HMIE operated for metastasis. Reflux (44%), fatigue (32%) and anxiety (32%) were the three main complains reducing HRQL more than 5 years (median 65.5 months) postoperatively.

In the French MIRO trial, a multicenter RCT comparing open Ivor-Lewis esophagectomy to HMIE no difference in survival was found after 3 and 5 years [62,65]. The 5-year overall survival among the 103 patients who underwent HMIE was 59% (95% CI, 48-68%) thus not significantly different from our data. The most common complications were anastomotic leaks in 11%, major pulmonary complications (pneumonia, severe respiratory failure) in 18% and cardiac arrhythmia in 12%, respectively. The 90-days mortality was 4% and the median hospital stay was 14 days (7-95 days).

Compared to the MIRO trial, we experienced a lower anastomotic leakage rate, higher frequency of pneumonia and a comparable 90-days mortality rate and length of hospital stay. Major postoperative (Clavien-Dindo ≥ 2) and pulmonary complications were found to be independent risk factors for decreased overall survival (OS) and disease-free survival (DFS) hypothesizing that HMIE is associated with increased OS and DFS due to reduced rate of complications [65]. In a meta-analysis of 14.592 patients operated with MIE (HMIE or TMIE, n=7358) and open esophagectomy (OE, n=7234) an 18% lower 5-year all-cause mortality was found in MIE compared to OE [66]. This is further supported by a bi-national cohort study from Finland and Sweden, including almost all patients who underwent elective MIE (n=470, 37.2%) or OE (n=794, 62.8%) from 2010-2016 also with an 18% reduction of all-cause 5-year mortality in MIE compared to OE [67]. Further subdividing, a 23% reduction was found in TMIE and 13% in HMIE, favoring the former. The mechanisms behind the reduced all-cause mortality in MIE is unknown, even when adjusting for R0 resection rate and number of lymph node extracted the better outcome after MIE did not change. In addition to the MIRO-trial it has been shown that major surgical complications and reoperations might be a

prognostic indication for decreased survival even when excluding those that dies within the first 90-days [68-69]. Further, MIE is associated with reduced perioperative complications, especially pulmonic complications compared to OE [70]. Thus, one possible explanation behind the reduced all-cause mortality, might be the lower rate of postoperative complications and reoperation, especially respiratory complications in MIE [67].

Surgical treatment of local recurrence is not standard treatment nor recommended by most guidelines for esophageal cancer [21]. In the literature, only small retrospective studies have been published, of which some show a survival benefit in a highly selective group of patients with isolated distant hematogenous recurrence [71].

In the MIRO trial, HRQL was measured by EORTC QLQ-C30 and QLQ-OES18 (not used in our studies) every 6 months for all patients (HMIE and OE) and compared with baseline values (before resection) [72]. In general, and at all time points for both HMIE and OE, the patients experienced decreased HRQL compared to baseline independently of surgical technique. However, patients who underwent HMIE seemed to experience less reduction in HRQL as measured by role and social functioning at 30 days, pain at 2 years postoperatively while the difference in social functioning lasted the first two years. Role function and pain was associated with postoperative complications. At 3 years postoperatively, there were no difference from baseline HRQL in neither HMIE nor OE. Similar long-term HRQL results, comparing Ivor-Lewis HMIE and OE, were found using data from the multicenter cross-sectional LASER-trial with no clinical difference in QLQ-C30 median 3.9 years postoperatively [73].

In paper 2 the primary aim was to "evaluate the outcome, including effectiveness and post-procedural HRQL in patients endoscopically treated for dysplasia or superficial esophageal cancer". All patients were treated with EMR and/or RFA. Histology revealed LGD in 26%, HGD in 51%, T1a in 15% and T1b in 7% with tumor regression or downstaging rates after a mean of 22.9 months and 1.5 treatments (0-4) of EMR and 0.7 (0-4) RFA, of 78%, 66%, 92% and 84%, respectively. Five patients underwent esophagectomy due to a suspected preoperative diagnosis of T1b, though only one ended up having a tumor more advanced than T1a (T1bN1M0). Postprocedural there were no perforations, 3% experienced bleeding, 8% developed a stricture that needed treatment and the 90-days mortality was 1%. After median 28 months, 88% of the patients experienced no dysphagia and compared to an age and gender similar non-cancerous European population, there were no difference in 11 out of the 15 variables in QLQ-C30 suggesting their post-procedure HRQL were satisfactory.

In a systematic review of 751 patients undergoing focal-EMR and RFA for HGD and/or T1a, complete eradication of HGD/EAC (CE-N) and intestinal metaplasia (CE-IM) was archived in 93.4% and 73.1%, respectively [74]. Postprocedural, 10.2% developed strictures, 1.1% bleeding and 0.2% perforations. Similar results were found in a recent publish single-center study by White J.R et al with 239 patients undergoing RFA out of which 183 had EMR [75]. CE-D and CE-IM was achieved in 90.4% and 89.8%, respectively. The median number of RFA was 3 per patient, 2.2% experienced bleeding, 5.4% strictures.

Compared to our series, 92% of the patients with T1a archived CE-N. Excluding patients not yet controlled (n=4), 55% (22/40) of patients with HGD and 83% (5/6) of patients with T1b experienced CE-N. However, this fraction includes patients still undergoing treatment (not included in the study by White JR et al). By including patients that had downstaging of histology or stable disease (no progression), 85% (34/40) did not progress. Initially, our treatment strategy in this subgroup of patients with significant comorbidity, was to prohibit neoplastic transformation and reduce the risk of postprocedural complications, primarily strictures. However, all summarized, we should have treated this subgroup more aggressively. Our complication rates are low, but in congruence with the literature.

In a meta-analysis, including 2752 patients from 22 different studies, the safety and efficiency of EMR compared to ESD was evaluated [76]. Patients undergoing ESD, independently of type of histology (EAC vs SCC), achieved significant higher rates of en-bloc resections, curative and R0 resections and lower recurrence rate. On the contrary, patients undergoing ESD experienced a significant longer procedure time and higher perforation rates, while no differences in post-procedural bleeding and stricture formations were found. For all variables, except procedure time, no difference in outcome was found when treating lesions $\leq$ 10 mm. Lesions $\geq$ 10mm had significant better outcome when treated with ESD, though the degree of complications and local recurrence between ESD and EMR was similar in lesions between 10-20 mm. Altogether, this meta-analysis supports EMR for lesions $\leq$ 10 mm, ESD for lesions $\geq$ 20 mm, while both techniques are validated for lesions between 10-20 mm.

In the first RCT comparing EMR to ESD (BE lesions < 3 cm without massive infiltration into the submucosa) ESD achieved a higher degree of en-bloc resections, R0 and curative resections than EMR. Regardless, patients treated with EMR did not have any increased need of esophagectomy nor less complete remission of neoplasia compared to ESD [30]. However, in a recent published retrospective study, including 243 BE patients with HGD/T1a, patients treated with ESD had significant fever local recurrence/residual disease than those treated

with EMR (3.5% vs 31.4%) [77]. Adjuvant to resection, 62.5% of patients treated with EMR and 69.4% of patients treated with ESD had RFA (p=0.4). Consequentially, patients treated with EMR needed more frequently additional treatments (EMR 24.2% vs ESD 3.5%).

Several ablative techniques exist in eradication dysplastic BE, including RFA, PDT, cryoablation and hybrid-APC. Even though the techniques yet have not been compared in a head-to-head RCT, RFA is the by far most well-documented technique, including efficiency and safety profile [78].

Very little is known about long-term QoL following endoscopic treatment of dysplastic BE/T1a cancer. Recently, a paper on long-term HRQL following endoscopic treatment for HGD/T1a (n=91) or esophagectomy (n=62) was published [79]. After median 6.8 years, HRQL was assessed using the EORTC QLQ-C30 and OES-18 (esophageal cancer specific module). At the end of follow-up, patients in the endoscopic group scored significantly worse on physical and role functioning than those in the surgical group. The rest of the functional and global health outcomes were similar among the groups. On the contrary, patients in the endoscopic group scored significantly better on several symptom outcomes, including diarrhea, eating difficulties, chocking, coughing and speech difficulties. No difference was found comparing the other symptom scores.

In a systematic review, 27 studies were identified evaluating HRQL in patients with BE using a total of 32 different PROMs [80]. This review showed that none of the studies used validated PROMs for measuring HRQL and none of them were measuring more than 9 out of 18 factors important for BE patients. Among these non-validated PROMs are the three used in paper 2, QLQ-C30, QLQ-OG25 and the Ogilvie dysphagia score as well as OES-18.

In paper 3 the aim was to “get insight into the long-term outcome including survival and postoperative HRQL in patients operated with TMIE for esophageal cancer”. After a median follow-up time of almost 5 years (58 months) the 5-year overall survival was 53% (R0-R2) and 57% (R0). The 90-days mortality was 2%, the anastomotic leakage rate was 14% and the median hospital stay was 16 days (10-104 days). Postoperatively, 37% had pneumonia and 14% arrhythmia. 80% of the patients received neoadjuvant (radio)chemotherapy. The three most common symptoms reducing long-term HRQL (after 5 years) was anxiety (35%), cough (32%) and insomnia (27%). The study encompasses all patients from the first 2.5 year following the introduction of TMIE at our hospital.

In recent years, the 5-year overall survival following Ivor Lewis TMIE has in the literature been reported between 55.9-61.8% with a 90-days mortality of 2-3%, a anastomotic

leakage rate of 15.1%-18.9% and a median hospital stay of 10 days (interquartile range (i.q.r): 8-16 days) [81-85]. Pneumonia and arrhythmia, two of the most common short-term complications, has been seen in 10% and 12% of the patients, respectively [86]. Compared to the literature, the patients in our study had similar 5-year overall survival, 90-days mortality and frequency of postoperative arrhythmia and anastomotic leaks, but were more frequently diagnosed with pneumonia.

Hopefully, the anastomotic leakage rate of 14% could be lowered once the associated learning curve following the introduction of a new surgical technique is overcome. In a multicenter cohort study, including 14 European hospitals and 2121 patients, the anastomotic leakage rate was reduced from 19.3%-14% after 131 cases and the frequency of an "ideal short-term outcome" (textbook outcome) was increased from 37.2% of the patients to 44% after 46 cases [87]. By looking isolated on hospitals performing ≥ 50 cases a year (high-volume centers) the learning curves was shorter than in hospital with < 50 cases a year (low-volume centers) and the plateau for leakage rate and textbook outcome was achieved after 85 and 38 and 89 and 115 cases, respectively. In high-volume centers the anastomotic leakage rate was reduced from 32.7%-13%, while in low-volume centers the leakage rate was stable at 15.3%-16%, indication no learning curve. Textbook outcome was decreased in the low-volume centers, going from 42.9%-37%, while centers with ≥ 50 cases experienced an increase from 31.7%-53%.

This study highlights the importance of learning curve and high-volume centers in order to achieve optimal outcome.

As discussed under "methodological considerations", the frequency of pneumonia could probably have been more accurate using a validated scoring system like the Utrecht Pneumonia Scoring system (UPSS). At least, the comparison between different patient series would have been more accurate.

Until now, no RCT exists that compares the outcome following Ivor Lewis TMIE and HMIE. However, recently a multicenter cohort study, consisting of 39 high-volume esophagectomy centers from 20 different countries (The International Esodata Study Group, IESG), using strict definitions classifying postoperative complications following Ivor Lewis TMIE (n=1472), HMIE (n=1364) and open surgery (n=1897), was published [82]. This study found a significant lower rate of anastomotic leaks, but higher rate of pneumonia and length of hospital-stay in patients operated with HMIE compared to TMIE. There was no difference regarding number of R0 resections, lymph nodes harvested, severity of anastomotic leaks or 90-days mortality.

Due to differences in use of neoadjuvant (radio)chemotherapy, pTNM stage and rate of anastomotic leaks in our two patient series (paper 1 and 3) one should be extremely careful comparing the two groups. However, in accordance with the literature, we experienced a higher anastomotic leakage rate in patients undergoing TMIE (14%), compared to HMIE (5%). Further, no differences were found regarding the number of R0 resections, lymph nodes harvested, 90-days mortality and postoperative pneumonia. An elevated anastomotic leakage rate in patients undergoing TMIE (18.9% vs 10%) and no differences in rate of postoperative pneumonia, complications, harvested lymph nodes or length of hospital stay was also found in a subgroup analysis of a meta-analysis comparing the two techniques [83]. That no difference in rate of pneumonia was found in the latter study could be related to greater heterogenicity in diagnostic criteria compared to the IESG-study where strict diagnostic criteria was used. Compared to our study, all variables, besides the in both series elevated frequency of pneumonia, are in accordance with the literature.

In the TIME trial, an RCT comparing Ivor Lewis TMIE to open surgery, HRQL measured using SF-36, EORTC QLQ-C30 and QLQ-OES18 was for found to be significantly better 1 year postoperatively, compared to preoperative values and at 6 week postoperative for both groups [88]. Further, patients in the TMIE group had at 1 year, significant better physical function (SF-36), Global health (QLQ-C30) and less pain (QLQ-OES18) than patients in the open surgery group. Thus, patient operated with TMIE had better HRQL at 1 year postoperatively compared to the OE group. At median 3.9 year postoperative no clinical significant difference were found in HRQL between TMIE, HMIE and OE, suggesting that the difference found after 1 year in the TIME trial gets dimmed out with time [73].

In paper 4 the primary aim was to "describe the prevalence of specific genetic and epigenetic markers in patients with Barrett's esophagus and esophageal cancer". Among the 108 EAC samples, *TP53* was mutated in 28% of the samples, 6% showed MSI and 5-62% had promotor hypermethylation in *APC*, *CDK2A*, *MGMT*, *TIMP3* and/or *MLH1*. In the 19 BE samples, none harbored mutations in *TP53* or showed MSI, 16-89% had promotor hypermethylation.

In a systematic meta-analysis, including 16 studies and more than 850 patients with EAC, *TP53* mutation was found in 57% (33-79%) of all patients and its presents was associated with significant reduced overall survival [89]. The discrepancy between our data and metanalysis could be explained be several factors. First of all, the meta-analysis includes a variety of patients (surgery alone, neoadjuvant/adjuvant treatment) with different sampling methods (surgical specimen, endoscopic biopsies) as well as different analysing modalities

(PCR sequencing, immunohistochemistry, single-strand conformational polymorphism). All these variables will influence the mutational status. By looking isolated on our patients that did not receive neoadjuvant treatment (surgery alone) 44% harbored mutations in *TP53* – a more similar mutational frequency compared to others.

In addition, and as explained under "Methodological considerations", we cannot preclude that the use of Sanger sequencing prohibits us from detecting mutations in samples with <15% tumor cells.

When addressing promotor hypermethylation of the five selected genes, there are some discrepancies between our results and the literature. Main reasons for this include primarily smaller study populations, tumor location and the PMR value used to classify gene as methylated or not. Even though larger studies have been conducted using genome-wide association study (GWAS), this is to our knowledge, the largest study using targeted analysis of five frequently altered genes as well as MSI status in a total of 108 patients with EAC/BE. We think this strengthens our data and makes them more robust.

Methodological considerations

Study design

All four studies in this thesis are observational, more specific cross-sectional studies.
The main advantages with this design includes inexpensiveness, easiness and being fast to conduct [90]. Typically, the aim is to measure the prevalence of one or more variables (e.g. number of patients receiving neoadjuvant treatment) at a given time.
On the other hand, the main disadvantage incudes the arduousness to validate causality between a specific outcome and exposure since both are measured at the same time. Further, cross-sectional studies are prone to bias (i.e. systematic errors), specific recall and nonresponse bias [90]. A recall bias occurs when patients systematically and inaccurately remembers the past, while nonresponse bias occurs when a group of patients that do take part in a survey significantly differ from those that do not.
However, cross-sectional studies may be used to generate hypothesis that further can be validated using other types of designs (e.g. RCT) and to monitor and evaluate treatment response, but not to evaluate trends (i.e. cannot calculate incidence rates).

Internal and external validity

Internal validity accesses whether the study has been conducted in a thorough way, using the correct methods, in order to be able to thrust the conclusion of the paper.
External validity focuses on whether the data from a single study is valid for other populations as well.

HMIE and TMIE (paper 1 and 3)

Study population

The two populations were significantly different with regard to the proportion of patients receiving neoadjuvant (radio)chemotherapy (60% HMIE, 80% TMIE) as well as the type of (radio)chemotherapy given, the anastomotic leakage rate (5% HMIE, 14% TMIE) and the median time of follow-up after R0 resection (76 months HMIE, 58 months TMIE).
Additionally, and based on the 6th edition of TNM, the tumors were more advanced in the HMIE group consisting of 41% stage II (T2-T3N0M0, T1-T2N1M0) and 47% stage III

(T3N1M0, T4anyNM0) tumors as compared to 55% and 31% in the TMIE group, respectively. These differences make a direct comparison between the two groups difficult.

Delayed surgery and late complications

During our late follow-up, and in conjunction with the survey on HRQL, all patients alive were asked if they had undergone any delayed surgery and if yes which procedure, at what hospital and at what time. In cases where the patient was uncertain about which procedure had been conducted, and the surgery had taken place at another hospital, the surgical report was obtained. This retrospective collection of data is prone to recall bias, which risk could have been decreased by registering data prospectively.

The survival data are accurate as these are verified using the official Norwegian Cause of Death Registry ("Dødsårsaksregisteret").

HRQL

The lack of preoperative data makes it impossible to study any changes in HRQL. Further, the comparison with the age and gender similar European non-cancerous population has a low external validation due to the limited number of patients included in our studies and their broad standard deviations. Further, each patient was sent more than 50 questions, which for some could have been too comprehensive, thus leading to not fulfilling (or inaccurate fulfilling) the survey (low internal validation).

On the other hand, 4/5 of the patients alive answered the two questionaries (QLQ-OG25, QLQ-C30), making nonresponse bias less likely.

Pneumonia

About 1/3 of our patients were classified as having postoperative pneumonia (33% HMIE, 37% TMIE). These prevalences are significantly higher than what is reported in a recent published multicenter cohort study, using strict definitions for complications, comparing Ivor Lewis TMIE (n=1472) and HMIE (n=1364) [82]. In this study, the prevalence of pneumonia was 10.9% and 16.3% (p=0.001) in the TMIE and HMIE group, respectively. After extensive surgery, it is not uncommon to experience a SIRS reaction (fever, leukocytosis, tachypnea, tachycardia) and we think this "normal reaction" might have misled some to put the wrong diagnosis of pneumonia. We could probably have gained a more accurate and comparable prevalence by using a validated pneumonia scoring system, e.g. as suggested by van der Sluis et al. by focusing on radiographic findings and leukocyte count and/or temperature – The

Utrecht Pneumonia Scoring System (UPSS) [39]. A slightly revised version of the UPSC has been internally and externally validated in two large esophageal centers in the Netherlands with a sensitivity of 79% and 83% and a specificity of 95% and 97%, respectively [91].

TNM Classification

In both papers we used the UICC TNM Classification edition 6 first published in 2002 and not the most recent edition 8 published in 2016 [92].

The main difference between the two editions is that edition 8 has separate classification systems for clinical (cTNM) and pathological (pTNM) stage as well as for SCC and EAC.

Initially, our secondary aim with the paper on TMIE (paper 3) was to compare the long-term outcome after TMIE with our previous paper on HMIE (paper 1). These papers included patients operated between 2007-2013 (HMIE) and 2013-2016 (TMIE), at which time the TNM 6th and 7th edition (published in 2009) were the two current classification systems, respectively.

In the 7th edition the separate classification systems for SCC and EAC was introduced, but not the separate clinical and pathological stage as applicable in the 8th edition. Due to our limited number of patients, a further subclassification according to TNM 7 or 8 would have weakened the power of the comparison between the groups.

However, due to the previous mentioned differences between the groups (tumor stage, use of neoadjuvant (radio)chemotherapy, anastomotic leakage rate) we were not able to compare the groups directly.

RFA and EMR (paper 2)

Study population

Patients resected for T1b cancer were only included in case of free resection margins. In order to increase the paper´s external validity, we should have included all patients resected for T1b, independently of resection margins. Especially, since the nature of EMR (piecemeal resection) prohibits the evaluation of the resection margins for larger lesions.

HRQL

This study has the same limitations as mentioned above for paper 1 and 3.

Molecular analysis (paper 4):

Study population

We are pleased with the selection process, in which tissue samples from the Biobank were selected with the goal of best mimicking a European population of surgical EAC patients. One might argue that we could further have fine-tuned this selection process by taking into account the N-stage, since it could be that tumors with the ability of metastasizing to lymph nodes, hold other genetic or epigenetic properties than those that have not metastasized. None of the included patients had at the time of surgery known distant metastasis and all included underwent surgery with curative intentions.

The selection of genes for analysis were based on a literature review, in which genes reported frequently altered (> 50%), and its frequency was described in at least three original articles, were considered for inclusion. Additionally, *MLH1* was selected in order to compare it to MSI status.

One of the most important methodological aspects with this paper is whether or not samples used for molecular analysing contains what they are said to contain. The histology of the EAC and BE samples were set according to the final pathology report from the surgery (EAC) and from the biopsies taken during the routine gastroscopy (BE). Meaning that the samples used in this paper (initially) were not accessed by a pathologist.

The BE samples were too small for both molecular analysing and validation of histology by a pathologist. However, all 19 BE patients had a prior history free of dysplasia.

After completing molecular analysis of all 145 EAC samples, 63 samples including all without molecular alterations, were analysed by a pathologist in order to calculate the fraction of tumor cells in each sample. Only samples ≥ 5% tumor cell were included. In addition to a biopsy from the tumor itself, a sample from adjacent (approx. 5-10 cm distally) macroscopically normal mucosa was taken. These macroscopically normal mucosa biopsies, as well as all biopsies from the BE patients (normal mucosa and BE lesion taken at routine gastroscopy) have not been verified by a pathologist.

Ideally, a pathologist should have analysed the tumor content of all samples prior to DNA extraction in order to surely verify that only samples containing sufficient tumor cells were included. Unfortunately, due to highly limited pathology recourses this was not possible. Specifically, samples from normal mucosa (BE and adjacent to EAC), the 19 BE patients as well as 82 samples with EAC were not analysed. However, the BE samples were taken from the exact same location as the “routine biopsies” at the BE check-up, that all had their

diagnosis verified by a pathologist. The 82 EAC patients had molecular alterations, thus normal mucosa seems unlikely and the normal mucosa biopsies were taken in great distance from the pathological mucosa.

In general, it is assumed that neoadjuvant (radio)chemotherapy, received by 81% of our EAC patients in paper 4, may alter the genetic and epigenetic changes in tumors. Specifically, an absence of mutations in *TP53* and epigenetic changes in *CDKN2A* or *TIMP* were found in patients receiving neoadjuvant (radio)chemotherapy. Though, when adjusting for age these differences were not present. Further, since the absolute number of patients not receiving neoadjuvant treatment is low, we cannot conclude whether or not neoadjuvant treatment influence the frequency of alterations. According to clinical guidelines, patients < 75 years of age with local advanced tumor (cT2-4 and/or N1-N3) without contraindications should receive neoadjuvant treatment as this has been shown to significantly increase long-term survival and the rate of R0 resections [21]. Thus, analysing only patients who did not receive neoadjuvant treatment would highly have biased the material.

In order to have made this study even more robust, more patients had to be included as well as verification of all samples by a pathologist.

TP53 mutational status

Sanger sequencing was used for accessing *TP53* mutational status. The drawbacks using this technique includes its reduced sensitivity in samples with low tumor burden. According to Illumina this technique requires at least 15% tumor cells in each sample in order to be able to detect any mutant alleles. However, our own data suggests a detection limit as low as 10% (data not shown). Accordingly, since we included samples with ≥ 5% tumor cells, we cannot preclude that the actual mutational frequency is higher.

Ethical considerations

All data collection and analysis in the four papers included in this thesis are approved by the Regional Ethics Committee (REK, "Regional etisk komite") under the application number 2018/720, REK NORD. Additionally, the research Biobank used in paper 4 is approved for EC (2012/2186/REK Sør-Øst B) and non-dysplastic BE (2017/1646/REK Nord for BE).
All patients suitable for inclusion were asked for a written informed consent, meaning that the consent is both voluntary and fully informed [93]. For the surgical patients, the surgeon in charge of the surgery the next day was the one asking the patient, for BE sampling the gastroenterologist performing the routine gastroscopy asked. Surgical patients had prior to inclusion received information in written at the outpatient clinic, the BE patients were given information per telephone prior to the examination. Patients in our survey on long-term HRQL received information per letter, including the consent to be signed.

As researchers we must be aware off the unequal position between ourselves and the patient, for instance when the surgeon himself asks the patient for consent. It could be that some patients feel that they will receive worse treatment if they do not sign. Additionally, the consent must be informed, meaning that the patient needs "… sufficient information about the methodic, purpose, expected results, possible side effects and how the results will be used" [93]. For the surgical patients there were no physical side effects, as the surgery itself is standardized independently of Biobank sampling or not. Additionally, tissue samples are taken from the removed surgical specimen and extra blood samples were taken from the anesthetized patient prior to surgery and through a routinely introduced arterial catheter. The BE patients had a theoretically higher possibility of side effects as extra esophageal biopsies were taken during routine gastroscopy and blood samples were collected (not necessary for the routine check-up). The patients in the HRQL-surveys received nearly 50 questions each that are time consuming to answer.

Further, the use of gene technology unlocks unlimited possibilities with regard to predicting the possibility of having (or receiving) a given disease. What should you do if you find out that one of the patients with non-dysplastic BE had a significantly increased risk of developing EAC? Or if you by "accident" find that one the patients had an increased risk of developing a deadly non-curable disease? Fortunately, the use and limitations of Biobanks is strictly legislated by Norwegian law ("Behandlingsbiobankloven") and none of the analysis in paper 4 can be used to predict the possibility of having (or receiving) a given disease, as specified in application 2012/2186/REK Sør-Øst B.

It is not possible (and perhaps not desirable) to eliminate all ethical dilemmas in research, but being aware of them, having the projects (and the consent) validated by REK and by having a strict legislation, lightens the decision making.

Conclusion

The 5-year survival of patients operated at OUH from 2007-2013 with HMIE was 49% and 53% for the total population and the R0-resected, respectively. R0 patients with stage IIB or III (lymph node metastasis and/or T3/T4 tumor) had significantly reduced survival, while patients who received neoadjuvant (radio)chemotherapy had increased survival. Five percent had anastomotic leak, 33% pneumonia. After more than five years, reflux, fatigue and anxiety were the three most common symptoms reducing long-term HRQL. Nighty percent had no problems with eating av normal diet (paper 1).

Patients with LGD, HGD, T1a or T1b with free resection margins, treated with EMR and/or RFA at OUH from 2014-2018, had median 2 years after treatment tumor regression or downstaging of histology in 78%, 66% and 89% of the cases, respectively. In general, there were few complications (bleeding, stricture) and that ended up needing esophagectomy. One out of ten patients had reduced HRQL two years after treatment (paper 2)

Patients operated with TMIE at OUH from 2013-2017 had a 5-year survival of 53% and 57% for the R0-R2 and R0 resected, respectively. As in paper 1, patients with stage IIB or III had significantly reduced survival. Postoperatively, 37% had pneumonia, 14% anastomotic leak. Anxiety, cough and insomnia were the three most common symptoms reducing long-term HRQL after five years. Eighty-four percent had no problems eating a normal diet (paper 3).

In paper 4 the prevalence of 2 genetic and 5 epigenetic promising alterations were identified in a series of 108 patients with EAC and 19 patients with non-dysplastic BE. In the EAC group, alterations in *TP53* were found in 28% and 6% showed MSI, while none of the BE patients had these alterations. Promotor hypermethylation of *APC*, *CDKN2A*, *MGMT* and *TIMP3* were frequently seen in EAC (21-62%) and BE (26-89%) (paper 4).

Future perspectives

Introduction of robotic surgery:

In many parts of surgery, especially when operating in regions with tight anatomical compartments, like the pelvic, robotic surgery has gradual replaced laparoscopic surgery.

The ROBOT trial, comparing robot-assisted Ivor-Lewis esophagectomy (RAMIE) to open transthoracic esophagectomy (OTE) in patients with EAC, showed less blood loss, pneumonic and cardiac complications, less pain and better short-term HRQL, in patients operated with RAMIE compared to the OTE [94]. There was no difference regarding the oncologic outcome after 40 months. The early results of the RAMIE-trial, comparing RAMIE to TMIE in patients with SCC, revealed that patients undergoing RAMIE had significantly shorter operation time and better lymph node dissection in patients receiving neoadjuvant therapy, compared to TMIE [95]. The ongoing ROBOT-2 trial, is comparing RAMIE to TMIE for patients with EAC [96]. Even tough further studies are needed, especially on oncological long-term outcome, it seems highly likely that robotic surgery will increasingly be used in esophagectomy.

Esophageal sparing resections:

During the last years there has been a gradual shift from esophagectomy to endoscopic resections for HGD and superficial EAC. It is likely that this shift will continue in favor of esophageal sparing surgery. Most guidelines, including the American Gastroenterological Association (AGA) and the European Society of Gastrointestinal Endoscopy (ESGE) now recommends endoscopic treatment as the choice of treatment for HGD/T1a [10,97]. Further, AGA finds endoscopic treatment as a feasible treatment strategy in patients with EAC T1bsm1 without lymph node metastasis and good/moderate differentiation [97].

Collaboration and precision medicine

In Norway there are approximately 350 new patients with EC yearly, unfortunately 70-80% are not eligible for curative surgery, primarily due to advanced disease and/or comorbidity. In order to improve survival and QoL, the Norwegian Esophageal Cancer Consortium (NORECa) was founded. By establishing a collaboration between hospitals doing esophageal surgery, high numbers of blood and tissue samples may be collected. These samples will be used in order to (hopefully) detect biomarkers that will detect EC in an earlier stage, thus increasing the rate of patients undergoing curative treatment. In addition to better prognosis,

detection of early EC may increase the number of patients that can be treated endoscopically, thus saving some from esophagectomy. Further, biomarkers are an important part of precession medicine, for instance to predict which patient may profit from adjuvant chemotherapy or perhaps more importantly detect those that do not.

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Popular scientific summary (in Norwegian)

Spiserørskreft er den 10. vanligste kreftformen på verdensbasis. Det finnes primært to undergrupper, plateepitelkarsinom og adenokarsinom. Førstnevnte er desidert vanligste og omfatter med enn 90% av alle pasientene. I Norden og Vest-Europa er det der i mot omvendt – adenokarsinom forekommer hos ca 90% av pasientene. Sannsynligvis stammer de alle fleste tilfellene av sistnevnte fra et forstadium med spesifikke celleforandringer i slimhinnen - Barretts øsofagus. Dette er en mye hyppigere tilstand enn kreft, men hvor en liten mindretall av pasientene på sikt vil utvikle kreft. Derfor må disse pasienter regelmessig gå til kontroller av spiserøret (gastroskopi). Skulle det tilkomme spesifikke celleforandringer (dysplasi eller overflatisk kreft) må disse fjernes (reseksjon) og slimhinnen rundt må kokes (ablasjon). Begge behandlinger blir gjort uten operasjon, men ved hjelp av et gastroskop. Skulle forandringene være mer omfattende (dyp kreft), må pasienten opereres hvor en fjerner mesteparten av spiserøret, og kobler gjenværende del av spiserøret til magesekken som «heiser opp» i brystkassen. Dette kan gjøres ved hjelp av flere forskjellige teknikker.

Målet med mitt doktorgradsarbeid har vært å se på langtidseffektene etter operasjon for spiserørskreft, utført ved hjelp av to forskjellige kirurgiske teknikker (HMIE og TMIE, hhv artikkel 1 og 3). Videre har vi sett langtidseffekten etter behandling av celleforandringer og overflatisk kreft hos pasienter med Barretts sykdom (artikkel 2). I det siste arbeidet har vi sett på forekomsten av bestemte forandringer i arvestoffet i kreftsvulster og hos pasienter med Barretts sykdom uten dysplasi (artikkel 4).

I den første artikkelen fant vi at ca halvparten av pasientene var i live 5 år etter operasjon (49-53%). 5% av pasientene fikk lekkasje fra skjøten mellom gjenværende del av spiserøret og magesekken (anastomoselekkasje). Hovedplagene til pasientene 5 år etter kirurgi var halsbrann (44%), slapphet (32%) og redsel (32%). I artikkel 3 fant vi igjen at rett over halvparten (53-57%) var i live etter 5 år. Anastomoselekkasje forekom hos 14% av pasientene og hovedplagene til pasientene etter 5 år var redsel (35%), hoste (32%) og innsovningsvansker (27%). I artikkel 2 ble pasientene behandlet uten operasjon for celleforandringer/overflatisk kreft i spiserøret. De aller fleste ble kvitt sine celleforandringer/overflatisk kreft og de det var generelt lite problemer til denne behandlingen. Ca 2.5 år etter behandling hadde de aller fleste pasientene god svelgfunksjon (88%) og var fornøyd med sin livskvalitet (87%). I artikkel 4 har vi beskrevet forekomsten av totalt 7 forandringer i arvestoffet til kreftsvulstene og hos pasienten med Barretts sykdom (uten dysplasi).

Appendix

EORTC QLQ-C30 (version 3)

We are interested in some things about you and your health. Please answer all of the questions yourself by circling the number that best applies to you. There are no "right" or "wrong" answers. The information that you provide will remain strictly confidential.

Please fill in your initials: ⌊ ⌋⌊ ⌋⌊ ⌋⌊ ⌋

Your birthdate (Day, Month, Year): ⌊ ⌋⌊ ⌋⌊ ⌋⌊ ⌋⌊ ⌋⌊ ⌋⌊ ⌋⌊ ⌋

Today's date (Day, Month, Year): 31 ⌊ ⌋⌊ ⌋⌊ ⌋⌊ ⌋⌊ ⌋⌊ ⌋⌊ ⌋⌊ ⌋

		Not at All	A Little	Quite a Bit	Very Much
1.	Do you have any trouble doing strenuous activities, like carrying a heavy shopping bag or a suitcase?	1	2	3	4
2.	Do you have any trouble taking a long walk?	1	2	3	4
3.	Do you have any trouble taking a short walk outside of the house?	1	2	3	4
4.	Do you need to stay in bed or a chair during the day?	1	2	3	4
5.	Do you need help with eating, dressing, washing yourself or using the toilet?	1	2	3	4

During the past week:

		Not at All	A Little	Quite a Bit	Very Much
6.	Were you limited in doing either your work or other daily activities?	1	2	3	4
7.	Were you limited in pursuing your hobbies or other leisure time activities?	1	2	3	4
8.	Were you short of breath?	1	2	3	4
9.	Have you had pain?	1	2	3	4
10.	Did you need to rest?	1	2	3	4
11.	Have you had trouble sleeping?	1	2	3	4
12.	Have you felt weak?	1	2	3	4
13.	Have you lacked appetite?	1	2	3	4
14.	Have you felt nauseated?	1	2	3	4
15.	Have you vomited?	1	2	3	4
16.	Have you been constipated?	1	2	3	4

Please go on to the next page

During the past week:	Not at All	A Little	Quite a Bit	Very Much
17. Have you had diarrhea?	1	2	3	4
18. Were you tired?	1	2	3	4
19. Did pain interfere with your daily activities?	1	2	3	4
20. Have you had difficulty in concentrating on things, like reading a newspaper or watching television?	1	2	3	4
21. Did you feel tense?	1	2	3	4
22. Did you worry?	1	2	3	4
23. Did you feel irritable?	1	2	3	4
24. Did you feel depressed?	1	2	3	4
25. Have you had difficulty remembering things?	1	2	3	4
26. Has your physical condition or medical treatment interfered with your family life?	1	2	3	4
27. Has your physical condition or medical treatment interfered with your social activities?	1	2	3	4
28. Has your physical condition or medical treatment caused you financial difficulties?	1	2	3	4

For the following questions please circle the number between 1 and 7 that best applies to you

29. How would you rate your overall health during the past week?

1	2	3	4	5	6	7
Very poor						Excellent

30. How would you rate your overall quality of life during the past week?

1	2	3	4	5	6	7
Very poor						Excellent

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EORTC QLQ – OG25

Patients sometimes report that they have the following symptoms or problems. Please indicate the extent to which you have experienced these symptoms or problems during the past week. Please answer by circling the number that best applies to you.

During the past week:	**Not at all**	**A little**	**Quite a bit**	**Very much**
31. Have you had problems eating solid foods?	1	2	3	4
32. Have you had problems eating liquidised or soft foods?	1	2	3	4
33. Have you had problems drinking liquids?	1	2	3	4
34. Have you had trouble enjoying your meals?	1	2	3	4
35. Have you felt full up too quickly after beginning to eat?	1	2	3	4
36. Has it taken you a long time to complete your meals?	1	2	3	4
37. Have you had difficulty eating?	1	2	3	4
38. Have you had acid indigestion or heartburn?	1	2	3	4
39. Has acid or bile coming into your mouth been a problem?	1	2	3	4
40. Have you had discomfort when eating?	1	2	3	4
41. Have you had pain when you eat?	1	2	3	4
42. Have you had pain in your stomach area?	1	2	3	4
43. Have you had discomfort in your stomach area?	1	2	3	4
44. Have you been thinking about your illness?	1	2	3	4
45. Have you worried about your health in the future?	1	2	3	4
46. Have you had trouble with eating in front of other people?	1	2	3	4
47. Have you had a dry mouth?	1	2	3	4
48. Have you had problems with your sense of taste?	1	2	3	4
49. Have you felt physically less attractive as a result of your disease or treatment?	1	2	3	4

Please go on to the next page

During the past week:	**Not at all**	**A little**	**Quite a bit**	**Very much**
50. Have you had difficulty swallowing your saliva?	1	2	3	4
51. Have you choked when swallowing?	1	2	3	4
52. Have you coughed?	1	2	3	4
53. Have you had difficulty talking?	1	2	3	4
54. Have you worried about your weight being too low?	1	2	3	4
55. Answer this question only if you lost any hair: If so, were you upset by the loss of your hair?	1	2	3	4

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Papers

I

II

III

IV

Targeted genetic and epigenetic profiling of esophageal adenocarcinomas and non-dysplastic Barrett's esophagus

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Abstract

Background: Despite the efforts to describe the molecular landscape of esophageal adenocarcinoma (EAC) and its precursor lesion Barrett's esophagus (BE), discrepant findings are reported. Here, we investigated the prevalence of selected genetic (*TP53* mutations and microsatellite instability (MSI) status) and epigenetic (DNA promoter hypermethylation of *APC*, *CDKN2A*, *MGMT*, *TIMP3* and *MLH1*) modifications in a series of 19 non-dysplastic BE and 145 EAC samples. Additional biopsies from adjacent normal tissue were also evaluated. State-of-the-art methodologies and well-defined scoring criteria were applied in all molecular analyses.

Results: Overall, we confirmed frequent *TP53* mutations among EAC (28%) in contrast to BE, which harbored no mutations. We demonstrated that MSI and *MLH1* promoter hypermethylation are rare events, both in EAC and BE. Our findings further support that *APC*, *CDKN2A*, *MGMT* and *TIMP3* promoter hypermethylation is frequently seen in both lesions (21-89%), as well as in a subset of adjacent normal samples (up to 12%).

Conclusions: Our study further enlightens on the molecular background of BE and EAC. To the best of our knowledge, this is one of the largest studies addressing a targeted analysis of genetic and epigenetic modifications simultaneously across a combined series of non-dysplastic BE and EAC samples.

Keywords

esophageal adenocarcinoma, Barrett's esophagus, *TP53* mutations, DNA methylation, microsatellite instability

Background

Esophageal cancer is the tenth most commonly diagnosed cancer worldwide causing more than 540,000 deaths annually.(1) Esophagectomy, combined with neoadjuvant radiochemotherapy or chemotherapy, is the mainstay of treatment of resectable tumors. The overall 5-year survival is 20% increasing to nearly 60% in the subgroup of patients undergoing surgery.(2) However, at the time of diagnosis, around 3/4 of the patients are not eligible for surgery due to either too advanced malignant disease or comorbidities.

The two major histological subtypes of esophageal cancer, squamous cell carcinoma and adenocarcinoma (EAC), are characterized by distinct etiologic factors and patterns of incidence, and differ not only histologically but also in their underlying molecular characteristics.(3) The incidence of EAC has increased in Western countries, where it currently represents around two-thirds of all esophageal cancers.(1) Most, if not all, EAC arise from a metaplastic lesion termed Barrett's esophagus (BE), whereby the squamous epithelium of the lower esophagus is replaced by specialized columnar intestinal epithelium typically as a consequence of chronic gastro-esophageal reflux. BE may subsequently progress into EAC through a multistep sequence involving increasing grades of dysplasia.(4) BE is therefore a well-recognized risk factor for the development of EAC, although only a small proportion of patients (<1%) with non-dysplastic BE develops cancer.(5)

Key genetic modifications including chromosomal instability, copy number alterations and mutations have been identified in EAC.(6-8) As for other solid cancer types, the *TP53* tumor suppressor is by far the most recurrently mutated gene in EAC, with reported frequencies from 7% to 83%.(6, 9-20) *TP53* mutations are rarely found in BE with no history of disease progression,(7, 21) but they have been reported in dysplastic BE as well as in non-dysplastic BE adjacent to EAC.(6, 8)

In addition to genetic aberrations, epigenetic alterations contribute to esophageal malignant transformation and tumor progression. These include histone modifications, aberrant expression of noncoding RNAs and DNA methylation alterations. Hypermethylation of selected gene promoters is observed already during the formation of non-dysplastic BE. Array-based methylation studies support that such DNA methylation changes are early events in EAC development, based on similar aberrations among BE and EAC, which are not found in normal squamous mucosa.(22-24) Among hypermethylated genes in EAC are *APC*, *CDKN2A*, *HPP1*, *RUNX3*, *MGMT* and *TIMP3*, which differ in the reported methylation frequencies.(25-33)

In contrast to other gastrointestinal cancers, *MLH1* promoter hypermethylation is infrequent in EAC.(26, 34, 35) Somatic hypermethylation of the *MLH1* promoter with consequent loss of protein expression is the main cause of defective mismatch repair during DNA replication in most sporadic tumors. As mismatch repair defects lead to microsatellite instability (MSI), this condition is, following *MLH1* promoter hypermethylation, expected to be rare in EAC. Only a limited number of studies have addressed MSI status in BE and EAC, reporting inconsistent frequencies.(16, 36-41)

Despite the efforts to describe the genetic and epigenetic landscape of EAC, discrepant findings are reported. Many of the studies in the field also rely on the analysis of relatively restricted cohort sizes. In the present study, we have investigated the prevalence of core genetic (*TP53* mutations and MSI status) and epigenetic (DNA promoter hypermethylation of *APC*, *CDKN2A*, *MGMT*, *TIMP3* and *MLH1*) modifications in a cohort of non-dysplastic BE and a large series of EAC samples.

Results

An overview of the results is shown in Figure 1 and detailed information about the sample selection process is illustrated in Figure 2.

Frequency, location and type of *TP53* mutations

TP53 mutations detected in BE and EAC are shown in Figure 3 and listed in Additional file 1: Table S1. A silent mutation with no amino acid change was detected in one BE (patient 13), which was classified as *TP53* wild type and therefore not considered as a mutation hereafter. The same silent mutation was detected in one EAC (patient 94), but this sample was still considered altered due to the presence of another *TP53* mutation.

Overall, none of the BE harbored *TP53* mutations whereas 30 out of 108 (28%) EAC samples carried mutations. One of the EAC samples (patient 83) harbored two different mutations. Seven of a total of 31 mutations (23%) were indels, while the rest were point mutations leading to amino acid substitution (missense mutation), four of them involving a stop codon (non-sense mutations). The 31 *TP53* gene mutations were distributed as follows: five in exon 4, five in exon 5, five in exon 6, seven in exon 7 and nine in exon 8. No mutation was found in exons 2-3 or 9-11. G:C to A:T single base transitions were predominant among point mutations (21 out of 24 mutations, 88%), eleven of which occurred at CpG dinucleotides.

A significant association was observed between *TP53* mutations and increased age (p=0.021, Wilcoxon's Test) and between *TP53* mutations and gender (p=0.0027, Fisher's Exact Test). In addition, neoadjuvant treatment of EAC patients was found to be significantly associated with the absence of *TP53* mutations (p=0.045, Fisher's Exact Test; Additional file 1: Table S2). Age is associated with the decision of treating patients with neoadjuvant therapy (p=3.4 x 10^{-10}). As age is a confounding factor when testing for potential association between *TP53*

mutation and neoadjuvant treatment, the patients were stratified into two subgroups, >75 (n=20) and ≤ 75 years old (n=88). No significant association was found between *TP53* mutations and neoadjuvant treatment in these subgroups.

MSI status and *MLH1* promoter hypermethylation

None of the BE lesions and seven out of 108 tumors (6%) showed MSI. Of the MSI tumors, three were scored as having high-degree of MSI (MSI-H) and four as having low-degree of MSI (MSI-L). All three MSI-H tumors had hypermethylated *MLH1* promoters (p=4.9 x 10^{-5}, Fisher's Exact Test). Among the microsatellite stable (MSS) samples, three BE (16%) and one EAC (1%) showed *MLH1* promoter hypermethylation. Methylation frequencies are shown in Figure 1 for BE and EAC samples, and in Additional file 1: Table S3 for normal samples matching EAC. The distribution of *MLH1* PMR values is illustrated in Figure 4. No significant associations were found between MSI-H status or *MLH1* promoter hypermethylation and clinicopathological data.

Promoter methylation frequencies of *APC*, *CDKN2A*, *MGMT*, and *TIMP3*

We examined the promoter DNA methylation status of four genes (*APC*, *CDKN2A*, *MGMT*, and *TIMP3*) in addition to *MLH1*. The distribution of PMR values is illustrated in Figure 4. A subset of normal samples adjacent to EAC (up to 12%) harbored promoter hypermethylation. For each gene, the promoter methylation frequency was significantly higher in BE or tumor samples (Figure 1) compared to the tumor adjacent normal counterpart (Additional file 1: Table S3) (p<0.05, Fisher's Exact Test if BE *vs* normal and McNemar's Test if EAC *vs* normal). Three BE (16%) and 8 EAC (7%) samples showed hypermethylation of all four genes simultaneously (Figure 1).

The PMR values for individual genes in BE and EAC patients are shown in Figure 5. Seventeen EAC patients (16%) had lower PMR values in the tumor compared with the matching adjacent mucosa for at least one gene. Nine of them (8% of all EAC patients) presented promoter hypermethylation in adjacent mucosa but not in the tumor for one or two of the genes. All but one of these pairs had other aberrations (mutations or hypermethylation) in the tumor sample, and pentanucleotide marker controls included in MSI analysis confirmed that EAC samples and normal counterparts belonged to the same patient. A single tumor presented no alterations (patient 103) despite a 20-30% tumor cell content.

A significant association was observed between *APC* promoter hypermethylation and male gender (p = 0.035, Fisher's Exact Test) in BE patients. In EAC patients, a significant association was found between *CDKN2A*, *MGMT* or *TIMP3* promoter hypermethylation and tumor location (p=0.034, p=0.0070 and p=0.013, respectively, Fisher's Exact Test). In addition, a significant association was observed between *TIMP3* and age (p=0.036, Wilcoxon's Test) or tumor stage (p=0.011, Fisher's Exact Test). The use of neoadjuvant treatment and the absence of *CDKN2A* or *TIMP3* promoter methylation were also found to be statistically associated (p=0.043 and p=0.0034, respectively, Fisher's Exact Test; Additional file 1: Table S2) when including all patients. However, these associations did not remain significant when patients were stratified by age.

Discussion

Description of molecular alterations in EAC is abundant in the literature, but discrepancies regarding frequency of these alterations have been observed across studies. In the present work, we analyzed key molecular features in a cohort of non-dysplastic BE and a large series of EAC tissue samples using robust methodologies and well-defined scoring criteria. Overall, our results confirmed frequent *TP53* mutations among EAC in contrast to non-dysplastic BE lesions, which harbored no mutations. Our findings also support that promoter hypermethylation is an early event in the multistep progression of EAC, and frequently seen in BE. Finally, we demonstrated that MSI and *MLH1* promoter hypermethylation are rare events in both lesions.

The series of EAC samples analyzed here was selected to be representative of the population operated for EAC at our institution, both in terms of neoadjuvant treatment status and of tumor-stage prevalence. As expected, many of the EAC samples with no detected molecular alterations had no or low tumor cell content (<5%; *n*=32), demonstrating the value of histopathological evaluation. These samples were left out when mutation and methylation frequencies were calculated, but otherwise kept in order to report the unbiased results of a representative series. Among the cases not evaluated by histopathology, but with one or more molecular alterations (Figure 2), the percentage of EAC samples with no or low tumor cell content would be expected to be lower than the evaluated cases. However, we cannot rule out that some of these EACs might have a lower tumor percentage than the limit of detection of the various molecular analyses, potentially lowering the frequencies reported.

Among molecular abnormalities in EAC, mutation of *TP53* tumor suppressor is one of the most common. We detected *TP53* mutations in 28% of the tumors, while most of the previous studies reported mutation frequencies above 40%.(6, 9-11, 13, 16, 17, 20) Some of this mutation frequency discrepancy may be explained by treatment status. In the present study we

found that tumors from treatment-naïve patients had 44% *TP53* mutations, which is closer to the frequencies reported in other studies including treatment-naïve patients only (11, 16). In contrast, neoadjuvant treated tumors harbored only half as many mutations. The lower *TP53* mutation frequency reported here may therefore be an effect of the sample series composition. In addition, we cannot exclude that some *TP53* mutations may have been missed due to the limit of detection in Sanger sequencing analyses. Although the number of studies on non-dysplastic BE is more limited, *TP53* mutations have been detected in this lesion when resected from tissue adjacent to the tumor,(6, 8) while they are rarely found in non-dysplastic BE of patients who have never developed cancer.(7, 21) In line with these observations, *TP53* mutations were not found in our series of non-dysplastic BE samples.

Here, all exons constituting the coding region of the canonical p53 protein (exons 2-11) were covered. Most of the previous studies span only exons 5-8, the region coding for p53 DNA-binding domain. However, although rare, mutations outside this region and in particular in exon 4 occur in EAC,(11, 13, 20) as well as in other cancer types.(42) In the present study, 16% of the detected mutations were found in exon 4. These findings demonstrate the importance of analyzing regions outside exons 5-8, and suggest that mutations of *TP53* in exon 4 may also play a role in EAC development. To the best of our knowledge, all of the point mutations identified in our study were previously described in EAC,(10-13, 15, 17, 19, 20, 43) with the exception of S127P, P128H (both in patient 83), Q136* (patient 51), T211I (patient 81) and Y220C (patient 62). Codon 220 has been reported as a “hotspot” for *TP53* mutations in other types of cancers.(42)

MSI has also been investigated in EAC by others. Differences in the number and nature of the evaluated markers, as well as in scoring criteria, may contribute to discrepancies in MSI prevalence observed across studies.(16, 36-41) Based on the markers recommended by the National Cancer Institute(36, 37, 39, 41) we found no BE MSI cases and low MSI-H

frequency in EAC (3%). In sporadic colorectal cancer, the MSI phenotype is associated with *MLH1* promoter hypermethylation, which is the most common mechanism of *MLH1* silencing in this cancer type.(44) These events have also been related in EAC.(34, 37) Here, we observed a low frequency of *MLH1* hypermethylation (5%), in agreement with the low prevalence of MSI. We further showed that *MLH1* promoter is hypermethylated in all MSI-H cases. On another hand, only one of the *MLH1* hypermethylated tumors was MSS, in line with the small fraction (<10%) observed in sporadic colorectal tumors.(44, 45) Interestingly, among the samples with *MLH1* promoter hypermethylation, *MLH1* PMR values were considerably lower in BE samples than in EAC. This may reflect the pre-neoplastic nature of BE lesions. Since all of the BE samples were scored as MSS, they clearly have a functioning mismatch repair system, indicating that the reported promoter methylation level for *MLH1* was not high enough to inactivate it.

We showed frequent promoter hypermethylation for most genes both in non-dysplastic BE and in EAC. In BE, these observations may be a consequence of the prolonged exposure to gastro-esophageal reflux, causing an inflammatory environment and tissue damage, often related to epigenetic alterations. Similarities in the methylation profiles of BE and EAC have been documented in several array-based methylation studies, including both non-dysplastic and dysplastic BE.(22-24) Interestingly, promoter methylation frequencies were higher in BE than in EAC for all genes except *CDKN2A*. Such a high methylation frequencies in non-dysplastic BE samples have previously also been reported by others.(35)

We also detected promoter hypermethylation in a subset of normal samples adjacent to EAC, as previously reported in histologically normal tissues adjacent to EAC.(9, 25, 28, 30)Notably, the highest methylation frequencies in normal mucosa were observed for *APC* and *MGMT*, two markers of field defect in prostate(46) and sporadic colorectal cancers,(47) respectively. For some of the EAC patients, lower PMR values were detected in the tumor

sample than in the normal counterpart. In the case of *APC* and *CDKN2A*, these findings may in part reflect the deletion of the methylated alleles attributable to loss of heterozygosity, which has been reported in EAC.(6, 17, 18)

We have found a statistically significant association between the use of neoadjuvant treatment in EAC patients and the absence of mutations in *TP53* or methylation of specific genes (*CDKN2A* or *TIMP)*. Moreover, 92% of the EAC patients showing no alterations across the set of markers had received neoadjuvant treatment. When stratifying patients by age, these associations lost their significance as age is a confounder of treatment. These observations are in line with a previous analysis of the DNA methylation patterns in EAC patients, which revealed no differences between patients receiving neoadjuvant chemotherapy or not,(48) regardless of age. Additional studies analyzing potential associations between neoadjuvant treatment and genomic or molecular aberrations in EACs are warranted.

In total, 13 tumor samples (12%) showed no alterations – including mutations or hypermethylation. It could be speculated that these samples correspond to lower stages of the disease. We have selected our set of samples based on the representativity of tumor-stage prevalence in EAC patients eligible for surgery, and therefore it inevitably comprises a low percentage of stage IV tumors. Nevertheless, we found no association between the absence of alterations and tumor stage (p=0.56, Fisher's Exact Test).

Overall, the prevalence of *TP53* mutations, as well as promoter methylation frequency of *APC*, *CDKN2A*, *MGMT* and *TIMP3*, observed in EAC show some discrepancies when compared to findings reported in other studies. Our results are based on a sample size larger than most of these, adding another layer of robustness to our analyses. Factors that may explain the inconsistencies in the methylation results may include differences in the prevalence of tumor location (distal esophagus *vs* gastroesophageal junction), and the thresholds used for distinction between methylated and unmethylated DNA. We have here

considered normal mucosa adjacent to BE samples as “methylation background” and defined the threshold for each gene individually.

Conclusions

The present study contributes to an improved characterization of the molecular background of EAC progression by analyzing a series of non-dysplastic BE, EAC, and matched normal samples. We reported a spectrum of genetic and epigenetic alterations occurring in these tissues and clarified discrepancies found in literature regarding frequency of these alterations. Our study derived its strength from a careful design, use of consensus markers, state-of-the-art methodologies and well-defined scoring criteria. To the best of our knowledge, this is one of the largest studies addressing a targeted characterization of genetic and epigenetic modifications simultaneously across a combined series of non-dysplastic BE and EAC samples.

Methods

Patients and tumor samples

This study included tissue samples from 19 BE patients without a current dysplasia or a history of dysplasia and from 145 EAC patients. BE biopsies were collected between November 2017 and February 2020 during routine gastroscopy at the Department of Gastroenterology, Oslo University Hospital, Ullevål. BE was defined as the presence of columnar epithelium in the distal esophagus containing specialized intestinal metaplasia with a minimum length of 1 cm.(49) Four-quadrant biopsies were taken every 2 cm within BE segment, in accordance with the current guidelines. Among these, multiple (2-4) samples were randomly chosen to be used in this study and pooled for DNA extraction. EAC samples were obtained from patients operated between September 2013 and May 2020 at the Department of Pediatric and Gastrointestinal Surgery, Oslo University Hospital, Ullevål. One hundred and seventeen (81%) EAC patients had received neoadjuvant radio(chemo)therapy. Only patients with macroscopic residual tumor left in the surgical specimen (stages T0-T4) were included in this study. Both neoadjuvant treatment status and tumor-stage prevalence are representative of the population operated for EAC at our institution.(50) For all patients (n=164), matched biopsies from adjacent macroscopically normal-appearing mucosa (5-10 cm from the tumor), hereafter referred to as normal samples, were included. Samples were taken immediately following specimen resection according to a predefined protocol. For all the paired BE and normal samples, as well as for 103 (71%) of the paired EAC and normal counterparts, patient identity was verified by short tandem repeat (STR) profiling according to the AmpFLSTR Identifiler PCR Amplification Kit (Thermo Fisher Scientific). Clinicopathological characteristics of the EAC patients are summarized in Additional file 1: Table S4.

Sixty-three EAC samples were subjected to histopathological evaluation as described in Figure 2. Of these, 37 were removed from frequency calculations due to absence of tumor or low tumor cell content (< 5%). The main series of this study therefore comprised samples from 108 patients (Figure 1). Clinicopathological characteristics of these patients are summarized in Table 1.

DNA extraction and bisulfite treatment

DNA from fresh frozen tissue samples corresponding to tumors and matched normal mucosa was extracted using the DNeasy Blood & Tissue Kit (Qiagen). For samples from BE biopsies (<30 mg) and adjacent normal mucosa, the AllPrep DNA/RNA Mini Kit (Qiagen) was used. DNA quantity and quality were measured using ND-1000 Nanodrop (NanoDrop Technologies). For the methylation analyses, 800 ng DNA of each sample was bisulfite treated using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's protocol. Bisulfite converted DNA was purified using the QIAcube automated pipetting system (Qiagen) and eluted in 40 µl elution buffer.

Selection of candidate genes for analysis

A literature search was performed in order to identify candidate genes in EAC (Additional file 1: Figure S1). Genes consistently reported as frequently altered (>50%) in at least three original papers were considered for inclusion. Based on this search, *TP53* was selected for mutation analysis, whereas *APC*, *CDKN2A*, *MGMT* and *TIMP3* were selected for DNA methylation analysis. In addition, *MLH1* promoter methylation, reported to be infrequent in EAC, was analyzed in order to relate it to MSI status.

TP53 mutation analysis

TP53 mutation status was assessed in all BE and EAC samples by Sanger sequencing. The entire coding region (exons 2–11)was analyzed using previously described primer sequences and reactions.(51) Mutation calling was performed independently by two of the authors, using the SeqScape V.2.5 and Sequencing Analysis V.5.3.1 softwares (both Applied Biosystems). All detected mutations were confirmed by sequencing of a new independent PCR product.

Microsatellite instability analysis

MSI status was assessed in all BE and EAC and compared to corresponding normal tissue by PCR-based analyses of the BAT-25, BAT-26, NR-21, NR-24 and MONO-27 mononucleotide markers using the MSI Analysis System, Version 1.2 (Promega) according to the manufacturer's instructions. Data was analyzed with Gene Mapper software (Applied Biosystems). Nuclease-free water replacing DNA as template was included in each run as control. All the paired samples (BE or EAC and normal counterparts) were confirmed to belong to the same patient by analyzing pentanucleotide marker controls available in the MSI Analysis System.

The results were scored independently by two of the authors following Bethesda guidelines for colorectal cancer.(52) MSI-H in BE or tumor DNA was defined if two or more markers showed aberrant peak profile, whereas one single unstable marker defined MSI-L. Samples with all loci exhibiting normal allelic ranges were regarded MSS. MSI status for each locus was confirmed by an independent run.

Quantitative methylation-specific PCR

APC, *CDKN2A*, *MGMT*, *TIMP3* and *MLH1* were analyzed for DNA promoter hypermethylation in all BE, EAC and adjacent normal samples using quantitative methylation

specific PCR (qMSP) and *ALU-C4* as a normalization control for DNA input. Primer and probe sequences have been reported previously.(35, 53) Primers were purchased from BioNordika (Oslo, Norway), and probes were obtained from Life Technologies (now Thermo Fisher Scientific).

The qMSP reactions were performed in triplicate and carried out as previously described (54) using ~30 ng bisulfite treated DNA per well. Methylation positive (*in vitro* methylated DNA; IVD Chemicon, Millipore), methylation negative (WGA non-methylated DNA; Zymo Research) and non-template (H_2O) controls were included, in addition to a standard curve consisting of a 5-fold serial dilution of IVD (32.5-0.052 ng).

Samples amplified after cycle 35 were censored in accordance with the recommendations from Life Technologies, and the median quantity value of the triplicates was used for data analysis. The qMSP results were calculated as percent of methylated reference (PMR) by dividing the *ALU-C4*-normalized quantity of the samples by the *ALU-C4*-normalized quantity of the positive control (IVD) and multiply by 100. To ensure high specificity for each qMSP assay, the thresholds for scoring samples as methylated were set according to the highest PMR value across the normal mucosa matching BE samples as shown in Figure 4. Samples with PMR values above the scoring threshold for each individual gene were considered to be methylated.

Statistics

Statistical analyses were conducted with R software version 3.6.2. Associations between gene alterations and the clinicopathological parameters listed in Table 1 were analyzed by Fisher's exact tests for categorical variables and by two-sided Wilcoxon's tests for continuous variables. Associations between genetic and epigenetic alterations were investigated using Fisher's exact tests or McNemar's tests. A p-value < 0.05 was considered significant. When

relevant, *p*-values were adjusted for multiple testing using the FDR criterion and Benjamini-Hochberg procedure. An adjusted p-value < 0.05 was considered significant.

List of abbreviations

BE, Barrett's esophagus

EAC, esophageal adenocarcinoma

IVD, *in vitro* methylated DNA

MSI, microsatellite instability

MSI-H, high-degree of microsatellite instability

MSI-L, low-degree of microsatellite instability

MSS, microsatellite stable

PMR, percent of methylated reference

qMSP, quantitative methylation specific PCR

STR, short tandem repeat

Declarations

Ethics approval and consent to participate

Written informed consent was obtained from all patients enrolled in the study. The study was approved by the Regional Ethics Committee (REK) and the research biobanks have been registered according to Norwegian legislation (2012/2186/REK Sør-Øst B for EAC and 2017/1646/REK Nord for BE). All experiments were performed in accordance with the standards set by the Declaration of Helsinki.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RP, MJ, EJ and GEL contributed to the conception and design. HDP, SHK, HH, SBW, MBF, TK, TrH and EJ contributed to the acquisition of data. RP, ToH, MJ, HDP, TM and GEL contributed to the analyses and interpretation of data. RP, MJ and GEL contributed to the drafting of the manuscript. All authors were involved in revision of the manuscript and have approved the final version.

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Figure Legends

Figure 1. Summary of genetic and epigenetic alterations in BE (*n*=19) and EAC (*n*=145) samples. *TP53* silent mutations with no amino acid change are not presented as alterations. In one sample (patient 83), two *TP53* missense mutations were found. All samples with no or a low percentage of tumor cells (<5%; *n*=37) have been removed from the main data set (see Figure 2) and the molecular alterations found in these samples are shown separately in the grey box. These samples were not used for determination of alterations frequencies. (*For MSI, the percentage refers to MSI-H tumors only.)

Figure 2. Flow diagram illustrating EAC samples selection process. 145 EAC patients were subjected to targeted molecular profiling, among which 37 were removed from the main data set due to absence of tumor or low tumor cell content (< 5%). Only samples from 108 patients were used for determination of alterations frequencies.

Figure 3. Graphical representation of *TP53* mutations identified in BE and EAC samples. The entire *TP53* coding region (exons 2–11) was analyzed by Sanger sequencing and mutations were found across exons 4-8. The silent mutation R213R found in one BE (patient 13) and one EAC (patient 94) sample was classified as *TP53* wild type.

Figure 4. PMR values distribution in BE (*n*=19), EAC (*n*=108) and respective normal adjacent mucosa (N). The thresholds for scoring the samples as methylated were set according to the highest PMR value across the normal mucosa matching BE samples. These thresholds were determined for each gene independently and are marked by dotted red lines.

Figure 5. Methylation levels of the evaluated genes in BE, EAC (T) and normal adjacent mucosa (N). EAC patients where normal mucosa presents PMR values higher than in the tumor are highlighted by grey bordered boxes. PMR values are shown in different color scales for each gene in order to facilitate visualization.

Table 1. Summary of clinicopathological characteristics of patient samples considered in this study after removal of the samples with no or a low percentage of tumor cells (<5%).

	BE (*n* = 19)	**EAC (*n* = 108)**
Age (years)		
Median (mean)	66 (62)	66 (66)
Range	35-84	34-82
Gender		
Male	15 (79%)	90 (83%)
Female	4 (21%)	18 (17%)
Barrett's segment length (cm)		
Median (mean)	4 (4.4)	-
Range	1-10	-
Location		
At or above carina	-	1 (1%)
Distal esophagus	-	37 (34%)
Gastroesophageal junction	-	70 (65%)
Tumor (T) stage		
T0	-	1 (1%)
T1	-	16 (15%)
T2	-	20 (18%)
T3	-	69 (64%)
T4	-	2 (2%)
Tumor length (cm)		
Median (mean)	-	3.0 (3.6)
Range	-	0.4-11
Lymph node metastases		
Yes	-	65 (60%)
No	-	43 (40%)
Neoadjuvant radio(chemo)therapy		
Yes	-	81 (75%)
No	-	27 (25%)

Figure 1

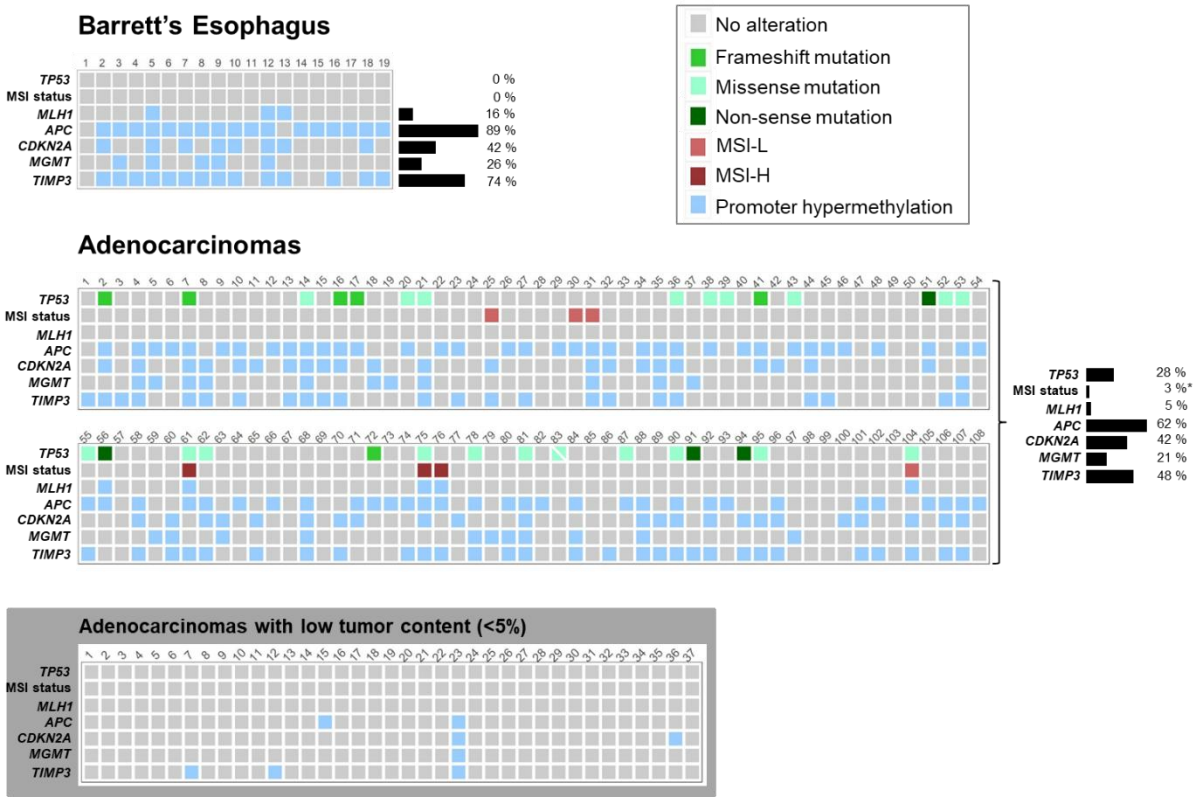
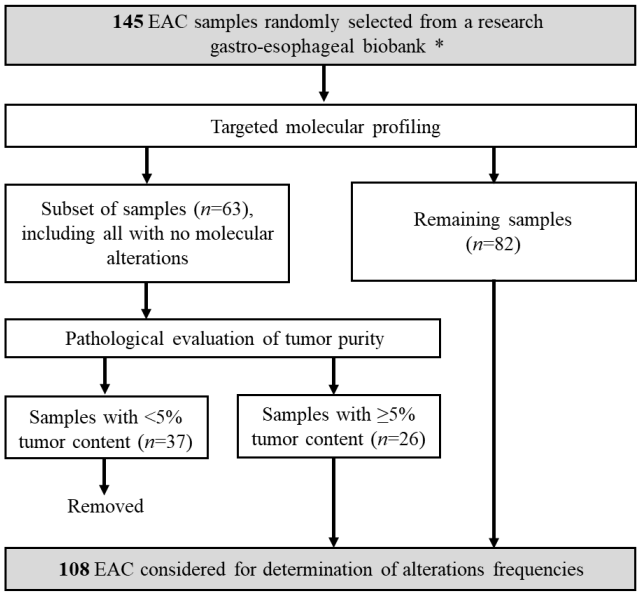

Barrett's Esophagus
TP53
MSI status
MLH1
APC
CDKN2A
MGMT
TIMP3
0 %
0 %
16 %
89 %
42 %
26 %
74 %
No alteration
Frameshift mutation
Missense mutation
Non-sense mutation
MSI-L
MSI-H
Promoter hypermethylation
Adenocarcinomas
TP53
MSI status
MLH1
APC
CDKN2A
MGMT
TIMP3
28 %
3 %*
5 %
62 %
42 %
21 %
48 %
Adenocarcinomas with low tumor content (<5%)
TP53
MSI status
MLH1
APC
CDKN2A
MGMT
TIMP3

Figure 2



* Both neoadjuvant treatment status and tumor-stage prevalence are representative of the population operated for EAC at our institution.

Figure 3

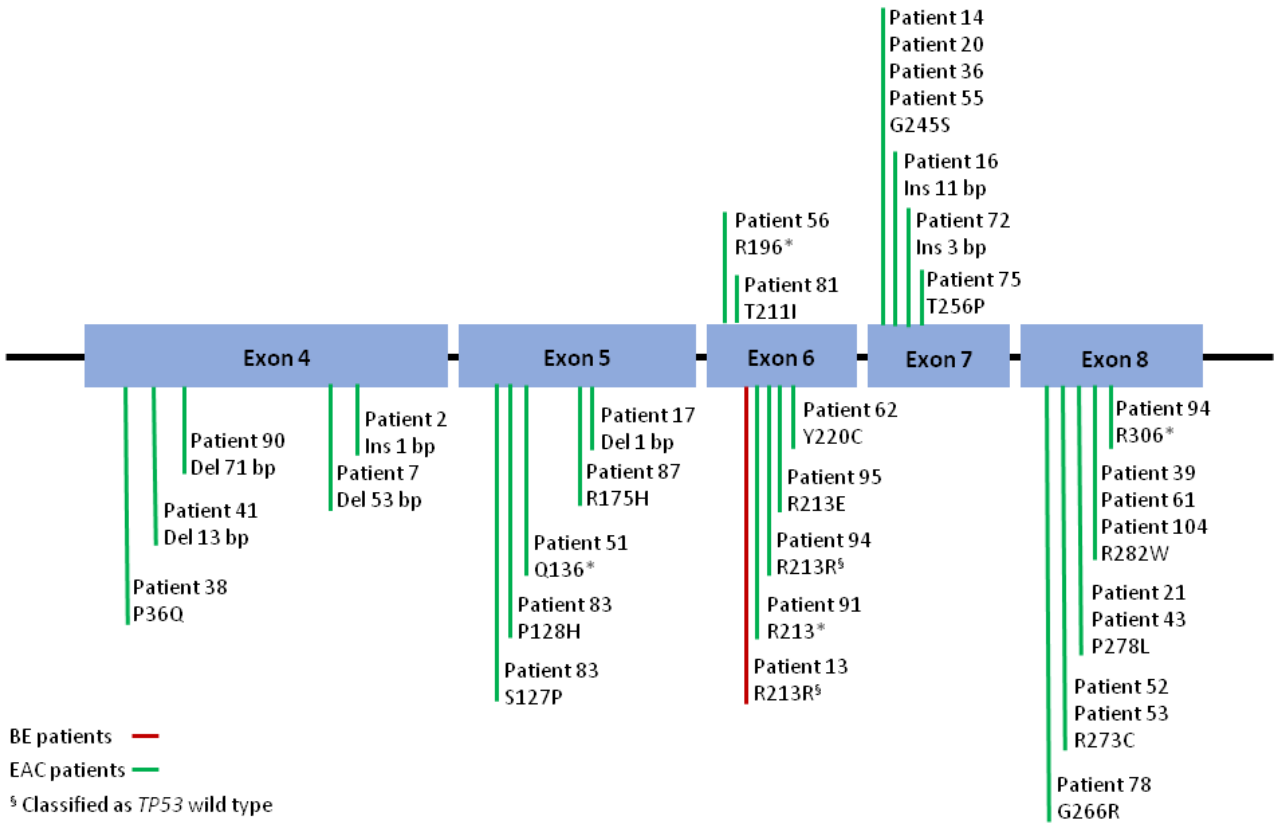

Exon 4
Exon 5
Exon 6
Exon 7
Exon 8
Patient 14
Patient 20
Patient 36
Patient 55
G245S
Patient 16
Ins 11 bp
Patient 72
Ins 3 bp
Patient 75
T256P
Patient 56
R196*
Patient 81
T211I
Patient 90
Del 71 bp
Patient 41
Del 13 bp
Patient 38
P36Q
Patient 2
Ins 1 bp
Patient 7
Del 53 bp
Patient 17
Del 1 bp
Patient 87
R175H
Patient 51
Q136*
Patient 83
P128H
Patient 83
S127P
Patient 62
Y220C
Patient 95
R213E
Patient 94
R213R§
Patient 91
R213*
Patient 13
R213R§
Patient 94
R306*
Patient 39
Patient 61
Patient 104
R282W
Patient 21
Patient 43
P278L
Patient 52
Patient 53
R273C
Patient 78
G266R
BE patients
EAC patients
§ Classified as TP53 wild type

Figure 4

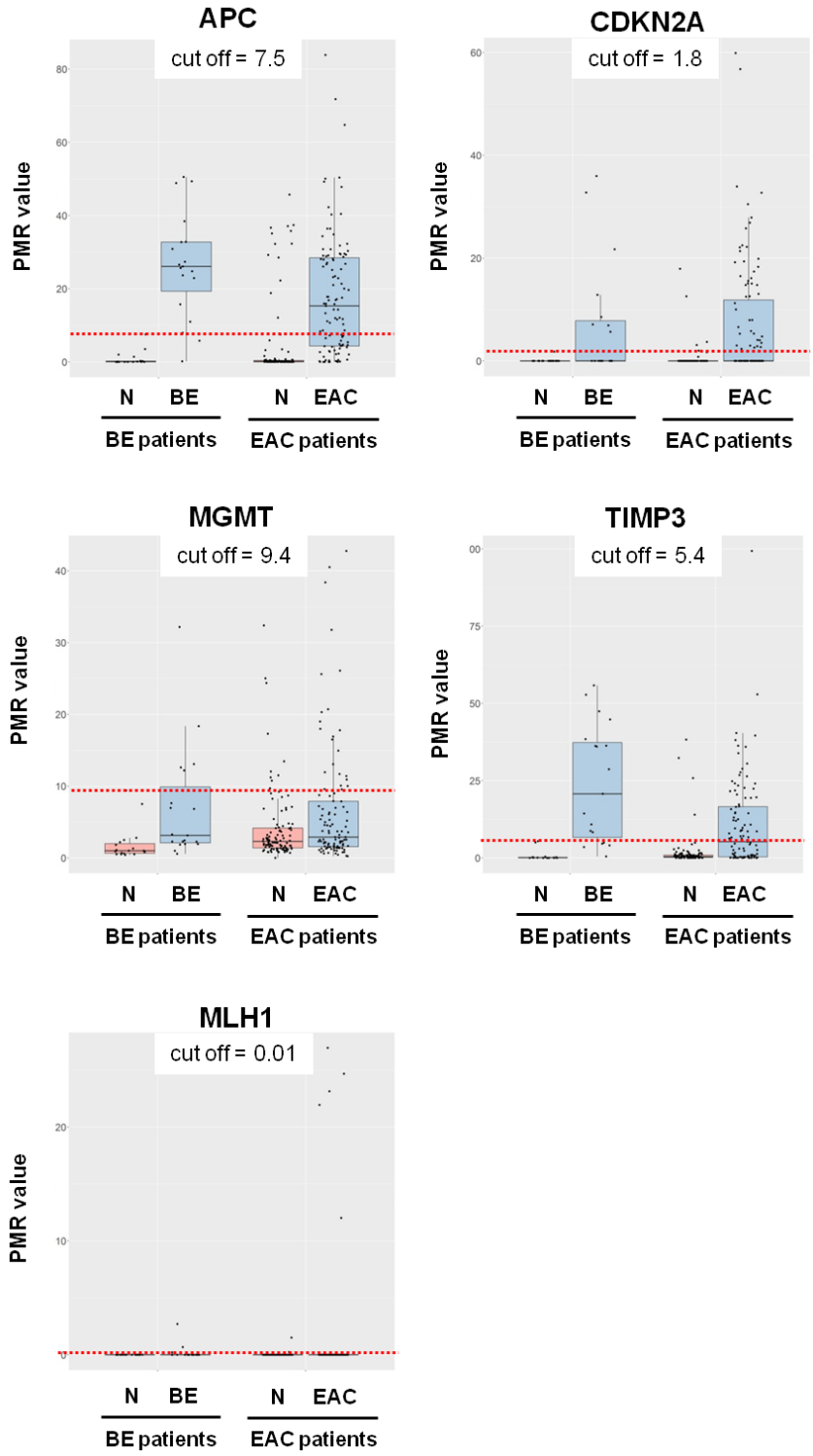

APC
cut off = 7.5
CDKN2A
cut off = 1.8
MGMT
cut off = 9.4
TIMP3
cut off = 5.4
MLH1
cut off = 0.01
PMR value
N
BE
N
EAC
BE patients
EAC patients

Figure 5

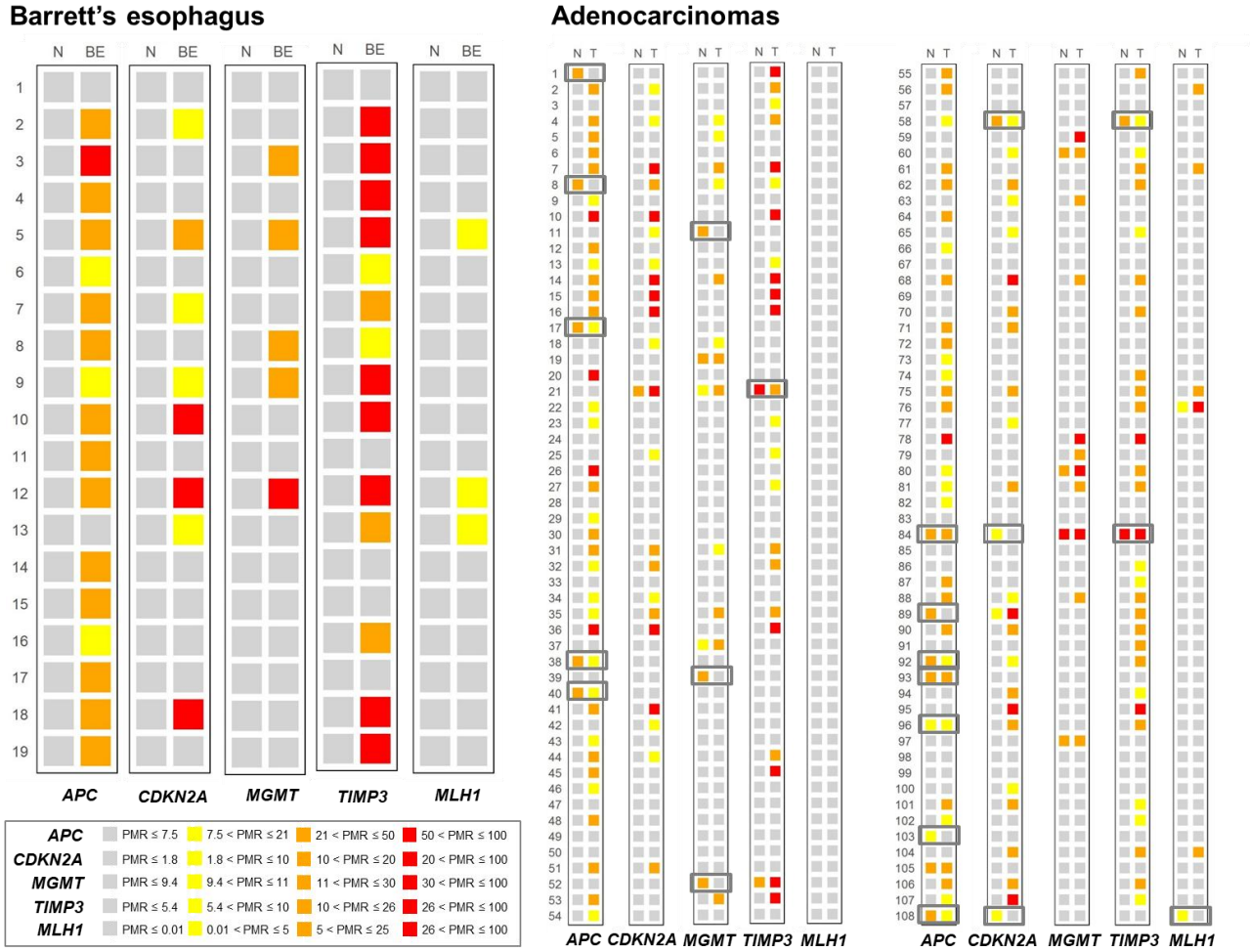

Barrett's esophagus
Adenocarcinomas
N BE
N T
APC
CDKN2A
MGMT
TIMP3
MLH1
APC PMR ≤ 7.5 7.5 < PMR ≤ 21 21 < PMR ≤ 50 50 < PMR ≤ 100
CDKN2A PMR ≤ 1.8 1.8 < PMR ≤ 10 10 < PMR ≤ 20 20 < PMR ≤ 100
MGMT PMR ≤ 9.4 9.4 < PMR ≤ 11 11 < PMR ≤ 30 30 < PMR ≤ 100
TIMP3 PMR ≤ 5.4 5.4 < PMR ≤ 10 10 < PMR ≤ 26 26 < PMR ≤ 100
MLH1 PMR ≤ 0.01 0.01 < PMR ≤ 5 5 < PMR ≤ 25 26 < PMR ≤ 100

Table S1. *TP53* mutations identified in BE and EAC samples.

Lesion	Patient	Exon	Codon	Mutation	Aminoacid
BE	13*	6*	213*	CGA → CGG*	Arg → Arg*
EAC	2	4	90	Ins 1 base	Frameshift
	7	4	–	Del 53 bases	Frameshift
	14	7	245	GGC → AGC	Gly → Ser
	16	7	–	Ins 11 bases	Frameshift
	17	5	–	Del 1 base	Frameshift
	20	7	245	GGC → AGC	Gly → Ser
	21	8	278	CCT → CTT	Pro → Leu
	36	7	245	GGC → AGC	Gly → Ser
	38	4	36	CCG → CAG	Pro → Gln
	39	8	282	CGG → TGG	Arg → Trp
	41	4	–	Del 13 bases	Frameshift
	43	8	278	CCT→ CTT	Pro → Leu
	51	5	136	CAA → TAA	Gln → STOP
	52	8	273	CGT → TGT	Arg → Cys
	53	8	273	CGT → TGT	Arg → Cys
	55	7	245	GGC → AGC	Gly → Ser
	56	6	196	CGA → TGA	Arg → STOP
	61	8	282	CGG → TGG	Arg→ Trp
	62	6	220	TAT → TGT	Tyr→ Cys
	72	7	246	Ins 3 bp	In-frame indel mutation
	75	7	256	ACA → CCA	Thr → Pro
	78	8	266	GGA → AGA	Gly→ Arg
	81	6	211	ACT → ATT	Thr → Ile
	83	5	127	TCC → CCC	Ser → Pro
			128	CCT → CAT	Pro → His
	87	5	175	CGC → CAC	Arg → His
	90	4	–	Del 71 bases	Frameshift
	91	6	213	CGA → TGA	Arg → STOP
	94	6*	213*	CGA → CGG*	Arg → Arg*
		8	306	CGA → TGA	Arg → STOP
	95	6	213	CGA → CAA	Arg → Gln
	104	8	282	CGG → TGG	Arg → Trp

* Classified as *TP53* wild type

Table S2. Summary of clinicopathological characteristics and the genetic and epigenetic alterations found in neoadjuvant treatment naïve and treated EAC patients.

	Neoadjuvant treatment-naïve EAC patients (*n* = 27)	**Neoadjuvant treated EAC patients (*n* = 81)**	***p*-value**
Age (years)			3.4×10^{-10}
Median (mean)	76 (75)	64 (62)	
Range	65-82	34-78	
Tumor (T) stage			NS
T0	0 (0%)	1 (1%)	
T1	6 (22%)	10 (12%)	
T2	6 (22%)	14 (17%)	
T3	15 (56%)	54 (67%)	
T4	0 (0%)	2 (2%)	
TP53 mutations			0.045
Yes	12 (44%)	18 (22%)	
No	15 (56%)	63 (78%)	
APC promoter hypermethylation			NS
Yes	21 (78%)	46 (57%)	
No	6 (22%)	35 (43%)	
CDKN2A promoter hypermethylation			0.043
Yes	16 (59%)	29 (36%)	
No	11 (41%)	52 (64%)	
MGMT promoter hypermethylation			NS
Yes	5 (19%)	18 (22%)	
No	22 (81%)	63 (78%)	
TIMP3 promoter hypermethylation			0.0034
Yes	20 (74%)	32 (40%)	
No	7 (26%)	49 (60%)	
MLH1 promoter hypermethylation			NS
Yes	2 (7%)	3 (4%)	
No	25 (93%)	78 (96%)	
MSI status			NS
MSI-H	1 (4%)	2 (2%)	
MSI-L/MSS	26 (96%)	79 (98%)	

NS: non-significant association

Table S3. Promoter methylation frequencies for the evaluated genes in normal mucosa adjacent to EAC samples.

Gene	Normal mucosa (*n* = 108)
APC	13 (12%)
CDKN2A	5 (5%)
MGMT	10 (9%)
TIMP3	4 (4%)
MLH1	2 (2%)

Table S4. Summary of clinicopathological characteristics of included EAC patient samples.

	EAC (*n* = 145)
Age (years)	
Median (mean)	66 (65)
Range	34-82
Gender	
Male	121 (83%)
Female	24 (17%)
Location	
At or above carina	1 (1%)
Distal esophagus	49 (34%)
Gastroesophageal junction	95 (65%)
Tumor (T) stage	
T0	2 (1%)
T1	26 (18%)
T2	30 (20%)
T3	85 (59%)
T4	2 (1%)
Tumor length (cm)	
Median (mean)	3.1 (3.6)
Range	0.4-11
Lymph node metastases	
Yes	73 (50%)
No	72 (50%)
Neoadjuvant radio(chemo)therapy	
Yes	117 (81%)
No	28 (19%)